Update on the Tuber Activation Pathway

The Multiple Signals That Control Tuber Formation1[OPEN]

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Under optimum environmental conditions, tuberization in potato (Solanum tuberosum) is activated by signals that either function in the leaf or arise in this organ and move down into stolon tips to induce tuber formation. Three major signals have been identified: CYCLING DOF FACTOR1 (StCDF1) for earliness and StBEL5 mRNA and SELF-PRUNING6A (StSP6A) protein as mobile signals originating in the leaf. Overexpression of StSP6A produces plants that tuberize earlier and under noninductive long days. Overexpression of StBEL5 leads to enhanced earliness and overall increased tuber yields. During the onset of tuberization, StBEL5 plus its StKNOX partner initiate signal activity in the leaf through transcriptional induction of both StCDF1 and StSP6A. Amplification of signals occurs on site in stolons, where StBEL5 again in tandem with its StKNOX partner induces the transcription of both StSP6A and StBEL5. In stolons, StSP6A interacts with a FLOWERING LOCUS D-LIKE protein to promote tuberization. In this model, StBEL5 is positioned upstream of a regulatory network that controls tuber formation. Overall, the results suggest that StBEL5 functions to directly activate the tuberization program and to amplify other pivotal signals in the pathway.

Potato (Solanum tuberosum) is the third most important subsistence crop grown worldwide. Based on calories generated for human consumption per acre, it is the most efficient food crop on the planet. Recent advances in biotechnology have established potato as an ideal crop for improvement through genetic enhancement, but increased yield has still not been addressed through biotech approaches (Halterman et al., 2016). With a better understanding of the signals that control tuberization, a molecular approach to yield enhancement may soon be within reach. Elegant studies over the past century established the existence of a photoperiodic signal responsible for activating the onset of tuberization (Garner and Allard, 1920; Werner, 1934). Under conditions of cool night temperatures and short days (SD), a graft-transmissible substance is transported from the leaves to stolon tips to activate tuber formation (Gregory, 1956; Chapman, 1958; Kumar and Wareing, 1973). In a benchmark experiment, heterografts using florally induced tobacco (Nicotiana tabacum) as the scion and noninduced potato as the stock demonstrated that some component of the mobile floral signal was capable of inducing tuberization (Chailakhyan et al., 1981). Despite years of innovative studies, however, the exact identity of this tuber-inducing stimulus, designated tuberigen, was only recently established.

A potato tuber is a specialized stem that arises from the underground organ known as the stolon (Jackson, 1999). It is not formed from a root. A stolon also can...
grow upward and emerge from the soil to form a new shoot. Under conditions inductive for tuberization, like SD, underground horizontal elongation of the stolon ceases and the stolon tip begins to swell to form the tuber. This early phase of tuberization is designated tuber initiation and is characterized by cell growth that arises from the pith and cortex just below the apical meristem of the stolon (Xu et al., 1998b). Following this process, cells begin to divide and expand in the perimedullary region of the stolon, supporting subsequent tuber enlargement that contributes to overall tuber yield (Xu et al., 1998b). Both of these progenitor cell regions are in direct contact with vascular connections and are readily accessible to the phloem and, therefore, to phloem-mobile signals (Table I). The expanding tuber accumulates a specific set of storage proteins and large amounts of starch (Ewing and Struik, 1992). The starch is synthesized from Suc that is transported from the leaf. In the various studies discussed in this review, tuber activity is measured primarily through three parameters: timing of tuber formation, capacity to tuberize under noninductive, long days (LD), and overall tuber yield (grams fresh weight plant−1). The underlying questions of this review are what is the phloem-mobile signal and what is its downstream action.

