Targets of the *StBEL5* Transcription Factor Include the FT Ortholog *StSP6A*[^OPEN]

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The *BEL1-like* family of transcription factors is ubiquitous in plants and plays important roles in regulating development. They function in tandem with KNOTTED1 types to bind to a double TTGAC motif in the upstream sequence of target genes. *StBEL5* of potato (*Solanum tuberosum*) functions as a mobile RNA signal that is transcribed in leaves, moves down into stolons in response to short days, and induces tuber formation. Despite their importance, however, very little is known about the targets of *BEL1-like* transcription factors. To better understand this network, we made use of a phloem-mobile *BEL5* induction model, an ethanol-inducible system coupled with RNA sequencing analysis, and a screen for tandem TTGAC cis-elements in the upstream sequence to catalog *StBEL5* target genes. Induction of *StBEL5* activated several genes that are also induced by *StISP6A* (*S. tuberosum* SELF-PRUNING 6A), a FLOWERING LOCUS T coregulator that functions as a signal for tuberization. Both enhancement and suppression of *StBEL5* expression were also closely linked to *StISP6A* transcriptional activity. Site mutagenesis in tandem TTGAC motifs located in the upstream sequence of *StISP6A* suppressed the short day-induced activity of its promoter in both young tubers and leaves. The expression profile of *StBEL5* induced in stolons from plants grown under long-day conditions revealed almost 10,000 differentially expressed genes, including important tuber marker genes and genes involved in cell growth, transcription, floral development, and hormone metabolism. In a random screen of 200 differentially expressed targets of *StBEL5*, 92% contained tandem TTGAC motifs in the upstream sequence within 3 kb of the transcription start site.

**BEL1-like** transcription factors (TFs) of plants are members of the three-amino acid-loop-extension (TALE) superfamily that includes KNOTTED1 (KN1) types and numerous members that are functional in animals and fungi. For example, several animal TALE TFs play significant roles in organogenesis and differentiation during embryogenesis (Lukowski et al., 2011; Sonnet et al., 2012) and in enhancing cell proliferation in certain cancer types (Rosales-Aviña et al., 2011; Okumura et al., 2014). All TALE types contain a highly conserved homeodomain composed of three helices with a Pro-Tyr-Pro link between helices 1 and 2. The plant types contain two conserved protein interaction regions, designated the BELL and SKY-box domains. Through these protein-binding domains, BEL-like homeodomain TFs act in tandem with KN1 types targeting the upstream sequence of select genes with tandem TTGAC cis-elements ([Müller et al., 2001; Chen et al., 2004; Hamant and Pautot, 2010]). They are ubiquitous in plants and are involved in meristem and floral development ([Rutjens et al., 2009; Ung et al., 2011]), inflorescence and leaf architecture ([Smith and Hake, 2003; Khan et al., 2012]), tuber formation ([Chen et al., 2003]), and embryo and ovule formation ([Brambilla et al., 2007; Pagnussat et al., 2007]).

Concordant with Arabidopsis, there are 13 BEL1 genes in potato (*Solanum tuberosum*; Sharma et al., 2014). One of the *StBELs*, *StBEL5*, functions as a signal mRNA in potato, is trafficked long distance through the sieve element system, and is involved in the activation of tuberization (Banerjee et al., 2006a). There is considerable information on the transcriptional function of *StBEL5* and one of its KNOX partners, POTH1 (POTATO HOMEBOX1), and their role as mobile signals ([Chen et al., 2003, 2004; Banerjee et al., 2006a; Mahajan et al., 2012; Lin et al., 2013]). The heterodimer of *StBEL5* and POTH1 binds to a TTGAC-TTGAC motif that is essential for regulating transcription ([Chen et al., 2004]). In potato, *StBEL5* and POTH1 regulate tuberization by targeting genes that control growth. RNA movement assays demonstrated that, under short days, *StBEL5* transcripts originating in the leaf move through the phloem to stolon meristems (Banerjee et al., 2006a, 2009) and that the RNA-binding proteins, StPTB1 (*S. tuberosum* POLYPYRIMIDINE TRACT-BINDING1) and StPTB6, mediate this movement ([Cho et al., 2015]). The movement and accumulation of *StBEL5* RNA have been consistently associated with enhanced tuberization even under long days ([Chen et al., 2003; Banerjee et al., 2006a]).

[^OPEN]: This work was supported by the National Science Foundation Plant Genome Research Program (grant no. 0820659).

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P.S. performed all of the experiments, wrote parts of the article, and implemented figure design; T.L. performed the analysis of the RNA-Seq data; D.J.H. wrote and edited portions of the article and designed most of the figures and tables.

[^OPEN]: Articles can be viewed without a subscription. www.plantphysiol.org/cgi/doi/10.1104/pp.15.01314

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FLOWERING LOCUS T (FT) is involved in controlling flowering and has been established as the universal flowering signal (Turck et al., 2008), but FT-like genes also function in a wide range of developmental events other than flowering (Shalit et al., 2009; Navarro et al., 2011; Pin and Nilsson, 2012). In addition to StBEL5 and POTH1, StSP6A, an FT ortholog of potato, also serves as a prime candidate for the mobile tuber signal (Navarro et al., 2011). Transcripts of StSP6A accumulate in both leaves and stolons in response to the inductive conditions of short days. Overall, the data available on StSP6A imply that it is a very likely candidate for a mobile tuber signal. Transgenic overexpression (OE) lines of StSP6A tuberized under noninductive long days, whereas suppression lines exhibited a decrease in tuber production under short days (Navarro et al., 2011). StSP6A-OE scions grafted onto wild-type stocks induced the stocks to tuberize, suggesting movement of the StSP6A protein into stolons similar to FT movement into shoot apices to induce flowering. In potato, the FT activator CONSTANS functions to inhibit tuberization (Martínez-García et al., 2002). This relationship has prompted the theory that CONSTANS acts to repress StSP6A expression. The candidate TF, however, that induces StSP6A expression in both leaves and stolons has not been identified. In summary, tuberization in potato is induced under short-day (SD) conditions by a phloem-mobile signal originating in the leaf. StSP6A, a coregulator, is assumed to move as a protein to stolons, where it is translated and functional (Banerjee et al., 2006a).

