Three amino acid loop extension (TALE) transcription factors that regulate potato
(Solanum tuberosum L.) tuberization

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For the Major Program
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CHAPTER 1. GENERAL INTRODUCTION

Potato is the fourth most important crop in the world (Fernie and Willmitzer, 2001). Formation of the tuber, the agronomic portion of the crop, represents a specific developmental process that has been studied intensively for many years. Many factors, such as light, photoperiod, gibberellins (GA), cytokinin and nutrition affect tuberization in potato (Ewing and Struik, 1992; Hussey and Stacey, 1984; Jackson, 1999). However, only a few genes that directly regulate tuberization have been identified.

Knox (knotted like homeobox) genes have been suggested to mediate the GA levels in plants (Hay et al., 2002; Kusaba et al., 1998a and b; Sakamoto et al., 2001; Tamaoki et al., 1997; Tanaka-Ueguchi et al., 1998). Overexpression of one potato knox gene, POTH1, enhances tuberization activity under both long- and short-day photoperiods in vitro (Rosin et al., 2003). These mutant plants exhibited decreased GA1 and GA20 levels, and increased GA19 level. Furthermore, mRNA of GA20 oxidase, which encodes the enzyme that catalyzes the conversion of GA19 to GA20, was down-regulated in these mutants (Rosin et al., 2003). Sakamoto et al. (2001) showed that tobacco KNOX protein, NTH15, directly represses the transcription of GA20 oxidase. It is very likely that GA20 oxidase may also be the direct target gene of POTH1.

The homeobox encodes a DNA-binding region designated the homeodomain
(HD) that is structurally related to the bacterial helix-turn-helix motif (Scott et al., 1989). Although HD proteins have a high degree of functional specificity in directing developmental programs, many HD proteins bind DNA weakly (Mann and Chan, 1996; Pellerin et al, 1994). Multiple HD proteins with different functions recognize the same consensus DNA sequence indicating that these proteins exhibit a high degree of redundancy in binding site specificity (Pellerin et al, 1994). Besides the low affinity and high redundancy in binding sites, on average, the 5-base binding site of HD proteins randomly shows up once every 1.0 kb in genomic DNA (Mann and Chan, 1996). To increase their binding affinity and specificity, HD proteins interact with other transcription factors. For example, while monomeric homeodomain proteins have modest specificity for DNA binding (Laughon, 1991), their specificity is greatly increased through cooperative binding with other DNA binding partners (Mann and Chan, 1996). Sakamoto et al (1999) reported that secondary structure in the protein-binding region of the tobacco KNOX proteins determines the severity of the mutant phenotype, suggesting protein-protein interaction is very important in regulating the activity of KNOX proteins in plants.

The objectives of this study are to: a) Identify the interacting partners of POTH1; b) Determine the function of StBEL5; and c) Investigate the possible role of the protein-protein interaction in regulating their putative target gene, ga20ox1.
Dissertation Organization

The dissertation is organized in the format consisting of two journal articles proceeded by a General Introduction and followed by a General Conclusion and a section of Literature Cited. The journal articles are formatted according to the requirements of each journal. The first article “Interacting transcription factors from the Three-amino acid loop extension superclass regulate tuber formation” was published in *Plant Physiology* (2003, 132:1391-1404). The second article “The tandem complex of BEL and KNOX partners is required for transcriptional repression of ga20ox1” has been submitted for publication in *The Plant Cell*. Hao Chen was the primary investigator for this work under the supervision of Dr. David J. Hannapel and is the first author of both articles.
CHAPTER 2. INTERACTING TRANSCRIPTION FACTORS FROM THE THREE-AMINO ACID LOOP EXTENSION SUPERCLASS REGULATE TUBER FORMATION

A paper published in *Plant Physiology* *

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Summary

Using the yeast two-hybrid system and a potato KNOX protein, designated POTH1, as bait, we have identified seven distinct interacting proteins from a vegetative meristem library of potato (*Solanum tuberosum* L.). All seven cDNAs are members of the *BEL1*-like family of transcription factors. Among these proteins,

there are at least three regions of high sequence conservation including the homeodomain, the proline-tyrosine-proline TALE, and a 120-amino acid region upstream from the homeodomain. Through deletion mutation analysis, we identified a protein-binding domain present in the carboxy-end of the KNOX domain of POTH1. The protein-binding domain in the BEL1 protein is located in the amino-terminal half of the 120-residue conserved region of the BELs. RNA blot analysis showed differential patterns of RNA accumulation for the BELs in various potato organs. Two subfamilies of the BELs exhibited mRNA accumulation throughout the plant and were developmentally regulated in tuberizing stolons. The level of StBEL-5 mRNA increased in response to short-day conditions in both leaves and stolons. Similar to sense mutants of POTH1, transgenic lines that overexpressed one of the BELs exhibited enhanced tuber formation even under noninductive conditions. Unlike POTH1 sense lines, however, these BEL lines did not exhibit the extreme leaf and stem morphology characteristic of KNOX overexpressers. Both BEL and POTH1 sense lines exhibited an increase in cytokinin levels in shoot tips. Our results demonstrate an interaction between KNOX and BEL1 transcription factors of potato that may potentially regulate processes of development.
Introduction

The primary developmental events of plants originate from apical meristems (Clark, 1997; Kerstetter and Hake, 1997). Many of these events are controlled at the molecular level by transcription factors. Transcription factors (TFs) are proteins that act as developmental switches by binding to the DNA (or to other proteins that bind to the DNA) of specific target genes to modulate their expression. An important family of TFs involved in regulating the developmental events in apical meristems is the knox (knotted-like homeobox) gene family (Reiser et al., 2000). Knox genes belong to the group of TFs known as the TALE superclass (Bürglin, 1997). These TFs are distinguished by a very high level of sequence conservation in the DNA-binding region, designated the homeodomain, and consisting of three α-helices similar to the bacterial helix-loop-helix motif (Kerstetter et al., 1994). The third helix, the recognition helix, is involved in DNA-binding (Mann and Chan, 1996). TALE TFs contain a three amino acid loop extension (TALE), proline-tyrosine-proline, between helices I and II in the homeodomain, that has been implicated in protein interactions (Passner et al., 1999). There are numerous TFs from plants and animals in the TALE superclass and the two main groups in plants are the KNOX and BEL types (Bürglin, 1997). Related genes in animal systems play an important role in regulating gene expression.
In animal developmental systems, members of the TALE superclass physically interact with other TFs to regulate gene expression via a direct effect on transcription of the target gene (Mann and Chan, 1996) or by determining the subcellular location of a key factor (Abu-Shaar et al., 1999; Berthelsen et al., 1999). Specific cooperative DNA binding is facilitated by the tandem protein complex of interacting cofactors (Mann and Chan, 1996; Pinsonneault et al., 1997). Extradenticle (EXD), a TALE TF, functions as a switch that changes homeobox (HOX) proteins from repressors to activators via protein/protein interaction (Pinsonneault et al., 1997). A structural analysis of the protein pairing of EXD and a HOX TF (Ultrabithorax) verified that the P-Y-P loop of EXD binds to a conserved sequence motif in Ultrabithorax to facilitate protein and DNA binding (Passner et al., 1999). EXD and Homothorax, another TALE TF, interact to facilitate nuclear localization of EXD (Rieckhof et al., 1997). The trimeric interaction of two TALE TFs (EXD and HTH) and a Hox protein facilitates specific binding to the target DNA (Ryoo et al., 1999). Protein interaction in these examples is mediated by specific conserved amino acid sequence motifs (Passner et al., 1999; Ryoo et al., 1999).

Expression patterns and functional analysis of mutations support the involvement of knox genes in specific developmental processes of the shoot apical meristem. Kn1 from maize, the first plant homeobox gene to be discovered
(Vollbrecht et al., 1991), is involved in maintenance of the shoot apical meristem and is implicated in the switch from indeterminate to determinate cell fates (Chan et al., 1998; Kerstetter et al., 1997; Clark et al., 1996). Transcripts of kn1 in maize (Jackson et al., 1994), OSH1 in rice (Sentoku et al., 1999), and NTH15 in tobacco (Tamaoki et al., 1997) were localized by in situ hybridization to undifferentiated cells of the corpus and the developing stem, but were not detected in the tunica or leaf primordia. Overexpression of kn1 in Arabidopsis (Lincoln et al., 1994) and in tobacco (Sinha et al., 1993), resulted in plants with altered leaf morphologies including lobed, wrinkled or curved leaves with shortened petioles and decreased elongation of veins. Plants were reduced in size and showed a loss of apical dominance. In plants with a severe phenotype, ectopic meristems formed near the veins of leaves indicating a reversion of cell fate back to the indeterminate state (Sinha et al., 1993). Overexpression of OSH1 or NTH15 in tobacco resulted in altered morphologies similar to the 35S-kn1 phenotype (Sato et al., 1996; Tamaoki et al., 1997).

Alterations in leaf and flower morphology in 35S-NTH15 or OSH1 transgenic tobacco were accompanied by changes in hormone levels. Whereas levels of all the hormones measured were changed slightly, both gibberellin and cytokinin levels were dramatically altered (Kusaba et al., 1998a; Tamaoki et al., 1997). RNA blot analysis revealed that the accumulation of GA 20-oxidase1 mRNA was reduced
severalfold in transgenic plants (Kusaba et al., 1998b; Tanaka-Ueguchi et al., 1998). A KNOX protein of tobacco binds to specific elements in regulatory regions of the GA 20-oxidase1 gene of tobacco to repress its activity (Sakamoto et al, 2001). GA 20-oxidase is a key enzyme in the GA biosynthetic pathway necessary for the production of the physiologically inactive GA20 precursor of active GA1 (Hedden and Kamiya, 1997). GA1 and other active GA isoforms are important regulators of stem elongation, the orientation of cell division, the inhibition of tuberization, flowering time, and fruit development (Jackson and Prat, 1996; Hedden and Kamiya, 1997; Rebers et al., 1999).

A homeobox transcription factor of potato (Solanum tuberosum L.) in the knox family (Reiser et al., 2000), designated POT1 (potato homeobox; GenBank accession #U65648) was isolated from an early tuber cDNA library of potato (Rosin et al., 2002). Sequence analysis indicates that POT1 is a member of the class I knox gene family (Rosin et al., 2002) and is also a member of the TALE superclass of homeobox genes (Bürglin, 1997). POT1 and related knox genes in tobacco and rice are involved in regulating plant growth by controlling gibberellin (GA) synthesis (Kusaba et al., 1998a; Rosin et al., 2002; Tamaoki et al., 1997). Overexpression of these KNOX genes produced plants with altered levels of intermediates in the gibberellin biosynthetic pathway and a reduction in bioactive gibberellins. These
mutants exhibited aberrant leaf formation, dwarfism, and, in the case of POTH1, enhanced tuber formation under both inductive and noninductive conditions (Rosin et al., 2002).