Several hormones have been implicated in the growth processes associated with the formation of a tuber (Xu et al., 1998a). For example, the change in cell growth orientation characteristic of tuber formation in potato are CYCLING DOF FACTOR1 (StCDF1), StSP6A, and StBEL5 (Banerjee et al., 2006; Hannapel et al., 2017). The activity of GA20 OXIDASE1 (GA20OX1), a biosynthetic enzyme, is repressed during tuberization, whereas GA2OX1, a catabolic enzyme, is strongly induced during the early stages of tuber formation (Kloosterman et al., 2007). Because of their importance in cell growth, both cytokinin and auxin metabolism also have been implicated as causal processes in tuberization (Roumeliotis et al., 2012, 2013b; Eviatar-Ribak et al., 2013; Kolachevskaya et al., 2015; Gao et al., 2016). Numerous reports of the enhanced activity of a particular hormone or receptor have been consistently linked to tuber formation. Specific genes like LONELY GUY (LOG), AGAMOUS-LIKE8 (AGL8), ISO-PENTENYL TRANSFERASE, the PINs, AUX/IAA, and POTATO LIPOXGENASE1 (POTLXI) have been correlated with growth processes associated with tuberization (Kolomiets et al., 2001; Rosin et al., 2003; Eviatar-Ribak et al., 2013; Lin et al., 2013; Roumeliotis et al., 2013b; Gao et al., 2016). These results alone, however, do not establish one hormone or another as the sole developmental switch for tuber formation. The same rationale would apply to other metabolites like Suc, oxylipins, tuber storage proteins, or tuberonic acid. Most of these molecules and metabolites are products of downstream processes in the pathway that follow the upstream switch for tuberization. They may contribute to aspects of tuber morphology but are not the causal developmental agent. Because of the transport capacity of the tuber signal, the search for the activating signal has focused on primary products like microRNAs, full-length mRNAs, and less abundant proteins that move through the sieve element system of the plant (Table I). Two important microRNAs, miR172 and miR156, have been implicated in potato development, but their exact mechanistic connection to tuber formation has not been established conclusively (Martin et al., 2009; Bhogale et al., 2014).

Based on published experimental evidence, the three most important signals that regulate the onset of tuber formation in potato are CYCLING DOF FACTOR1 (StCDF1), StSP6A, and StBEL5 (Banerjee et al., 2006; Hannapel et al., 2017).

<table>
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<tr>
<th>Signal Type</th>
<th>Example</th>
<th>Function</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Protein</td>
<td>FT</td>
<td>Flowering</td>
<td>Corbesier et al. (2007)</td>
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<td></td>
<td>FT ortholog SP6A</td>
<td>Tuberization</td>
<td>Navarro et al. (2011)</td>
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<td>Full-length mRNA</td>
<td>StBEL5, StBEL11, StBEL29</td>
<td>Tuberization</td>
<td>Hannapel and Banerjee (2017)</td>
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<td></td>
<td>CmGAI</td>
<td>Leaf growth</td>
<td>Haywood et al. (2005)</td>
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<td></td>
<td>AtFT</td>
<td>Flowering</td>
<td>Li et al. (2011)</td>
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<td>ATC</td>
<td>Root growth</td>
<td>Huang et al. (2012)</td>
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<td>Aux/IAA18, Aux/IAA28</td>
<td>Vegetative growth</td>
<td>Notaguchi et al. (2012)</td>
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<td>MicroRNA</td>
<td>mi156</td>
<td>Vegetative growth</td>
<td>Mahajan et al. (2012)</td>
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<td></td>
<td>mi172</td>
<td>Vegetative growth</td>
<td>Bhogale et al. (2014)</td>
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<td>mi395</td>
<td>Tuberization</td>
<td>Martin et al. (2009)</td>
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<td>mi399</td>
<td>Sulfur signaling</td>
<td>Buitz et al. (2010)</td>
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<td>Small-interfering RNA</td>
<td>24-nucleotide small RNAs</td>
<td>DNA methylation</td>
<td>Lewsey et al. (2016)</td>
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<td></td>
<td>Cosuppression of nitrate reductase</td>
<td>Nitrate metabolism</td>
<td>Palauqui et al. (1997)</td>
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Navarro et al., 2011; Kloosterman et al., 2013). StCDF1 and StBEL5 are transcription factors (TFs), whereas StSP6A is a member of a family of coregulators. This review will illuminate our current understanding of the tuberization pathway by focusing on the spatial and temporal regulation of these three crucial signals and their dynamic interactions. For StCDF1 and the FT ortholog, StSP6A, information on their Arabidopsis (Arabidopsis thaliana) counterparts and their roles in flowering will be initially considered. Highlighting the role of StSP6A, recent reviews have addressed the similarities between photoperiodic signaling in both flowering and tuberization (Suárez-López, 2005; Rodríguez-Falcón et al., 2006; Abelenda et al., 2014). Whereas valuable information on floral signaling in solanaceous species has been thoroughly summarized (Abelenda et al., 2014), Arabidopsis is used here as a more detailed, comprehensive model for examining floral signals and comparing these with similar components that function in tuberization. Figure 1 elucidates the origin of these signals and their interactions in the leaf. StCDF1 interacts with proteins that measure daylength in the leaf to mediate earliness. StSP6A protein and StBEL5 mRNA are both mobile signals that originate in the leaf and, under favorable conditions, move underground to the stolon tip to initiate tuber formation (Banerjee et al., 2006; Navarro et al., 2011). In this review, we will discuss how these signals regulate the timing and development of tuberization.