Numerous reports have linked BEL1 TFs and their KN1 partners to regulating the expression of genes involved in hormone activity. Using chromatin immunoprecipitation sequencing to define the cis-acting regulatory elements of the maize (Zea mays) genome specifically recognized by KN1, Bolduc et al. (2012) showed that KN1 targets are strongly enriched for genes participating in metabolic pathways for cytokinins, GAs, and auxin. Here, we show that StBEL5 of potato controls the expression of numerous genes, including the FT ortholog StSP6A, that mediate hormone activity and signaling during early stages of tuber formation. Using a phloem-mobile induction system, an ethanol-inducible system coupled with RNA sequencing (RNA-Seq), and identification of the upstream tandem TTGAC cis-element, a catalog of StBEL5 target genes has been assembled. The expression profile of StBEL5 reveals many genes activated in the tuberization pathway, including the important marker genes AGA2 oxidase1 (StGA2ox1), AGL8 (AGAMOUS-LIKE8), SCD1 (S. tuberosum CYCLING DOF FACTOR1), POTLX-1 (POTATO LIPOXYGENASE-1), and StLONELY GUY1 (LOG1).

RESULTS

The StBEL5 and StSP6A Signal Pathways Appear to Overlap

Because StSP6A and the full-length mRNA of StBEL5 are currently the two best candidates for phloem-mobile signals that regulate tuber formation, we compared their inductive capacity for a select group of tuber...
marker genes (Kloosterman et al., 2008). Utilizing GALACTINOL SYNTHASE (GAS):BEL5 transgenic lines in a phloem-mobile system, the capacity of StBEL5 expression to activate several of these marker genes known to be induced by SISP6A in stolons (Navarro et al., 2011) was tested (Fig. 1A). The GAS promoter of melon (Cucumis melo) is specific to the minor veins of the leaf mesophyll (Ayre et al., 2003) and to detect transgenic RNA in any other organ indicates movement of the RNA (Lin et al., 2013). Using the GAS:BEL5 transgenic line allows us to closely mimic the temporal events of tuberization and the activity profile of the native promoter of StBEL5. The GAS promoter is expressed in minor veins of the leaf, whereas the StBEL5 promoter is active in leaf veins and petioles (Banerjee et al., 2006a). The onset of tuber formation occurs approximately 8 to 10 d after inductive SD conditions are initiated. This time frame allows significant movement and localization of StBEL5 into stolons and provides for an assessment of its effect on target gene activity. In this model under short days, StBEL5 RNA moves down into stolons, where it is translated and, in tandem with its KNOX partner, POTH1 (Chen et al., 2004), StBEL5 regulates genes involved in the synthesis or activity of GA, auxin, and cytokinin, strongly suggesting that it is an important developmental signal for controlling growth in potato. StGA2ox1 is particularly interesting because it contains six tandem TTGAC elements (Table I; Lin et al., 2013) and is induced up to 50-fold at the onset of tuber formation (Kloosterman et al., 2007).

To confirm the interaction of StBEL5 and its KN1-like partner, POTH1 (Chen et al., 2004), with the tandem TTGAC motifs listed in Table I, gel-shift assays were performed with sequence from four select target genes (Supplemental Fig. S1). Bait DNA for StLOG1, AGL8, StSISP6A, and StPIN4 each contained the tandem elements in one of the four structural configurations of the motif: tail-to-head on either the + or − strand, head-to-head, and tail-to-tail. Linker sequence between motifs ranged from zero (StSISP6A) to 24 (AGL8) nucleotides. For all four, the strongest interactions occurred with the BEL5/POTH1 complex and, in the case of StLOG1, with the StBEL5 protein alone (Supplemental Fig. S1, arrows, lane 4). A shifted band was also observed in interactions with StBEL5 protein alone for the other three targets (Supplemental Fig. S1, arrows, lane 2).

**Table I. Tuberization marker genes that contain tandem TTGAC motifs in their upstream sequence**

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Motif</th>
<th>Orientation</th>
<th>Nucleotides Upstream</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>StBEL5</td>
<td>GTCAAgtgTTGAC*</td>
<td>HtH 820 (TSS)</td>
<td>BEL5/POTH1 bind</td>
<td></td>
</tr>
<tr>
<td>StPIN1</td>
<td>TTGACcactcattttcgtgTTGCA</td>
<td>TtT 1,249 (AUG)</td>
<td>Induced in roots</td>
<td></td>
</tr>
<tr>
<td>StPIN4</td>
<td>TGAcactcactTTGCA*</td>
<td>TtT 914 (AUG)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARF8</td>
<td>GTCAAactcactaaATTTGCA</td>
<td>TtT 486 (AUG)</td>
<td>Induced in roots</td>
<td></td>
</tr>
<tr>
<td>FPF</td>
<td>GTCAactgaaactaaatattattacttactaactagaaagtTTGAC</td>
<td>HtH 1,240 (AUG)</td>
<td>Flowering promoter factor1</td>
<td></td>
</tr>
<tr>
<td>CDC</td>
<td>GTCAaactgaaacteTTGCA</td>
<td>TtT 1,614 (AUG)</td>
<td>Cell division cycle function</td>
<td></td>
</tr>
<tr>
<td>SUT1</td>
<td>TTGACactgaaTTGCA</td>
<td>HtT 1,383 (AUG)</td>
<td>Auxin response factor</td>
<td></td>
</tr>
<tr>
<td>AGL8</td>
<td>GTCAactgaaactTTGCA</td>
<td>TtT 1,090 (TSS)</td>
<td>POTM1</td>
<td></td>
</tr>
<tr>
<td>StGA2ox1</td>
<td>TTGACaattTTGCA</td>
<td>TtT 1,249 (TSS)</td>
<td>Contains six tandem motifs</td>
<td></td>
</tr>
<tr>
<td>StLOG1</td>
<td>TTGACTGCAactactTTGAC</td>
<td>TtT 1,718 (TSS)</td>
<td>Contains six tandem TTGAC motifs</td>
<td></td>
</tr>
<tr>
<td>StLOG2</td>
<td>TTGACactactTTGAC</td>
<td>TtT 1,249 (TSS)</td>
<td>Cytokinin synthesis</td>
<td></td>
</tr>
<tr>
<td>StLOG3</td>
<td>TTGACagctTTGAC</td>
<td>HtH 1,029 (AUG)</td>
<td>Cytokinin synthesis</td>
<td></td>
</tr>
</tbody>
</table>

