Identification and functional characterization of *KNOTTED*-like homeobox genes in potato

A Thesis

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Doctor of Philosophy

By

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CERTIFICATE

Certified that the work incorporated in the thesis entitled **"Identification and functional characterization of *KNOTTED*-like homeobox genes in potato"** Submitted by Mr. Ameya S Mahajan was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other university or institution.

Dr. Anjan K. Banerjee
Supervisor

Date:

DECLARATION

I declare that this written submission represents my ideas in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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Synopsis

Title: Identification and functional characterization of *KNOTTED*-like homeobox genes in potato.

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Introduction:

Plants are sessile in nature and this demands them to stand and respond to the fluctuations in the environment. Thus, plasticity in responses to a changing environment is vital for plant survival. One of the important traits that allows plants to generate developmental plasticity is meristem mediated post-embryonic development. Meristems are the pool of undifferentiated cells that have potential for self-renewal as well as for generating different plant organs. A mature plant embryo does not have complete adult organs formed, instead it just has the adult body plan laid out. This body plan is later executed by the meristems during the course of plant life cycle to generate various adult organs. For example, the shoot apical meristem formed in plant embryo gives rise to organs such as stem, leaves and flowers whereas the root apical meristem gives rise to root tissue. Maintenance of meristems and development of various organs from them is regulated by an complex interplay of intrinsic (genetic) and extrinsic (environmental) factors. This interplay is central to the plasticity of plant responses to the environment. One of the important tools in the plant genetic toolkit that regulates the meristem activity is a family of homeobox genes namely knotted like homeobox (KNOX) genes. Thus, understanding of KNOX gene functions is imperative to understanding of plant development.

Homeobox genes play major roles in the development in all the eukaryotes. The first homeobox gene to be discovered in plants was knotted-1 (*KN-1*) from maize (Vollbrecht et al. 1991). Since then, KNOX genes have been identified from various

plant species and are shown to be ubiquitous to green plant lineage. In unicellular algae *Chlamydomonas*, KNOX protein is required for 'minus' identity of the 'm' gamete and it regulates mating as well as zygotic development (Lee et al. 2008). Over the course of evolution, KNOX genes have undergone duplication and diversification. In higher plants, KNOX genes form a multimember family of transcription factors (Hay & Tsiantis 2010). Based on their expression pattern and intron positions, KNOX genes are grouped in two subclasses; class I and II (Kerstetter et al. 1994). Class I KNOX genes are involved in diverse vegetative and reproductive processes like meristem maintenance, leaf development, floral development, tuberization, bulbil formation and so on (Hay & Tsiantis 2010; Ragni et al. 2008; Uchida & Tasaka 2010). For example, the KNOX gene shoot meristemless *(STM)* of *Arabidopsis* is essential for initiation and maintenance of shoot apical meristem (Barton & Poethig 1993). *STM* is also required for proper patterning of organ initiation and determination of leaf morphology (Gallois et al. 2002).

KNOX proteins are shown to interact with BEL1-Like homeobox proteins (BELL). The KNOX-BELL interactions are selective, thus dimers of different KNOX-BELL members regulate a different array of downstream genes (Hay & Tsiantis 2010). The KNOX-BELL transcription factor module is known to be involved in downregulation of gibberellin and lignin biosynthetic pathways and upregulation of cytokinin biosynthetic pathway. For example in potato, a KNOX protein POTH1 and its BELL partner *St*BEL5 form dimer that binds to *ga20ox1* promoter and downregulate its activity (Chen et al. 2003; Chen et al. 2004). This results in reduction of GA levels in stolons and thus promotes tuberization. Other studies have also shown that KNOX-BELL downregulate GA20-oxidase (GA anabolism enzyme) expression and upregulate GA2-oxidase (GA catabolic enzyme) expression thus resulting in low GA levels in the specific tissue where they are active (Hay et al. 2002; Bolduc & Hake 2009). Recently, Bolduc et al (2012) have performed a comparative RNA-seq and ChIP-seq on the inflorescence of wild type and *knotted1* mutants of maize. This study indicates that *KN1* regulates large number of genes including several important transcription factors and hormone pathway components. Thus by modulating the levels of key phytohormone and transcription factors KNOX genes regulate various developmental processes. As for the regulation of KNOX expression only a handful of factors have been identified. The components that repress KNOX expression are chromatin modifying proteins such as ARP family proteins and polycomb repressor complex proteins (PRC1-like and PRC2 family proteins) (Hay & Tsiantis 2010). Auxin and *YABBY* are also known to repress the

KNOX activity but if the repression is direct is still not known (Hay et al. 2006; Kumaran et al. 2002). KNOX genes are shown to auto-upregulate their expression (Tsuda et al. 2011). Amongst other activators are proteins such as *JAGGED LATERAL ORGANS (JLO)* and *CUP-SHAPED COTYLEDON (CUC)* (Hay & Tsiantis 2010).

Given the diverse roles of KNOX genes in plant development, our knowledge of KNOX gene regulatory network is far from complete. Further studies are necessary to unravel the significance of KNOX nexus in plant developmental processes. This study is one such initiative to understand the role of KNOX genes in plant development. We have used potato (*Solanum tuberosum* ssp *andigena*) as a model system for this purpose. The primary goal of this study is identification and functional characterization of KNOX genes in potato. The study is divided in three objectives -

1. **Identify, validate and clone the *KNOX* genes from potato**
2. ***Potato Homeobox 1* (*POTH1*) - Studying the regulation and transcript mobility**
3. ***Potato Homeobox 15* (*POTH15*) – Regulation, over-expression and target gene identification**

1. Identify, validate and clone the *KNOX* genes from potato

KNOX form a multimember family of genes in higher plants with 4 members in *Physcomitrella*, 9 in *Arabidopsis*, 16 in rice and 7 in tomato (Hay & Tsiantis 2010). Before this study began, Potato Homeobox 1 (*POTH1*) was the only potato KNOX gene to be identified and studied. It was demonstrated to be involved in regulation of vegetative development (Rosin et al. 2003). Using potato genome sequence data and tools at The Potato Genome Sequencing Consortium (2011), we have identified and validated six novel KNOX genes from potato. Further, full length sequences of these novel KNOX genes were obtained by performing 5' and 3' Rapid Amplification of cDNA End (RACE). The full length transcript sequences of these genes were then cloned and their sequences were submitted to NCBI. Preliminary characterization of these genes shows that three out of six KNOX genes belong to Class I and two belong to Class II while one is a mini-KNOX.

2. *Potato Homeobox 1* - Studying the regulation and transcript mobility

POTH1 is a KNOX-I gene that is located on chromosome number 5 of potato and it codes for 345 amino acid protein. Earlier studies have shown that POTH1 regulates vegetative development in potato. POTH1 along with its protein partner, StBEL5, binds to specific target genes and modulates hormone levels. *POTH1* transcripts have previously been detected in phloem by Yu et al (2007) and Campbell et al (2008).

In this study, we have investigated the regulation of *POTH1* expression as well as mobility of *POTH1* transcripts. Further the role of *POTH1*-UTRs in regulation of its translation and transport has been addressed. The promoter analyses demonstrate that the upstream sequence of *POTH1* is rich in light regulatory motifs. Consistent with this observation, the *POTH1* promoter drives β-glucuronidase expression in response to light. Qualitative GUS staining in transgenic $_{prom}$POTH1::GUS lines shows that *POTH1* promoter has widespread expression pattern including shoot tips, axial nodes, stolons, leaves, stamens and roots including phloem cells of both petioles and stems.

As POTH1 transcripts were detected in phloem, long-distance movement of its mRNA was tested. *In vitro* micrografts of wild type and *POTH1* over-expressing lines of potato were generated. RT-PCR for transgenic *POTH1* transcript in the micrografts demonstrated unilateral movement of *POTH1* RNA in a rootward direction. To validate the *POTH1* transcript mobility, heterografts of soil grown wild type and *POTH1* over-expression lines of tobacco were generated. Analysis of these heterografts confirmed the movement of *POTH1* transcript across a graft union into leaves, stem and roots of wild type stocks. Leaves from the wild type stock containing the transgenic *POTH1* RNA exhibited a reduction in leaf size relative to leaves from wild type grafts.

Long distance transport of RNAs through phloem occurs as ribonucleoprotein complex (Ham et al. 2009). Mobile RNAs have specific sequences where these RNA binding proteins (RBPs) bind (Huang & Yu 2009; Banerjee et al. 2009). *In silico* analysis of *POTH1* transcript revealed that both 5' and 3' untranslated regions (UTRs) of *POTH1* are rich in such protein binding sites. Thus, effect of these UTRs on regulation of translation was studied. Both the UTRs of *POTH1* when fused to an expression marker β-glucuronidase, repressed its translation in tobacco protoplasts. Further, RNA/protein binding assays demonstrated that the UTRs of *POTH1* bind to two RNA-binding proteins indicating role of *POTH1* UTRs in transcript mobility. These results

demonstrate that *POTH1* expression is light regulated and *POTH1* mRNA could act as a mobile signal in regulating development (Mahajan et al. 2012).

3. *Potato Homeobox 15* (*POTH15*) – Regulation, over-expression and target gene identification

Potato Homeobox 15 (*POTH15*) is one of the novel KNOX-I gene that we have identified and cloned from potato genome. *POTH15* is located on chromosome number 2 of potato and it codes for 343 amino acid protein. *POTH15* is an ortholog of *SHOOT MERISTEMLESS* (*STM*) gene of Arabidopsis. *STM* is shown to be essential for initiation and maintenance of shoot apical meristem (Barton & Poethig 1993). *STM* orthologs from a number of species such as rice, maize, tobacco, tomato are studied (Vollbrecht et al. 1991; Matsuoka et al. 1993; Janssen et al. 1998; Tanaka-ueguchi et al. 1998; Bolduc et al. 2012). These studies show that *STM* orthologs are important for development of various plant tissues like apical and axillary meristems, leaves, inflorescence, and fruit (Matsuoka et al. 1993; Parnis et al. 1997; Nishimura et al. 2000; Sakamoto et al. 2001; Takacs et al. 2012). Thus, in this study we have taken an initiative to understand the functions of *POTH15* in potato development.

Tissue-specific qRT-PCR analysis exhibited a higher abundance of *POTH15* mRNA in shoot tips and stolons under tuber inducing short day conditions. Upstream sequence of *POTH15* drives β-glucuronidase expression in apical and axillary meristems, petiole, tuber eyes and tuber pith indicating its role in meristem maintenance, leaf development and tuberization. In accordance with this, over-expression of *POTH15* in potato altered multiple morphological traits including leaf development and exhibited early tuberization under *in vitro* conditions. To understand the basis of such a dramatically different phenotype of *POTH15* over-expression lines in contrast to wild-type plants, a comparative RNA-seq approach has been undertaken. Comparative transcriptomic analysis of wild-type and *POTH15* over-expression lines identified 435 differentially expressed genes including 79 novel target genes with unknown functions. Functional analysis of these genes revealed their involvement in key biological processes such as metabolism, biotic and abiotic stress responses, regulation of transcription, and signal transduction. qRT-PCR of selected candidate genes validated their differential expression in wild-type and *POTH15* over-expression lines. Overall, this study indicates the role of *POTH15* in controlling diverse developmental processes in potato.

Summary:

1. We have identified, validated and cloned six novel KNOX genes from potato genome. The full-length transcript sequences of these genes are submitted to NCBI **(Mahajan AS, Amit K and Banerjee AK. 2014)**. Functional characterization studies of these genes will be continued to understand the KNOX network in potato.
2. We have demonstrated that *POTH1* expression is light regulated and *POTH1* transcript is phloem mobile. Further, we investigated the role of *POTH1*-UTRs in regulation of translation and transport of *POTH1* transcript **(Mahajan et al. 2012)**.
3. Our results reveal that, *POTH15* mRNA abundance is affected by photoperiod and its promoter has a widespread expression pattern. Over-expression of *POTH15* drastically altered plant morphology and a comparative RNA-seq analysis revealed 435 putative target genes of *POTH15*, suggesting its role in diverse developmental processes in potato. **(Mahajan et al. 2015, *under review*)**

List of Publications:

1. **Mahajan A,** Bhogale S, Kang IH, Hannapel DJ, Banerjee AK. (2012) The mRNA of a Knotted1-like transcription factor of potato is phloem mobile. *Plant Molecular Biology* **79**, 595-608.
2. **Mahajan AS,** Rajabhoj MP, Kondhare KR, Ghate T, Ravindran N, Kumar A, Habib F, Siddappa S, Banerjee AK. Regulation, over-expression and target gene identification of *Potato Homeobox 15* (*POTH15*) - a class-I *KNOX* gene in potato. (Under review)

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Abbreviations used:

1. β-glucuronidase	GUS
2. BEL1-like	BELL
3. BREVIPEDICELLUS	BP
4. Curly	Cu
5. Gibberellic acid	GA
6. Glycerol Responsive Element	GRE
7. Homeobox	HOX
8. Isopentyl Transferase	IPT
9. KNOTTED1	KN1
10. KNOTTED-like homeobox	KNOX
11. Mouse ear	Me
12. Murashige and Skoog's	MS
13. PETROSELINUM	PTS
14. Polycomb Repressor Complex	PRC
15. Polypyrimidine Tract Binding	PTB
16. POTATO HOMEOBOX1	POTH1
17. POTATO HOMEOBOX15	POTH15
18. POTATO HOMEOBOX20	POTH20
19. Rapid Amplification of cDNA Ends	RACE
20. Shoot Apical Meristem	SAM
21. SHOOT MERISTEMLESS	STM
22. *Solanum tuberosum* KNOTTED1	*StKn1*
23. *Solanum tuberosum* HOMEOBOX1	*St*Hox1
24. *Solanum tuberosum* HOMEOBOX2	*St*Hox2
25. *Solanum tuberosum* PETROSELINUM	*St*PTS
26. Three Amino acid Loop Extension	TALE
27. Untranslated Region	UTR
28. Transcription factor	TF
29. Tomato KNOTTED	TKn
30. Yeast Three Hybrid	Y3H
31. RNA Binding Protein	RBP