Another plant homeobox gene family that is closely related to the knox genes is the BEL (BELL) family (Chan et al., 1998; Bürglin, 1997). BEL TFs have been implicated in flower and fruit development (Reiser et al., 1995; Dong et al., 2000). Genetic analysis of BEL1 in Arabidopsis showed that expression of this TF regulated the development of ovule integuments and overlaps the expression of AGAMOUS (Ray et al., 1994; Reiser et al., 1995; Western and Haughn, 1999). In COP1 mutants, the photoinduced expression of ATH1, another BEL TF of Arabidopsis, was elevated, indicating a possible role in the signal transduction pathway downstream of COP1 (Quaedvlieg et al., 1995). Recently, the interaction of BEL1 proteins with KNOX proteins was reported in barley, Arabidopsis, and maize (Müller et al., 2001; Bellaoui et al., 2001; Smith et al., 2002). Here we report interactions between a potato KNOX protein involved in the regulation of growth in potato and seven distinct proteins from the BEL1-like family of transcription factors.
Results

Isolation of potato KNOX interactive proteins

Making use of the two-hybrid selection system in yeast, we screened approximately $10^6$ transformants from a stolon cDNA library of potato using POTH1 in the GAL4-binding domain vector, pBridge (Clontech), as bait. We identified thirty-eight positive clones that grew on selective media. Of the thirty-eight that were sequenced, twenty-three clones could be grouped into seven unique genes encoding different members of the TALE superclass of transcription factors (Chan et al., 1998). All seven, designated StBEL-05, -11, -13, -14, -22, -29, and -30 (GenBank accession numbers AF406697, AF406698, AF406699, AF406700, AF406701, AF406702, AF406703, respectively) showed selective interaction when tested in the yeast system both for nutritional markers and for lacZ activation (Figure 1A and 1B). Interaction occurred also when the prey cDNAs were cloned into pBridge and transformed with POTH1 in a GAL4-activation domain vector (data not shown). In vitro binding experiments verified the results of the two-hybrid selection. POTH1 pulled down three representative StBEL proteins (5, 13, and 30) synthesized by in vitro transcription/translation in immunoprecipitation assays (Figure 1C).

The proteins that interact with the potato KNOX protein are members of the BEL family of transcription factors.
A phylogenetic analysis of the sequences of the seven interacting proteins identified them as members of the BEL1-like family of transcription factors (Figure 2). The amino acid sequence of StBEL-5 has 56% similarity to BLH1 of Arabidopsis that interacts with KNAT1 (GenBank accession number AAK43836), StBEL-13 matches an apple BEL (Dong et al., 2000, GenBank accession number AAF43095) at 74% similarity, and StBEL-30 matches another Arabidopsis BEL (GenBank accession number T05281) at 59% similarity. The close match of all seven to the conserved homeodomain and the presence of the proline-tyrosine-proline (P-Y-P) loop between helices I and II (Figure 3A) distinguish these novel proteins as BEL types in the TALE superclass (Bürglin, 1997). The homeodomain region is nearly identical among these seven (Figure 3A, encompassing helices I, II, and III). Other regions of conserved sequence identity are shown schematically in Figure 3A. These include the amino-terminal SKY-box consisting of 20 aa (from ser-207 to lys-226 in StBEL-5), the 120-aa domain starting at leu-272 of the StBEL-5 sequence, and the carboxy-terminal VSLTLGL-box beginning at val-620. Three α-helices were predicted from the conserved 120-aa region of the BEL protein StBEL-5 (underlined sequence of Figure 3B). Among the seven BELs, the percent similarity of the amino acid sequence in this conserved 120-aa domain ranged from 55 to 90%. Bellaoui et al. (2001) refer to this region as the BELL domain.
The deduced lengths of the seven original cDNAs are 688 aa for StBEL-5, 535 aa for StBEL-11, 586 aa for StBEL-13, 589 aa for StBEL-14, 612 aa for StBEL-22, 511 aa for StBEL-29, and 645 aa for StBEL-30. Five’-RACE was used to verify the full-length of StBEL-5, -13, -14 and -30. For blot hybridizations, we used a representative clone from each of the four subgroups (StBEL-5, -13, -14, and -30). Southern blot analysis revealed that these genes are unique or belong to small gene families, based on the complexity of bands detected by gene-specific probes from each of the cDNAs (Figure 4).

**Patterns of mRNA accumulation for the potato BELs**

The BEL1-like gene represented by StBEL-5 exhibited mRNA accumulation in all organs examined with the greatest levels in leaves and stems (Figure 5A). Transcript accumulation of StBEL-11 and StBEL-29 was similar to the pattern of StBEL-5 (data not shown). The greatest accumulation of StBEL-30 was in flowers with detectable levels in all organs examined. For StBEL-14, transcripts were detected in flowers, leaves, roots, and stolons. Transcripts for StBEL-13 accumulated to the highest levels in the SAM and in fully formed flowers but were barely detectable in other organs (Figure 5A). The autoradiographs for StBEL-13 were exposed two-times longer than the other StBELs. To examine more closely the
dynamics of StBEL expression during tuber induction, a temporal study was undertaken for the accumulation of StBEL-5 transcripts in leaves and stolons of the photoperiod-sensitive potato species S. tuberosum ssp. andigena grown under short-day conditions. Steady state levels of StBEL-5 mRNA increased in both leaves and stolons after exposing the plants to short-day (SD) conditions (Figure 5B). Visible tuber formation for the plants grown under SD conditions was observed between 10-14 days. In this study, the accumulation of mRNA for the BEL cDNA, StBEL-5, was linked to the induction of tuber formation in the leaves and stolons of a potato species responsive to a SD photoperiod.

Determining the protein binding regions in POTH1 and the BEL1-like proteins

Interaction with StBEL-5 was observed with all deletions outside the KNOX domain, with pBHD2 (missing the amino-terminus and the first 48 aa of the KNOX domain, Figure 6A), with pBHD6 (missing the carboxy terminus and 52 aa of the carboxy-end of the KNOX domain), and with pBHD-9 (first amino-terminal 114 aa but no KNOX domain sequence). No interaction was observed with pBHD3 (missing all of the KNOX domain and the first 114 aa). Control experiments identified the first 114 aa of the N-terminus (pBHD9) as a transcriptional activator. This construct transformed alone into AH109 exhibited nutrient selection on -histidine,
tryptophan, and adenine medium. Cotransformation of pBHD9 with an empty pGAD cassette produced transformants capable of growth on histidine, tryptophan, adenine, and leucine medium and induction of lacZ (data not shown). None of the other constructs containing this domain were capable of growing on selection media without StBEL-5. Using the in vitro binding protocol, both the pBHD6 construct, containing the amino-terminal half of the KNOX domain, and the pBHD9 construct were unable to pull-down StBEL-5 (data not shown). When the pBDH9 construct was cloned into the pGAD vector, no interaction was observed with StBEL-5 in pBridge.

Fusion constructs of StBEL-5 that dissected the 120-aa domain (pAD5-2, -3, -4, -9, and -11) were tested because this is one of the regions that is conserved in BEL TFs from other plant species (Bellaoui et al., 2001; Figure 3B). Interaction with POTH1 was observed with all constructs that had deletions exclusively outside of the conserved 120-aa box (Figure 6B). The only exception to this was with pAD5-9 that demonstrated an interaction and included a 43-aa deletion from the carboxy end of the 120-aa domain. Even with as little as a 27-aa deletion from the amino end of the 120-aa domain, interaction did not occur (Figure 3B, Figure 6B, pAD5-2). Similar to the results of Bellaoui et al. (2001), deletion of the SKY box (construct
pAD5-1) resulted in a 55% decrease in the induction of the lacZ marker as measured by β-galactosidase activity relative to the full-length construct, StBEL5 (Figure 6B).

Enhanced tuber formation in transgenic plants that overexpress the BEL cDNA, StBEL-5

To examine the function of the potato BELs, we analyzed transformed potato plants (Solanum tuberosum ssp. andigena) that over expressed StBEL-5 from a strong, constitutive promoter. This BEL gene was selected because of its moderate level of activity in stolons and tubers (Figure 5). For these experiments, we used a 2000-bp fragment of the coding sequence of StBEL-5 in a sense orientation driven by the CaMV-35S promoter in the binary vector pCB201 (Xiang et al., 1999). Transformants were identified by PCR analysis of genomic DNA and by detection of the accumulation of sense transcripts of StBEL-5 in RNA samples from vegetative meristems. From among approximately twenty-five positives, four independent lines with the highest levels of StBEL-5 mRNA accumulation (Figure 7A) were selected for evaluation of tuber formation in vitro under both inductive (SD) and noninductive (LD) conditions. The highest expressers of StBEL-5 sense transcripts (lines 11, 12, 14, and 19) exhibited tuber formation under LD conditions (Figure 7B). Control plants (WT and line 6) produced tubers only under SD conditions. The highest overexpressers of StBEL-5 also produced more tubers than control plants.
over the course of this experiment and were more responsive to inductive conditions. After 7 days under SD conditions, the control plants had produced no tubers, whereas the overexpression mutants (lines 11, 12, 14, and 19) had produced 10, 8, 15, and 4 tubers, respectively (Figure 7B). After 14 days under SD, controls had increased to 6 and 4 tubers, whereas the overexpression lines had increased to 12, 14, 24, and 10 tubers, respectively. Tuber yields (fr wt) also increased in the overexpression lines. The greatest tuber production was exhibited by lines 12 and 14 with a five- and sixteenfold increase, respectively, relative to WT (Table I). Tubers from the overexpression lines grew larger than controls (Figure 7C). Select tubers from line 14 reached fresh weights of almost 700 mg, whereas the largest control tuber reached only 140 mg. Seven overexpressing sense lines (lines 7, 11, 12, 14, 16, 19, and 20) also exhibited tuber activity (swollen stolons or tuber formation) on soil-grown plants under LD greenhouse conditions. Five of these plants produced tubers, whereas control plants exhibited no tuber activity.

With whole plants grown in soil under SD conditions for 14 d, StBEL overexpression lines produced an average of three- to fivefold more tubers plant$^{-1}$ and more than a threefold greater tuber yield plant$^{-1}$ than controls (Table II). Similar to $POTH1$ overexpressers (Rosin et al., 2003), these results show that the accumulation of $StBEL-5$ mRNA is correlated with an increased rate of tuber
formation. Other than this enhanced capacity for tuberization, the StBEL-5 overexpression lines in Table II did not exhibit the phenotype characteristic of KNOX gene overexpressers, including extreme dwarfism and abnormal leaf morphology (Figure 8). The abnormal phenotype of KNOX overexpressers is mediated by changes in hormone levels, specifically, a reduction in gibberellins and an increase in cytokinins (Rosin et al., 2003; Sato et al., 1996; Tamaoki et al., 1997; Frugis et al., 2001). With the exception of two StBEL-5 sense mutants (lines 11 and 20), the leaf and stem growth of the StBEL-5 overexpression lines was comparable to WT (Figure 8). Lines 11 and 20 exhibited a slight reduction in leaf size. To examine the mechanism for this abnormal leaf morphology, cytokinin analysis was performed on shoot apices down to the fourth true leaf. Comparable to POTH1 overexpressers, shoot tips of both StBEL-5 lines 11 and 20 exhibited a two-to fivefold increase in the bioactive forms of cytokinin (Table III). The overall magnitude increases in the cytokinin types among the four StBEL and POTH1 mutant lines was remarkably consistent.

POTH1 sense lines had increased levels of GA_{33} and GA_{19} and decreased levels of GA_{20} and GA_{1} in shoot tips, implicating a down-regulation of the biosynthetic enzyme GA 20-oxidase1 (Rosin et al., 2003). Using a probe for the potato GA 20-oxidase1 gene (Carrera et al., 1999), we observed a reduction in GA 20-
oxidase1 mRNA in shoots of the most severe mutant phenotypes for POTH1 sense lines (Rosin et al., 2003). To determine the effect of overexpression of the POTH1 partner, StBEL-5, RNA levels for GA20 oxidase1 were examined in the stolons of StBEL-5 sense lines grown under long-day conditions. Three of the sense lines examined that produced tubers under LD conditions exhibited a reduction in GA20 oxidase1 mRNA in stolon tips comparable to controls (Figure 9).

To determine the effect of upregulating StBEL-5 mRNA levels on POTH1 RNA accumulation, northerns were performed on total RNA extracted from StBEL-5 sense lines 12, 14, 19, and 20 using POTH1 as a probe. There were no changes in the levels of POTH1 mRNA in both shoot tips and stolon tips of these StBEL-5 lines relative to WT (data not shown). These results indicate that the enhancement of tuber formation in StBEL-5 overexpression lines is not mediated by an indirect increase in POTH1 expression.