StBEL5 also induces the activity of three LOG genes of potato (Fig. 1B), which encode for enzymes involved in cytokinin activation. Overexpression of LOG1 in tomato (Solanum lycopersicum) generated tubers from axillary stem buds (Eviatar-Ribak et al., 2013), suggesting that this enzyme is a critical component of the tuberization pathway. Of the 13 genes induced by StBEL5 or SISP6A listed in Table I, all except one contain the tandem TTGAC motif within 2 kb of the start codon. Some contain more than one upstream motif (e.g. StGA2ox1, StPIN1, and StSISP6A). All four orientations of the double motif are represented in this group with linker regions ranging from zero (StSISP6A) to 41 (FPF, FLOWERING PROMOTER FACTOR1) nucleotides. Like StGA2ox1 (Lin et al., 2013) and StGA20ox1 (Chen et al., 2004), StBEL5 regulates genes involved in the synthesis or activity of GA, auxin, and cytokinin, strongly suggesting that it is an important developmental signal for controlling growth in potato. StGA2ox1 is particularly interesting because it contains six tandem TTGAC elements (Table I; Lin et al., 2013) and is induced up to 50-fold at the onset of tuber formation (Kloosterman et al., 2007).
lines of potato. Thirty lines were screened for StBEL5 RNA interference (RNAi) suppression in leaves, and five lines were selected for further analysis. All five lines exhibited a reduction in tuber production (Supplemental Fig. S2C), despite control levels for shoot fresh weight for three of the five lines (Supplemental Fig. S2B). Line B5-RNAi-18 exhibited a 35-fold reduction in tuber yield after 21 d of SD conditions. Target gene activity from suppression lines E and L was quantified in stolons from plants grown for 12 d under SD conditions (Fig. 2). Line 18 was not included in this assay because, unlike lines E and L, no stolons or new tubers were observed after 12 cycles of SD conditions. Significant reduction of mRNA accumulation occurred for the tuber marker genes, StSP6A, StPIN2, AGL8, and StLOG1, in close correlation with the suppression of StBEL5 activity (Fig. 2). These results coupled with electrophoretic mobility shift assays (Supplemental Fig. S1; Hannapel et al., 2013) and overexpression (Fig. 1; Hannapel et al., 2013) data suggest that the observed yield decreases were the result of target gene repression in response to StBEL5 suppression. To further explore StBEL5’s effect on StSP6A expression and to validate a potential transcriptional relationship with StSP6A, the effect of StBEL5 overexpression on StSP6A RNA levels was assessed. To mimic the activity of the native promoter of StBEL5, we again made use of the GAS:BEL5 transgenic line in the phloem-mobile signal induction system described previously (Fig. 1). In the GAS:BEL5 line, StBEL5 induces the accumulation of StSP6A mRNA in both stolons and roots under short days (Fig. 3). Induction of StSP6A is almost 9-fold greater than control levels in roots of the GAS:BEL5 line. The pattern of photoperiod induction mediated by StBEL5 is consistent with the SD-induced accumulation of StSP6A observed in stolons of wild-type plants, the putative autoregulatory loop (Navarro et al., 2011). Accumulation of StSP6A in roots in response to StBEL5 activity was completely unexpected. Unlike StBEL5 (Lin et al., 2013), a role for FT in root development has not been documented previously. Consistent with these results, in leaves from the wild type and GAS:BEL5 and StBEL5-RNAi lines grown under short days, StSP6A transcripts accumulated in direct correlation with the activity of StBEL5 (Fig. 4).

### Upstream TTGAC Elements Regulate StSP6A Gene Activity

Expression assays confirmed that suppression of StBEL5 reduced StSP6A expression (Fig. 2), whereas overexpression of StBEL5 enhanced StSP6A RNA levels (Figs. 3 and 4). Gel-shift assays confirmed a potential transcriptional relationship between StSP6A and the StBEL5/POTH1 complex (Supplemental Fig. S1). To examine the molecular mechanism of this putative regulation, we undertook an analysis of the upstream sequence of StSP6A to better understand the StBEL5-mediated control of StSP6A activity. Searching for tandem TGAC motifs within 3.2 kb of StSP6A upstream sequences, five tandem motifs were identified with linkers ranging from zero to 60 nucleotides (Fig. 5A). No CONSTANS motifs (TGTG[N2-3]ATG) were observed within this promoter region. Site mutagenesis was performed on several of these motifs by deleting the internal G and A (Fig. 5B), both confirmed to be critical for binding to the StBEL5/POTH1 complex (Chen et al., 2004). As an example, using a transient expression assay, a single point mutation in the tandem TTGAC motif, G→C, resulted in the complete abolishment of StBEL5’s effect on the activity of the SIGA20ox1 promoter (Chen et al., 2004). The StSP6A mutated promoter of Figure 5B and the wild-type sequence were fused to a GUS marker and transformed into potato plants (S. tuberosum ssp. andigena). Approximately 20 lines of each StSP6A construct type were screened for GUS activity in leaves, roots, and stolons. All of the mutated promoter lines evaluated contained all seven of the site modifications shown in Figure 5B in their upstream sequence. In evaluating select transgenic lines, GUS activity was very robust in young tubers from 8-d-old SD plants expressing the wild-type promoter (Fig. 6A). No visual GUS activity was observed, however, in tubers from the mutated promoter lines (Fig. 6B). Transverse sections of these tubers revealed a high concentration of GUS expression in the vascular bundles of tubers from wild-type promoter lines but none from the mutated promoter lines (Fig. 6C, arrows). Quantification of GUS activity in 8-d swollen stolons in two independent lines for each promoter type demonstrated as much as a 25-fold difference in activity (Fig. 6D). These results clearly show that the targeted

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**Figure 2.** Effects of StBEL5 suppression on select target gene activity in stolons of RNAi lines E and L. Transgenic plants of Solanum tuberosum ssp. andigena were grown under long-day (LD) conditions for 4 weeks and then shifted to short days. Stolons were harvested after 12 d of SD conditions. RNA was extracted from stolons, and RT-qPCR with genespecific primers was used to calculate the relative amount of RNA for each target gene. Each sample was measured in duplicate and normalized against StACTIN8 mRNA. The fold change in RNA levels was calculated as the \(2^{-\Delta \Delta C_t}\) value relative to the mean values obtained in the wild-type (WT) sample. \* values of two biological replicates are shown, with asterisks indicating significant differences (*, \(P < 0.05\) and **, \(P < 0.01\)) using Student’s t test.
deletion of specific nucleotides in the TGAC motifs in the upstream sequence of StSP6A severely repressed its gene activity in stolons and young tubers.