Chapter 1

Introduction

Plants are sessile in nature and this demands them to stand and respond to the fluctuations in the environment. The plant kingdom exhibits an enormous range of forms with increasing complexity that have evolved to deal with sessile life. The evolution of such complex macroscopic plant forms required an increase in the number of their specialized cell types that can perform specific functions in diverse environmental conditions.All the cells of adult plants are ultimately derived from the meristems. Meristems are the pool of undifferentiated cells that have potential for self-renewal as well as to generate new cell types. In higher plants, the formation of the plant body occurs in two developmental stages. The embryogenesis stage where the juvenile/seedling body plan is established and the post-embryonic growth stage where the body plan is executed to form new plant tissues and organs. For example, the embryonic structure of higher plants, such as *Arabidopsis* includes cotyledons, hypocotyl and root. The embryo also contains two populations of undifferentiated cells termed as Shoot Apical Meristem (SAM) and Root Apical Meristem (RAM)necessary for post-embryonic growth. RAM is located at the basal end of the embryo and is the source of all subsequent root tissues, whereas, SAM is located at the apical end of the embryo and is the source of allaerial plant tissues. There are only 5 cell typesin chlorophycean algae, 26 cell types in Moss and approximately 44 cell types in vascular plants (Hedges et al., 2004). Such increase in the complexity of the organismcorrelated with an increase in the number of Transcription Factor (TF) genes. The genomes of unicellularalgaehave fewer than 200 TFs, the moss *Physcomitrella patens* contains ~ 1000 and the seed plants, such as *Arabidopsis* and rice contain ~2000 TF genes (Cock et al., 2010). Among the most well-known and functionally important TFs are the Homeoboxtranscription factors, which have only a few members in chlorophytes, 23 members in moss and 60-80 in vascular plants (Cock et al., 2010).

1.1.Homeobox genes

The Homeobox (Hox) genes were first discovered in the fruitfly*Drosophila melanogaster* through mutation studies that resulted inthe transformation of certain body parts into other structures.For example, the *antennapedia*mutation results in development of legs from head instead of antennae. Albertset al., (1994) have shown that homeotic genes are crucial in patterning the body axis in *Drosophila* by specifying differences

between body segments along the head to rear axis.Hox genes have been discovered in diverse of animal taxa (from nematodes to mammals) and they have been shown to play an important role in body patterning (Burglin; 1995). The Hox genes code for homeodomain proteins that act as transcription factors and regulate gene expression to control various developmental processes. The hallmark of all homeodomain proteins is a highly conserved homeobox sequence that encodes a 60 amino acids long DNA binding domain (Gehring et al., 1994). The homeodomain is made of three helices. The third helix binds in the major groove of DNA and along with the N terminal arm of the protein, regulates transcription of the target gene (Lewin, 1995).Homeobox genes have been isolated from a number of different plant species ranging from unicellular algae to angiosperms and along with body patterning; they are shown to play important roles in other developmental and response processes (Mukherjee at al., 2009). Based on the sequence of the homeobox and of other conserved domains, the homeobox genes from plants are divided into following seven families - *HD-KN1, HD-ZIP I*, *HD-ZIP II*, *HD-ZIP III*, *HD-GL2, HD-BEL* and *HD-PHD*. The *HD-KN1* family is also referredto as *KNOTTED-1* like homeobox genes *(KNOX).* This gene family is the focus of the current study.

1.2.*KNOX* genes

Knotted-like homeobox (KNOX) genes belong to Three Amino acid Loop Extension (TALE) superclass of homeobox transcription factors (Burglin, 1997). The TALE homeobox genes in plants fall into two clades, the *BEL-like (BELL)* and the *KNOX,* and they playkey roles in development and patterning.Vollbrecht et al. (1991)discovered the first plant KNOX gene *viz. KNOTTED1*(*KN1*) from maize (*Zea mays*).Maize*kn1*was an over-expression mutant that resulted from the insertion of a transposon into a regulatory intron. Further it was demonstrated that ectopic expression of the KN1protein in the maize leaves results in atypical cell proliferation which leads to knotted like appearance of the leaf blade (Fig.1.1; Vollbrecht et al., 1991; Kerstetter et al., 1997). Since then, *KNOX* genes from diverse plant species ranging from unicellular algae to angiosperms have been discovered and they are found to be a vital tool in the plant developmental tool-kit as reviewed by Hake et al. (2004), Hay and Tsiantis (2010), Hamant and Pautot (2010), Giacomo et al. (2013).

1.3.Conserved domains in *KNOX* family genes

The key feature of the *KNOX* genes is the homeobox region, which binds to DNA. This region is located at the extreme 3' of the coding sequence in the *KNOX* genes. The characteristic of the TALE superclass members is a homeodomain that has three additional amino acids (Proline, Tyrosine, Proline) between helices 1 and 2 (Burglin, 1997). Upstream of the homeobox is the ELK domain, named so because of the three conserved amino acids in this region in several KNOX family members are E (glutamic acid), L (leucine) and K (lysine) (Vollbrecht et al., 1991; Kerstetter et al., 1994). ELK domain has a nuclear localization signal (Meisel and Lam, 1996) and it is also proposed to function in protein-protein interactions (Kerstetteretal., 1994). Located at 5' end of the gene, upstream of the homeobox and the ELK domains, is the MEINOX domain (Myeloid Ecotropic viral Integration knotted-like homeobox). The MEINOX domain has been hypothesized to function in protein-protein interactions (Mushegian and Koonin, 1996;Burglin, 1997). Based on their expression pattern, intron positioning and the domains present in protein,*KNOX*genes can be classified in three main groups: class-I, class-II and KNAT-M (Kerstetteretal., 1994;Magnani and Hake, 2008). The hallmark of KNAT-M-like proteins is absence of ELK and homeodomain complex (Magnani and Hake, 2008). Class-I KNOX and KNAT-M-like proteins have MEINOX domain that consists of two KNOX motifs, KNOX-A and KNOX-B connected by a short linker (Fig. 1.2; Burglin et al., 2009). Class-II KNOX proteins have an additional KNOX motif called as KNOX-C (Fig.1.2; Mukherjee et al., 2009).

1.4.KNOX – BELL interactions:

KNOX proteins form heterodimers with another TF family from TALE superclass of homeobox TFs, *viz* the BELL proteins. The MEINOX domain on the KNOX proteins interacts with SKY and BELL domains on the BELL proteins to form heterodimers. The homeodomain of each of the two proteins in the heterodimer binds to target DNA sequence to regulate gene expression (Bellaoui et al., 2001; Smith et al., 2002). In plants, each KNOX and BELL family has several members and they interact in a highly connected network that determines their subcellular localization and transcriptional activity

(Bellaouiet al., 2001; Bhatt et al., 2004; Cole et al., 2006; Hackbusch et al., 2005; Smith et al., 2002). Thus, different KNOX-BELL heterodimers regulate different set of target genes in a tissue specific manner giving rise to a complex regulatory circuit that orchestrates diverse developmental programs. For example, in *Arabidopsis,* heterodimers of STM or BP interact with the BELL protein BELLRINGER (BLR) and play a role in SAM maintenance and also inflorescence and fruit patterning (Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003). Whereas in potato, a KNOX protein POTH1 and its BELL partner *St*BEL5, form tandem dimer that binds to GA*20OX1* promoter and down-regulate its activity. This results in reduction of GA levels in the stolon and promotes tuberization (Rosin et al., 2003; Chen et al., 2003, 2004).

1.5.*KNOX* gene functions

KNOX genes are ubiquitous to green plants. Unicellular algae like *Chlamydomonas* have only one KNOX gene, whereas higher plants have multiple members in the KNOX family. In each of the specific clade, members of KNOX genes family perform various functions as described below.

1.5.1. *KNOX* gene functions in lower plants

In unicellular alga,*Chlamydomonasreinhardtii*, the plus and minus mating type each express a particular homeodomain protein. The plus mating type expresses *GSP1*, a *KNOX*-type protein, whereas the minus mating type GSM1, expresses BEL-like protein. An interesting study by Lee et al. (2008) showed that, upon fusion of plus and minus gametes GSP1 and GSM1 hertero-dimerize and the hetero-dimer translocates to the nucleus and activates the transcription of zygote specific genes. Further, it was demonstrated that the ectopic expression of GSP1 in minus-type gametes is sufficient to induce zygote specific gene expression and development in haploid gamete (Kurvari et al., 1998, Lee et al., 2008). Based on these studies it was concluded that *KNOX* genes regulate alternation of generation and zygotic development in *Chlamydomonas*. Functions of *KNOX* genes in plants up the evolutionary tree are derived from this ancestral function of regulating alternation of gametophytic and sporophytic generations (Sakakibara et al., 2013). Along with the evolution of a multicellular sporophyte body plan, *KNOX* genes underwent an increase in gene number. Based on their expression pattern and intron

positioning, Burgin (1997) categorized the*KNOX*genes into two classes, Class-I and Class-II (or *KNOX-I* and *KNOX-II*). Sakakibara et al., (2013) have shown that in early-diverging land plants, the *KNOX-II*genes play a role in regulating genes that orchestrate sporophytic development as well as repress gametophytic development in the sporophyte. The authors further demonstrated that the *KNOX-II* knock-out mutants in *Physcomitrella patens* developed a diploid gametophyte-like tissue from the sporophyte signifying that expression of *KNOX-II* genes is essential for maintenance of sporophytic state. The *KNOX-II* gene (*CRKNOX3)* of the fern *Ceratopterisrichardi* is shown to express throughout the sporophytic generation but not in the haploid gametophytic generation, indicating its role in sporophytic identity and development (Sano et al., 2005).

*KNOX-I*genes are shown to regulate development in the sporophytic generation. The sporophytic stage of *P. patens* is determinate whereas, the gametophytic stage is indeterminate. *P. Patens* has three *KNOX-I*genes and the triple *KNOX-I* knockouts result in reduced size and abnormal development of the sporophyte, but the gametophytic development is not affected in these mutants (Sakakibara et al., 2008). Based on these observations, authors concluded that the developmental defects in the sporophytes of the triple mutants appear to result from reduced cell divisions in the determinate meristems of the developing moss sporophyte (Sakakibara et al., 2008).

1.5.2. *KNOX* gene functions in higher plants

In vascular plants, the diploid sporophytic generation is the dominant phase in the life cycle of the organism. The SAM is the source of all the post-embryonic shoot tissues in the sporophyte of vascular plants. *KNOX-I* gene expression in SAM is conserved across all the tested vascular plants. Various reports have established that *KNOX-I*genes are key regulators of meristem initiation and maintenance (Long et al., 1996; Kerstetter et al., 1997; Harrison et al., 2005; Sano et al., 2005; Kawai et al., 2010; Larsson et al., 2012). It has been demonstrated that *KNOX-I* genes play a critical role by initiating lateral organ development through the down-regulation of their expression at sites of incipient organ primordium (Smith et al., 1992; Lincon et al., 1994; Waites et al., 1998; Nishimura et al., 1999). *KNOX* genes from various plant species have been studied so far. From these studies, it can be inferred that along with meristem maintenance, *KNOX* genes regulate

diverse vegetative and reproductive processes like leaf development, floral development, tuberization, bulbil formation and so on (Hay and Tsiantis, 2010; Ragni et al, 2007; Uchida et al, 2010). A detailed list on KNOX gene functions have been provided in Table 1.1.Although, there are numerous studies carried out of KNOX genes in plants, for the purpose of the present study, a short list of 71 reports has been provided.

1.5.2.1. Meristem maintenance and role of *KNOX* genes

Role of *KNOX* genes in meristem maintenance was first elaborated by *shoot meristemless* mutant (*stm-1*) of *Arabidopsis* (Long et al., 1996; Barton, 2010). *Arabidopsis* plants with the *stm-1* allele could not form or maintain a functional SAM and only produced two cotyledons, often fused at their base (Clark et al., 1996; Endrizzi et al., 1996). Similarly, Vollbrecht et al. (2000) have demonstrated that *kn1* mutant of maize failed to initiate a shoot apical meristem indicating vital role of KNOX genes in meristem identity. On the other hand, a number of reports have shown that *KNOX* over-expression mutants often produce meristems on their leaf surface signifying the *KNOX* gene function in cell fate indeterminacy (Vollbrecht et al., 1991; Sinha et al., 1993; Jannsen et al., 1998; Hake *et al.*, 2004; Hay and Tsiantis, 2010),

1.5.2.2. Role of *KNOX* genes in leaf development

KNOX genes play an important role in leaf development. Leaves can be broadly classified into two categories – simple leaves and compound leaves. Simple leaves have a single leaf blade and a petiole whereas, compound leaves have multiple blade units called as leaflets attached to a rachis. In simple leaf species, the expression of class-I *KNOX* genes is usually confined to meristems and stems, whereas in compound leaf species, they are expressed in leaf primordia as well (Fig 1.3;Hareven et al. 1996; Chen et al. 1997; Janssen et al. 1998; Bharathan*et al*., 2002; Uchida *et al*., 2007). This correlation of KNOX-I gene expression and leaf shape holds true for naturally occurring compound leaf species indicating that KNOX expression provides an indeterminate environment in the developing leaf primordia of compound leaf species. A number of studies on ectopic expression of *KNOX-I* genes in different simple leaf species resulted in development of leaves with outgrowths, prominent lobes and ectopic shoots (Muehlbauer et al. 1999; Schneeberger et al. 1995; Sinha et al. 1993; Vollbrecht et al. 1991). On the other hand, over-expression of *KNOX-I* genes in compound-leafed plant usually results in increased leaf complexity

(Hareven et al. 1996; Janssen et al. 1998; Barkoulas et al. 2008). For example when KN1 was overexpressed in tomato, it developed severely dissected leaves having up to 1000 leaflets (Hareven et al. 1996; Janssen et al. 1998). Thus *KNOX-I* genes play a pivotal role in leaf development by regulating the determinate vs. indeterminate cell fate within developing leaf primordia.