Discussion

Seven BEL1 proteins interact with a KNOX protein of potato

Using a yeast two-hybrid library screen, we have identified seven unique proteins from potato stolons that interact with the knotted-like protein, POTH1. Sequence analysis revealed that these interacting proteins are from the BEL1-like
family in the TALE superclass of homeodomain proteins. These proteins have conserved regions in common with other TALE proteins, including the homeodomain (comprised of three α-helices) and the proline-tyrosine-proline "TALE" (Bürglin, 1997). These sequences have been implicated in DNA-binding and protein/protein interactions, respectively (Mann and Chan, 1996; Passner et al., 1999). A second conserved region of 120 aa just upstream from the homeodomain (designated the BELL domain by Bellaouï et al., 2001) was identified among BEL proteins by using a BLAST analysis (Figure 3B, bold letters). Sequence analysis of the predicted secondary structure of this domain reveals the presence of three putative α-helices within the 120 residues (Figure 3B, underlined sequence). Not all BEL proteins conserve the third helix, however, including the Arabidopsis BEL, ATH1 (Quaedvlieg et al., 1995) and the barley BEL, JUBEL2 (Müller et al., 2001). Protein interaction using the two-hybrid system demonstrated that the first 80 aa of this domain (up to QVKAT of the StBEL-5 sequence and comprising the first two predicted helices of this region) are necessary to mediate interaction with POTH1 (interaction of construct pAD5-9 with POTH1). Deletion of as little as the first 20 aa of this domain (comprising a major portion of the first predicted helix) interfered with the interaction with POTH1 (Figures 3B and 6B, construct pAD5-2). Our results also showed that deletion of 43 aa from the carboxy-end of the 120-aa domain (see
Figure 6B, construct pAD5-9; comprising the third helical structure) did not affect protein interaction. Deletion of the two carboxyl-terminal helices in this region (construct pAD5-11) resulted in a loss of interaction. It is conceivable that all three helical structures contribute to specific binding affinity for POTH1 but that only the amino-terminal two-thirds of the 120-aa domain are necessary for binding to occur. Müller et al. (2001) identified a coiled-coil region in a barley BEL protein that was involved in the interaction with KNOX proteins. This coiled-coil domain overlaps with 48 of the 80 aa (and comprising the first helix) that we identified as essential for interaction to occur.

Sequence differences in this putative protein binding region could contribute to the regulation of POTH1 activity by affecting binding affinity to a shared partner. In the interaction between PIF3, a basic helix-loop-helix factor, and phytochrome A and B, phytochrome B has tenfold greater binding affinity for the PIF3 partner than phytochrome A (Zhu et al., 2000). A comparison of this 120-aa domain in the potato BELs revealed that StBEL-5 (expressed ubiquitously) has a 58% similarity match to StBEL-13 (expressed predominately in the SAM and flower only) and that StBEL-13 has a 63% match to StBEL-30 in this conserved region. Such differences in sequence may mediate binding affinities to shared partners and, coupled with expression patterns, could reflect cell-specific differences in function.
Conservation in sequence among these seven proteins can also be identified in two short amino acid sequence motifs, one near the carboxyl-end of the protein (VSLTLGL) and another just upstream of the BELL domain (SKY box, Figure 3A). Both of these regions are conserved among other plant BELs. Protein interaction studies showed that the VSLTLGL box is not involved in protein interaction with POTH1 and its function remains unknown. Consistent with Bellaoui et al. (2001), we observed that, whereas binding occurred without the 229 aa at the amino terminus of StBEL-5 (construct pAD5-1), this 229 aa sequence alone, containing the SKY box, was sufficient to mediate an interaction with POTH1 (and other class I KNOX proteins) but not a class II KNOX protein (data not shown). Müller et al. (2001) identified the SKY-box sequence in the barley BEL protein to be a part of the KNOX-interacting domain. Our deletion analysis indicates that the SKY box enhances the binding affinity of StBEL-5 to KNOX partners.

The protein binding region of POTH1

In addition to the homeodomain, KNOX TFs also contain a conserved region of approximately 100 aa, upstream from the homeodomain, known as the KNOX (MEINOX) domain, and postulated to be involved in protein/protein interaction (Bürglin, 1998). Using deletion mutants in the two-hybrid yeast system, we have
identified regions of amino acid sequence in the KNOX domain of the class I KNOX protein, POTH1, that are involved in an interaction with the BEL TFs. Binding to the BEL partner is mediated by the KNOX domain but is not dependent on the presence of the first half of the 120 aa KNOX region (Figure 6A). Similar results were obtained by Müller et al. (2001). Sakamoto et al. (1999) showed by using chimeric proteins that the second half of the KNOX domain (designated KNOX2) of a tobacco KNOX protein (NTH15, with 63 % similarity to POTH1 in the KNOX region) was most important for determining the severity of the mutant phenotype. Their results indicated that this conserved domain was even more important in determining the phenotype than the DNA-binding domain. The deletion analysis for POTH1 in the present study combined with the results of Sakamoto et al. (1999) suggest that the interaction of the BEL proteins with the KNOX domain may be a prominent control mechanism for mediating KNOX activity and maintaining stable development of the vegetative meristem. KNOX2 contains 18 aa that form a conserved α-helix among all tobacco and potato KNOX proteins. POTH1 has a close sequence match to members of the family of KNOX proteins of tobacco (Nishamura et al., 2000), with an overall sequence similarity ranging from 60 to 73 % and an even greater match in the conserved KNOX and homeodomain regions. Using the two-hybrid system, all seven potato BELs interacted with four other tobacco class I-type KNOX proteins.
Unlike KNOX proteins of barley (Müller et al., 2001) and rice (Nagasaki et al., 2001), however, POTH1 did not form homodimers in vitro (data not shown). Structural similarities to the MEIS domain of animal homeodomain proteins (Bürglin, 1998) suggest that sequences in the KNOX domain of plants are important for interactions with other proteins (Sakamoto et al., 1999). Our results confirm the function of this domain in an interaction with a BEL1-like protein of potato.

The function of the BEL/POTH1 interaction

Through both molecular and genetic analyses, the BEL proteins are known to function in the development of ovules. Reiser et al. (1995) showed that BELL1 of Arabidopsis was involved in the pattern formation of ovule primordium. More specifically, NOZZLE (a nuclear protein and putative TF) and BELL are genetically linked to determine distal-proximal pattern formation during ovule development. Both are chalazal identity genes that share overlapping functions (Balasubramanian and Schneitz, 2000). In bel1 mutants, the chalazal domain undergoes altered development and growth of the integuments is replaced by irregular outgrowths (Modrusan et al., 1994). Overexpression of an apple BEL gene (MDH1) in Arabidopsis produced plants that were dwarf, had reduced fertility, and exhibited changes in both carpel and fruit shape (Dong et al., 2000). Overall, these results suggest that
BEL proteins function in controlling the formation of carpellate tissues and plant fertility. Overexpression of a cDNA of a barley BEL in tobacco produced plants that were dwarf and exhibited malformed leaves and reduced apical dominance (Müller et al., 2001). This BEL1-like cDNA isolated from floral meristems produced a sense phenotype similar to a class I knox overexpresser (Chan et al., 1998). All seven of the potato BEL TFs in this study were isolated from stolons, a vegetative organ. Based on these results and the patterns of mRNA accumulation in potato, it is likely that the BEL1 TFs of potato play a diverse role in plant growth by regulating the development of both reproductive and vegetative meristems.

Because the BEL1s of potato and POTH1 interact, the function of one may provide a clue to the function of the other. The KNOX protein of tobacco, NTH15, affects plant growth by regulating GA levels through a direct interaction with a specific motif in regulatory sequences of the GA 20-oxidase1 gene, a key GA biosynthetic enzyme (Sakamoto et al., 2001). NTH15 directly suppresses the expression of GA 20-oxidase1 within specific cells of the SAM to maintain the indeterminate state of corpus cells. The knotted1-like protein of potato, POTH1, is also involved in the regulation of GA synthesis and acts as a developmental switch during tuber formation. Transgenic plants that overexpressed POTH1 had reduced levels of GA 20-oxidase1 mRNA, altered levels of GA intermediates, and exhibited a
phenotype that could be partially rescued by GA3 treatment (Rosin et al., 2003). These plants were dwarf and developed malformed leaves. Under both short-day (inductive conditions) and long-day (noninductive) photoperiods, POTH1 overexpressing lines produced more tubers than controls (Rosin et al., 2003). These sense lines exhibited a capacity for enhanced tuber formation. All four lines that overexpressed StBEL-5 produced tubers even under LD in vitro conditions, whereas control plants produced tubers only after 10 days of SD conditions. Overall, the BEL sense lines produced more tubers at a faster rate than controls even on soil-grown plants. After only 14 d of SD conditions, soil-grown StBEL-5 overexpressers exhibited a threefold increase in tuber production relative to WT (Table II). In addition to enhanced tuber production, select StBEL lines exhibited increases in cytokinin levels and a reduction in GA20 oxidase1 mRNA similar to POTH1 overexpression lines. GA is involved in regulating cell growth in a tuberizing stolon (Xu et al., 1998) and in contributing to the control of the photoperiodic response of tuber formation (Kumar and Wareing, 1974; Jackson and Prat, 1996, Martínez-García et al., 2001). Low levels of GA in the stolon tip are correlated with tuber induction (Xu et al., 1998). Tuberization is also affected by cytokinin accumulation, with high levels inhibiting and moderate levels promoting tuber formation (Gális et al., 1995; Romanov et al. 2000). Local accumulation of cytokinins in axillary buds of transgenic
tobacco produced truncated, tuberizing lateral branches (Guivarc'h et al., 2002).

Through an interaction with POTH1, the BEL protein encoded by StBEL-5 may also function to regulate hormone levels in stolons or leaves to favor the formation of tubers.

Whereas, all seven BELs may act to regulate growth during stolon and tuber formation, it is possible that some of the potato BELs are functional only in other organs. The expression patterns of StBEL-5, -13, -14, and -30 (Figure 5) suggest such a specialization of function. The interaction of POTH1 (and other KNOX proteins of potato) and the various BELs could represent unique complexes with different affinities for DNA-binding motifs. This modification in structure coupled with the regulation of protein accumulation could mediate the activity of POTH1 and determine binding to a select target gene. Third-partner protein interaction could also affect the activity of the KNOX/BEL complex via structural modification or subcellular localization. In preliminary studies with two-hybrid screening, we have identified other proteins that interact with StBEL-5. The interaction of HOX proteins with MEIS and PBC class proteins produces a complex regulatory network, where even slight changes in protein levels can have profound phenotypic effects (Azpiazu and Morata, 1998). Throughout the plant, the various BELs may regulate growth by acting as either activators or repressors of POTH1 activity. Saleh et al. (2000)
suggested a model whereby the complex of two homeodomain proteins, HOX and PBX, can act as a repressor or activator of transcription via interaction with a third partner. In this system, a protein kinase modifies a CREB-protein to facilitate its binding to a HOX/PBX complex to activate transcription of the target gene (Saleh et al., 2000).

The results of this study suggest that the physical interaction between the KNOX and BEL1 proteins provides a molecular basis for regulating processes of growth in the potato and that overexpression of each partner alone enhances tuber development.

Experimental Procedures

Two-hybrid selection and deletion analysis

The Matchmaker two-hybrid system (Clontech, CA) was used for the yeast two-hybrid screen. Yeast transformation and plasmid rescue into DH5-α E. coli cells were according to the manufacturer's instructions. Full-length POTH1 was cloned into the pBridge (Clontech, CA) vector and used as bait to screen the potato (S. tuberosum 'Desireé') stolon cDNA library in pAD-GAL4-2.1 (Stratagene, CA). Positive interactions were confirmed by cotransforming yeast strain AH109 with each purified pAD plasmid and pBridge: POTH1 and plating on -leucine /-tryptophan
(transformation control) and -leucine/-tryptophan/-histidine/-adenine (selection) nutrient medium. Induction of the AH109 reporter gene, lacZ, was measured with a yeast β-galactosidase assay kit (Pierce Chemicals). β-galactosidase activity (Figure 1B) was determined from a known density of yeast cells and calculated as 1000 × OD₄₂₀/time of color reaction (min) × volume of yeast culture (ml) × OD₆₀₀.