With the wild-type StSP6A promoter, GUS activity was observed in primary and secondary leaf veins, in petioles, and in primary roots (Fig. 7, A and C, arrows). No GUS staining was observed in leaves, petioles, or roots from plants expressing the mutated promoter (Fig. 7, B, D, and E). Because StSP6A is regulated by SD conditions (Navarro et al., 2011), the effect of photoperiod on the activity of the two promoter types was assessed. Leaf samples were harvested from SD plants at 0, 2, 4, and 8 d, and GUS activity was quantified (Fig. 7F). Wild-type promoter activity was strongly enhanced by SD conditions in two separate lines, whereas the mutated promoter lines exhibited a minimal response to the SD conditions (Fig. 7F). The data in Figure 7F represent relative changes in activity based on 0-d levels, and it should be noted that the quantitative differences between the two types were approximately 12- and 70-fold for the two independent lines in 8-d samples (Supplemental Fig. S3). Induction in roots was comparable with differences between the two types at approximately 6-fold in 8-d samples (Supplemental Fig. S4). Overall, these data suggest that the targeted changes in the TGAC motifs strongly affected the SD response of the StSP6A promoter and that StBEL5 activity mediated the photoperiod-controlled transcription of StSP6A in both leaves and stolons.

An Ethanol-Inducible Promoter Driving StBEL5 Activity

To assess StBEL5’s transcriptional control over its target genes within a short temporal window, we made use of the AlcA/ALC5 ethanol-inducible system in transgenic lines to drive StBEL5 expression in stolons submerged in dilute ethanol for 6 h. This is a two-component system that operates with an ethanol-activated transcription factor, designated ALC5, that drives expression of the ALC5-responsive promoter, AlcA (Deveaux et al., 2003). This induction system was used previously with potato, and the parameters of induction were optimized and worked very efficiently for StSP6A induction (Navarro et al., 2011). Because StBEL5 is normally functional under SD conditions (Banerjee et al., 2006), activity in induced stolons from both LD and SD plants was evaluated (Fig. 8). Consistent enhanced activity of several select marker genes, including StSP6A, StPIN2, AGL8, and StGA2ox1, was observed in treated stolons from LD plants (Fig. 8A). A transgenic line expressing a pAlcA:GUS construct was used as a baseline control. It is important to clarify here that among the RNAs quantified in Figure 8, only the enhanced levels of StBEL5 are the direct result of the 6-h ethanol treatment. These results demonstrate that the transcriptional partner of StBEL5 is available in stolons under both LD and SD conditions.

Transcriptional Profile of StBEL5

To provide a more comprehensive, in-depth characterization of the transcriptional profile of StBEL5, ethanol induction of StBEL5 in stolons from LD transgenic plants was performed and replicate RNA libraries were subjected to RNA-Seq analysis. Sequence reads were aligned to the potato genome (Xu et al., 2011), and differentially expressed (DE) genes were identified. The average number of reads among replicate samples was approximately 31,000,000 for both the GUS control and StBEL5. On average, just under 90% of all reads for each library were mapped to the genome uniquely (Supplemental Table S1). Of the approximately 39,000 genes contained in the potato genome, nearly 25,000 genes were detected with at least one read in each of the three replicates of both sample types (Supplemental Table S2). The six libraries were normalized with the upper quantile and analyzed with a generalized linear model using QuasiSeq. P values were calculated with QLSpine based...
on negative binomial distribution. Q-values were obtained by adjusting the P value for family-wise error rate (Lin et al., 2015). Approximately 10,000 potential target genes were identified in the DE gene pool (Supplemental Table S3). Of these, 5,619 were up-regulated and 4,339 were down-regulated. To visualize functional relationships in this diverse expression profile, the 9,958 DE genes were analyzed for their Gene Ontology (GO) categories (Supplemental Fig. S5). GO terms for biological process, cellular component, and molecular function were applied to the DE pool. In general, StBEL5 induction influenced a wide range of gene activity in all three biological categories (Supplemental Fig. S5). GO distribution was analyzed with GOseq to identify over-represented GO groups. With adjusted $P < 0.05$, GO terms for 28 biological processes and 14 cellular components were significantly enriched in the DE gene pool (Fig. 9). Among the overrepresented GO terms were several involved in cell division, including anaphase, cell proliferation, ribosome biogenesis, cytokinesis by cell plate formation, regulation of G2/M transition of the mitotic cell cycle, and cyclin-dependent protein Ser/Thr kinase regulator activity. Two defense terms were over-represented: response to salt stress and defense response to bacterium. StBEL5 activity also preferentially regulates the expression of genes involved in plasmodesmatal biology. StBEL5 RNA is mobile through cells from leaves to stolons and roots (Banerjee et al., 2006a), and this cell-to-cell trafficking is most likely accomplished via the plasmodesmata.