1.5.2.3.*KNOX* genes and reproductive development

KNOX-I genes are shown to regulate inflorescence and flower development. In *Arabidopsis*, a KNOX-I loss-of-functionmutant*brevipedicellus (bp)* has defects in the inflorescence architecture, wherein the pedicels become downward-pointing (Douglas et al., 2002; Venglat et al., 2002). Ragni et al., (2008) showed that this phenotype was the result of increased expression of two KNOX-I genes, *viz. KNAT2* and *KNAT6,* in pedicels. Authors further demonstrated that double mutations in KNAT2 and KNAT6 can completely rescue the pedicel phenotype in the *bp* mutant. When KNAT2 or STM is ectopically expressed during flowering, ectopic carpels are formed and the ovules are converted to carpels (Pautot et al., 2001; Belles-Boix et al., 2006; Scofield et al., 2007). Moreover, Scofield and co-authors (2007) have shown that *STM* is essential for the development of the central floral whorl, where it functions to maintain the central floral stem cells and later plays a role in the formation of carpels as well as the placental tissues that give rise to ovules. It was also demonstrated that STM along with two BELL genes PNY and PNF regulate the maintenance of boundaries between the inflorescence meristem and initiating floral primordia as well as floral specification and internode patterning (Karnar et al., 2006; Yu et al., 2009). In *Antirrhinum*, Golz et al. (2002) have shown that the spontaneous mutations in *KNOX* genes result in novel floral structures. They also demonstrated that dominant mutations at two loci *Hirzina* (*HIRZ)* and *Invaginata* (*INA*) resulted in spur like outgrowths from *Antirrhinum* petals. Spurs are considered to be an important innovation because of their ability to attract pollinators. Similarly in *Linaria*, Box et al. (2011) demonstrated that expression of two petal associated KNOX genes *viz. LvHIRZ* and *LvINA* is sufficient to induce sac like outgrowth in heterologous host. Based on these studies, it was proposed that changes in *KNOX-I* expression pattern could be a shared feature of spur development in angiosperms. Potato *KNOX-I* gene, *POTH1*, was

shown to act as a negative regulator of GA biosynthesis (Rosin et al. 2003; Chen et al. 2004). Over-expression of *POTH1* in potato modulated vegetative development and enhanced *in vitro* tuberization, wherein the number of tubers as well as rate of tuberization was shown to be increased (Rosin et al. 2003). In *Agave tequilana*, it was demonstrated that spatial and temporal control of *KNOX* genes is essential during bulbil formation (Abraham-Juarez et al., 2010).

In summary, *KNOX-I*genes are a key part of the developmental tool kit in plants. The important functions they play in plant development make this gene family a particularly interesting target of study. Future studies on KNOX gene would help us in understanding the extent of their functions in shaping the plant development.

Table 1.1: *KNOX* gene discovery, functions and regulation

Sr. No.	Plant species	Genes studied	Research interest / Findings	Reference
1	*Zea mays*	*KN1*	*KN1* is a homeobox gene	Vollbrecht et al., 1991
2	*Zea mays*	*KN1*	*KNOTTEDl*-like homeobox genes	Kerstetter et al., 1994
3	------	TALE superclass genes	Conserved domains in TALE superclass genes	Bürglin., 1997
4	*Zea mays*	*KN1*	Shoot meristem maintenance	Kerstetter et al., 1997
5	*Nicotianatabacum*	*NTH15*	Ectopic expression of *NTH15* alters leaf morphology and hormone levels	Tamaoki et al., 1997
6	*Lycopersicumesculentum*	*LeT6*	Ectopic expression of *LeT6* reveals indeterminate features in compound leaf	Janssen et al., 1998
7	*Nicotianatabacum*	*NTH15*	Down Regulation of GA biosynthesis by KNOX	Tanaka-Ueguchi et al., 1998
8	*Nicotiana tabacum*	*KN1*	Leaf senescence	Ori et al., 1999

Sr. No.	Plant species	Genes studied	Research interest / Findings	Reference
9	*Solanumlycopersicum (Xa and Me mutants)*	*LeT6*	Developmental changes due to long-distance movement of a fusion transcript PFP-LeT6	Kim et al., 2001
10	*Nicotianatabacum*	*NTH15*	NTH15 directly suppresses the expression of *ga20-oxidase* in SAM development	Sakamoto et al., 2001
11	Comparative study across eudicots	*KNOX-I*	KNOXI expression correlates with complex leaf primordia	Bharathan et al., 2002
12	*Arabidopsis thaliana*	*STM*	STM and WUS initiate the progression from stem cells to organ initiation.	Gallois et al., 2002
13	*Nicotianatabacum*	*NTH 1,NTH 9, NTH15, NTH 20, NTH22*	Ectopic expression of tobacco KNOX alters leaf morphology	Nishimura et al., 2002
14	*Arabidopsis thaliana*	*STM*	Shoot apical meristem maintenance	Barton et al., 2003
15	*Solanumtuberosum*	*POTH1*	Vegetative development and tuberization	Rosin et al., 2003
16	*Arabidopsis thaliana*	CLASS III HD-ZIP GENES, YABBY, FIL and AS1 & 2	Lateral organ polarity	Engstrom et al., 2004
17	*Streptocarpus species*	Streptocarpus*STM*-like	Comparison of leaf forms and Evolution of leaf morphological novelty	Harrison et al., 2005
18	*Arabidopsis thaliana*	*KN1*	Intercellular protein and mRNA trafficking, trichome development	Kim et al., 2005
19	*Arabidopsis thaliana*	*KNAT1,KNAT2,KNAT6, STM*	Leaf and inflorescence morphology in tae mutants	Lebedeva et al., 2005
20	*Nicotianatabacum*	*KNOTTED*-like 1	Over-expression of *KNOTTED-I* in tobacco causes a switch from determinate to indeterminate cell fates	Sinha et al., 2005
21	*Arabidopsis thaliana*	*BP*	*AS1* and auxin repress *BP* to regulate leaf development	Hay et al., 2006

Sr. No.	Plant species	Genes studied	Research interest / Findings	Reference
22	*Arabidopsis thaliana*	*BP* and *STM*	*BP* and *STM* along with *PNY* and *PNF* regulate inflorescence architecture	Kanrar et al., 2006
23	*Nicotianatabacum, Nicotianaglauca*and*Solanumtuberosum*	Tobacco *KN1*, *KN2*, and *BARLEY KNOX3*	Constitutive KNOX expression Epiphyllous shoots	Muller et al., 2006
24	*Taraxacumofficinale*	*BARLEY KNOX3*	Constitutive expression of *BKN3* changes leaf morphology from simple to compound	Muller et al., 2006
25	*Arabidopsis thaliana* and*Zea mays*	WUSCHEL HOMEOBOX LIKE (WOX), SHOOTMERISTEMLESS (STM), DORN-ROESCHEN (DRN) and CUP SHAPED COTYLEDON (CUC) genes	Plant development revolves around KNOX-ARP axes	Chandler et al., 2007
26	*Lycopersicumesculentum*	*LeT6*	LeT6 regulates leaf compounding	Jasinski et al., 2007
27	*Arabidopsis thaliana*	*BP*	*BP* along with *SAW1* and *SAW2* regulates leaf margin serration	Kumar et al., 2007
28	*Physcomirella patens*	moss KNOX homologues (KNOX)	Ancestral roles of KNOX	Singer et al., 2007
29	*Arabidopsis thaliana*	*STM*	Regulation of upstream non coding DNA sequence in leaf development	Uchida et al., 2007
30	*Arabidopsis thaliana* and*Cardaminehirsuta*	*KNOX*	Simple and dissected leaf species development	Barkoulas et al., 2008
31	*Solanumcheesmaniae, Solanumgalapagense*	*PETROSELINUM* (PTS)	Leaf Morphology	Kimura et al., 2008

Sr. No.	Plant species	Genes studied	Research interest / Findings	Reference
32	*Chlamydomonas*	Homeoproteins*Gsp1* and *Gsm1*	Evolution of KNOX/BELL	Lee et al., 2008
33	*Arabidopsis thaliana*	*KNATM*	KNAT-M homologs in *Medicagotruncatula*, *P. tricho-carpa*, and *Solanumesculentum* KNATM- BELL protein interaction	Magnani and Hay, 2008
34	*Arabidopsis thaliana*	*KNAT2, KNAT6, BP*	*KNAT2, KNAT6, BP* along with *PNY* regulate inflorescence development	Ragni et al., 2008
35	*Kohleria*	*KNOX* genes	Leaf development	Barth et al., 2009
36	*Zea mays*	*KN1*	*KN1* directly regulates the gibberellin catabolism gene *ga2ox1*	Bolduc et al., 2009
37	*Solanumlycopersicum*	*TKN1,TKN2*,	*KNOXI* proteins act at specific stages within the compound-leaf development program to delay maturation and enable leaflet formation	Shani et al., 2009
38	*Arabidopsis thaliana and Lycopersicumesculentum*	KNOX, BELL, PIN1, GA20ox and GA2ox	Plant Development	Hay and Tsiantis., 2010
39	*Arabidopsis thaliana*	KNAT1,KNAT2,KNAT6 and AS1 and AS2	LEAF development	Ikezaki et al., 2010
40	*Agave tequilana*	AtqKNOX-I, AtKNOX-II	Bulbil formation	Juárez et al., 2010
41	*S. Rexii, S. glandulosissimus*	SrARP and KNOX-I	caulescent and acaulescent stem species - Leaf development	Nishii et al., 2010
42	*Hordeumvulgare*	*HvKNOX3*, BERF1, BEIL1, BGRF1	KNOX and ethylene pathway crosstalk	Osnato et al., 2010
43	*Arabidopsis species*	STM	Leaf form	Piazza et al., 2010
44	*Myriophyllumaquaticum*	KN1	Lobes in simple leaf development	Bourque et al., 2011

Sr. No.	Plant species	Genes studied	Research interest / Findings	Reference
45	*L. Vulgaris, A.majus*	LvHirz,LvIna,AmHirz and AmIna, KNOX	Petal spur development	Box et al., 2011
46	*Amborellatrichopoda, Cabombaaquatica, Ginkgo biloba*	CUC, NAM, miR164	Gymnosperm and angiosperm evolution	Guiraud et al., 2011
47	*Arabidopsis thaliana*	CUC 1, CUC2, and CUC3	leaf serration suppression	Hasson et al., 2011
48	*Arabidopsis thaliana*	KNAT2, KNAT 6 and ATH1	Regulation of plant inflorescence architecture	Li et al., 2011
49	*Arabidopsis thaliana*	LFY and AG	Floral development Bio physical model	Moyroud et al., 2011
50	*Medicagotruncul ata*	Fused compound leaf 1, single leaflet1 (sgl1), Palmate like pentafoliata 1	Regulation of compound leaf development	Peng et al., 2011
51	*Arabidopsis thaliana*	BREVIPEDICELLUS	Floral abscission	Shi et al., 2011
52	*Arabidopsis thaliana*	Flowering Locus T-FD, PNY,PNF,STM	Inflorescence Development	Smith et al., 2011
53	*Arabidopsis thaliana*	CUC1, miR164 and STM	STM regulation	Spinelli et al., 2011
54	*Nicotianatabacu m, Prunusdomestica*	KNOX-I	Ectopic expression	Srinivasan et al., 2011
55	*Oryza sativa*	OSH1 and OSH15	SAM maintenance	Tsuda et al., 2011
56	*Arabidopsis thaliana*	Chaperonin containing TCP1, KN1	Chaperonin in stem cell maitenance	Xu et al., 2011
57	*Medicagotruncat ula*	Slm1 and Sgl1	Compound Leaf development	Zhou et al., 2011
58	*Solanumtuberosu m*	CONSTANS and StFT/StSP6A	Photoperiodic tuberization	González-Schain et al., 2012

Sr. No.	Plant species	Genes studied	Research interest / Findings	Reference
59	*Arabidopsis thaliana*	TCP, AS2 and KNOX	repression of KNOX for leaf organogenesis	Li et al., 2012
60	*Arabidopsis thaliana*	KNAT1, KNAT2 and KNAT6.	Floral organ abscission	Butenko et al., 2012
61	*Zea mays*	*KN1*	Regulatory network of KN1	Bolduc et al., 2012
62	*Arabidopsis thaliana*	JLO, LBD, AS2, BP, PIN	Regulation of expression of KNOX and PIN in shoot and root meristems	Rast et al., 2012
63	*Arabidopsis thaliana*	WUS, CLAVATA and AG	Stem Maintenance in SAM	Perales et al., 2012
64	*Arabidopsis thaliana*	STM, WUS and CLV3	Maintenance of Stem Cell homeostasis	Uchida et al., 2012
65	*Zea mays*	Maize *KNOX* genes	Ontogeny of Maize SAM	Takacs et al., 2012
66	*Solanumtuberosum ssp. Andigena*	*POTH1*	Mobility of *KNOX* mRNA in phloem	Mahajan et al., 2012
67	*Physcomitrella patens*	*PpKNOX-II*, *PRC2*, *PpCLF* and *PpFIE*	Regulation of the Haploid-to-Diploid Morphological Transition in Land Plants	Sakakibara et al., 2013
68	*Arabidopsis thaliana*	AS, PRC2, KNAT2 and BP	Repression of KNOX homeobox genes	Lodha et al., 2013
69	*Arabidopsis thaliana*	BELL -KNOX	Leaf Development	Giacomo et al., 2013
70	*Oryza sativa*	OSH1	Shoot apical meristem maintenance	Tsuda et al., 2014
71	*Arabidopsis thaliana*	STM	Regualtion of STM expression	Aguilar-Martinez et al., 2015

1.6. *KNOX* gene targets

In order to understand the functions of KNOX genes, it is necessary to identify their target genes. Till now, only a few KNOX-I target genes have been identified (Fig 1.4). First of the downstream targets of *KNOX-I*-TFs to be identified were genes from cytokinin

and gibberellin pathways as has been illustrated by various authors (Frugis et al., 1999; Ori et al., 1999; Sakamoto et al., 2001; Jasinski et al., 2005; Yanai et al., 2005; Bolduc and Hake, 2009).