The StBEL-5 deletion constructs were amplified by PCR, then cloned into the vector, pGAD, in-frame with the GAL4 activation domain. POTH1 deletion constructs were amplified by PCR, and cloned into pBridge (Clontech) in-frame with the GAL4 binding domain. Sequencing of selected cDNAs and constructs was performed at the Iowa State University DNA Facility. For deletion analysis, modified constructs of POTH1 were cloned into the pBridge vector for fusion with the DNA-binding domain of GAL4 (Figure 6A). For StBEL-5, constructs were cloned into the pGAD vector for fusion with the activating domain of GAL4 (Figure 6B). Deletion constructs were made from both the amino and carboxy termini. These mutants were then tested for interaction in the yeast two-hybrid system by cotransforming into yeast strain AH109 with the corresponding full-length partner (StBEL-5 in pGAL4 or POTH1 in pBridge). All constructs were sequenced to verify that they were in-frame. Positive interactions were verified for lacZ induction by using a β-galactosidase assay (Pierce Chemical Company). For POTH1, seven
deletion constructs were tested (Figure 6A). For the BEL TFs, a fusion construct of
StBEL-5 (653 aa of StBEL-5 sequence) and nine deletion constructs were tested
(Figure 6B).

GenBank accession numbers for StBEL-5, -11, -13, -14, -22, -29, and -30 are AF406697, AF406698, AF406699, AF406700, AF406701, AF406702, AF406703, respectively.

In vitro binding assay

In vitro binding experiments were performed as described by Ni et al. (1998). Full-
length sequence for POTH1 was cloned into a pET17b/GAD fusion cassette and
transcribed under the control of the T7 promoter. The BEL cDNAs were cloned into
pGEM11Z vectors and were transcribed under the control of the T7 promoter. 35S-
methionine labeled bait and prey proteins were synthesized using the TnT in vitro
transcription-translation kit (Promega) according to the manufacturer’s protocols.
Each 50 μl TnT reaction contained 2.0 μg of template plasmid DNA and 20 pmol
(20μCi) of labeled 35S-methionine. The POTH1: GAD/BEL complex was
immunoprecipitated with anti-GAD antibodies (Santa Cruz Biotechnology, CA). The
proteins from the pellet (one-half the fraction) and for the prey (one-fourth of the
reaction volume) were resolved on a 10% SDS-PAGE gel and visualized by autoradiography.

Hybridization blot analysis

Genomic DNA extraction and Southern analysis were performed as described by Kolomiets et al. (2000). DNA was extracted from shoot tips of *Solanum tuberosum* ssp. *andigena*. Total RNA was extracted from various organs of *andigena* plants grown under a long-day photoperiod by using TRI REAGENT® according to the manufacturer's manual (Molecular Research Center, Inc., Cincinnati, OH). Swollen stolons and tubers were harvested from short-day plants. For Figure 5B, RNA was extracted from leaves and stolons that were harvested from the photoperiod-responsive species *Solanum tuberosum* ssp. *andigena* grown under a short-day photoperiod. Total RNA was size-fractionated via electrophoresis through a 1.4% agarose gel that contained 5.0 mM methyl-mercury hydroxide and transferred onto a MagnaGraph nylon membrane (Micron Separations Inc., Westboro, MA). Hybridization and washing conditions were the same as described by Kolomiets et al. (2001). For autoradiography, membranes were exposed to X-ray film with intensifying screens for three to six days at -80° C. A 1.2 kb wheat 18S ribosomal RNA probe was used to confirm uniform loading of RNA for the blots in Figure 5.
Blots presented are representative examples of at least two independent experiments.

**Plant transformation**

Transformation and regeneration of plants was undertaken on leaf sections from *Solanum tuberosum* ssp. *andigena* as described by Liu et al. (1995). The sense constructs were made from a 2.0 kb fragment from the StBEL-5 cDNA or the full-length POTH1 sequence and cloned into the binary vector pCB201 (Xiang et al., 1999) driven by the constitutive CaMV-35S promoter. Constructs were checked by using PCR with clone-specific primers. Positive recombinants were transferred to the *Agrobacterium tumefaciens* strain GV2260 by using the procedure of direct transformation (An et al., 1988). Control plants in the tuberization study were *andigena* plants regenerated in vitro. Functional transformants were identified by PCR analysis of genomic DNA and by detection of the accumulation of sense transcripts of StBEL-5 or POTH1 in shoot tip samples. From among these positives, the eight independent transformants (lines 11, 12, 14, and 19 for StBEL-5 and lines 11, 15, 18, and 29 for POTH1) used in this study were selected on the basis of abundant accumulation of sense mRNA in shoot tips.
Evaluation of tuber formation

For in vitro tuberization, cultured transgenic plants were grown on a Murashige and Skoog medium with 6.0 % sucrose under a long-day photoperiod (16h light, 8h dark) in a growth chamber for two weeks and then transferred to a short-day photoperiod (8h light, 16h dark). For tuber induction, plants were evaluated daily for tuber formation. Soil-grown plants were grown in 10-cm pots under long days until they reached the 16-leaf stage and then transferred to short days. After 14d under short days, plants were evaluated for tuber formation.

Acknowledgements

We thank Uwe Sonnewald for the stolon two-hybrid library and Makoto Matsuoka for providing us with the NTH cDNAs. We also thank Phil Becraft and Dan Voytas for critical reviews of the manuscript and Harry Van Onckelen for performing the cytokinin analysis.

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Zhu Y, Tepperman JM, Fairchild CD, Quail PH (2000) Phytochrome B binds with greater affinity than phytochrome A to the basic helix-loop-helix factor PIF3 in a reaction requiring the PAS domain of PIF3. Proc Natl Acad Sci USA 97: 13419-13424
Table I. Rate of tuberization in vitro for overexpression lines of StBEL5. Plants were grown under short days (8 h light, 16 h dark) on nutrient media supplemented with 6.0% sucrose for 21 d and then evaluated for tuber formation. Twenty-four plants per independent transgenic line were evaluated for tuber formation, thirty-five for WT. Tuber yield plant\(^{-1}\) for the StBEL overexpressers was determined by dividing the total fresh weight of tubers harvested after 21 d by the number of plants per line.

<table>
<thead>
<tr>
<th>Plant line</th>
<th>No. tubers plant(^{-1})</th>
<th>Tuber yield plant(^{-1}) (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>0.4</td>
<td>18</td>
</tr>
<tr>
<td>StBEL5-6</td>
<td>0.4</td>
<td>28</td>
</tr>
<tr>
<td>StBEL5-11</td>
<td>0.6</td>
<td>18</td>
</tr>
<tr>
<td>StBEL5-12</td>
<td>1.0</td>
<td>95</td>
</tr>
<tr>
<td>StBEL5-14</td>
<td>1.3</td>
<td>292</td>
</tr>
<tr>
<td>StBEL5-19</td>
<td>0.9</td>
<td>50</td>
</tr>
</tbody>
</table>

Table II. Rate of tuberization for overexpression lines of StBEL5 under soil-grown, short-day conditions. Plants were grown in 10-cm pots under long days (16 h light, 8 h dark) until they reached the 16-leaf stage and then transferred to short days (8 h light, 16 h dark). After 14 d under short days, four plants per independent line were evaluated for tuber formation. Standard errors of the mean are shown.

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Number tubers plant(^{-1})</th>
<th>Tuber yield plant(^{-1}) (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>2.2 ± 1.4</td>
<td>1.4 ± 0.9</td>
</tr>
<tr>
<td>StBEL5-12</td>
<td>8.0 ± 0.8</td>
<td>5.4 ± 1.3</td>
</tr>
<tr>
<td>StBEL5-14</td>
<td>8.3 ± 0.9</td>
<td>4.6 ± 1.3</td>
</tr>
<tr>
<td>StBEL5-19</td>
<td>11.5 ± 2.1</td>
<td>4.7 ± 1.4</td>
</tr>
</tbody>
</table>
Table III. Cytokinin content (picomoles g fr wt⁻¹) in shoot tips of POTH1 and STBEL5 overexpression lines grown under long days (16 h light, 8 h dark) in the greenhouse. Wildtype is nontransformed *Solanum tuberosum* ssp. *andigena*. Zeatin types include zeatin, zeatin riboside, dihydrozeatin, and dihydrozeatin riboside. Isopentenyl types include isopentenyl and isopentenyladenine. Standard error was calculated on three replicates.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zeatin types</th>
<th>Isopentenyl types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>10.5 ± 1.0</td>
<td>12.0 ± 1.5</td>
</tr>
<tr>
<td>POTH1-15</td>
<td>42.5 ± 15</td>
<td>35.5 ± 7.0</td>
</tr>
<tr>
<td>POTH1-29</td>
<td>34.0 ± 12</td>
<td>30.0 ± 6.0</td>
</tr>
<tr>
<td>STBEL5-11</td>
<td>55.5 ± 30</td>
<td>31.5 ± 11</td>
</tr>
<tr>
<td>STBEL5-20</td>
<td>30.5 ± 6.0</td>
<td>29.5 ± 6.5</td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1.** Specific interaction of POTH1 with seven BEL-like proteins of potato.

A) Selection on a nutrient carbon medium minus histidine, leucine, tryptophan, and adenine. The pAD plasmid provides leucine selection, the pBD plasmid (pBridge) provides tryptophan selection, and histidine and adenine selection are activated from the host strain (AH109) chromosomal DNA. The asterisk (*) designation indicates yeast growth with both plasmids transformed together, whereas the pAD plasmids (designated 5, 11, 13, 14, 22, 29, 30) are transformed alone (no growth). SIR4 is a positive control and pBHD is POTH1 in pBridge alone. B) POTH1 interacts with all seven BELs as determined by a quantitative yeast two-hybrid assay. LacZ induction in the yeast strain AH109 was assayed in transformed yeast cultures using a quantitative yeast β-galactosidase assay method (Pierce Chemical Company). For each pair, the dark bars on the left represent the pAD or pBHD plasmid alone transformed into yeast. The white bars on the right in each pair represent both plasmids (pAD and pBHD) transformed together. The standard error of the mean is represented by error bars. C) Immunoprecipitates of the in vitro binding of POTH1 to BEL proteins of potato. 35S-labeled GAD: POTH1 fusion protein and the three BEL1 proteins (p11Z-5, -13, and -30) were synthesized in separate in vitro transcription/translation reactions (lanes 2, 3, 6, and 9, respectively). Each of the three BEL1 proteins were incubated with the GAD:POTH1 protein and
immunoprecipitated with anti-GAD antibodies (lanes 5, 8, and 11). None of the three BEL1 proteins bound to the GAD protein alone (lanes 4, 7, and 10). Labelled proteins were visualized by autoradiography after separation by SDS-PAGE. Molecular size markers are shown on the right.

**Figure 2.** Phylogenetic tree of the BEL-like proteins of potato (*Solanum tuberosum* L.). The amino acid sequence of the seven known potato BEL-like proteins was analyzed and compared to BEL proteins of plants. These data were organized into a phylogenetic tree with the ME-Boot program of the MEGA package (Kumar et al., 1993) and the neighbor-joining program (Saitou and Nei, 1987). The numbers listed at the branching points are boot-strapping values which indicate the level of significance (%) 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. Databank accession numbers are listed on the dendrogram and the common name of the species is listed in the right-hand column.