To validate the expression values of the RNA-Seq data, 18 genes from the DE pool were selected based on their novelty or importance as developmental regulators. All 18 contained at least one tandem TTGAC motif within 3 kb of the TSS, and several contained three or more separate motifs (Supplemental Table S4). Five of the 18 were down-regulated, and the other 13 were up-regulated (Fig. 10). With all 18 genes, the RT-qPCR results were consistent with the RNA-Seq values. SCARECROW and SHORT-ROOT are TFs from the GRAS family, and both play critical roles in the maintenance of the root apical meristem (Helariutta et al., 2000; Sabatini et al., 2003). The NODULATION...
SIGNALING PATHWAY1 protein is a GRAS TF related to both SCARECROW and SHORT-ROOT. NAC2 and NAM are members of a large family of plant-specific TFs that function in both development and stress responses (Puranik et al., 2012). OBERON3 is a PHD-finger protein that functions as a transcriptional coregulator and is crucial for the activation of MONOPTEROS-dependent gene expression during embryonic root meristem initiation (Saiga et al., 2012). CLAVATA1 is a receptor protein kinase that maintains the population of stem cells in shoot meristems (Nimchuk et al., 2015), and BAK1 is a BRASSINOSTEROID INSENSITIVE1-associated receptor kinase (Wang et al., 2008). ELF4, CONSTANS-LIKE, and APETALA2 all play roles in floral development. Several lipoxygenases were regulated by StBEL5, both 9- and 13-lipoxygenase types, but the 9-lipoxygenase selected, POTLX-1, plays a significant role in controlling tuber formation (Kolomiets et al., 2001). KNOLLE is a cytokinesis-specific syntaxin that functions to stabilize the trans-SNARE complex during membrane fusion (Park et al., 2012). CLASP is associated with mitosis and the initial central spindle assembly through its microtubule polymerase activity (Maton et al., 2015). Both the LOB domain protein and LATD/NIP function in activating root growth (Harris and Dickstein, 2010; Majer et al., 2012). Similar to previous results identifying KN1 targets (Bolduc et al., 2012), StBEL5 induced seven StBEL genes, including itself (Supplemental Table S5). StBEL5 also induced StCDF1, a member of the CYCLING DOF FACTOR family (Fig. 10). StCDF1 contains six upstream tandem TGAC motifs (Supplemental Table S4, line 24), and its induction in correlation with enhanced StBEL5 activity was also observed in leaves of S. tuberosum ssp. andigena (Supplemental Fig. S6). StCDF1 is a transcriptional regulator that controls plant maturity and mediates the onset of tuberization by down-regulating StCONSTANS expression and activating the StSP6A tuberization signal (Kloosterman et al., 2013). StBEL5 also regulates numerous genes involved in floral development, cell growth, transcription, signal transduction (Supplemental Table S6), and hormone metabolism (Supplemental Table S7). Among the many genes (877) related to hormone synthesis or...
signaling, almost 30% were involved in auxin metabolism.

Verification of Tandem Motifs in Genes Induced by StBEL5

A random screen of the 3-kb upstream sequence of 200 genes from the DE pool revealed that approximately 92% contained at least one tandem TGAC motif (Supplemental Table S8). This data set of 200 genes was proportionately selected from representative expression groups relative to their overall frequency in the DE gene pool. Several contained as many as six to nine motifs. Of course, those targets without motifs could have them located outside the 3-kb sequence or within an intron. The average number of motifs for the 200 selected genes was approximately 3- to 4-fold more than the average for non-regulated genes (Table II). Up-regulated genes averaged approximately 2.2 motifs, whereas down-regulated genes averaged three to 3.5 motifs per gene. Our results demonstrate that the 6-h ethanol induction of StBEL5 in stolons creates a reliable profile for identifying putative transcriptional targets. This is consistent with the high percentage of double TGAC motifs observed in the upstream sequence of genes from the DE pool. Differences observed among induction profiles obtained from the GAS:BEL5 lines (Figs. 1 and 3), RT-qPCR, and RNA-Seq are most likely the result of the rate of transcription, transcript stability, and timing of expression. In addition, with sample reads under 50,000,000, RT-qPCR is much more sensitive than RNA-Seq (Hellemans, 2014).

DISCUSSION

Do StBEL5 and StSP6A Work Together to Regulate Tuberization?

By utilizing transcriptome analysis and a phloem-mobile BEL5 induction system, thousands of potential targets of the StBEL5 transcription factor have been identified and validated in this study, including the FT ortholog of potato, StSP6A. There has been considerable work establishing StSP6A and StBEL5 as signals involved in both the onset and development of tuber growth in potato, but to date, no connection between the two has been confirmed. The suppression of each signal represses tuberization (Fig. 2; Supplemental Fig. S2; Navarro et al., 2011), whereas overexpression activated earliness and enhanced tuber yields (Chen et al., 2003; Banerjee et al., 2006a, 2009; Navarro et al., 2011). Our results demonstrate that both signals activate common tuber genes and that both may function in a complex, dynamic partnership. StBEL5 enhances StSP6A activity in stolons in a photoperiodic response similar to the activation of its own gene in response to SD-enhanced movement of its mRNA (Lin et al., 2013). In this evolving model, StBEL5 augments two tuber signals at the site of tuber formation. This creates a
quandary, however, for the signaling dynamics of StSP6A. If the mobile StBEL5 RNA signal moves down from leaf to stolon to activate StSP6A transcription and the tuberization process, what is the need for a mobile protein signal in the form of an FT ortholog? Due to technical limitations with immunodetection, there is no direct evidence confirming that the StSP6A protein moves from leaf to stolon (Navarro et al., 2011). Indeed, StSP6A is expressed in leaves and stolons under SD conditions, and this activation appears to be controlled by StBEL5 expression, as modification of tandem TGAC motifs in the upstream sequence of StSP6A largely suppressed its SD-induced promoter activity (Figs. 6 and 7). It is conceivable that, upon translation in stolons, StBEL5 acts locally as an additional enhancer of both signals to ensure that the tuberization process proceeds in an efficient and timely manner.