1.6.1. *KNOX* genes up-regulate cytokinin biosynthesis

Cytokinins are known to induce cell divisions and assist SAM formation in plants. Several studies have shown that the plants over-expressing *KNOX-I*genes accumulate high levels of cytokinin (Frugis et al., 1999, 2001; Ori et al., 1999; Hewelt et al., 2000). Using Arabidopsis and rice models, it was shown that *KNOX-I* genes positively regulate cytokinin biosynthesis through induction of *ISOPENTENYL TRANSFERASE* genes (Jasinski et al., 2005; Yanai et al., 2005; Sakamoto et al., 2006). Frugis et al (2001) demonstrated that over-expression of *KNAT1*in lettuce results in over production of cytokinin along with initiation of ectopic leaves. Similarly, ectopic expression of *IPT7* in tomato leaves resulted in formation of super-compound leaves, whereas decrease in the cytokinin levels resulted in reduced leaf complexity (Shani et al., 2010). These two studies correlated the KNOX-I expression and leaf complexity to local cytokinin levels.

1.6.2. KNOX genes down regulate Gibberellin levels

Gibberellins (GA) promote polar cell expansion and organ outgrowth and *KNOX* genes are shown to regulate levels of active GA. Induction of tobacco *KNOX* protein NTH15 resulted in repression of transcript levels of *NTC12*, the GA 20-oxidase gene (Tanaka-Ueguchi et al., 1998). Later, Sakamoto et al. (2001) demonstrated that *NTH15* binds specifically to a sequence in the first intron of *NTC12*. Similarly, *KN1* induction in *Arabidopsis* resulted in repression of the *AtGA20ox1* transcript levels (Hay et al., 2002). Chen et al.(2003, 2004) have shown that a KNOX protein POTH1 and its BELL partner StBEL5 in potato, form tandem dimer that binds to *ga20ox1* promoter and down-regulate its activity resulting in reduction in GA levels. The dominant mutants of tomato, *Mouse ears* (*Me*) and *Curly* (*Cu*), that over-express the KNOX gene *TKN2*, displayed reduced levels of *LeGA20ox1* transcript (Hay et al., 2002). The expression of *GA2ox2* which encodes an enzyme involved in inactivation of bioactive GA was shown to be up-regulated in response to STM induction in *Arabidopsis* (Kessler et al., 2006). Similarly, Bolduc and

Hake (2009) demonstrated that*GA2ox2* transcripts accumulated at higher levels in leaves of the dominant maize mutant *Kn1-N.* Thus, *KNOX* genes maintain low levels of active GA through direct repression of GA biosynthesis gene, GA20-ox1 and up-regulation of GA catabolism gene *GA2ox2*.

1.6.3. KNOX genes modulate auxin and brassinosteroid levels

Bolduc et al (2012) have performed a comparative RNA-seq and ChIP-seq on maize *KN1* loss-of-function (*kn1-e1*) or gain-of-function (*Kn1-N*) mutants. This study indicated that KN1 binds to several thousand loci including 643 genes that are modulated in one or more types of tissues. The most significant functional category enriched in these genes was hormone metabolism. The ChIP-seq results showed preferential binding of KN1 near genes involved in hormone metabolism such as GA, brassinosteroid, and auxin pathways. In addition, the genes involved in auxin biosynthesis, transport as well as signalling were found to be bound and modulated, especially in leaves of *Kn1-N*. KNOX-I and auxin have an antagonistic relationship and it is found to be crucial to promote organ initiation and maintain organ boundaries (Hay and Tsiantis 2010; Giacomo et al., 2013). Thus identification of auxin as a main hormonal pathway regulated by KN1 is of enormous interest. Recently,Tsuda et al (2014) showed that a rice *KNOX*gene *OSH1* represses the brassinosteroid (BR) phytohormone pathway through activation of BR catabolism genes and concluded that local control of BR levels by *KNOX* genes could be a key regulatory step in SAM function.

1.6.4. Transcription factors as targets of KNOX

KNOX genes are also shown to regulate several transcription factors. Spinelli and co-authors (2011) performed a transcript profiling of transgenic *Arabidopsis* plants in which STM expression was induced and it revealed CUC1 as a direct target of *STM.* In the ChIP-seq/RNA-seq analysis performed by Bolduc et al. (2012) on maize *KN1* mutants, many *NAM-ATAF1,2-CUC2 (NAC)* genes were bound and modulated by *KN1.*The authors found thatKN1 direct targets were strongly enriched for transcription factors. Primarily, the members of homeobox (HB) family were enriched and they comprised several *BLH* and 10 *KNOX* genes, including *KN1*. This indicates that a complex auto-feedback mechanism may exist to regulate *KNOX* and *BLH* expression (Bolduc et al., 2012). Similar auto-feedback

regulation was previously demonstrated in rice for *OSH1* by Tsuda et al (2011). In the study performed by Bolduc and co-authors (2012), members of the *HOMEODOMAIN LEUCINE ZIPPER* (*HD-ZIP*) and *MADS-box* family of transcription factors such as homologs of *PHABULOSA* (*PHB*), *REVOLUTA* (*REV*), and *YABBY* were also bound by *KN1*. These TFs regulate leaf adaxial/abaxial polarity and blade outgrowth. The link between KNOX genes and leaf polarity genes may be crucial for leaf initiation and lamina outgrowth.

1.6.5. KNOX genes regulate cell wall related proteins

Another key set of *KNOX* targets are the cell wall related proteins. Lignin deposition is a characteristic of irreversible cell differentiation. Mele et al. (2003) performed a microarray analysis of KNOX mutant *bp*-9 of *Arabidopsis,* which revealed lignin biosynthetic pathway to be a target of the KNAT1/BP. Authors demonstrated that KNAT1/BP prevents premature cell differentiation by repressing lignin biosynthesis. In addition to lignin biosynthetic enzymes, other genes involved in metabolism of cell wall components such as cellulose and pectin were also altered in *bp-9* mutant (Mele et al., 2003). Spinelli and co-authors (2011) have shown that several genes involved in pectin, cellulose and lignin metabolism were altered in transgenic *Arabidopsis* lines in which *STM* was induced, while Townsley et al. (2013) demonstrated that *KN1*over-expressing plants exhibited a reduction in lignin content in both maize and tobacco.

In summary, all of the above studies establish that KNOX genes regulate various hormonal pathways, transcription factors and cell wall related proteins and play a pivotal role in plant development. Despite of these studies mentioned above, our knowledge of KNOX targets and the developmental pathways they regulate is still incomplete. Identification of KNOX target genes from different species would therefore illuminate how *KNOX* genes shape the plant development across diverse plant species.

1.7. KNOX regulation

Ectopic expression of KNOX genes causes severe pleiotropic effects and thus repression of KNOX genes outside their normal domain is crucial for lateral organ development. As KNOX genes are key regulators of meristem activity and leaf development, most of the studies are restricted on the regulation of KNOX expression in SAM and leaves. A number of studies have revealed several epigenetic regulators that

repress KNOX expression (Fig 1.4). The first upstream regulators of KNOX gene to be discovered were the myb-type proteins ARP -ASYMMETRIC LEAVES1 (AS1) in *Arabidopsis*, ROUGH SHEATH2 in maize and PHANTASTICA in snapdragon (Byrne et al., 2000; Hay and Tsiantis, 2006; Tattersall et al., 2005; Timmermans et al., 1999; Tsiantis et al., 1999). Schneeberger et al. (1998) reported that mutations in ARP genes result in ectopic expression of KNOX genes in leaves, but the boundary between KNOX-expressing and non-expressing cells in the SAM was still maintained. Two other reports later demonstrated that AS1 forms a heterodimer with the LATERAL ORGAN BOUNDARIES (LOB) domain protein AS2 and the AS1-AS2 protein dimer binds the promoters of *BP* and *KNAT2*,which was hypothesized to form a repressive chromatin complex through recruitment of the histone chaperone HIRA (Phelps-Durr et al., 2005; Guo et al., 2008). Another epigenetic pathway that restricts the KNOX gene expression to the meristem, involves polycomb-group proteins CURLY LEAF (CLF), SWINGER (SWN) and FERTILISATION INDEPENDENT ENDOSPERM (Katz et al., 2004; Schubert et al., 2006). Schubert et al. (2006) also established that CLF and SWN act as histone methyltransferase and CLF-containing PRC2 adds a trimethylation mark on histone H3 at lysine 27 upstream of the KNOX gene (H3K27me3). Further, a PRC1-like complex, containing the chromodomain protein LIKE HETEROCHROMATIN PROTEIN1 and two RING domain proteins were shown to bind to these methylated histones and repress the transcription of KNOX genes (Xu and Shen, 2008). Another protein family known as YABBY are shown to promote lateral organ formation in plants. Mutation in YABBY genes resulted in ectopic expression of STM, BP and KNAT2 and produced ectopic meristems on leaves as demonstrated by Byrne et al. (2000) and Kumaran et al. (2002). Thus, similar to AS1/AS2, the YABBY genes function to restrict KNOX expression to the SAM.

Auxin is also demonstrated to repress the KNOX expression in the periphery of the SAM (Benkova et al., 2003; Reinhardt et al., 2003). Conversely, ectopic expression of KNOX genes in *Arabidopsis* leaves was shown to alter the auxin activity gradients, which lead to the dramatic changes in leaf shape, suggesting that KNOX and auxin activities may form a feedback loop that functions to maintain a boundary between meristem and leaf (Hay et al., 2006). Interestingly, Borghi et al. (2007) showed that JAGGED LATERAL

ORGANS (JLO; a LOB domain protein in *Arabidopsis*), could activate STM and BP expression and repress PIN auxin efflux transporters. This regulation of KNOX and auxin activities seems to be crucial to the demarcation of boundary between the SAM and lateral organ primordia. In plants with simple leaves, KNOX genes are found to be expressed only in the meristem and stem, but in the compound leaf species, they are also expressed in leaf primordia, suggesting their role in determination of leaf morphology (Bharathan et al., 2002; Uchida et al., 2007). Interestingly, the function of *KNOX-I* genes in leaf compounding is hypothesized to be independent of their ability to induce SAM. This is thought to be achieved through differential regulation of their expression in SAM and leaf primordia. During tomato leaf development, TRIPINNATE (TP) and CLAUSA (CLAU) were shown to act together to restrict the expression level and domain of the KNOX genes *Tkn1* and *LeT6*/*Tkn2* (Jasinski et al., 2007). These authors also showed that loss of TP or CLAU activity resulted in increased KNOX expression and it was indicated that CLAU and TP may participate in a domain-specific KNOX repressive system that restricts the ability of the tomato leaf to generate leaflets. Remarkably, Uchida et al. (2007) found two conserved non-coding sequences, the K-box and the RB box, in the promoters of STM orthrologs from diverse plant species. These two sequences were later shown to differentially regulate the expression of STM in leaf sinuses and SAM (Aguilar-Martínez et al., 2015). KNOX genes play key role in SAM maintenance, however, not much is known of the mechanisms that activate KNOX gene expression. NO APICAL MERISTEM (NAM) and CUP-SHAPED COTYLEDON (CUC) boundary genes were demonstrated to activate STM expression during SAM establishment and compound leaf development, where a feed-forward loop between KNOX and CUC transcription factors was shown to regulate leaflet development (Blein et al., 2008; Takada et al., 2001). In another interesting study, Tsuda et al. (2011) demonstrated that OSH1, a rice KNOX-I gene, positively regulates the expression of five rice KNOX genes in a direct manner through evolutionarily conserved *cis*-elements. Authors further showed that the positive auto-regulation of OSH1 is essential for the expression of itself and SAM maintenance in rice.