**Figure 3.** A) Schematic of the amino acid sequence of the BEL-like proteins of potato. Boxed regions represent conserved sequences identified by aligning all seven BELs. Helices I, II, and III of the homeodomain are designated. The proline-tyrosine-proline (PYP) loop extension is located between helices I and II. B) Predicted helices of the putative protein binding region (BELL domain) of the BEL1 protein StBEL-5.
The bold letters represent amino acids conserved in other plant BEL1 proteins based on a BLAST analysis of StBEL-5. The underlined portion of the sequence represents a predicted α-helix. A consensus for the prediction of the sequence structure was derived by using three software programs for amino acid sequence analysis: sspal, ssp, and nnssp (http://www.softberry.com/protein.html). Four deletion constructs from Figure 6B are designated with arrows. Construct pAD5-1 contains aa 230 through 653 of pAD-05 (interaction with POTH1), and pAD5-2 contains aa 257 through 653 of pAD-05 (no interaction). Construct pAD5-11 consists of aa 1 through 286 of pAD-05 (no interaction), and pAD5-9 consists of aa 1 through 315 (interaction with POTH1).

**Figure 4.** Southern blot analysis of BEL-like genes of potato. Genomic DNA (10 μg per lane) was digested with EcoRI, HindIII, and PstI. Each blot was hybridized with a 32P-labeled gene-specific probe from each of the four StBEL cDNAs. DNA size markers in kilobases are indicated on the right.

**Figure 5.** A) Northern blot analysis of the accumulation of mRNA for four BEL-like cDNAs (StBEL-5, -13, -14, and -30) in potato organs. Ten μg of total RNA from flowers, shoot tips (SAM), leaves, stems, roots, unswollen stolons (U stolon), swollen stolons (S stolon), and tubers were loaded per lane. A probe for the 18S ribosomal RNA was used to verify equal loading of RNA samples (bottom panel). B) Northern
blot analysis of the accumulation of the mRNA of StBEL-5 in leaves and stolons of WT plants grown under long days (LD, 16 h light, 8 h dark) and short days (SD, 8 h light, 16 h dark). Ten µg of total RNA from stolons were loaded per lane. Leaves and stolons were harvested from the photoperiod-responsive potato species, Solanum tuberosum ssp. andigena, 4 and 8 days after the plants were transferred to short-day conditions. A gene-specific probe for each BEL cDNA was used.

Figure 6. Deletion analysis of the binding regions of POTH1 and a potato BEL-like protein using the yeast two-hybrid system. A) Deletion constructs of POTH1 in pBridge were tested for expression in the yeast strain AH109 and cotransformed with the full-length BEL cDNA, StBEL-5, in pGAL4 to test for interaction. B) Deletion constructs of StBEL-5 in pGAL4 were cotransformed with the full-length cDNA of POTH1 in pBridge. Interaction was verified with both nutritional selection and β-galactosidase activity. The white box indicates the homeodomain. The gray box indicates the putative protein/protein interaction region (For POTH1, this is the conserved KNOX domain, for StBEL-5, the BELL domain). The black boxes are conserved sequences identified in the BEL proteins (see Figure 3A) and the diagonal hatched boxes in POTH1 represent the ELK domain. The numbers in parentheses represent the aa of the full-length sequence included in each construct.
Figure 7. In vitro tuberization of transgenic plants that overexpress sense transcripts of *StBEL-5*. Northern blot analysis for the accumulation of mRNA for *StBEL-5* was performed by using 10 μg of total RNA/lane from vegetative meristems of in vitro plantlets and gene-specific probes for *StBEL-5*. (A). Equal loading of RNA samples was verified by visualizing ethidium bromide-stained rRNA bands with UV light (not shown). The rate of tuberization (days to tuberize) was determined by the first appearance of tubers from among twenty-four replicates (B). The number of tubers was scored after 2 weeks of LD conditions (0d), and after 7 (7d) and 14 d (14d) of SD conditions (B). Tubers were harvested after 21d (C) from the *StBEL* overexpression lines (24 plants each) and WT (35 plants). Cultured transgenic plants of *Solanum tuberosum* ssp. *andigena* were grown on a Murashige and Skoog medium with 6.0 % sucrose under a long-day photoperiod (16 h light, 8 h dark) in a growth chamber for two weeks. For tuber induction, plants were transferred to an M and S medium supplemented with 6.0 % sucrose and evaluated daily for tuber formation under a short-day photoperiod (8 h light, 16 h dark) in the growth chamber until tubers formed. All numbered lines were verified as transgenic by using PCR with transgene-specific primers. Control plants were both nontransgenic (WT) and transgenic (*StBEL-5* line 6).
Figure 8. Overexpression mutant lines for the potato KNOX gene, POTH1 (lines 18 and 15), and for the BEL-like protein, StBEL5 (lines 14 and 19). Other than the enhanced capacity for tuber formation, these StBEL5 sense lines have a phenotype similar to wildtype (WT) plants. These are 8-week plants grown under long-day conditions in the greenhouse. The StBEL5 plants ranged in height from 34 to 39 cm, whereas, the POTH1 lines were 7 to 10 cm in height.

Figure 9. Northern blot analysis of the accumulation of the mRNA of the GA20 oxidase1 gene of potato (Carerra et al., 1999) in WT plants and sense lines 11, 12, and 20 of StBEL5. Total RNA was extracted from the 2.0 mm distal tip of stolons from plants grown under LD conditions (16 h light, 8 h dark). WT RNA was extracted from two separate pools. Ten μg of total RNA were loaded per lane. A gene-specific probe for GA20 oxidase1 was used for hybridization. These StBEL5 lines all exhibited enhanced tuber formation.
Figure 1A, B.
<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td>GAD</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GAD/POTH1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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</table>

**Figure 1C.**
Figure 2.
A.

**SKY box**

**BELL domain**

120-124 aa

**Homeodomain**

I  

II  

III

Met

---

---

PYR


B.

**pAD5-1**

N-terminus ---- SSRQ KNEVAELT *TAQRQELQMKKAKLAMLEEVEQRYRQ*

**pAD5-2**

**pAD5-11**

**pAD5-9**

YHHQMQIIIVLSFEQVAGIGS AKSYTQLALHAISQFRCLKDAIAEVKAT SKSL

**GEEGLGGKIEGS RLKFVDHHLRQQRALQQI GMMQ** ---- C-terminus

Figure 3.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>StBEL5</th>
<th>StBEL13</th>
<th>StBEL14</th>
<th>StBEL30</th>
</tr>
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<tbody>
<tr>
<td>EcoRI</td>
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Figure 4.
Figure 5A.
Figure 5B.
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Figure 6.
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* from twenty-four plantlets per line, thirty-five for WT

Figure 7A, B.
Tuber yields after 21d for StBEL-5 sense lines

Figure 7C.
Figure 8.
Figure 9.
CHAPTER 3. THE TANDEM COMPLEX OF BEL AND KNOX PARTNERS IS REQUIRED FOR TRANSCRIPTIONAL REPRESSION OF GA20OX1

A paper submitted to *The Plant Cell*

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SUMMARY

Homeodomain (HD) proteins play an important role in cell fate determination and body plan definition by binding to regulatory regions of key target genes. Two interacting atypical HD three amino acid loop extension (TALE) proteins of potato (*Solanum tuberosum* L.), StBEL5 and POTH1, mediate developmental processes by regulating phytohormone levels. Overexpression of either partner alone increased tuber yield and with StBEL5, enhanced the rate of growth. Gel-shift assays demonstrate that StBEL5 and POTH1 bind to the regulatory region of *ga20ox1* from potato, a gene that encodes a key enzyme in the GA biosynthetic pathway. In tandem, StBEL5 and POTH1 had a greater binding affinity for the *ga20ox1* promoter than either alone. The StBEL5-POTH1
heterodimer bound specifically to a composite sequence

\((T/A)GA(C/G)(T/A)(T/A)GAC\) containing two TGAC cores. Using a
transcription assay with a GUS marker, StBEL5 and POTH1 alone suppressed
the activity of the \(ga20ox1\) promoter by more than 50%, in tandem, 80%.

Dominant negative constructs containing amino-terminal protein binding
domains of StBEL5 or POTH1 blocked the repression activity of StBEL5 or
POTH1, respectively. The mutated \(ga20ox1\) promoter that could be bound by
StBEL5 or POTH1 individually but not by the StBEL5-POTH1 heterodimer also
abolished the repression activity of StBEL5, POTH1, and the StBEL5-POTH1
heterodimer. These results suggest that the tandem interaction of StBEL5 and
POTH1 is essential for regulation of the expression of their target gene.

**INTRODUCTION**

Since the first identification of conserved homeobox sequences in
homeotic genes of *Drosophila* (McGinnis et al., 1984; Scott and Weiner, 1984),
numerous homeobox genes have been found in fungi, animals, and plants.
Proteins encoded by homeobox genes comprise a large superfamily of
eukaryotic DNA-binding proteins that regulate the transcription of numerous
important genes in development (Geerts et al., 2003; Gehring et al., 1994a;
Leemans et al., 2001). Genetic analyses have shown that homeobox genes play
crucial roles during development to define the body plan and to determine cell fate (Hughes and Kaufman, 2002; Ito et al., 2002; Krumlauf, 1994). For example, the difference between executing a pathway that results in a leg or an antenna in *Drosophila* is dependent on whether the homeobox Antennapedia protein is present or not (Mann and Chan, 1996). The first plant homeobox gene to be cloned was *KNOTTED1* (*KN1*) from maize (Vollbrecht et al., 1991). Loss-of-function mutants implicated class I *knox* genes in the determination of cell fate and in patterning in the meristem, whereas gain-of-function mutants illustrated *KN1*'s capability to alter plant morphology profoundly (Reiser et al., 2000).

The homeobox encodes a DNA-binding region designated the homeodomain (HD) that is structurally related to the bacterial helix-turn-helix motif (Scott et al., 1989). The typical homeobox is 180 bp long and encodes a HD of 60 amino acids. X-ray crystallographic and nuclear magnetic resonance spectroscopic studies revealed that HDs contain three helical regions that are folded into a compact, globular structure, with the third helix binding to the major groove of DNA (Gehring et al., 1994b). There are also atypical homeodomain proteins with more or less than 60 amino acids in the homeodomain region. One group of such proteins is called the TALE (three amino acid loop extension) superclass because of the three conserved residues
proline-tyrosine-proline located between the first and second α-helix of the HD (Burglin, 1997). There are two groups of TALE HD proteins in plants: KNOX and BEL1-like proteins.

Several studies have implicated the involvement of KNOXs in regulating the level of gibberellin (GA). Overexpression mutants of the knox genes, NTH15 or OSH1, in tobacco plants exhibited decreased GA1 and GA20 levels, and increased GA19 level (Kusaba et al., 1998a; Tamaoki et al., 1997). Correspondingly, mRNA of GA20 oxidase, the gene encoding the enzyme that catalyzes the conversion from GA19 to GA20, was down regulated in these mutants (Kusaba et al., 1998b; Tanaka-Ueguchi et al., 1998). Hay et al. (2002) showed that SHOOT MERI5TEMLESS, a KNOX protein, inhibits GA synthesis in the meristem and that other KNOX genes of tomato are implicated in the regulation of leaf architecture. Overexpression of POTH1 in potato also exhibited a GA-deficient phenotype, including dwarfism, malformed leaves, and enhanced tuber activity. Consistent with such GA-deficient phenotypes, overexpression mutants of POTH1 exhibited reduced levels of bioactive GA and GA20 oxidase mRNA (Rosin et al., 2003). Direct evidence for KNOX-mediated repression of GA20 oxidase gene expression was demonstrated for NTH15 of tobacco (Sakamoto et al., 2001). Using a transcription assay, NTH15, a KNOX
protein of tobacco, repressed the transcriptional activity of the *GA20 oxidase* promoter by binding to the first intronic sequence. This consensus intronic sequence acted as a *cis*-element to repress reporter gene activity in protoplasts.