AtCDF1 FUNCTIONS IN THE LEAF AS A REGULATOR OF FLOWERING

CDF1 is a transcription regulator from the zinc finger DOF (DNA-binding with one finger) family. This family of TFs is involved in numerous aspects of development, including regulation by light, hormone biology, and seed metabolism (Yanagisawa, 2002). Under non-inductive conditions in Arabidopsis, AtCDF1 directly represses CONSTANS (CO) transcription in the leaf, leading to a reduction in FT and a suppression of flowering (Fig. 1A). Under optimum conditions for flowering, AtCDF1 is degraded through its interaction with FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1) and GIGANTEA (GI) proteins, mediating clock output in response to daylength (Sawa et al., 2007). Consistent with this role, overexpression lines of AtCDF1 flower later than the wild type (Imaizumi et al., 2005). So AtCDF1 functions as a repressor of flowering,
and its stability determines the activity of CO. AtCDF1 binds to conserved motifs (the Dof-binding site) in the CO promoter. Degradation of AtCDF1 in conjunction with the FKF1/GI complex regulates CO levels to be active during the critical time for flowering. In Arabidopsis, AtCDF1 binds to both CO and FT promoters, repressing their activity and blocking flowering (Imaizumi et al., 2005; Song et al., 2012). Under LD, both CO and GI also directly bind to the promoter of FT to activate its expression (Tiwari et al., 2010; Sawa and Kay, 2011).

Genetic analyses have confirmed that several AtCDFs of Arabidopsis function redundantly to suppress CO activity, inhibit FT transcription, and repress the floral transition (Fornara et al., 2009). These CDFs, in turn, are removed by the GI/FKF1/CDF complex that targets the CDFs for degradation. GI is central to this process and is required for removal of the AtCDF proteins. GI also enhances FT mRNA levels through the activation of miRNA172, a microRNA that blocks activity of TARGET OF EAR1 (TOE1) by down-regulating the stability of the TOE1 transcript. TOE1 represses FT activity by reducing CO protein levels (Zhang et al., 2015) and by acting as a transcriptional repressor to directly inhibit FT activity (Yamashino et al., 2013). Overall, FKF1 has three roles in regulating FT expression that lead to enhanced flowering: (1) it degrades CDF1 to induce CO transcription; (2) it degrades CDF1 to induce FT transcription; and (3) under blue light, it helps to stabilize CO, increasing FT expression (Song et al., 2012). This blue light perception stabilizes the GI/FKF1 complex, so that longer photo-periods cause enhanced accumulation of the GI/FKF1 complex and increased CO mRNA levels through the reduction of CDF1 protein levels (Fig. 1A).

Accumulation of CDF1 mRNA levels is regulated by the transcription-repressing PRR protein family (Nakamichi et al., 2007, 2012). Mutations of the PRRs resulted in elevated expression of CDF1 (Nakamichi et al., 2007; Ito et al., 2008). PRR proteins function in the clock circuit by directly regulating the timing of expression of key TFs involved in clock-output pathways (Nakamichi et al., 2012; Hayama et al., 2017). In Arabidopsis, the PRRs act to promote the floral transition by increasing the rate of FT transcription (Hayama et al., 2017). Based on genetic analysis, activation of AtCDF1 expression is likely controlled by CCA1 and LHY proteins (Seaton et al., 2015). This model proposes that the CCA1/LHY component regulates both CDF1 and FKF1 transcription (Seaton et al., 2015; Fig. 1A). In the following section, the role of StCDF1 in the signaling network of tuberization in potato will be addressed.

IDENTIFICATION OF CDF1 ALLELES IN POTATO

In potato, earliness and plant maturity are critical aspects of development that control tuber onset, flowering, and senescence. Using two diploid potato populations and screening with existing genetic markers, the plant maturity quantitative trait locus was mapped recently (Kloosterman et al., 2013). Phenotyping and detailed mapping narrowed down the locus responsible for this trait to a region of approximately 110 kb on chromosome 5. Potential candidate genes were screened, and a CDF homolog, designated StCDF1, was identified (PGSC003DMG400018408). Sequence analysis of the StCDF1 gene in the very-late-maturing line showed homozygosity for the StCDF1 allele, designated StCDF1.1. In contrast, a very-early-maturing line, CE3130, had two different truncated alleles, designated StCDF1.2 and StCDF1.3. Both of these genes contained an insertion that interrupted the coding sequence in its C terminus. Overexpression of the early-maturing/tuberizing StCDF1.2 gene variant in the photoperiod-dependent potato subspecies andigena produced tubers under noninductive LD and also resulted in early tuber formation in a late-maturing line (Kloosterman et al., 2013). Consistent with these results, Morris et al. (2014) reported that a Neo-Tuberosum accession adapted to tuberization in LD, but not the andigena subspecies, contained an allele that encoded for a truncated StCDF1 protein modified in the C-terminal region (similar to StCDF1.2). These genetic analyses confirm an important role of StCDF1 in controlling the timing of tuber formation.