Owing to a large diversity of KNOX genes in higher plants, it is apparent that various mechanisms and numerous factors would possibly be involved in regulation of KNOX gene expression depending on the species and pathway they regulate. As KNOX

gene expression confers indeterminate cell-fate, it would be interesting to understand how their expression is regulated across different pathways, tissues and plant species to generate diverse plant forms.

1.8. KNOX: The mobile signals in plants

Intercellular as well as long distance trafficking of bio-molecular signals is vital for integrating environmental inputs and regulating plant development. Macromolecules such as proteins and mRNAs have been shown to act as such long distance signals. *KNOX* proteins and transcripts were also found to contribute to this intercellular and long distance signalling pathways in plants.

The possibility of *KN1* trafficking was suggested by *in situ* hybridization and immunolocalization experiments, which showed that *KN1* protein is detected outside the domain of its mRNA expression (Jackson et al., 1994). Later, maize homeodomain protein, *KN1*and its mRNA have been shown to traffic cell-to-cell (Lucas et al., 1995). Using microinjection studies, of fluorescently labeled KN1 protein, the authors showed that KN1has the ability to traffic between mesophyll cells, to increase the size exclusion limit of plasmodesmata and to specifically transport its mRNA. Further, it was shown that a GFP-tagged *KN1* fusion is able to traffic in the leaf and SAM in *Arabidopsis* (Kim et al., 2002). Same group later demonstrated that *KNOX* homeodomain (HD) is necessary and sufficient for intercellular trafficking, and it also promotes the trafficking of the *KN1* mRNA (Kim et al., 2005). Xu et al. (2011) then demonstrated that a specific group of chaperons is essential for cell-to-cell trafficking of KN1. It would be interesting to investigate what functions the mobility of KN1 provides in the maize SAM. Moreover, if the KNOX proteins from other plant species also have the ability for cell-to-cell trafficking still remains to be discovered.

An interesting example of long distance trafficking of *KNOX* transcript is the *LeT6/TKN2* mRNA in tomato (Kim et al 2001). The *Me*mutant of tomato produces a chimeric transcript produced by a fusion between PYROPHOSPHATE-DEPENDENTPHOSPHOFRUCTOKINASE (PFP) and *KNOX* gene *LeT6*. In this report, authors showed that the strong promoter activity of PFP controls the expression of the chimeric transcript and this leads to an over-expression of the chimeric gene. Grafting

studies of *Me* and WT tomato plants further revealed that the *Me* transcript expressed in the mutant rootstock could specifically accumulate in the shoot apex and leaf primordia of the scion and this altered the leaf morphology of the wild type scion. In addition, the *Me* mRNA was detected in phloem cells by *in situ* reverse-transcriptase PCR indicating that it may be transported via phloem. In another study, Campbell et al (2008) demonstrated that the *KNOTTED1*-like RNAs were present in phloem-enriched sap of potato. As a part of the current study, we have demonstrated that the mRNA of potato KNOX transcription factor *POTH1* is phloem mobile (Mahajan et al., 2012), which is elaborated in Chapter 3. In pumpkin, Ham et al. (2009) found the *STMP* transcript to be a component of phloem mobile ribonucleoprotein complex indicating its phloem mobility.

Future studies on mobility of the *KNOX* transcripts and proteins across various plant species would help to unravel their role as mobile signals.

1.9. Potato as a model system

Potato belongs to solanaceae family in the asterid clade of eudicot plants. Potatoes produce underground tubers (enlarged modified stems) as storage organs and means of vegetative reproduction (Fig. 1.5A). Tubers are edible and are rich in nutrients; hence potato was domesticated long back as a food crop. Currently, it is the third major food crop in the world and thus, it is the focus of major agricultural and food science studies. Potato is also used as a model system to study plant developmental biology and plant pathology. There are more than 5000 different cultivated varieties of potato and approximately 200 different wild species providing a diverse germplasm for research. With availability of genome sequence and protocols for raising transgenics, potato can be used as suitable model system for all molecular genetic studies.

One of the widely studied aspects of potato biology is the tuberization process. Tubers are formed from the specialized type of horizontal shoots that arise from the basal axillary nodes of the plant called as stolons. The stolon to tuber transition is regulated by numerous environmental and internal signals. In *Solanumtuberosum*ssp. *Andigena,*tuberization is strictly photoperiod dependent, where tubers develop only under short day conditions (Fig. 1.5B). A few genetic players that fine tune this day-length dependent tuberization have been discovered. Phytochrome protein-PHYB, Flowering

locus-T/CONSTNAS (*St*SP6A/*St*CO) module, *St*BEL5, POTH1, miR172, and miR156 have been implicated to regulate the tuberization pathway (Jackson et al., 1996; Rosin et al., 2003; Banerjee et al., 2006; Martin et al. 2009; Navarro et al., 2011; Gonzalez-Schain et al., 2012; Bhogale et al. 2014).

It has been established that *KNOX* genes are key regulators of plant development. The literature survey suggests that role of KNOX genes in regulation of tuberization as well as other developmental pathways in potato is not clearly understood. The only potato *KNOX* gene characterized prior to this study, *POTH1*, was shown to regulate vegetative development and accelerate tuberization under *in vitro* conditions (Rosin et al., 2003). Moreover, the transcript of *POTH1* was shown to be present in the phloem (Yu et al., 2007; Campbell et al., 2008).

Current study is an initiative to understand the role of *KNOX* genes in orchestrating development in potato. As only one KNOX gene from potato was identified so far, the first objective of this work was to identify, validate and obtain full-length sequence for other potato KNOX genes. In parallel, the regulation of *POTH1* expression and phloem mobility of its transcript was also explored. Further, the functional characterization studies with one of the novel *KNOX-I* gene, *viz. POTH15,* were performed.

Hence, the objectives of the current study were:

1. To identify, validate and obtain full-length sequence for potato KNOX genes
2. To study the regulation of POTH1 expression and to investigate the mobility of *POTH1* mRNA
3. To understand the regulation, over-expression and to identify the target genes of *POTATO HOMEOBOX15 (POTH15).*

Figures

Chapter 1

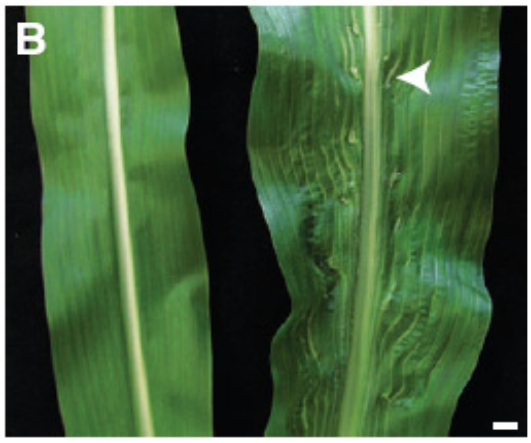


Fig. 1.1 –KN1 over-expression produced knots on leaves. Wild-type maize leaf blade (left) and a *Kn1-N leaf blade (right).* Dominant *Kn1-N mutants express kn1 inappropriately in leaves* and alter leaf development such that proximal cell fates, such as ligule, differentiate in distal positions in the blade (arrowhead), and cells overgrow to form knots. (Adapted from Hay and Tsiantis, 2010)

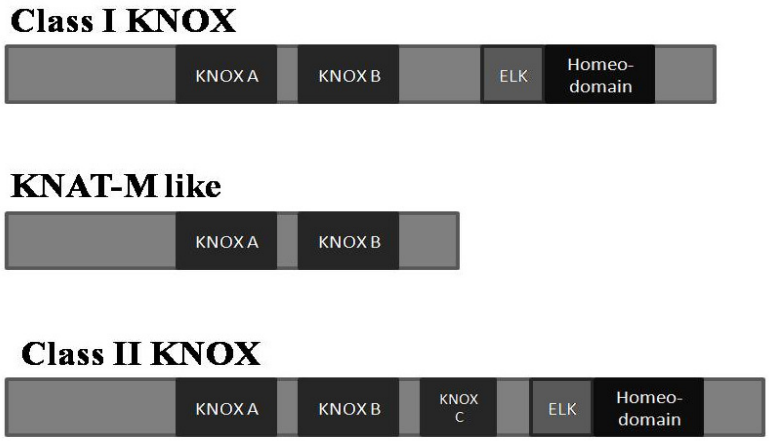


Fig.1.2– Domain sequence in the primary structure of the KNOX proteins; following Mukherjee et al. (2009)

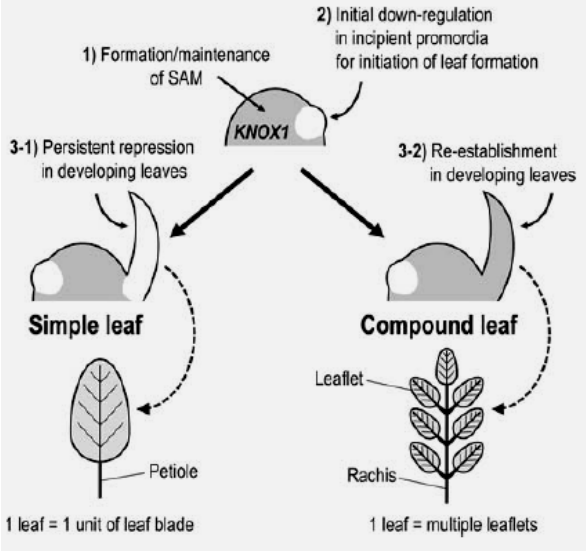


Fig. 1.3- Regulation of KNOTTED1-LIKE HOMEOBOX (KNOX-I) genes in leaf development.KNOX-I expression domains are shown in gray. (Adapted from Uchida et al., 2010)

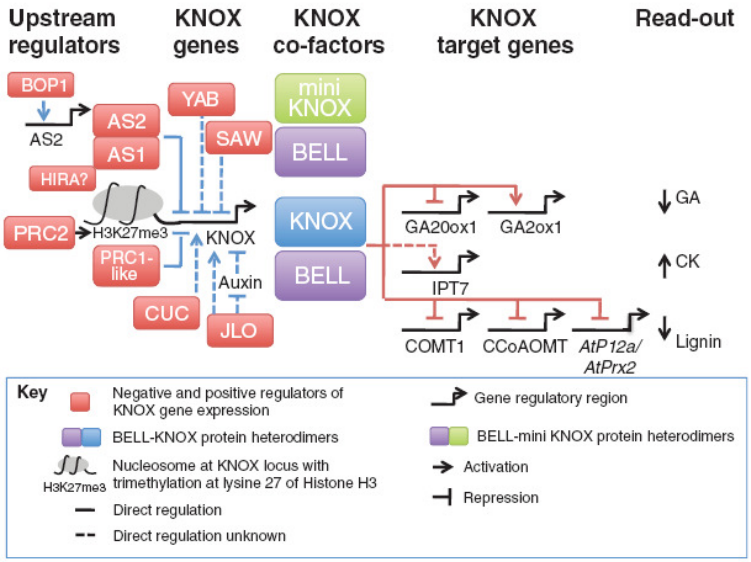


Fig. 1.4 - **KNOX gene regulatory network.** The upstream regulators and targets of KNOX genes identified so far. (Adapted from Hay and Tsiantis, 2010)

Abbreviations: AS1, ASYMMETRIC LEAVES1; BELL, BEL1-like homeodomain family; BOP1, BLADE-ON-PETIOLE1; CUC, CUP-SHAPED COTYLEDON; H3K27me3, trimethylation of histone H3 at lysine 27; HIRA, histone regulatory protein A; JLO, *JAGGED LATERAL ORGANS*; PRC2, polycomb repressive complex 2; SAW, SAWTOOTH; YAB, YABBY.

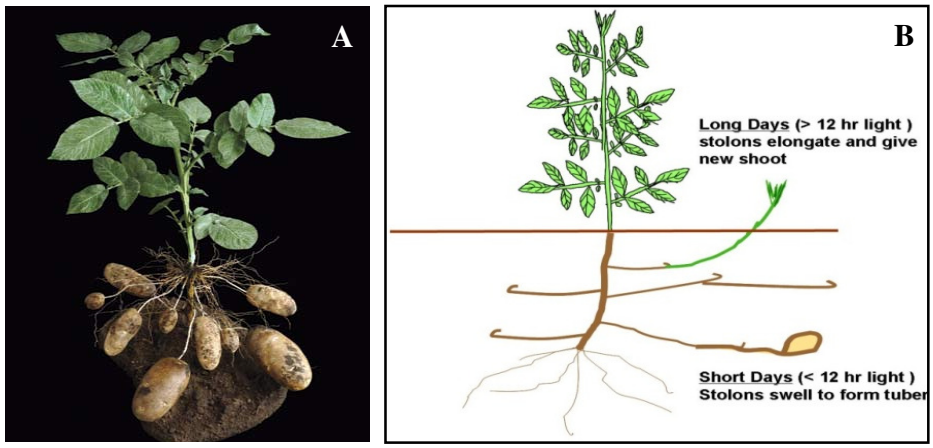


Fig. 1.5–Potato as a model system.(A) A typical potato plant with tubers. (B) Graphic depicting photoperiod dependent tuberization in *Solanumtuberosum*ssp*Andigena*.