To understand this dual signaling system, it is important to note the functional differences between StBEL5 and StSP6A. StBEL5 is a TF and works in tandem with its KN1-like partner (Chen et al., 2004). In the approaches described in this study, targets of StBEL5/POTH1 can be readily identified and screened for the presence of the BEL/KNOX TGAC motif. StSP6A is a coregulator and a member of the phosphatidylethanolamine-binding protein family. It requires a TF partner (like bZIP TF, FD, in the floral pathway) to function as a transcriptional switch. So an important issue to address is the identity of the stolon-expressed TF partner that complements StSP6A activity and initiates the tuberization pathway. Recent work on the floral signal demonstrated that the FT ortholog of rice (Oryza sativa), Hd3a, functions in hexameric floral activation complex composed of two Hd3a molecules, a 14-3-3 dimer and two OsFD1 peptides (Taoka et al., 2011). In this complex, Hd3a and OsFD interact physically with the 14-3-3 protein but not each other. An analogous complex may be functional to regulate tuber formation. In this putative tuber activation complex model, only the partner TF of StSP6A would come in contact with the target DNA. Through changes of its partners that regulate transcriptional targets, this model provides the molecular basis for creating multiple functions for FT beyond flowering (Tsuji and Taoka, 2014). As examples, in aspen (Populus spp.), FT/partner complexes have been implicated in regulating developmental transitions distinct from flowering (Azeez et al., 2014; Tylewicz et al., 2015). It is feasible that tuberization may be activated by an StBEL/StKNOX complex and by another complex mediated by the coregulator, StSP6A, and that both function as redundant signaling pathways controlling tuber initiation and development. That StGA2ox1 and other tuber genes containing upstream tandem TGAC motifs may be regulated by both StBEL5/KNOX and an StSP6A complex implies that both signals may function in transcriptional complexes with common components.

Figure 8. Effects of ethanol induction of pAlc:StBEL5 in stolons from both LD-grown (A) and SD-grown (B) plants on the activity of select target genes. Transgenic potato plants containing pAlc:StBEL5 were grown under LD conditions (16 h of light/8 h of dark) for 5 weeks or under SD conditions (5 weeks of LD conditions plus 10 d of 8 h of light/16 h of dark). Stolons were then submerged in 1% ethanol for 6 h to induce target gene expression. Gene targets selected are induced during early stages of tuber formation, and each contained identifiable TGAC cis-elements in its upstream sequence. RNA levels were calculated using RT-qPCR with three biological replicates. SE values of three biological replicates are shown, with asterisks indicating significant differences (*, P < 0.05, **, P < 0.01, and ***, P < 0.001, respectively) using Student’s t test. Stolons from induced transgenic plants containing the pAlc:GUS construct were used as a control.
StBEL5 Regulates Hormone Metabolism and Growth Processes

The transcriptional profiling and expression analysis presented here clearly demonstrate that StBEL5 regulates numerous genes involved in hormone metabolism and cell growth processes. Previous experimental work has shown the importance of TALE TFs in the activation and synthesis of hormones. Hormone measurements correlated with TALE TF activity strongly suggest that BEL1-like TFs and their KNOX partners regulate growth by directly controlling the transcriptional activity of genes involved in hormone synthesis (Tanaka-Ueguchi et al., 1998; Chen et al., 2003; Rosin et al., 2003; Bolduc and Hake, 2009). Overexpression of StBEL5 and KN1-like TFs affects hormone levels in several plant species. StBEL5 expression lowers GA activity and enhances cytokinin levels (Chen et al., 2003). StBEL5 activity has also been associated with genes that encode for auxin synthesis and signaling (Hannapel et al., 2013; Lin et al., 2013; Figs. 1 and 2). A study by Bolduc et al. (2012) combining chromatin immunoprecipitation and RNA-Seq identified over 5,000 genes potentially targeted by KN1 in maize. Their results demonstrated preferential binding of KN1 to sequence near genes belonging to the GA, cytokinin, brassinosteroid, and auxin pathways. These included BRII, LOG, YABBY,
GA2ox1, GA20ox1, DELLA, AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA), BELs, and 10 other KNOX genes. Among all of the bound hormonal genes, however, differential gene regulation occurred preferentially for auxin-related genes, including TFs involved in auxin signaling like AUX-IAAs and ARFs (AUXIN RESPONSE FACTORS) and numerous IAA synthesis genes. KN1 binds to almost half of the AUX-IAA and ARF genes annotated in the maize genome. Overall, this study conclusively demonstrated that KN1 directly modulates genes involved in hormone synthesis and signaling, particularly those in the auxin pathway. These results are consistent with the preponderance of auxin-related genes observed in the DE pool of StBEL5 targets (Supplemental Table S7).

Comparison of the StBEL5 Transcriptome with Early Tuberization Profiles

The RNA-Seq data presented here provide both depth and range and helped identify approximately

**Table II.** Frequency of the occurrence of tandem TGAC motifs in upstream sequence (3 kb from the TSS) of several categories of DE genes (Supplemental Table S8)

<table>
<thead>
<tr>
<th>Fold Level Change</th>
<th>Total No. of Genes</th>
<th>Average No. of Motifs</th>
<th>( P )</th>
<th>Mean Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>( &gt;10x )</td>
<td>18</td>
<td>2.11</td>
<td>0.0026</td>
<td>2.64</td>
</tr>
<tr>
<td>( \geq 2x ) to (&lt; 10x )</td>
<td>109</td>
<td>2.28</td>
<td>0.0002</td>
<td>2.84</td>
</tr>
<tr>
<td>( &gt;0.3 ) to ( 0.5x )</td>
<td>47</td>
<td>3.04</td>
<td>&lt;0.0001</td>
<td>3.80</td>
</tr>
<tr>
<td>( 0.1 ) to ( &gt;0.3x )</td>
<td>26</td>
<td>3.50</td>
<td>&lt;0.0001</td>
<td>4.37</td>
</tr>
<tr>
<td>Regulated total</td>
<td>200</td>
<td>2.60</td>
<td>&lt;0.0001</td>
<td>3.25</td>
</tr>
<tr>
<td>Nonregulated</td>
<td>20</td>
<td>0.80</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
10,000 genes regulated by StBEL5. Whereas this is not an analysis of early tuber genes per se, a comparison with previous genomic studies on the tuberization process is certainly warranted. Using publicly available ESTs, a unigene assembly was generated and a microarray was used to examine gene expression during early stages of tuber formation (Kloosterman et al., 2008). This study identified the up-regulation of genes encoding auxin-dependent regulators (EBP1, ErbB-3 epidermal growth factor receptor-binding protein), numerous genes involved in carotenoid biosynthesis, cyclins, and a carotenoid cleavage dioxygenase (CCD). CCD8, a key enzyme in the strigolactone pathway, has been implicated in regulating the morphology of stolons (Pasare et al., 2013). Two EBPI genes, several CCDs, and genes involved in the carotenoid pathway, including phytoene synthase, lycopene epoxidase cyclase, and 9-cis-epoxycarotenoid dioxygenase, are regulated by StBEL5 (Supplemental Table S3). In their transcriptome analysis of potato using Digital Gene Expression tag profiling, Shan et al. (2013) identified several novel genes potentially involved in the photoperiod control of tuberization. These included a LOV KELCH REPEAT PROTEIN2, a DOF domain zinc finger TF, and a syntaxin-like protein. Thirteen syntaxin genes are regulated by StBEL5, including KNOLLE (Fig. 10; Supplemental Table S3). Over 100 zinc finger TFs are regulated by StBEL5, including the StCDF1 gene (Fig. 10; Supplemental Table S9, line 22) that encodes a CYCLING DOF protein known to regulate the photoperiodic timing of tuberization by down-regulating CONSTANS (Kloosterman et al., 2013).