In addition to the DNA-binding DOF domain, CDF proteins share conserved domains in their C terminus, designated domains II and III (Kloosterman et al., 2013). These basic domains are likely involved in interactions with FKF1 and GI proteins. The modifications that distinguish StCDF1.2 and StCDF1.3 result in the absence of domain III (49 amino acids in length) in both truncated proteins. Yeast two-hybrid interaction studies demonstrated that, whereas the intact protein from the StCDF1.1 allele interacts with both StGI and StFKF1, the truncated versions encoded by the early alleles were unable to bind StFKF1 yet retained their capacity to bind the StGI protein (Kloosterman et al., 2013). This lack of interaction with StFKF1 stabilizes both truncated StCDF1 types leading to reduced StCO activity. In summary, these results suggest that StFKF1 regulates the levels of StCDF1 and that the inability of the StCDF1.2 and StCDF1.3 proteins to bind to the GI/FKF1 complex inhibits their degradation and increases their activity. This increase in StCDF1.2 and StCDF1.3 activity is correlated with an enhanced repression of StCO, an increase in StSP6A expression, and enhanced earliness and tuberization under noninductive LD. In potato, under SD, enhanced StCDF1 levels result in CO repression and the activation of tuberization (Kloosterman et al., 2013). Conversely, LD and CO activity inhibit tuberization, whereas, in Arabidopsis, LD and enhanced CO activity induce flowering (Fig. 1).

Unlike its positive effect on flowering, over-expression of AtCO in potato produced plants that were impaired in tuber development under inductive SD (Martínez-García et al., 2002). In a study on native CO genes of potato, StCO also functioned in a repressive manner in tuberization in potato (González-Schán...
et al., 2012). Overexpression lines of StCO tuberized later than wild-type plants, whereas silencing StCO expression promoted tuberization under both repressive and weakly inductive photoperiod conditions. These results show that StCO represses tuberization in a photoperiod-dependent manner and that StCO inhibits the accumulation of StSP6A and StBEL5 RNAs in leaves. The effect of StCO on tuber induction was transmitted through grafts, suggesting that StCO is involved in the expression of long-distance regulatory signals. These observations are consistent with the role of StCDF1 in repressing StCO transcription and enhancing StSP6A activity (Fig. 1B). Recent work by Abelenda et al. (2016) showed that the suppression of StSP6A also is controlled by a second potato FT ortholog, StSP5G. StSP5G transcription is induced directly by StCOL1/StCO2, which is stabilized by phytochrome B in leaves under LD, leading to a reduction in StSP6A expression and the inhibition of tuber formation. In summary, in the models of Figure 1, upon exposure to inductive conditions, the respective signals are primed in the source leaf and ready for transport. In Arabidopsis, FT is mobilized to the shoot apical meristem to activate flowering, whereas in potato, StSP6A and StBEL5 are mobilized to the underground stolon tip, the site of tuber formation.

**StSP6A, an FT Ortholog of Potato, Is a Signal for Tuberization**

Historically, the best example of a long-distance mobile signal that regulates development is FT. Under the control of CO, FT is expressed in the leaf under conditions inductive for flowering (Fig. 1A). It then moves as a protein from the leaf to the shoot apical meristem through plasmodesmata and the sieve element system (for review, see Turck et al., 2008). FT is a member of the phosphatidylethanolamine-binding protein (PEBP) family with a tertiary structure similar to that of mammalian PEBPs (Ahn et al., 2006). FT is a coregulator of transcription and likely does not function without a TF partner. In the shoot apex, FT binds to the bZIP TF, FLOWERING LOCUS D (FD), and this complex activates floral genes like APETALA1 (API) and LEAFY (LFY), leading to induction of the floral pathway (Wigge et al., 2005; Corbesier et al., 2007; Tamaki et al., 2007; Yoo et al., 2013).