Chapter 2

KNOX genes in potato

(*Solanum tuberosum* ssp *Andigena*)

2.1. Introduction

The first KNOX gene to be discovered was *KNOTTED1* (*KN1*) from maize (*Zea mays;* Vollbrecht et al., 1991). Since then, KNOX genes from diverse plant species ranging from unicellular algae to angiosperms have been discovered and they are found to be ubiquitous to green plant lineages. In unicellular algae *Chlamydomonas*, only one KNOX member is present and it is required for 'minus' identity of the 'm' gamete and regulates mating as well as zygotic development (Lee et al. 2008). Over the course of evolution, KNOX genes have undergone duplication and diversification. *KNOX* form a multimember family of genes in higher plants with 5 members in *Physcomitrella*, 9 in *Arabidopsis*, 16 in rice and 7 in tomato (Hay &Tsiantis 2010). It is now well establish that *KNOX* genes regulate diverse developmental processes like regulating alternation of gametophytic and sporophytic generations, meristem maintenance, leaf development,floral development, tuberization, bulbil formation and so on (Hay et al, 2010; Ragni et al, 2007; Uchida et al, 2010).

Our interest is to investigate the KNOX gene function in potato development. The only potato *KNOX* gene characterized prior to this study, *POTH1*, was shown to regulate vegetative development and accelerate tuberization under *in vitro* conditions (Rosin et al., 2003). Moreover, the transcript of *POTH1* was shown to be present in the phloem (Yu et al., 2007; Campbell et al., 2008). Apart from the above studies, no other literatures were available about the KNOX gene functions in potato development. To explore the existence and functions of additional KNOX genes in potato, we mined Potato Genome Sequence Database. Following approaches were taken to study the KNOX genes in potato:

(i) Identification of putative KNOX genes from potato genome.
(ii) Validation of expression of potato KNOX genes.
(iii) Obtaining the full-length sequence for potato KNOX genes.

2.2 Material and Methods

2.2.1. Identification of putative KNOX genes from potato genome

The mRNA sequences forseventomato KNOX genes *viz.Tkn1*(NM_001246878.1), *Tkn2* (NM_001247012.1), *Tkn3* (NM_001246998.2), *Tkn4* (NM_001279346.1),

Petroselinum (NM_001247449.2), *Thox1* (NM_001247016.1), and *Thox2* (NM_001247004.1) were used as a query in BLAST against the potato genome sequence. The best match for each of the sequences was obtained and labelled as a putative KNOX gene. Further, the potatoExpressedSequence Tags (ESTs) matching to each of the tomato KNOX gene were obtained by performing BLAST against *S. tuberosum*EST database. Multiple ESTs that matched to the entire length of the query were selected and their sequences were then assembled to obtain a putative mRNA sequence for each of the potato KNOX genes.

2.2.2.RT-PCR analysis to validate the potato KNOX genes

To validate the expression of the identified putative KNOX genes, RT-PCR analysis was performed. Total RNA from leaves of eight weeks old potato plants was extracted with Trizol (Invitrogen) and RT-PCR was performed using one step RT-PCR kit (Invitrogen). The primer pairs used for each of the genes are given in table 2.1. Reaction conditions for all the genes were 55^{o}C for 60 min, 95^{o}C for 2 min followed by 30 cycles of 95^{o}C-30 s, 55^{o}C-30 s and 72^{o}C-2 min

2.2.3. 5' RACE of KNOX genes

To obtain the full length sequences of the newly identified potato KNOX genes, 5' Rapid Amplification of cDNA Ends (RACE) was performed using ClontechSMARTer RACE kit (Cat. No. 634923) following manufacturer's instructions. Detail protocol for 5'RACE is given in appendix. The primers used for RACE of each the genes are mentioned in table 2.2. The reaction conditions for primary as well as secondary PCR reactions were as 5 cyclesof94°C 30 sec, 72°C 3 min followed by 5 cycles of 94°C 30 sec, 70°C 30 sec, 72°C 3 min followed by 27 cycles of 94°C30 sec, 68°C 30 sec and 72°C 3 min for all the KNOX genes.

Table 2.1 - List of primers used for RT-PCR reactions to validate the expression of newly identified *KNOX* genes from potato.

Gene	Primers used for RT-PCR	
POTH15	POTH15-qF	CTCAATCCTCACTACCATCG
	POTH15-qR	GCATCTCACAATAAGCCTCC
POTH20	POTH20-qF	CTGGTGCTGGTGAAGTAATTG
	POTH20-qR	AACATCTCTGTCGGTCAACG
StKn1	StKn1-qF	AGGCTGAGGCTGAGGATGAG
	StKn1-qR	TGATGTCATTCCTTGCGGTGC
StPTS	*StPTS* –qF	CACACACAATGGTTCATCAAGCAG
	StPTS–qR	GGTGTGGAAGTAGAAGTAGGAAG
StHox1	StTHox1qF	CGCATCCTCACCTTCCTCAG
	StTHox1qR	CCTCCTCCGCTATGCTGTTG
StHox2	StTHox2qF	CATCTATGATGATTTCTTCGGAG
	StTHox2qR	ATGATGAGATTGCGAGAGCTGAGC

Table 2.2 - List of primers used for 5'RACE reactions to identify the sequence on the 5' end of the transcripts.

Gene	Primers used for 5'RACE	
POTH15	POTH15 5'RACE	CCATTTCCATTTCCATTACCATTTCC
	nPOTH15 5'RACE	CAAGATGTACTAGTATTTCCACTAG
POTH20	POTH2 5' RACE	CAATCTCCACCTCTTCCATAAATAGG
	nPOTH2 5RACE	CTGAAGTCTCCGTACTCTCTTGATG
StKn1	StKn1 5'RACE	GATCTGACATATTGGTGCTGGATTTAG
	nStKn1 5'RACE	CCTGTTGTTGTTGTCTACTGCGTG
*St*PTS	StPTS 5'RACE	GGTGTGGAAGTAGAAGTAGGAAG
	nStPTS 5'RACE	TCAAACAATCCAAGTGAGTCTGAACC
*St*Hox1	StTHox1 5'RACE	GTAGGTGAGAGCCACTGGTTAGAG
	nStTHox1 5'RACE	CTGACTGTGTTGTTGACGAAGGAGA
*St*Hox2	StTHox2 5'RACE	ATGATGAGATTGCGAGAGCTGAGC
	nStTHox2 5'RACE	GAAATATCACCGCCACCTAAACCAG

2.2.4. Cloning and phylogenetic analysis of potato KNOX genes

Based on the sequence obtained from 5'-RACE, primers were designed to obtain the full-length amplicons for all the newly identified potato KNOX genes. Total RNA from leaves of eight weeks old potato plants (*S. tuberosum*, ssp. Andigena) was extracted with Trizol (Invitrogen). The reverse transcription was performed with 2ug of RNA and oligodT primer usingSuperScript III Reverse Transcriptase. The PCRs were performed using this cDNA and the gene specific primer pairs. The details of the primers are mentioned in table 2.3. Reaction conditions used for PCR were 95^{o}C for 2 min followed by 30 cycles of 95^{o}C-30 s, 55^{o}C-30 s and 72^{o}C-2 min. The amplicons were then sub-cloned into pGEM-Teasy vector (Promega) and the clones were sequenced to obtain the potato KNOX sequences.The open reading frames of each of the six KNOX genes were predicted using the NEBcutter tool. This tool also provided the putative protein sequence for these KNOX genes which were further aligned to tomato KNOX protein sequences for checking the extent of similarity. The phylogenetic tree for KNOX proteins from potato, tomato,and*Arabidopsis* was generated by the Neighbor-Joining method using MEGA6(Tamura et al. 2013).

Table 2.3 - List of primers used to amplify full-length transcript sequences for potato KNOX.

Gene	**Primers used to amplify full-length transcript sequence**	
POTH15	POTH15-FP	AGGAATATTGGGTGTTTGCTT
	POTH15-RP	ACGTACAAACTAATAAATTTTATAAGAG
StKn1	StKN1-FP	CTTTTTCCAGGTGTCGATCTTTATC
	StKN1-RP	CTACAGAAATTTTGTATTTTGAAC
POTH20	POTH20-FP	GTTTCTAGAGGGTTGTCTTTTTCTTATTTAATGG
	POTH20-RP	AAAGAGCTCCATAAATATTACTGACCCAAACG
StPTS	StPTS-FP	ATGGAGACAAAAAGTGATAATT
	StPTS-RP	TCATTTGTTGCCACGGGCCT
StHox1	StHOX1-FP	CAACAGTTATAGTAAACGCTG
	StHOX1-RP	CAGTGGAACAAGTAATTATAATC
StHox2	StHOX2-FP	CTGCTTATGTGTTTAGAGTTTTCAG
	StHOX2-RP	CATTGAAATACTAAGCAATTAC

2.3 Results

2.3.1. Potato genome codes forseven*KNOX* genes

For all theseventomato KNOX genes that we have used as a query, homologous sequenceswere found in the potato genome database as well as in potato EST database (Table 2.4). Analysis of these sequences revealed six novel KNOX genes from potato and one previously identified potato *KNOX1* gene POTH1(NM_001288425). Tomato *KNOX1* gene, *Tkn3* was found to be homologous to *POTH1*. The nomenclature for the newly identified potato KNOX genes is provided in table 2.5. RT-PCR analysis of these genes revealed that all of them are expressed in *S. tuberosum* ssp *Andigena.*Further, the 5'RACE analysis provided the full length transcript sequences for these KNOX genes. Details of each of the newly identified potato KNOX gene are provided in the following sections:

Table 2.4 – Identification of KNOX genes from potato genome using tomato KNOX genes as a query.

Tomato KNOX gene used as a query	**Best match in the potato genome sequence (Accession number and sequence co-ordinates)**	**Best matches in the potato EST database (Accession numbers of ESTs covering entire query length)**
TKn1 (NM_001246878.1)	AEWC01025829.1	CK254926.1; CK252205.1
TKn2 (NM_001247012.1)	AEWC01015278.1	CX699376.1; DV624511.1
Tkn3 (NM_001246998.2)	AEWC01025117.1	No match found
TKn4 (NM_001279346.1)	AEWC01010836.1	CK262271.1; CK262270.1
Petroselinum (NM_001247449.2)	AEWC01014074.1	No match found
Thox1 (NM_001247016.1)	AEWC01030278.1	CK276863.1; DN939399.1
Thox2 (NM_001247004.1)	AEWC01029270.1	DR036708.1; CO502360.1

Table 2.5 – Nomenclature of potato KNOX genes

Tomato KNOX gene	Nomenclature for potato KNOX homolog
TKn1 (NM_001246878.1)	POTATO HOMEOBOX 20 (*POTH20*; KP335125)
TKn2 (NM_001247012.1)	POTATO HOMEOBOX 15 (*POTH15*; KJ477687)
Tkn3 (NM_001246998.2)	POTATO HOMEOBOX1 (*POTH1*; NM_001288425.1)
TKn4 (NM_001279346.1)	*Solanum tuberosum KNOTTED1* (*StKN1;* KJ477691)
Petroselinum (NM_001247449.2)	*Solanum tuberosum Petroselinum* (*StPTS;* KJ477688)
Thox1 (NM_001247016.1)	*Solanum tuberosum Homeobox1* (*StHox1;* KJ477689)
Thox2 (NM_001247004.1)	*Solanum tuberosum Homeobox2* (*StHox2;* KJ477690)

2.3.1. 1. *POTATO HOMEOBOX15 (POTH15)*

Potato ortholog of tomato *KNOX1* gene*Tkn2* was identified from potato genome and labelled as *POTATO HOMEOBOX 15 (POTH15)*. The amplicon of 194bpspecific to the predicted*POTH15* transcript was obtained by RT-PCR and this validated the expression of *POTH15* mRNA in potato (Fig. 2.1). 5' RACE identified the 5' end of the transcript and full-length *POTH15* mRNA sequence was then obtained by RT-PCR followed by sequence verification (Fig.2.2). The POTH15 gene is found to be located on chromosome number 2 of potato and its PGSC accession number isPGSC0003DMT400043083 (Fig. 2.4A). Further analysis revealed that POTH15 has a transcript length of 1544 bases (Fig. 2.4 B) having 5'UTR of 296 bases and 3'UTR of 215 bases and is predicted to code for 343 amino acids protein (Fig. 2.4 C). POTH15 protein sequence is 89% identical to its tomato homolog TKn2. The NCBI accession number for the POTH15 mRNA isKJ477687 and for POTH15 protein is AHX00579.

2.3.1. 2. *POTATO HOMEOBOX20 (POTH20)*

Homology search for tomato *KNOX1* gene *Tkn1*identified a KNOX gene from potato genome,which was labelled as *POTATO HOMEOBOX20* (*POTH20*). A 189

bpamplicon specific to the predicted *POTH20* transcript was obtained by RT-PCR and this validated the expression of *POTH20* mRNA in potato (Fig.2.1). Further, 5' RACE identified the 5' end of the transcript and full-length *POTH20* mRNA sequence was then obtained by RT-PCR followed by sequence verification (Fig. 2.2). The POTH20 gene is located on chromosome number four of potato and its PGSC accession number is PGSC0003DMT400012712(Fig. 2.5A).Further analysis revealed that POTH20has a transcript length of 1682 bases (Fig. 2.5B) with 5'UTR of 351 bases and 3' UTR of248 bases and is predicted to code for 360 amino acids protein (Fig. 2.5C). Our analysis revealed that POTH20 protein sequence is 93% identical to its tomato homolog TKn1. The NCBI accession number for the POTH20mRNA is KP335125.