Tuber formation begins in the subapical region of the meristem of the stolon. Upon induction in response to the arrival of the mobile tuberization signal, cell division and enlargement are activated in a discrete and narrow region between the pith and the cortex, designated the perimedullary zone, and a change occurs in the orientation of cell division from transverse to longitudinal (Xu et al., 1998b). Overall, these changes result in an increase in the radial expansion of the subapical region, leading to tuber formation. It is assumed that decreased levels of GA produce the change in the orientation of cell division and that cytokinins and auxins are responsible for the enhanced cell growth. For example, as a stolon elongates under noninductive conditions, GA levels are relatively high in the apical region of the meristem. Upon induction, as the stolon begins to swell in the subapical region, GA levels can decrease by as much as 40-fold (Xu et al., 1998a). Our current understanding of tuber development envisions a finely tuned process of cell growth that is tightly regulated both spatially and temporally. A mobile TF like StBEL5 that interacts with a partner protein would be an ideal signal to regulate a complex network of cellular growth, expansion, and quiescence. From these results, we can see that StBEL5 functions as both an activator (cytokinin and auxin pathways) and a repressor (GA pathway, AGL8, KNOLLE, CLASP, LATD/NIP, and LOB DOMAIN-CONTAINING PROTEIN41) of growth. Similar to KN1, StBEL5 appears to be positioned upstream of an intricate regulatory network involving hormonal pathways and transcriptional regulators that controls the onset of tuber formation. The remarkable observation that StBEL5 induces the transcription of both STS6A and StCDF1 supports this premise.

MATERIALS AND METHODS

Plasmid Constructs

The GAS-BEL5 construct transformed into S. tuberosum ssp. andigena was described previously (Banerjee et al., 2006a). To clone the STS6A promoter sequences, the promoter region of STS6A (3,208 bp) was PCR amplified from genomic DNA of potato (Solanum tuberosum ssp. andigena) using primers designed from the upstream sequence of the STS6A transcript (PCSS00003D4T400060557). Sequence upstream of the STS6A transcript was retrieved from the Potato Genome Sequencing Consortium Data Release (http://potatogenomics.plan biology.msu.edu/). The PCR-amplified fragment of 3,208 bp upstream sequence was cloned into pCRII-TOPO vector (Invitrogen) and sequenced. For promoter mutagenesis experiments, modification of seven TGAC elements, present at five different tandem sites on the upstream sequence of STS6A, was completed using the GeneArt Site-Directed Mutagenesis PLUS System (Invitrogen). The oligonucleotide primers (Supplemental Table S10) for multisite mutagenesis were designed using the GeneArt Primer and Construct Design Tool (https://maidenr. invitrogen.com/oligoDesigner). Mutagenesis was performed as described in the instruction manual, and the product was verified by sequencing. The promSTS6A:GUS and mutpromSTS6A:GUS constructs were generated by cloning the 3,208-bp wild-type promoter and the 3,192-bp mutant promoter fragments of STS6A into the pB101.1-GUS binary vector (Jefferson, 1987), upstream of the uidA (GUS) gene, by transcriptional fusion. Sall and BanHI sites were introduced into primer sequences for cloning into the pB101.1-GUS binary vector.

The StBEL5 ethanol-inducible construct (expression of the StBEL5 protein is driven by the ALa/Aker ethanol-inducible system) was generated by cloning the StBEL5 complementary DNA (from the translational start site up to 462 bp of the 3' untranslated region) into the pENTR/D-TOPO plasmid (Invitrogen) and further insertion into the pBluescript-GW destination vector (kindly provided by Salome Prat) by recombination with the LR Clonase II enzyme (Gateway Technology; Invitrogen).

For the StBEL5 RNAi construct, a gene-specific amplicon (93 bp) was PCR amplified using primers spanning the 3' untranslated region of the StBEL5 gene and including the #H hairpin recombinase sites upstream of the 5' and 3' gene-specific priming sequences. The target gene fragment was then cloned into the pDONR221 entry vector (Invitrogen) using BP Clonase enzyme-mediated recombination according to the manufacturer's protocol. Cloned target sequences were further transferred into an intron-containing hairpin RNA (ihpRNA) producing the pCAPD destination vector (Filichkin et al., 2007) containing the potato PIV2 intron (Vancanneyt et al., 1990), using Gateway LR Clonase II enzyme mix (Invitrogen). The orientations of sense and antisense target gene fragments in the final expression vector were confirmed by sequencing using primers directed against the 35S promoter and the ribonuclease terminalase. Final constructs were verified by sequencing, and the primer sets used to generate these constructs are provided in Supplemental Table S10.