**FT-like** genes also function in a wide range of nonfloral developmental events. These include bud dormancy in *Vitis vinifera* (Vergara et al., 2016), seasonal control of growth in *Populus* spp. (Böhlenius et al., 2006; Tylewicz et al., 2015), bud dormancy in conifers (Gyllestrand et al., 2007), meristem growth termination in tomato (*Solanum lycopersicum*; Shalit et al., 2009), and tuberization in potato (Navarro et al., 2011). Overall, these observations suggest that FT orthologs may function as mobile signals in meristem-associated transitions in nonfloral pathways. Because of the economic importance of potato, the role of a specific FT ortholog, designated *StSP6A*, as a prominent tuber signal has been carefully studied. There are four FT clade members in potato, *StSP6A*, *StSP5G*, *StSP5G-like*, and *StSP3D*. *StSP3D* controls the floral pathway, *StSP6A* regulates tuber initiation, and *StSP5G* represses tuber formation by suppressing *StSP6A* activity (Abelenda et al., 2016). Utilizing transgenic analysis and heterografting experiments, *StSP6A* has been established as a candidate for the mobile tuber signal (Navarro et al., 2011). Overexpression lines of *StSP6A* tuberized earlier and under noninductive LD. In the grafting experiments, transgenic scion material that overexpressed *StSP6A* was grafted onto wild-type stock. These *StSP6A-ox* scions induced the stocks to tuberize, suggesting movement of the *StSP6A* protein similar to FT movement into shoot apices to induce flowering. Silencing *StSP6A* delayed tuberization under SD, but final yields were comparable to those of the wild type. Overall, these results suggest that *StSP6A* is involved in the initial activation of tuberization but probably not long-term tuber morphology. Consistent with this premise, *StSP6A* expression was strongest in an early-maturing variety and relatively lower in a late-maturing variety (Navarro et al., 2011). Induction of *StSP6A* using an inducible promoter led to the activation of several tuber marker genes, including *SIA20X1*. In a photoperiod-responsive variety, *StSP6A* exhibited an SD-induced accumulation of its mRNA in both leaves and stolons. The mechanism for this transcriptional control, however, was not clearly established at the time.

Supporting the common theme with flowering, overexpression of the rice (*Oryza sativa*) FT ortholog, *Heading date3a* (*Hd3a*) in potato, produced plants with enhanced flowering and tuberization. The *Hd3a* protein moved through a graft into wild-type potato stolons and increased tuber production (Navarro et al., 2011). *Hd3a* functions in a hexameric floral activation complex (FAC) composed of three homodimers consisting of *Hd3a*, OsFD, and a 14-3-3 protein that functions as a scaffold (Taoka et al., 2011). Through interactions of FT with a different protein partner that regulates a unique set of targets, the FAC model provides the molecular basis for creating multiple functions for FT beyond flowering (Tsuij and Taoka, 2014). Similar interactions among *StSP6A*, a 14-3-3 protein, and an StFD1-like protein appear to be functional as the tuber activation complex (TAC; Teo et al., 2017). Transgenic lines that constitutively express either *StSP6A* or *Hd3a* exhibited enhanced production of flowers from the shoot apical meristem and tubers from the stolon tips (Navarro et al., 2011). During activation of these FT genes, some unknown mechanism blocks the floral pathway in stolons and facilitates the formation of tubers. In rice, a 14-3-3 protein has been identified that, upon interaction with *Hd3a*, functions as a negative regulator of flowering (Purwetari et al., 2009). A related 14-3-3 protein of potato could function in a similar fashion to block flowering and promote tuberization.
Recent work by Teo et al. (2017) has elucidated important aspects of a specific TAC that promotes tuber induction. Protein-binding assays demonstrated that, similar to the FAC of rice, the tuberigen complex that functions locally in stolon tips is composed of StSP6A, a 14-3-3 protein, and an FD-like (FDL) protein. Yeast two-hybrid analyses demonstrated that StSP6A protein interacted with both 14-3-3 and FDL proteins of potato. Consistent with previous results (Navarro et al., 2011), overexpression of StSP6A induced early tuberization in a 14-3-3-dependent manner, whereas suppression of StFDL1 delayed tuberization. Overall, these results strongly suggest that the components of this complex are a local trigger for tuber formation. No effect on tuberization was reported, however, upon overexpression of StFD1 (Teo, 2015). In the more recent study (Teo et al., 2017), tuberization was measured by the percentage of plants that tuberized at discrete time points without regard for overall tuber yields. This suggests that the components of this complex regulate the onset of tuberization but do not necessarily affect overall tuber yield. FDL proteins normally activate floral genes like API or LFY. In contrast to StBEL5 targets (Sharma et al., 2016), however, no downstream tuber marker genes (e.g. StGA2OX1, StGA2OX1, or StLOG) putatively regulated by the TAC have been identified.

**STBEL5, A MOBILE SIGNAL CONTROLLING DEVELOPMENT IN POTATO**

BEL1-like TFs are members of the ubiquitous TALE (for three-amino acid loop extension) superclass (Bürglin, 1997). TALE TFs are distinguished by a high level of sequence conservation in the homeodomain, consisting of three α-helices (Bürglin, 1997). In plants, BEL1-like proteins function in a tandem complex with KNOX-type TFs to regulate numerous aspects of growth and development (Chen et al., 2003; Smith and Hake, 2003; Brambilla et al., 2007; Rutjens et al., 2009; Khan et al., 2012). Related genes from the TALE class in animals also control important processes of growth and differentiation (Machon et al., 2015; Marcos et al., 2015; Merabet and Galliot, 2015; Villaescusa et al., 2016). The physical interaction between KNOX and BEL1 proteins and its functional significance have been documented in numerous plant species (Bellaoui et al., 2001; Müller et al., 2001; Smith et al., 2002; Chen et al., 2003, 2004).