Interaction between KNOX and BEL1-like proteins has been reported in barley (Müller et al., 2001), *Arabidopsis* (Bellaoui et al., 2001; Byrne et al., 2003; Smith and Hake, 2003), and maize (Smith et al., 2002). Seven BEL1-like proteins that interacted with *POTH1* also have been identified in potato (Chen et al., 2003). Similar to other BEL1 proteins, all seven contained the conserved HD and BELL domains. Overexpression of one of these *BELs*, *StBEL5*, produced plants that exhibited enhanced growth and tuber activity. This phenotype was accompanied by decreased *GA20ox1* mRNA levels in stolon tips and increased cytokinin levels in shoots (Chen et al., 2003). Overexpression of an apple BEL1-like gene (*MDHT*) in *Arabidopsis* (Dong et al., 2000), or overexpression of a barley BEL cDNA (*JuBel2*) in tobacco (Müller et al., 2001) produced dwarf plants with malformed floral organs, suggesting that BEL1-like proteins may also be involved in the regulation of GA biosynthesis.

To understand the mechanism of these interacting transcription factors (TFs) in regulating potato development, DNA-protein binding assays with the regulatory sequences of the potato *ga20ox1* gene were performed. To examine
the effect of the binding of StBEL5 and POTH1 to \textit{ga20ox1} promoter sequence, promoter activity was quantified by using a transcriptional assay system in tobacco protoplasts. Our results showed that cooperative interaction between the TALE TFs, StBEL5 and POTH1, directly represses \textit{ga20ox1} promoter activity by binding to a specific promoter sequence.

**RESULTS**

**StBEL5 and POTH1 bind to the regulatory regions of \textit{ga20ox1}**

Recombinant StBEL5 protein expressed from \textit{E. coli} retarded the mobility of all three promoter sequences and the first intron (Fig. 1a-c). POTH1 formed a complex with P1, but not with P2, P3 or the first intron. StBEL5 and POTH1 together produced a supershifted band with P1, which had stronger signal intensity and migrated much slower than either the StBEL5-P1 or POTH1-P1 complexes (Fig. 1b). Competition assays were performed with labeled P1 and unlabeled P1 or unlabeled P3. With increased unlabeled P1, the P1-StBEL5 complex quickly diminished (Fig. 2a). With unlabeled P3, however, even at a concentration 100-fold more than labeled P1, the shifted band was still present (Fig. 2a). Unlabeled P1 also reduced the P1-POTH1 complex formation, but unlabeled P3 had no effect on the P1-POTH1 complex (Fig. 2b). Consistent with the increased signal intensity of the StBEL5-POTH1-P1 complex, the dissociation rate of this complex was much slower than
either the StBEL5-P1 or POTH1-P1 complexes (Fig. 3). Even after a 30 min incubation, substantial association of the tandem complex to P1 was still evident, compared to only trace amounts for either protein alone. Although StBEL5 could bind to P2, P3, and the intron fragments, there was no supershifted band formed when both StBEL5 and POTH1 were incubated with these three DNA fragments (Fig. 1b-c). These results suggest that the binding of both StBEL5 and POTH1 to the P1 DNA fragment is required for StBEL5-POTH1-P1 complex formation. Based on these results, at least two TALE homeodomain binding sites may be present in P1.

In support of this premise, excessive amounts of a truncated protein containing only the HD portion of StBEL5 produced a supershifted band similar to the POTH1-StBEL5-P1 complex (Fig. 4). Apparently, there are two binding sites recognized by StBEL5 in P1. No supershifted band was detected, however, when P1 was incubated with excessive amounts of either full-length StBEL5 or POTH1 alone (data not shown). This suggests that the two binding sites in P1 (345 nt in length) are in close proximity to one other and that two full-length StBEL5 molecules cannot bind to both sites at the same time because of size constraints.

The StBEL5-POTH1 heterodimer binds specifically to the (T/A)GA(C/G)(T/A)(T/A)GAC site

DNase I footprinting identified a 20-bp region protected from digestion (Fig.5). The greatest degree of protection was displayed in the reactions containing
both proteins (POTH1 + StBEL5 lanes). This putative StBEL5-POTH1 heterodimer binding site, TTGACTTGAC, is similar to the Arabidopsis KNOX-BEL heterodimer binding site TGACAG(G/C)T (Smith et al., 2002) and the TGAC binding core confirmed for MEINOX proteins (Smith et al., 2002; Tejada et al., 1999).

Oligonucleotides with serial point mutations across this site were used as probes in gel-retardation assays in the presence of StBEL5, POTH1, or both. Point mutations across this site (Fig. 6a) did not affect the binding of either StBEL5 or POTH1 alone (data not shown.). With the exception of T in the fifth position, all other mutations in TGACTTGAC either reduced or abolished the binding by StBEL5-POTH1 heterodimer (Fig. 6b). Single point mutations at positions 2 and 3 (m4 and m5, Fig. 6a) and at postions 7, 8, and 9 (m9, -10, and -11, Fig. 6a) completely abolished binding to the heterodimer (Fig. 6b). Based on these cumulative results, the consensus sequence of the StBEL5-POTH1 heterodimer binding site was identified as (T/A)GA(C/G)(T/A)(T/A)GAC.

Repression of ga20ox1 promoter requires the interaction of StBEL5 and POTH1

POTH1 encodes for a 345-residue protein estimated to have a mass of 37.95 kDa. The coding sequence of the protein includes the 97-aa KNOX domain and the 64-aa homeodomain consisting of three helices (Fig. 7a). The KNOX domain of POTH1 contains two conserved regions, designated Knox I and II. The Knox II
region mediates interaction with protein partners (Chen et al., 2003; Bellaoui et al., 2001; Müller et al., 2001). StBEL5 is 688 aa in length with an estimated mass of 75.68 kDa. The coding sequence of StBEL5 contains the conserved SKY box, BELL domain, homeodomain, and the proline-tyrosine-proline (P-Y-P) loop between helices I and II (Fig. 7b). The BELL domain is 120 aa in length and the HD of StBEL5 is 64 aa. Regions of the BEL domain mediate interaction with protein partners (Chen et al., 2003; Bellaoui et al., 2001; Müller et al., 2001).

To examine the effect of StBEL5 and POTH1 on ga20ox1 transcription, we utilized a protoplast assay with a marker gene. GUS, driven by a CaMV enhancer and the ga20ox1 promoter, was used as the marker and luciferase as an internal control. The various constructs and vectors used in this assay are shown in Figure 8a. When co-transfected with the effector p35S::StBEL5, p35S::POTH1, or both, relative GUS-LUC activity of the pGAOP::GUS reporter construct decreased by more than half (Fig. 8b, left side). Neither StBEL5 nor POTH1 showed any effect on the activity of the CaMV 35S promoter (Fig. 8b, right side). To eliminate the possibility that endogenous BEL1-like or KNOX proteins cooperatively interact with POTH1 or StBEL5, respectively, truncated forms of StBEL5 and POTH1, StBEL5ΔC295 and POTH1ΔC122 (Fig 9a), were generated to use as dominant negatives in the transcription assays. StBEL5ΔC295 and POTH1ΔC122 contain the
intact protein-binding domain, but lack the carboxy-terminal region including the homeodomain. StBEL5ΔC295 and POTH1ΔC122 can interact with endogenous KNOX or BEL1-like proteins, respectively, but cannot bind to DNA. Such heterodimers are not functional due to the lack of the homeodomain from the truncated proteins. In transcription assays with pGAOP::GUS as reporter, StBEL5ΔC295 had little effect on the activity of the ga20ox1 promoter (Fig. 9b, BEL). As expected, StBEL5 alone repressed expression (Fig. 9b, StBEL5). When co-transfected with StBEL5, StBEL5ΔC295 abolished almost all of the repression activity of StBEL5 (Fig. 9b, BEL + StBEL5). POTH1ΔC122 had a similar effect on the repression activity of POTH1 (Fig. 9b, right side).

The binding site in the ga20ox1 promoter acts as a cis-element for the repression by the StBEL5-POTH1 heterodimer

To investigate whether the StBEL5-POTH1 binding site identified through EMSA studies functions as a cis-element, a reporter construct with a point mutation (G → C) in the binding site was used for the transcription assay (Fig. 10a). The G in the seventh position of the TGACTTGAC motif was selected for the mutation because this is one of the bases that were critical for binding by StBEL5-POTH1 heterodimer, but not for individual StBEL5 or POTH1 protein binding (Fig. 6). Constructs containing this single mutation exhibited no detectable repression of
promoter activity when co-transfected with either StBEL5, POTH1, or both (Fig. 10b).

**DISCUSSION**

Cooperative interaction between StBEL5 and POTH1 mediates binding affinity for the ga20ox1 promoter

This study examines the mechanism for the StBEL5-POTH1 heterodimer in regulating gene expression and plant growth. Previous studies have shown that these two TALE TFs have a major impact in controlling the development of potato (Rosin et al., 2003; Chen et al., 2003). Genetic analyses showed that by mediating hormone levels, both partners enhanced yields and in the case of StBEL5, increased the rate of growth (Chen et al., 2003).

Gel-retardation assays showed that both StBEL5 and POTH1 bound to the promoter region of the potato ga20ox1 gene. StBEL5 could also bind with the first intron sequence (Fig. 1). Unlabeled P3 competed with the StBEL5-P1 complex, but not as effectively as unlabeled P1 (Fig. 2a), whereas P3 had no competition effect with the POTH1-P1 complex (Fig. 2b). These results indicated that the interaction between these two TALE HD proteins and P1 was specific and that StBEL5 bound to P1 more strongly than to P3. It is highly likely then that P1 contains the cis element that functions with this protein complex in planta. The tobacco KNOX protein,
NTH15, binds to both the promoter and the first intron of \textit{GA20 oxidase}, but with higher affinity to the first intron (Sakamoto et al., 2001). NTH15 is not the tobacco ortholog of POTH1 and this may explain the disparity in binding specificities. No tobacco BEL partners were tested for binding with the tobacco KNOX protein or the \textit{GA20 oxidase} promoter.

Consensus binding sites for KNOX proteins have been identified in the target gene promoter for tobacco (Sakamoto et al., 2001) and by in vitro binding site selection for barley (Krusell et al., 1997) and rice (Nagasaki et al., 2001). Because the homeodomains of these KNOX proteins are almost identical, it is not surprising that the consensus sequences recognized by them share a core motif, TGTCAC (Nagasaki et al., 2001). Two interacting TALE proteins of vertebrates, Meis1 and Pbx1, dimerize and bind to the composite DNA sequence, TGATTGACAG, containing 5'-Pbx and 3'-Meis half sites (Chang et al., 1997). The consensus sequence, TGACAG(G/C)T, was reported for the \textit{Arabidopsis} BEL-KNOX heterodimeric complex (Smith et al., 2002). Because the StBEL5-POTH1-P1 transcription complex requires both proteins to bind the target DNA, and increased amounts of the StBEL5 homeodomain lead to a supershifted band, it is probable that there are two closely located TALE homeodomain binding sites in the P1 region similar to the two half binding sites for Meis1 and Pbx1 (Chang et al., 1997). Based on these results and
those from the DNase protection assay (Fig. 5), a potential StBEL5-POTH1 binding site, TTGACTTGAC, has been identified in the P1 fragment. Gel-retardation assays confirmed that this oligo was sufficient for binding to StBEL5, POTH1, and the StBEL5-POTH1 complex (Fig. 6). Mutational gel-retardation analysis of this BEL-KNOX binding site showed that the StBEL5-POTH1 heterodimer recognizes the 9-bp sequence, $\text{(T/A)GA(C/G)(T/A)(T/A)}\text{GAC}$, containing two TGAC cores. StBEL5 and POTH1 could bind to either one of the TGAC cores, because serial mutations had no effect on the DNA-binding ability of StBEL5 or POTH1 (data not shown). This is consistent with the prediction of Tejada et al. (1999) on the composition of the core motif. Based on highly invariant key amino acids, such as Asn51, Ile50, and Asn47, in the third $\alpha$-helix of the homeodomain region of BEL, CUP, Hac-ATYP, Kn, KNOX, and MEIS proteins (Burglin, 1997), it was proposed that proteins from the TALE superclass (including BEL and KNOX types) would have a binding affinity for the TGAC core (Tejada et al., 1999).