2.3.1. 3. *Solanum tuberosum Knotted1 (StKn1)*

Anortholog of tomato *KNOX1* gene *Tkn4* was identified from potato genome and it was labelled as *Solanum tuberosum Knotted1* (*StKn1*). A 225 bp amplicon specific to the predicted *StKn1* transcript was obtained by RT-PCR and this validated the expression of *StKn1* mRNA in potato (Fig. 2.1). 5' RACE identified the 5' end of the transcript and full-length*StKn1* mRNA sequence was then obtained by RT-PCR followed by sequence verification (Fig. 2.2). The StKn1 gene is found to be located on chromosome number one of potato and its PGSC accession number isPGSC0003DMT400007159 (Fig. 2.6A).Further analysis revealed that *StKn1*has a transcript length of 1680 bases (Fig. 2.6B) with 5'UTR of 313 bases and 3' UTR of 135 bases and the transcript is predicted to code for 323 amino acids protein (Fig. 2.6C). The StKn1 protein is 88% identical to its tomato homolog Tkn4. The NCBI accession number for the *StKn1* mRNA isKJ477691 and for StKn1 protein isAHX00583.

2.3.1.4. *StPetroselinum (StPTS)*

Potato ortholog of tomato mini-KNOX gene *Petroselinum* was identified from potato genome and was labelled as *StPetroselinum (StPTS)*. RT-PCR resulted a 220 bpamplicon specific to the predicted *StPTS*transcript, which validated the expression of *StPTS* mRNA in potato (Fig. 2.1). 5' end of the transcript was obtained by 5' RACE and the full-length (510 bases)*StPTS* mRNA sequence was detected by RT-PCR followed by sequence verification (Fig. 2.2). The *StPTS* gene is positioned on chromosome number one of potato and its PGSC accession number is PGSC0003DMT400066158

(Fig. 2.7A).Further analysis showed that *StPTS*has a transcript length of 510 bases (Fig. 2.7B) without any UTRs and is predicted to code for 169amino acidsprotein(Fig. 2.7C). The StPTS protein is observed to be 88% identical to its tomato homolog PTS. The NCBI accession number for the *StPTS* mRNA and StPTS protein is KJ477688and AHX00580 respectively.

2.3.1.5. *Solanum tuberosum Homeobox1 (StHox1)*

Homology search for tomato *KNOX2* gene *Thox1* identified a KNOX2 gene from potato genome and it was labelled as *StHox1*. A 225 bp amplicon specific to the predicted *StHox1* transcript was obtained by RT-PCR and this validated the expression of *StHox1* mRNA in potato (Fig. 2.1). 5' RACE resulted the 5' end of the transcript and RT-PCR identified the full-length*StHox1* mRNA (1821 bp) which was later verified by sequencing (Fig. 2.2). *StHox1* gene is found to be located on chromosome number seven of potato and its PGSC accession number is PGSC0003DMT400078990 (Fig. 2.8A).Further analysis revealed that *StHox1*has a transcript length of 1821 bases (Fig. 2.8B) having 5'UTR of 140 bases and 3' UTR of 399 bases and is predicted to code for 426 amino acids protein (Fig 2.8C). Our analysis showed that the StHox1 protein is 95% identical to its tomato homolog Thox1. The NCBI accession number for the *StHox1* mRNA is KJ477689 and for StHox1 protein isAHX00581.

2.3.1.6. *Solanum tuberosum Homeobox2 (StHox2)*

An ortholog of tomato*KNOX2* gene *Thox2* was identified from potato genome and was labelled as *StHox2*. A 144 bp amplicon specific to the predicted *StHox1* transcript was obtained by RT-PCR and this validated the expression of *StHox2* mRNA in potato (Fig. 2.1). 5' RACE identified the 5' end of the transcript and full-length*StHox2* mRNA sequence (1479bp) was then obtained by RT-PCR followed by sequence verification (Fig. 2.2). The *StHox2* gene is located on chromosome number eight of potato and its PGSC accession number is PGSC0003DMT400058528 (Fig 2.9A).Further analysis revealed that *StHox2*has a transcript length of 1479 bases (Fig 2.9B) with 5' and 3' UTR of270 and 269 bases respectively. The transcript is predicted to code for 312 amino acids protein (Fig 2.9C). We found that the StHox2 protein is 94% identical to its tomato homolog Thox2. The NCBI accession number for the *StHox2* mRNA is KJ477690 and for StHox2 protein isAHX00582.

In the current study, six novel KNOX genes from potato genome were identified, validated and cloned. Thus including POTH1, the number of KNOX genes identified from potato is now seven.Further, a phylogenetic analysis of potato KNOX proteins along with tomato and *Arabidopsis* KNOX proteinsrevealed that four of them belong to Class-I (POTH1, POTH15, POTH20, and StKn1), two belongs to Class-II (StHox1 and StHox2) and one (StPTS) is a mini-KNOX(Fig.2.10).

2.4 Discussion

Knotted like homeobox (KNOX) genes are ubiquitous in green plant lineages and they control cell fate determination and development (Hay and Tsiantis; 2010). Based on expression pattern and intron position, KNOX genes were grouped in two subclasses; class I and II (Kerstetter et al, 1994). Later, Magnani and Hake (2008) introduced a new class of KNOX proteins, which possessed a MEINOX domain but not a homeodomain and hence this class is sometimes referred as mini-KNOX.KNOX-I genes are known to regulate diverse developmental mechanisms in plants. For example, in Arabidopsis four KNOX-I genes; STM, KNAT1, KNAT2, and KNAT6, regulate various aspects of development such as embryo patterning, meristem maintenance, leaf development, floral development and so on,whereas in tomato, TKn2has been demonstrated to regulate compound leaf development (Kim et al 2001, Shani et al 2009). The members of mini-KNOX clade, KNATM in Arabidopsis (Magnani and Hake, 2008) and PTS in tomato (Kimura et al., 2008), are implicated in regulation of leaf development and their role in other developmental pathways are not yet understood. On the other hand, studies on function of class II KNOX genes are rare and their functions remain mostly unknown (Hay and Tsiantis, 2010).

Previous to this study, only one potato KNOX gene *viz*. POTH1 was identified and it was found to be a class-I KNOX gene. In 2011, the potato genome sequence was published (The Potato Genome Sequencing Consortium; 2011) and it revealed that the 844megabases genome codes for approximately 40,000 protein coding genes.
Availability of genome data provides opportunities to explore novel genes from potato.

In the present study, potato KNOX genes were identified by performing homology searches for tomato KNOX genes in the potato genome sequence data.Tomato genome codes for seven KNOX genes - four KNOX-I genes (TKn1,

TKn2, TKn3 and TKn4), two KNOX-II genes (Thox1 and Thox2) and one mini-KNOX gene (*PTS*).Same as tomato, potato genome was also found to code for seven KNOX genes, four of which belong to class-I, two belong to class-II and one is a mini-KNOX (Fig 2.10).The discovery of these KNOX genes from potato genome is the next step towards unravelling their role in potato development.Out of the seven potato KNOX genes, POTH1 was earlier shown to regulate vegetative development and accelerate *in vitro* tuberization by regulating GA levels (Chen et al., 2003; 2004; Rosin et al., 2003). As part of the present study, we have investigated the regulation and transcript mobility of POTH1 which has been described in chapter 3, whereas the functional characterization of POTH15 has been described in chapter 4. Future interest could be to investigate the functions of remaining KNOX genes in potato.

Chapter 2

Figures

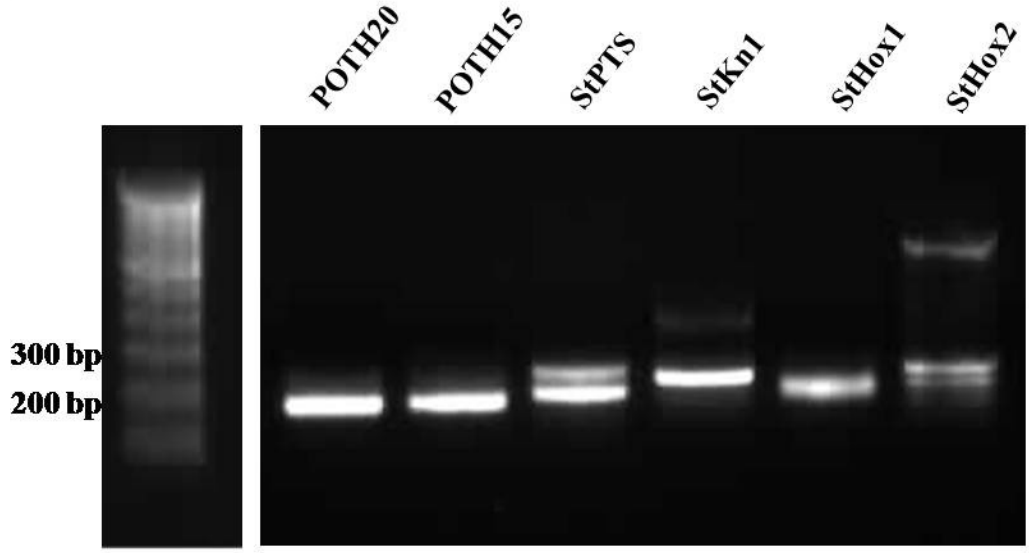


Figure 2.1 – Validation of KNOX genes from potato. RT-PCRs of six novel KNOX gene from potato using gene specific primers and total RNA isolated from shoot tips of 8 weeks old potato plants

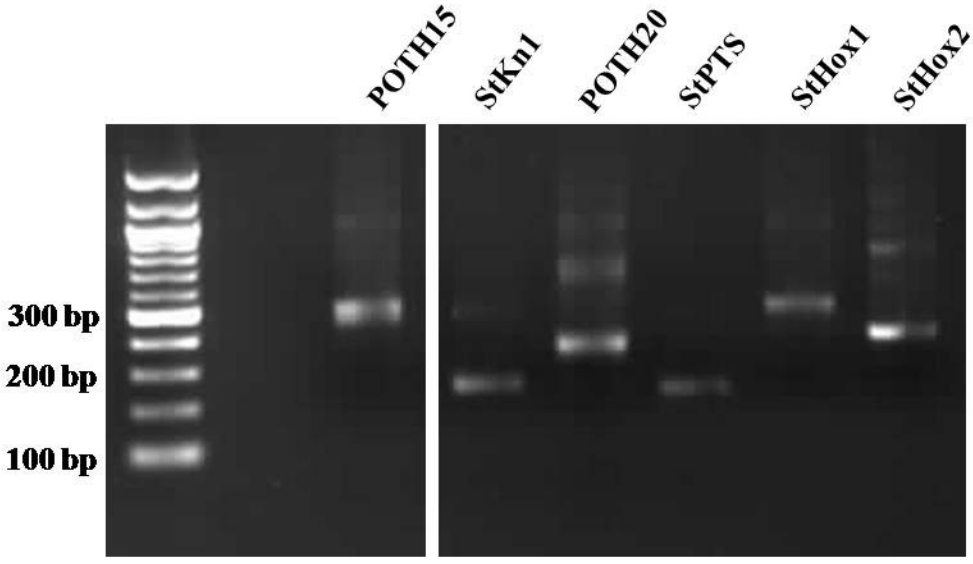


Figure 2.2.5' RACE of six KNOX genes from potato. The amplicons obtained for each of the gene were sequence verified.