The potato BEL1 gene, STBEL5, functions to regulate tuber and root growth (Chen et al., 2003; Lin et al., 2013). Operating as a mobile signal, its full-length transcript has the capacity to move long distances through the sieve element system (Banerjee et al., 2006). Its expression is stimulated in leaves by low levels of blue and red light but not by daylength. The movement, stability, and localization of the STBEL5 RNA are controlled by two RNA-binding proteins from the polypyrimidine tract-binding (PTB) family of proteins, designated StPTB1 and StPTB6 (Cho et al., 2015). These RNA-binding proteins bind to a CU-rich sequence present in the 3’ untranslated region (UTR) of the STBEL5 transcript, and their activity is enhanced by conditions favorable for tuber formation. In this way, the movement of STBEL5 RNA is induced by SD and the presence of its 3’ UTR. Long-distance transport of STBEL5 mRNA has been verified using RNA movement assays in both whole plants and heterografts (Banerjee et al., 2006, 2009; Lin et al., 2013; Cho et al., 2015). Movement through the phloem also has been confirmed for a BEL5-like transcript in Arabidopsis (Notaguchi et al., 2015). STBEL5 augments its own transcription through autoregulation in newly tuberizing stolon tips (Fig. 2). This autoregulation was verified through site mutagenesis in an upstream sequence of the STBEL5 gene (Lin et al., 2013) and helps to explain the SD-induced activity of the STBEL5 promoter in stolons growing in the dark (Chatterjee et al., 2007).

There are numerous documented examples of STBEL5’s effect on tuberization in both a commercial cultivar and the photo-period-responsive subspecies anigena (Chen et al., 2003; Banerjee et al., 2006, 2009; Cho et al., 2015). Overexpression and enhanced accumulation led to early, increased tuberization, whereas RNA interference suppression of STBEL5 significantly reduced tuber yields in several independent lines (Sharma et al., 2016). Overexpression lines of full-length STBEL5 RNA tuberize under noninductive LD, whereas constructs that contain only the coding sequence of STBEL5 minus both UTRs do not (Banerjee et al., 2006). These results reinforce the biological significance of the STPTB RNA-binding proteins and their role in enhancing STBEL5 activity. Even when STBEL5 RNA levels increased indirectly due to a relative increase in STPTB activity, tuber yields of soil-grown plants were enhanced approximately 2-fold (Cho et al., 2015).

**TRANSCRIPTIONAL TARGETS OF STBEL5**

Consistent with its role in enhancing tuber formation, the STBEL5/StKNOX complex inhibits the transcription of GA20OX1 and promotes the activity of GA20OX1. In both cases, STBEL5 is functional in tandem with its KNOX partner, POTH1. This complex recognizes a specific cis-element, a double TTGAC motif present in STBEL5 target genes. Site mutagenesis and gel-shift assays have demonstrated the importance of the fidelity of both motifs in facilitating the binding of the BEL1/KNOX complex (Smith et al., 2002; Chen et al., 2004; Lin et al., 2013). This double motif ensures specificity during the regulation of BEL1/KNOX target genes.

Recent work utilizing STBEL5 induction systems coupled with RNA profiling have expanded our understanding of the range of targets and pathways modulated by STBEL5. Using an ethanol-inducible promoter and RNA profiling, thousands of BEL5 target genes were identified in stolon tips after just 6 h of induction, many involved in cell growth, signal
transduction, transcription, and hormone metabolism (Sharma et al., 2016). A few examples include StISP5G, BRASSINOSTEROID INSENSITIVE1 and BRASSINOSTEROID INSENSITIVE1-ASSOCIATED RECEPTOR KINASE, numerous lipoxygenases (e.g. POTLX1), SCARECROW, SHORT-ROOT, CLAVATA1, and flowering genes like EARLY FLOWERING4, CONSTANS-LIKE, and APETALA2. In a screen of 200 differentially expressed targets of StBEL5, 92% contained the tandem TTGAC motifs in their upstream sequence, some with as many as nine copies (Sharma et al., 2016). Including its first intron, SIGA2OX1 contains six of these tandem motifs. Using whole-plant induction assays, numerous genes highlighted for their involvement in tuberization also have been identified as targets of StBEL5. These include GA2OX1 and GA20OX1, AGL8, three members of the LOG family, several PINs and AUX/IAA genes, and seven StBEL genes (Sharma et al., 2016), including two that suppress tuberization, StBEL11 and StBEL29 (Ghate et al., 2017). Only those target genes that contain the tandem TTGAC elements present in their upstream promoter sequence were considered for further study. Many of the motifs from these target genes were confirmed to bind to the StBEL5/StKNOX complex using gel-shift assays (Chen et al., 2004; Lin et al., 2013; Sharma et al., 2016).