Plant Material and Growth Conditions

Transgenic S. tuberosum ssp. andigena plants containing promSTS6A:GUS, mutpromSTS6A:GUS, the StBEL5 ethanol-inducible plasmid, and the StBEL5 RNAi constructs were generated by Agrobacterium tumefaciens-mediated transformation of leaf explants as described previously (Banerjee et al., 2006b). For the promoter GUS lines, transgenics were verified for the presence of the GUS gene, and we selected four to five lines based on GUS activity in leaves, roots, and stolons. Wild-type and transgenic plants were cultured in vitro on Murashige and Skoog medium (Murashige and Skoog, 1962) and maintained in a growth chamber under 16-h-light/8-h-dark photoperiod conditions at 24°C. Soil-grown plants were maintained in a growth chamber under either an LD (16 h of light/8 h of dark) or an SD (8 h of light/16 h of dark) photoperiod at
300 μmol m⁻² s⁻¹ with 26°C day temperature and 18°C night temperature. To analyze the tuber-specific target gene expression, wild-type plants, the GAS: BEL5 transgenic line (Banerjee et al., 2006a, 2009), and the StBEL5 RNAi transgenic lines were grown under LD conditions for 3 weeks and then transferred to SD conditions for 10 d. Young tubers were harvested, frozen in liquid nitrogen, and stored at −80°C until RNA extraction. The tuberization phenotype was analyzed for the StBEL5 RNAi lines by growing both the StBEL5 RNAi and wild-type plants in soil under LD conditions in growth chambers for 4 weeks and then transferring to SD inductive conditions for another 3 weeks. The StBEL5 ethanolic-inducible plants and the transgenic control Alc-uid plants (expressing GUS under the control of the Alc/AlcR inducible system; provided by Salomé Prat) were transferred to soil and maintained in a growth chamber for 5 weeks under LD conditions. For induction, the underground portions of the StBEL5 ethanolic-inducible transgenic plants and the transgenic controls were submerged in a 1% (v/v) ethanol solution for 6 h. Stolons were then harvested, immediately frozen in liquid nitrogen, and stored at −80°C until further analysis.

RNA Isolation and RT-qPCR
For expression analyses, total RNA was extracted from harvested samples using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. The RT-qPCR analysis was performed with the qScript One-Step SYBR Green qRT-PCR Kit (Quantabio) following the protocol described previously by Lin et al. (2013). All reactions were performed in two independent biological replicates using the Illumina Eco qPCR machine. The relative gene quantification (comparative threshold cycle) method (Livak and Schmittgen, 2001) was used to calculate the expression levels of the target RNAs. Each sample was measured and normalized against 5ACTINS RNA. RNA values were calculated as the 2⁻ΔΔCt value relative to the mean values obtained from wild-type control samples. The list of primer sets used for RT-qPCR is provided in Supplemental Table S10.

Histochemical and Fluorometric Assays
Leaves, young tubers, and roots were harvested from soil-grown promStSP6A::GUS and mutpromStSP6A::GUS plants. Plants were grown in a growth chamber under LD conditions (16 h of light/8 h of dark) for 5 weeks (until the 12- to 13-leaf stage) and then transferred to SD conditions (8 h of light/16 h of dark) at 300 μmol m⁻² s⁻¹ with a 26°C day temperature and 18°C night temperature. Leaves were harvested at 0, 2, 4, and 8 d of SD induction. Stolons and roots were harvested on day 8 of SD conditions. Samples were frozen in liquid nitrogen immediately after harvest and stored at −80°C until further use. For histochemical staining of GUS, fresh samples were incubated overnight at 37°C in the GUS substrate 5-bromo-4-chloro-3-indolyl-glucuronide containing 1M NaPO₄, pH 7.0, 2.5M EDTA, pH 8, 10% Triton X-100, 1 mM 5-bromo-4-chloro-3-indolyl-beta-glucuronic acid, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide. The tissues were then washed three times with 75% ethanol prior to photodocumentation with an Olympus E-500 digital camera. For GUS quantification, the fluorometric assay was performed using total soluble protein fractions extracted from frozen samples stored at −80°C as described previously (Butler and Hannapel, 2012). The relative fluorescence was measured using a fluorometer (FluroMax-2) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The specific GUS activity was calculated using 4-methylumbelliferone as a standard.

Analysis of RNA-Seq Data
RNA was isolated from ethanol-treated stolons in triplicate, and complementary DNA libraries were prepared using 2 μg of total RNA and sequenced using the HiSeq2500 (Illumina) at the DNA Facility, Iowa State University. Each library was sequenced with mRNA-Seq HiSEQ High Output 100-Cycle S.E. Reads were processed and aligned to the potato genome as described previously (Lin et al., 2015). The number of concordant unique reads in each library was counted with HTseq, and the libraries were normalized with the 0.75 quantile to eliminate the differences caused by sample scale and sequencing depth. DE genes were identified using QuasiSeq (Lund et al., 2012) as described by Lin et al. (2015). The count of each gene is the mean value of the normalized reads of the three replicates. GO analysis was performed with Gosteq (Young et al., 2010) as described previously (Lin et al., 2015).

Sequence reads of the RNA-Seq analysis have been deposited at the National Center for Biotechnology Information Short Read Archive (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA301499).

Supplemental Data
The following supplemental materials are available.
Supplemental Figure S1. Gel-shift assays of representative tandem TGAC core motifs.
Supplemental Figure S2. Phenotypes of StBEL5 suppression lines.
Supplemental Figure S3. GUS activity for wild-type and mutated StSP6A promoters in leaves.
Supplemental Figure S4. GUS activity for wild-type and mutated StSP6A promoters in roots.
Supplemental Figure S5. GO analysis.
Supplemental Figure S6. StBEL5 induces StCDF1 in leaves.
Supplemental Table S1. Summary of RNA-Seq reads.
Supplemental Table S2. GUS and BEL5 RNA-Seq data output.
Supplemental Table S3. DE gene pool.
Supplemental Table S4. List of 18 target genes of BEL5 validated through RT-qPCR.
Supplemental Table S5. StBEL genes induced by StBEL5.
Supplemental Table S6. Select GO categories of DE genes.
Supplemental Table S7. DE genes involved in hormonal pathways.
Supplemental Table S8. Verification of tandem motifs in genes induced by StBEL5.
Supplemental Table S9. Zinc finger TFs regulated by StBEL5.
Supplemental Table S10. Primer sequences.

ACKNOWLEDGMENTS
We thank Dr. Salomé Prat for providing the pBinSRSNA-GW destination vector and the pAlc/GUS transgenic potato line and for sharing experimental insights on the use of the ethanol-inducible system, Dr. Andrew Severin for assistance in organizing the RNA-Seq data, Dr. Sung Ki Cho for help with the regression analysis, and Kathleen Rey for running the Poisson generalized linear model analysis.

Received August 21, 2015; accepted November 6, 2015.

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Sharma et al.
Transcriptional Targets of StBEL5


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