It has been a paradox for HD proteins regarding their high level of functional specificity in directing developmental programs and their high degree of redundancy in binding site specificity (Pellerin et al., 1994). Besides the low affinity and high redundancy in binding sites, the 5-base consensus sequences recognized by HD proteins randomly occur every 1.0 kb in eukaryotic genomes (Mann and Chan,
Therefore, it is likely that interaction with other DNA-binding transcription factors is necessary for HDs to affect binding affinity and specificity. Monomeric HD proteins have modest specificity for DNA binding (Laughon, 1991), but their specificity is greatly increased through cooperative binding with other DNA binding partners (Mann and Chan, 1996). In humans, the TALE TF, Prep1, forms a stable complex with Pbx independent of DNA binding. Heterodimerization of Prep1 and Pbx results in a strong DNA binding affinity towards the TGACAG target site of the urokinase plasminogen activator promoter (Berthelsen et al., 1998a and b). A Pbx1 and Prep1 heterodimer are also required to generate a strong transcriptional activation of the somatostatin gene promoter (Goudet et al., 1999). EXD binds cooperatively with HOM-C proteins to DNA target sites, thereby increasing their DNA binding specificity (Mann and Chan, 1996).

Our EMSAs showed that StBEL5 and POTH1 in tandem formed a complex with P1 with greater stability than either the POTH1-P1 or StBEL5-P1 complexes (Fig. 1), and that the StBEL5-POTH1-P1 complex had a slower dissociation rate (Fig. 3). Together these results indicate that the BEL-KNOX heterodimer has a greater binding affinity for the target element than either partner alone.
The StBEL5-POTH1 heterodimer mediates the repression of the ga20ox1 promoter

Previous studies showed that both StBEL5 and POTH1 overexpression mutants exhibited decreased ga20ox1 mRNA levels in stolons and leaves, respectively (Chen et al., 2003; Rosin et al., 2003). Gel-retardation assay results showed that these two transcription factors bound to the promoter and that StBEL5 could also bind to the first intron of ga20ox1. These results suggest that StBEL5 and POTH1 may directly repress ga20ox1 transcription by binding to its promoter region. Results from the transcription assay showed that either StBEL5 or POTH1 alone could repress reporter gene activity by more than 50%. That neither POTH1 nor StBEL5 affected CaMV 35S promoter activity (Fig. 8) confirmed that such repression was not due to inhibition of the general transcription machinery. Direct repression of GA20 oxidase gene transcription by the KNOX protein NTH15 alone has also been reported in tobacco (Sakamoto et al., 2001).

Although either StBEL5 or POTH1 could repress ga20ox1 promoter in the transcription assay, it is highly possible that KNOX-BEL heterodimers were still formed with endogenous partners to function in tobacco protoplasts. There are three lines of evidence to support this possibility. First, of the seven BEL proteins identified in potato, all seven interacted with four tobacco KNOX proteins (Chen et al., 2003). Although, to date, no BEL1-like TFs have been identified in tobacco, their
existence is almost a certainty. Second, the protein binding domains of the tobacco
KNOX NTHs were most important in determining the severity of transgenic plant
phenotypes (Sakamoto et al., 1999), implying that interaction with protein partners,
most probably the BEL1-like proteins, is essential for KNOX function. Third, the
identification of BEL-KNOX binding sites (Smith et al., 2002) and the StBEL5-POTH1
binding site in this study, further supports the premise that the BEL-KNOX
heterodimer is involved in the regulation of target genes. In our transcription
assays, constructs of the dominant negatives, StBEL5ΔC295 or POTH1ΔC122,
abolished the repression activity of StBEL5 or POTH1, respectively (Fig. 9).
Therefore, StBEL5 or POTH1 alone are not sufficient for the repression of ga20ox1
promoter. The BEL-KNOX heterodimeric complex is required for repression of
transcription to occur.

Actually, it is very common for animal HD proteins to interact with each
other to control the regulation of their target genes. The interaction of the TALE
proteins, EXD or PBX, with HOX proteins is required for the specific control of the
transcription of their target genes (Pinsonneault et al., 1997; Saleh et al., 2000;
Sanchez et al., 1997). Sometimes, even a trimeric complex of homeodomain proteins
is essential for the modulation of the activity of HOX target genes (Berthelsen et al.,
1998b; Kobayashi et al., 2003; Ryoo et al., 1999; Shen et al., 1999).
Unlike POTH1, NTH15 of tobacco binds to the first intron of GA20 oxidase \((Ntc12)\) with greater affinity than to the promoter of this gene. The first intron also acts as a \textit{cis}-element in planta. A mutation in the binding site of the first intron blocked NTH15's capacity to repress mRNA accumulation of \(Ntc12\) in the SAM (Sakamoto et al., 2001). Our results showed that the mutated P1 binding site of the \textit{ga20ox1} promoter did not respond to StBEL5-POTH1-mediated repression (Fig. 10), suggesting that this binding site functions as a \textit{cis}-element for the StBEL5-POTH1 heterodimer. Based on the results from gel-retardation analysis of serial mutations in this site, the mutated promoter was capable of binding with StBEL5 or POTH1 separately, but not the StBEL5-POTH1 heterodimer. Here is further evidence that it is the BEL-KNOX heterodimer and not the individual BEL or KNOX proteins that affect repression.

The interaction of the StBEL5-POTH1 complex to its binding motif is summarized in the model of Figure 11. The third \(\alpha\)-helix of StBEL5 and POTH1 fits into the major groove of TTGACTTGAC site, with each helix occupying a half site. The partner proteins interact through conserved protein binding domains. For StBEL5, this includes the two amino-terminal helices of the BELL domain and the sky box (Chen et al., 2003). For POTH1, this includes the KNOX domain with Knox II playing the most significant role (Sakamoto et al., 1999). The SKY box also
contributes to the tandem formation and interacts weakly with Knox I (Chen et al., 2003). Interaction between the respective protein binding domains and the spatial arrangement of the first two helices of the homeodomain bring the third helices of both TFs together. Specificity is then provided within the spatial constraints of the three components (StBEL5, POTH1, and the DNA target) through recognition of the binding motif. DNA-binding specificity is then imparted through charged contacts of the phosphate backbone with conserved amino acids in the third helix (Tejada et al., 1999). In this case, the BEL/KNOX complex may repress transcription by interfering with the binding of critical components of the transcriptional machinery.

In Drosophila, even-skipped (EVE), the most extensively studied HD repressor protein, represses target genes by at least three mechanisms: interacting with TATA binding protein and thereby preventing the formation of TFIIID/TATA box complex (Austin and Biggin, 1995); interacting with histone deacetylase and thereby inducing chromatin condensation (Mannervik and Levine, 1999); and recruiting certain corepressors to achieve its potent repressive activity (Zhang et al., 2002). Other BEL/KNOX complexes may affect gene expression differentially by recognizing other cis-elements as a result of slight modifications in protein structure or recruiting different corepressors/coactivators. Besides POTH1, five other knotted1-like proteins in the Solanaceae that interact with the seven StBELs have been identified.
The results of this study suggest that similar to HDs in animals, collaboration of HD proteins to modulate the expression of target genes also occurs in plants. The interaction of HD proteins not only enhances their DNA-binding affinity, but also imparts another level of regulation to these complexes in fine-tuning developmental processes. It is very likely that the numerous potential BEL/KNOX protein interactions participate in a comprehensive system of regulation that coordinates plant growth.

METHODS

Plant Materials

Tobacco 'Petit Havana' plants were maintained in Murashige and Skoog basal medium (1962) supplemented with 2% sucrose and incubated at 25 °C, under 16-h photoperiods for three to four weeks.

Protein Expression and Purification in E. coli

Glutathione S-transferase (GST) fusion constructs were generated by introducing full-length cDNAs of StBEL5 and POTH1 in frame with GST into the pGEX-5X-2 expression vector (Roche, Indianapolis, IN) and transformed into BL21 (DE3) E. coli cells (Stratagene, La Jolla, CA). Cells were grown at 30 °C until the OD600 reached 0.6, induced with 1.0-mM isopropyl-β-D-thiogalactopyranoside, and cultured for 5 h. The manufacturer's protocol (Roche) was followed for cell lysis.
and affinity purification by using glutathione sepharose 4B beads. The GST portion of the fusion protein was cleaved by Factor Xa protease (Promega, Madison, WI). Purified StBEL5 and POTH1 protein were frozen in liquid N₂ and stored at -80 °C.

**Gel Retardation Assay**

The first intron with partial flanking exon sequence (450 bp) of potato ga20ox1 and its promoter (981 bp, provided by Dr. Salomé Prat, CSIC Cantoblanco Campus, Univ. of Madrid, Spain) were used for gel mobility shift assays. Polymerase chain reaction (PCR) was used to amplify three regions of the promoter: -981 to 636 (P1), -660 to 307 (P2), and -331 to 0 (P3). About a 25-bp overlap was maintained between P1 and P2 or P2 and P3 in the chance that the protein-binding site would span the overlapped region. The first intron of this gene was amplified from potato genomic DNA by using PCR and the oligos 5'-GGATCCTTGAAGTGGCTCTTCTCTG-3' and 5'-AATCTAGAGACACTCTCTTTTTCGT-3' as primers. These primers were designed based on the site of the first intron of the tobacco GA20 oxidase gene NtG12. The four fragments were purified on a 1.4% agarose gel and labeled with α³²P-dATP using Klenow fragment. DNA-binding reactions were set up on ice in 20 μL containing 10-mM Tris-HCl (pH 7.5), 5% glycerol, 0.5-mM EDTA, 0.5-mM DTT, 0.05% NP-40, 50-mM NaCl, 50-ng • L⁻¹ poly (dG-dC) • poly (dG-dC) (Amersham Pharmacia Biotech, Piscataway, NJ), 100-ng protein, and 1-fmol labeled DNA. After
incubation on ice for 30 min, the reactions were resolved on a 6% native polyacrylamide gel in 1X TGE (Tris-Glycine-EDTA) buffer. The gel was dried and exposed to X-ray film.

In the competition assays, unlabeled double-stranded DNA fragments (10X, 25X, 50X, 100X) were incubated with the recombinant protein before the addition of the radioactive probe. The dissociation rates were determined by adding 500-fold more cold DNA fragments to the DNA-binding reactions that were being incubated on ice, and loaded onto the running gel every 10 min. Mutated oligos for binding sites were synthesized by the DNA Sequencing and Synthesis Facility, Iowa State University (Ames, IA).

DNase I Footprinting

The DNA-binding reactions were prepared on ice as in the gel retardation assays, but scaled up to 50 µl. After 30 min incubation, 50 µl Ca²⁺/Mg²⁺ solution (5mM CaCl₂, 10mM MgCl₂) was added into reactions, and followed by addition of 0.5 unit DNase I (Promega). After exact 1 min, DNase digestion was terminated with 100 µl stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, 100 µg/ml yeast RNA). DNA was extracted with 200 µL phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with 500 µL 100% ethanol, run on 8% acrylamide sequencing gel and detected with autoradiography. The protected regions were mapped with reference
to the migration of Maxam-Gilbert A-G sequencing products with the same DNA fragments (Maxam and Gilbert 1977).