Besides these many targets identified through RNA profiling of StBEL5 induction, three important tuber signals also are induced by StBEL5. As discussed earlier, StBEL5 autoregulates its own gene in stolon tips, where promoter activity is induced by SD (Chatterjee et al., 2007; Lin et al., 2013). Deletion of one of the TTGAC motifs present in the StBEL5 upstream sequence essentially eliminated its promoter activity in stolons and new tubers (Lin et al., 2013). Gel-shift assays verified StBEL5/POTH1 binding specificity to this double motif present in the StBEL5 promoter. The second tuber signal regulated by StBEL5 is StSP6A. There are several lines of evidence that support this premise. Developmental studies with StBEL5 overexpression and suppression lines demonstrated a strong positive correlation between StBEL5 and StSP6A gene activity (Sharma et al., 2016). Along with several other tuber marker genes, StSP6A RNA levels were enhanced in stolons upon induction of StBEL5 activity or the movement of StBEL5 RNA. Close examination of the upstream sequence of StSP6A revealed five TTGAC tandem motifs (Fig. 3A). After site mutagenesis of the motif sequences, the mutated promoter was fused to a GUS marker (Fig. 3B). Both wild-type and mutated StSP6A promoters were transformed into potato lines for analyses. As expected, the wild-type StSP6A promoter was active in new tubers in response to SD, whereas the mutated promoter lines exhibited very little activity in new tubers (Figs. 3, C and D). These results strongly suggest that StSP6A is a transcriptional target of StBEL5 in stolons and help to elucidate the mechanism for the autoregulatory loop formulated to explain the SD-induced accumulation of transcripts of StSP6A in stolons (Navarro et al., 2011). Finally, StBEL5 appears to activate StCDF1, the earliness gene, for tuberization (Kloosterman et al., 2013). This was first observed in the RNA profile using the StBEL5-induced system and was validated subsequently with reverse transcription-quantitative PCR (Sharma et al., 2016). Induction was then confirmed in leaves of

Figure 2. Mutation of the StBEL5/StKNOX upstream tandem TTGAC element suppresses StBEL5 promoter activity in new tubers. A, Schematic of the modification of the wild-type (WT) StBEL5 promoter sequence of potato. To create the mutated StBEL5 promoter, one of the tandem TTGAC cis-elements (underlined and boldface) that make up the binding motif for StBEL5 and its StKNOX-like partner, POTH1 (Chen et al., 2004), was deleted. To facilitate cloning, this five-base motif plus the TGC linker and an additional 8 bp (all in brackets) were removed and replaced by the ctgcag sequence. The intact wild-type double motif sequence begins 820 nucleotides upstream from the start of the StBEL5 promoter (Chatterjee et al., 2007). The wild-type StBEL5 promoter sequence analyzed here was 2,002 nucleotides in length. B and C, StBEL5 promoter activity is shown in newly formed tubers of transgenic lines containing either the wild-type StBEL5 promoter (B) or the mutated form fused to a GUS marker sequence (C). The potato subspecies andigena was used for transformations and was grown under SD to generate the small tubers shown here. These results are representative of several independent lines (Lin et al., 2013). The GUS staining technique has been described in greater detail (Lin et al., 2013; revised with permission of the authors). Bars = 5 mm.
an StBEL5 whole-plant induction system. Consistent with its role as an StBEL5/StKNOX target, the upstream sequence of the StCDF1 gene contains six TTGAC tandem elements (Sharma et al., 2016). Considering the abundance of the upstream tandem TTGAC motif in putative targets of StBEL5 (Sharma et al., 2016), an analysis of target elements utilizing an approach like chromatin immunoprecipitation (Bolduc et al., 2012) would be invaluable in verifying these observations.

In summary, StBEL5 enhances tuberization by functioning upstream of StSP6A and StCDF1 (Fig. 1B) and by regulating thousands of genes that mediate tuber formation (Sharma et al., 2016). An analysis of target elements utilizing an approach like chromatin immunoprecipitation (Bolduc et al., 2012) would be invaluable in verifying these observations.

CONCLUSION

The perception of daylength is a mechanism of regulation shared by both flowering and tuberization. Both processes measure daylength through blue light perception in the FKF1/GI complex that modulates CDF1 stability, which, in turn, controls CO expression (Sawa et al., 2007; Song et al., 2012; Kloosterman et al., 2013). As shown earlier (Fig. 1), CO enhances flowering in Arabidopsis but represses tuberization in potato. Whereas there are a number of signaling components common to both flowering and tuberization, several unique features in this updated tuber signaling model

Figure 3. Mutagenesis of StBEL5/StKNOX upstream tandem TGAC elements suppresses StSP6A promoter activity in new tubers. A and B, Transgenic lines with either the wild-type 3.2-kb upstream sequence of StSP6A (A) or with mutated TTGAC sequences in the same 3.2-kb sequence (B) driving GUS expression were generated and analyzed. Five TTGAC motifs (BEL5/KNOX element) were detected in the StSP6A upstream sequence (red triangles in A). The exact mutagenesis of critical nucleotides (strike-through letters) of these TTGAC motifs is shown in B. Transgenic plants (subspecies andigena) were grown under SD for 8 d to generate new tubers before harvest and staining. C and D, Whole new tubers (C) and longitudinal sections of new tubers (D) are shown with arrows designating the site of internal vasculature. Similar results were observed for several independent lines for each construct (Sharma et al., 2016). Details of GUS staining are provided by Sharma et al. (2016). Quantification of GUS activity confirmed these visual observations. Reduction in GUS activity with the mutated StSP6A promoter lines also was observed in leaves (Sharma et al., 2016; revised with permission of the authors). TSS, Transcription start site; WT, wild type. Bars = 1 mm.
can be enumerated. For example, tuber formation is additionally regulated by SD activation of the StPTB proteins (Cho et al., 2015). This activation enhances the stability and transport of StBEL5 transcript, directing this signal RNA to stolon tips. This mechanism explains the photoperiod-regulated accumulation of both StBEL5 and StSP6A in tuberizing stolons (Fig. 4). The three key signals of tuberization are StSP6A, StCDF1, and StBEL5. StBEL5 induces transcription of the FT ortholog, StSP6A, but at least two components, the full-length mRNA of StBEL5 and the StSP6A protein, function as long-distance mobile signals. StBEL5 also may target StCDF1, the TF that controls tuber maturity. The movement, stability, and location of the StBEL5 mRNA are mediated by the RNA-binding proteins, StPTB1 and StPTB6.
But despite the recent insights into this process, there are still critical questions that remain (see Outstanding Questions). There is some evidence, for example, that suggests that StBEL5 and StSP6A may work together (Sharma et al., 2016). StSP6A and StBEL5 are coexpressed in tuberizing stolons, and both activate some of the same downstream targets (Sharma et al., 2016). Both induce SIGA2OX1, but StSP6A, functioning as a coregulator, cannot mediate this induction on its own. Remarkably, the upstream sequence of SIGA2OX1 contains six tandem TTGAC elements, suggesting that an StBEL/STKNOX or StBEL/StSP6A complex functions in its regulation. Gel-shift assays confirmed StBEL5/POTH1 binding specificity to these tandem motifs in the upstream sequence of SIGA2OX1. This transcriptional mechanism appears to be conserved, as GA2OX1 of both potato and maize (Zea mays) contain the double TTGAC motif in their regulatory sequences. In maize, a KNATTED1 complex activates this gene (Bolduc et al., 2012). It is feasible that homodimers of StSP6A, StBEL5 (or an FDL TF), and a tuber-specific 14-3-3 protein interact to form the TAC, comparable to the FAC but unique to tuber initiation (Fig. 4; Teo et al., 2017).

In the current model (Fig. 4), StBEL5 is positioned upstream of a regulatory network involving hormonal pathways and transcriptional regulators that controls the onset of tuber formation. StBEL5 appears to directly activate tuberization and, at the same time, to amplify other indispensable signals in the pathway. This process is implemented by front-loading both StCDF1 and StSP6A activity in the leaf, mobilizing the transport of StBEL5 mRNA to stolons, and by expanding the activity of specific tuber signals in stolons through auto-regulation and the enhancement of StSP6A expression. In this way, StBEL5 can putatively circumvent the StCDF1 protein pathway in leaves even when StCDF1 is degraded, because StBEL5 activates the transcription of both StSP6A and StCDF1. Therefore, the effect of StBEL5 on StSP6A and tuberization is 3-fold. With its KNOX partner, it activates StSP6A directly in both leaves and stolons and increases StCDF1 levels, which repress CO, enhancing StSP6A expression in leaves. The current model proposes the existence of two TACs working in concert, an StBEL5/STKNOX complex and another composed of StSP6A, StFDL1, and a 14-3-3 protein (Fig. 4; Teo et al., 2017).

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