Transcription Assay

The cauliflower mosaic virus (CaMV) 35S promoter in pBI221 (Clontech, Palo Alto, CA) was replaced by an enhancer fragment (-832 to -50) of the 35S promoter plus 980 bp of the ga20ox1 promoter to generate the pGAOP::β-glucuronidase (GUS) reporter construct. With this construct, the reporter GUS transcription level is augmented but its transcription may still be affected by the ga20ox1 promoter. A CaMV 35S promoter-driven luciferase (LUC) construct 35S-LUC (obtained from Dr. Takahashi, Dept. of Biological Sciences, Graduate School of Science, Univ. of Tokyo, Japan) was used as an internal control. Effector constructs were also generated by using pBI221 vector as a backbone, with the GUS gene replaced by the full-length cDNAs of either StBEL5 or POTH1, downstream of the CaMV 35S promoter. Truncated cDNAs that encode the N-terminal protein-binding domains of StBEL5 or POTH1 were used to generate the dominant negative constructs, StBEL5ΔC295 and POTH1ΔC122, respectively. The reporter construct with the mutated promoter was generated by site-directed PCR mutagenesis with oligos 5’-CTATTTGACTTC*ACACGGTTATTTT-3’ and 5’-AAATAACCGTGCT*AAGTCAAATAG-3’.
Fully expanded leaves from three- to four-week-old tobacco plants were excised and placed in K3 basal media (Kao and Michayluk, 1975) supplemented with 0.4 M sucrose, 0.25% (w-v) cellulases (Karlan Research Products, Santa Rosa, CA), and 0.05% (w-v) macerases (Calbiochem, La Jolla, CA) and incubated for overnight at 28°C. After incubation, the liberated protoplasts were filtered through sterile cheesecloth into a Babcock bottle, and centrifuged for 10 min at 1000 rpm. Protoplasts were collected from the bottleneck area and washed once in K3 media with 0.4 M sucrose and resuspended in K3 media containing 0.4 M glucose to a final concentration of 4 x 10⁶ protoplasts per milliliter.

For each transfection analysis, 700 µL of tobacco protoplasts (prepared as described above) were mixed with 30 µL 2 M KCl and plasmid DNA in an electroporation cuvette with 0.4-cm electrode gap. The plasmid DNA was a mixture of 2 µg of the pGAOP::GUS reporter construct, 0.1 µg of the 35S-LUC construct as internal control, and a different combination of 2 µg of each effector plasmid. After electroporation (voltage = 170 V, capacitance = 125 µF, Gene Pulser Transfection Apparatus; Bio-Rad, Hercules, CA), 4.0 mL of Murashige and Skoog (1962) basal media was added, and the protoplasts were incubated in the dark at room temperature for 40 to 48 h before conducting GUS and LUC activity assays. Transfections were performed three times for each effector combination.
Luciferase assays were performed by injecting 100-μL luciferase substrate (Promega, Madison, WI) into 20 μL of extract and measuring the emitted photons for 15 s in a TD-20 luminometer (Turner Designs, Sunnyvale, CA). Fluorometric GUS assays were performed as described (Jefferson, 1987). A fluorescence multiwell plate reader, Fluoroskan II (MTX labs, Vienna, VA), was used to measure GUS activity at 365 nm (excitation) and 455 nm (emission). Each sample was measured three times for both LUC and GUS activity. Relative GUS-LUC activity was calculated by dividing the ratio of GUS activity to LUC activity from different effectors with the ratio from reporter plasmid alone. Relative activities calculated from three transfection replications were presented as a mean ± SE.

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Figure Legends

Figure 1. Binding of StBEL5 and POTH1 to ga20ox1 regulatory sequences. a) schematic of potato ga20ox1 promoter. Gel-retardation assay of ga20ox1 promoter P1 and P2 (b), P3 and first intron sequences (c) with StBEL5 (lane 2 and 6), POTH1 (lane 3 and 7) or both (lane 4 and 8). Lane 1 and 5 are labeled probe without protein to show the position of the free probe. The DNA-protein complexes are indicated with arrowheads.

Figure 2. Competition gel-retardation assay of P1 with cold P1 or P3 in the presence of StBEL5 (a) or POTH1 (b). Lane 1 is labeled P1 alone, lane 2 is the labeled P1 with either StBEL5 (a) or POTH1 (b). Increased amount (10X, 25X, 50X, 100X) of unlabeled P1 or P3 were added to lanes 3 to 6 and 7 to 10, respectively. The DNA-protein complexes are indicated with arrowheads.

Figure 3. Analysis of the dissociation rate of StBEL5-P1, POTH1-P1, and StBEL5-POTH1-P1 complexes. Labeled P1 was incubated on ice for 30 min with recombinant proteins, as indicated on the top. Then a 500-fold molar excess of unlabeled P1 was added and aliquots analyzed by gel mobility shift assay after indicated time. The arrows indicate the DNA-protein complexes.
Figure 4. Gel retardation assays using P1 with increased amounts of the homeodomain portion of StBEL5. Labeled P1 was incubated on ice for 30 min with increased amounts (25, 100, 300ng) of protein. The arrows indicate the DNA-protein complexes.

Figure 5. DNase I footprinting of StBEL5-POTH1 heterodimer, POTH1, and StBEL5 binding site. One end labeled P1 was protected with proteins as indicated on top. Protected regions were marked with solid lines.

Figure 6. Gel-retardation with mutated binding site oligos. a), The synthesized oligos used for probes, underlined base represents the point mutation; b), Retardation with StBEL5-POTH1 heterodimer. The 9-base motif is represented by oligos m3 through m11. The first lane on the left represents WT sequence without any protein.

Figure 7. Protein structures of POTH1 (a) and StBEL5 (b). Conserved regions include the protein-binding regions for POTH1, KNOX I and KNOX II, and for StBEL5, the Sky box and the BELL domains. The DNA-binding domains (HD) consisting of three helices and the characteristic proline-tyrosine-proline TALE are also designated.
POTH1 is 345 aa in length, whereas StBEL5 is 688 aa. The schematics of protein structure presented here are not drawn to scale to enhance visual clarity.

**Figure 8.** The repression effect of StBEL5 and POTH1 on ga20ox1 promoter. a) schematic of constructs; b), relative GUS/LUC activity in transcription assay. The construct with LUC gene under the control of cauliflower mosaic virus (CaMV) 35S promoter was used as control. Each transfection was performed three times. Relative GUS-LUC activity was calculated with reporter alone set as 100%. Data are means ± SE.

**Figure 9.** The effect of dominant negative constructs of either StBEL5 or POTH1 on the repression activity of StBEL5 or POTH1, respectively. a), schematic of constructs; b), relative GUS/LUC activity in transcription assay. The construct with LUC gene under the CaMV 35S promoter was used as control. Each transfection was performed three times. Relative GUS-LUC activity was calculated with reporter alone set as 100%. Data are means ± SE.

**Fig. 10.** Mutation in StBEL5-POTH1 heterodimer binding site deprived the ga20ox1 promoter of its response to StBEL5 and POTH1 repression. a) schematic of mutated
base in 10-bp motif; b), relative GUS/LUC activity in transcription assay. The construct with LUC gene under the CaMV 35S promoter was used as control. Each transfection was performed three times. Relative GUS-LUC activity was calculated with reporter alone set as 100%. Data are means ± SE.

Figure 11. Model of the interaction of the homeodomains of the StBEL5/POTH1 hetordimer with its target DNA. The homeodomain structures of StBEL5 (green) and POTH1 (red) were generated using Swiss Model (http://swissmodel.expasy.org/). The DNA structure was generated using ChemSite Pro 5.1 (ChemSW Inc., Fairfield, CA) with 5′-TATTTGACTTGACACG-3′. Proteins and DNA were assembled with Swiss Deepviewer 3.7 (Guex and Peitsch, 1997) using Ubx-Exd-DNA structure (PDB ID: 18BI) as template. Helices I, II, and III of the homeodomains are indicated. Based on the TALE TF model for homeodomain structure, StBEL5 has a conserved arm in its HD adjacent to helix III.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
a) TTTGACTTGACA
m1 ATTGACTTGACA
m2 TATGACTTGACA
m3 TTAGACTTGACA
m4 TTTCACTTGACA
m5 TTTGTCTTGACA
m6 TTTGAGTTTGACA
m7 TTTGACATTGACA
m8 TTTGACTAGACA
m9 TTTGACCTCACA
m10 TTTGACTTGACA
m11 TTTGACTTGAGA
m12 TTTGACTTGACT

b) BEL5+POTH1

Figure 6.
Figure 7.
Figure 8.
Figure 9.

[Graph showing relative GUS/LUC activity (%)]

- KNOX+POTH1
- KNOX
- POTH1
- None
- BEL+SigelS
- BEL
- SigelS

Relative GUS/LUC activity (%)
a) TTGACTTGAC
   \[\rightarrow\]
   TTGACTTTGAC (mutated)

b) Figure 10.
Figure 11.
CHAPTER 4. GENERAL CONCLUSIONS

In this study, seven interacting partners of POTH1, a potato KNOX protein, were isolated from the potato stolon library using two-hybrid yeast system. BLAST analysis showed that all seven cDNAs had high similarity to Arabidopsis BEL1, which encodes a TALE HD protein (Reiser et al., 1995). Therefore, these potato BELs were named StBELs, indicating BELs from Solanum tuberosum. Recently, protein-protein interactions between BEL and KNOX have also been reported in Arabidopsis (Bellaoui et al., 2001; Byrne et al., 2003; Smith and Hake, 2003), barley (Müller et al., 2001), and maize (Smith et al., 2002).

The identified StBELs can be clustered into four groups based on their sequence and mRNA accumulation patterns. They also exhibit selective interaction to KNOX proteins, suggesting that these StBELs may function as switches to regulate KNOX protein activity during potato development. One of these StBELs, StBEL5, showed increased transcript level under short-day conditions, and its overexpression lines exhibited enhanced tuberization activity under both long day and short day conditions.

Consistent with previous work (Dong et al., 2000; Hay et al., 2002; Kusaba et al., 1998a and b; Müller et al., 2001; Rosin et al., 2003; Sakamoto et al., 2001; Tamaoki et al., 1997; Tanaka-Ueguchi et al., 1998), StBEL5 overexpression lines also decreased
ga20ox1 mRNA levels in stolon tips, suggesting the interaction between StBEL5 and POTH1 may specifically regulate tuberization by working on ga20ox1 to modulate GA levels.

EMSA showed that both proteins could bind to the regulatory sequence of ga20ox1 individually or in tandem. In tandem, however, the protein complex possesses a higher affinity for their target DNA sequence. Based on the regions protected in DNase footprinting and known TALE protein consensus sequences (Nagasaki et al., 2001; Smith et al., 2002; Tejada et al., 1999), one putative binding site, TGACTTGAC, for the StBEL5-POTH1 heterodimer binding was identified. Mutation analysis showed that the heterodimer specifically recognizes this sequence. Although Sakamoto (2001) showed that NTH15 alone is sufficient for repressing tobacco ga20ox transcription, our results showed that the repression of ga20ox1 promoter by either StBEL5 or POTH1 could be abolished by the protein-interacting domain of the corresponding protein. Therefore it is the StBEL5-POTH1 complex that repressed the transcription of ga20ox1 promoter. Many animal HD proteins have been shown to interact with each other to specifically control their target genes (Berthelsen et al., 1998; Kobayashi et al., 2003; Saleh et al., 2000; Shen et al., 1999). No repression was detected by either StBEL5 or POTH1 or StBEL5-POTH1 heterodimer when using the mutated ga20ox1 promoter in the transcription assay,
suggesting that the identified binding site acts as a cis-element in vivo for StBEL5-POTH1. Because the mutated site is recognized by StBEL5 or POTH1 individually but not by the StBEL5-POTH1 heterodimer, this further proves that StBEL5-POTH1 complex is necessary for repression of the target gene.

In conclusion, StBEL5, one of seven BEL proteins that interact with POTH1, is involved in potato tuberization just as its interacting partner, POTH1 (Rosin et al., 2003). These two proteins most likely promote tuberization activity by working as a complex to repress the ga20ox1 transcription, and in this way, directly mediate GA biosynthesis.
LITERATURE CITED


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