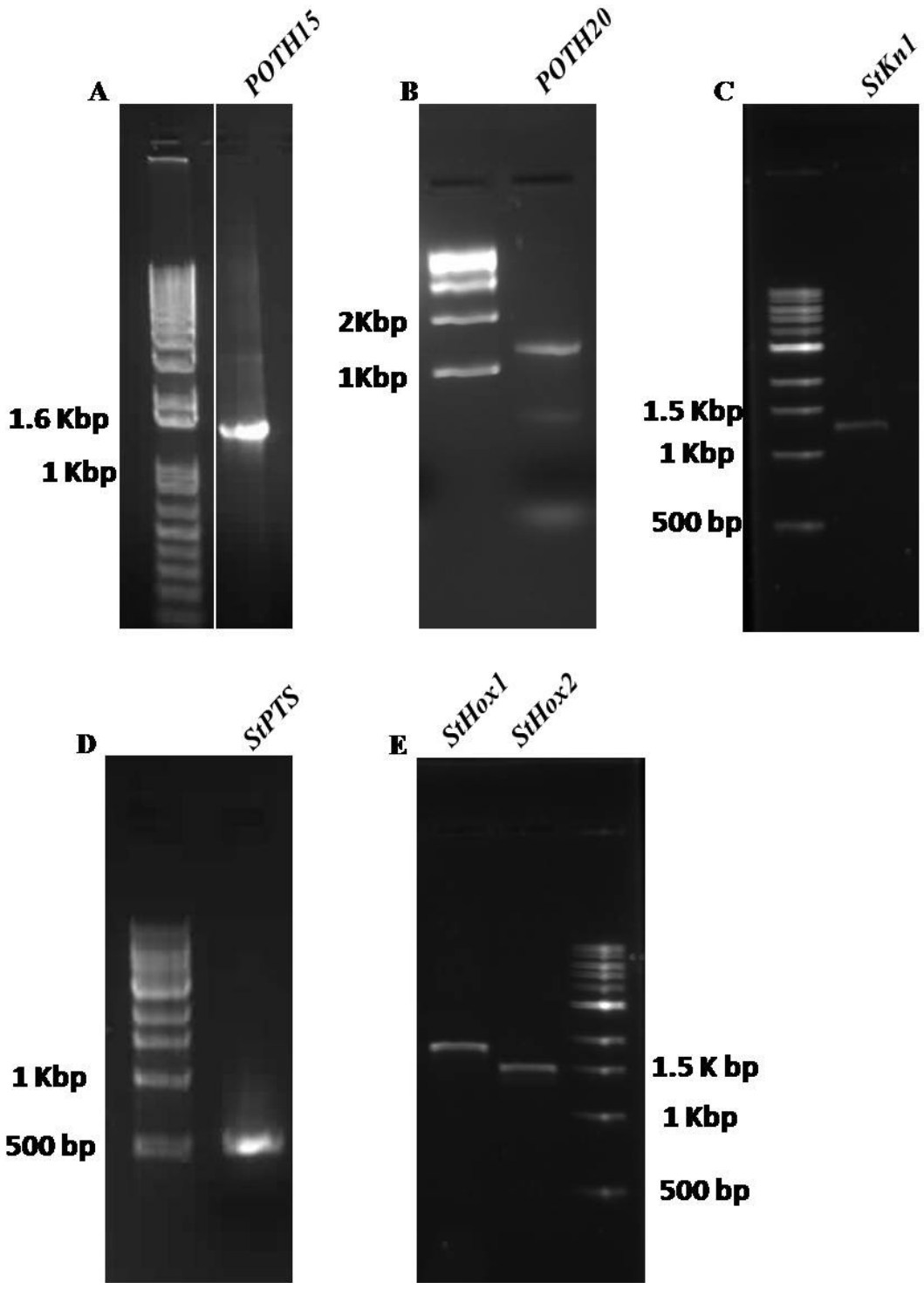
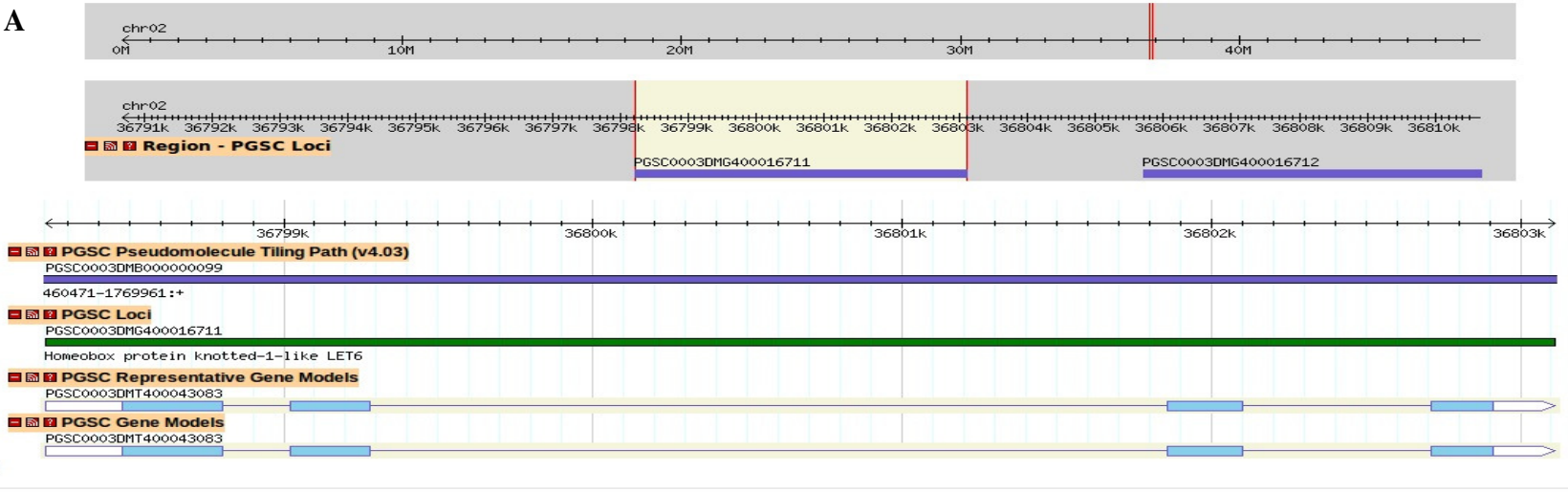


Figure 2.3.Cloning potato KNOX genes. Amplification of full length mRNA for *POTH15* (A), *POTH20* (B), *StKn1* (C), *StPTS* (D), *StHOX1* and *StHOX2* (E) from total RNA isolated from shoot tips of 8 weeks old potato plants.

A



B >POTH15[organism= *Solanum tuberosum*] ssp *andigena* class-I *KNOX* gene full length cDNA sequence

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ACTTACACACACACCCTCCCCAAAAAGAGAGAGAGATTTACTATTAAACAGAGTTTTTTTTTTTATTACATTATTTATTGTGTGTGTGTGTGAGAGAGAGAGATGGTTTTCAT
AGGCAAAAACAAATAGAAAGGAACAAAATTTAGACTCAAGAAGAAAGTGTGTGTGAGAGAGAGGAAGAATAATGGAAGGTGGAAATACTAATACATCTTGTTTAATGATG
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GGATAGAAATGGATCATCTGATGAAGAGGTTGACGTGAATAACAGTTTCATCGACCCTCAGGCTGAGGATAGAGAGCTCAAAGGTCAATTGTTGCGTAAGTACAGTGGGTA
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C

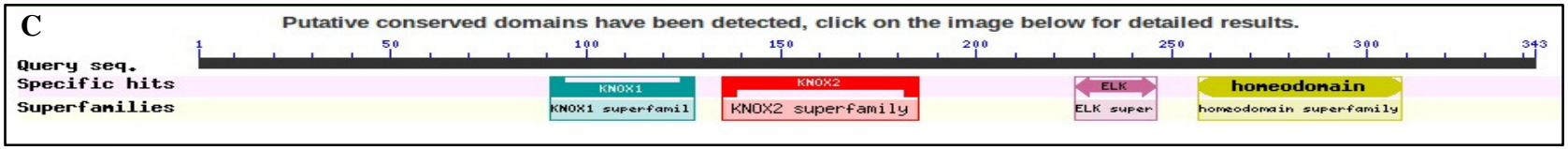

Fig. 2.4 Location of POTH15 gene in potato genome with sequence of POTH15 transcript (B) and domains in POTH15 protein (C)

POTATO HOMEBOX 20 (POTH20)

A

B

>POTH20[organism= *Solanum tuberosum*] ssp *andigena* class-I *KNOX* gene full length cDNA sequence

GATATTTTAATATTCTTTTCCAATATATATATATATTCTTCAAGGAAGAACGTCTTATCACCCTACGCAGCGCTACCAGTAACACCACTATCTACCCCTTTTTTTGGTTCTCTT
GCTCACCACAATAATCTTCTAAGATCCCTTCCCTTTTCTATCTTTTGATAATAATAAGTTTATTTACTTCTTTAGTGCAAAAATAATATCCATTTTAATTTATTTTCTTGGTTTG
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TCCAACCTTTTGGACGCTTACATGGGCTGCCAAAAGGTGGGTGCACCGCCGGAAGTGGCGGCGAGACTGTCGGCGGTACGGCAAGAGTTTGAGGCGAGGCAACGAGCTTC
GTTGACCGACAGAGATGTTTCCAAGGACCCAGAACTTGATCAGTTCATGGAAGCATATTATGACATGCTAGTGAAGTATAGAGAGGAGTTAACAAGGCCTCTTCAAGAAGC
AATGGAATTCATGCAAAAGATTGAAGCTCAGCTTAATATGCTTGGTAATGCTCCTGTTCGGATCTTCAACTCTGAGGACAAGTGTGAGGGTGTTGGATCATCAGAAGAGGAT
CAAGACAACAGTGGTGGAGAAACTGAACTTCCTGAAATAGATCCAAGAGCAGAAGATAGAGAACTGAAGAATCACTTGTTGAGAAAATACAGTGGCTATTTAAGTAGCCT
CAAACAAGAACTTTCAAAGAAGAAGAAAAAAGGAAAATTGCCCAAAGATGCTAGGCAGAAGTTGATCACTTGGTGGGAGTTGCACTACAAGTGGCCATACCCTTCGGAGT
CAGAGAAGGTTGCATTGGCAGAATCAACAGGATTGGACCAGAAACAAATAAATAACTGGTTCATTAACCAAAGGAAAAGACACTGGAAACCTTCAGAAGACATGCAATTT
ATGGTGATGGATGGTCTTCATCCACAAAGTGCTGCTGCTCTTTATATGGAAGGTCACTACATGGGTGAAGCCCCTTTTCGTTTGGGTCAGTAATATTTATGATTTTTAATGAA
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C

C

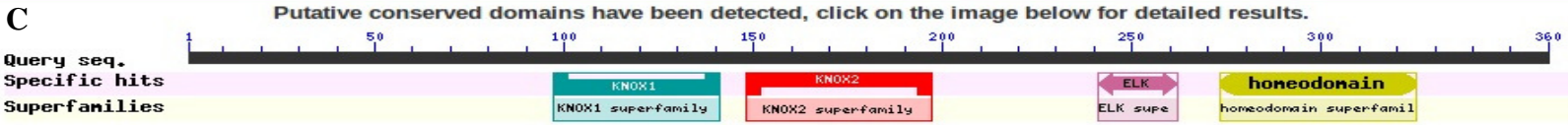
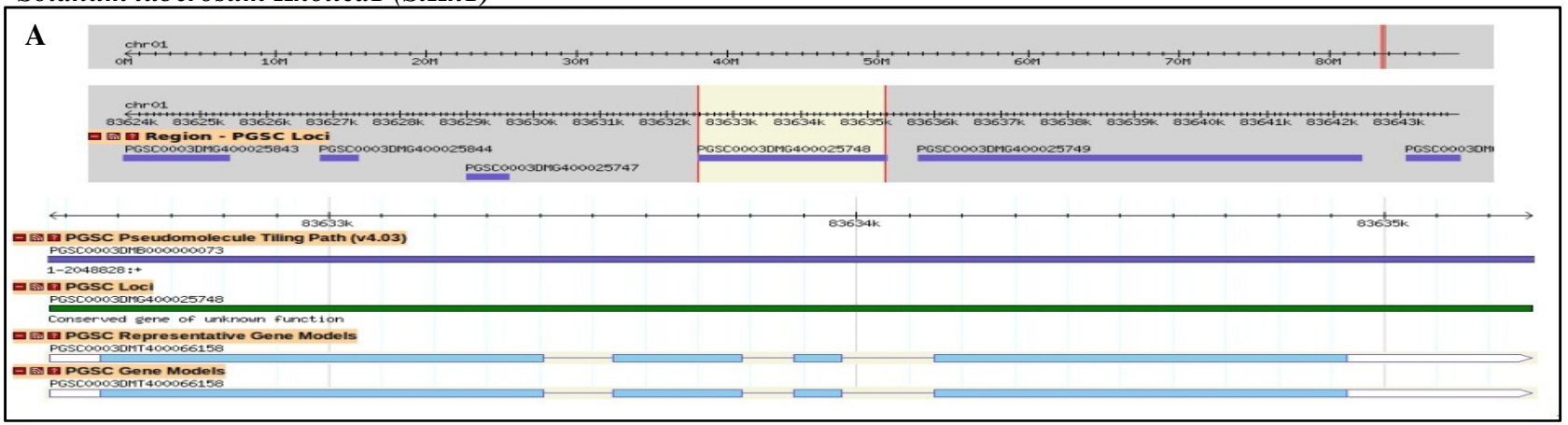


Fig. 2.5 Location of POTH20 gene in potato genome with sequence of POTH20 transcript (B) and domains in POTH20 protein (C)

Solanum tuberosum Knotted1 (StKn1)



C >StKN1[organism= *Solanum tuberosum*] ssp andigena Class-I *KNOX* gene full length cDNA sequence

CTTTTTCCAGGTGTCGATCTTTATCTTCTTTTTTTGGGTTTAAAGAAACGTACTCCATATATATACAGCCAGATTATAGCTAAGTATTTTTAATTACAAGTTATTGTTCACATA
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ACAATTATTAATAACACTTTCCTATGCAGCATTATCTGTCAATTATGAAGCCGCTGATCATTAAAATAATACTAAATCCTGCACCAATATGTCAGATCTTATTAAAGCTCAGA
TTGCAAATCATCCTCTTTATCCTAACTTACTTTCTGCTTATCTTCAATGTCGTAAGGTTGGAGCACCGCAAGGAATGACATCAATTCTCGAAGAAATTAGCAACGAGAACAA
CTTAATATCCAGCTCATGTCACAGTAGTGAAATTGGAGCTGATCCAGAACTTGATAAGTTTATGGAATCATATTGTGCGGTATTAGTGAAATACAAAGAGGAGCTTTCAAAG
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CTGATGAAGCAGGTGGTACCTCAGAGGAGGATCTAGGCTGTGAGGAGATGGAAGCAGCGGATAGTCCTGCTAACCGTGAAGGCGATAATGAGCTAAAAGAAATGCTGATG
CGCAAATATAGTGGCTATCTTAGCAGTTTGAGAAAGGAATTTCTGAAGAAGAGGAAGAAAGGCAAGTTACCTAAGGAAGCAAGAATTGTATTGCTGGACTGGTGGAATACT
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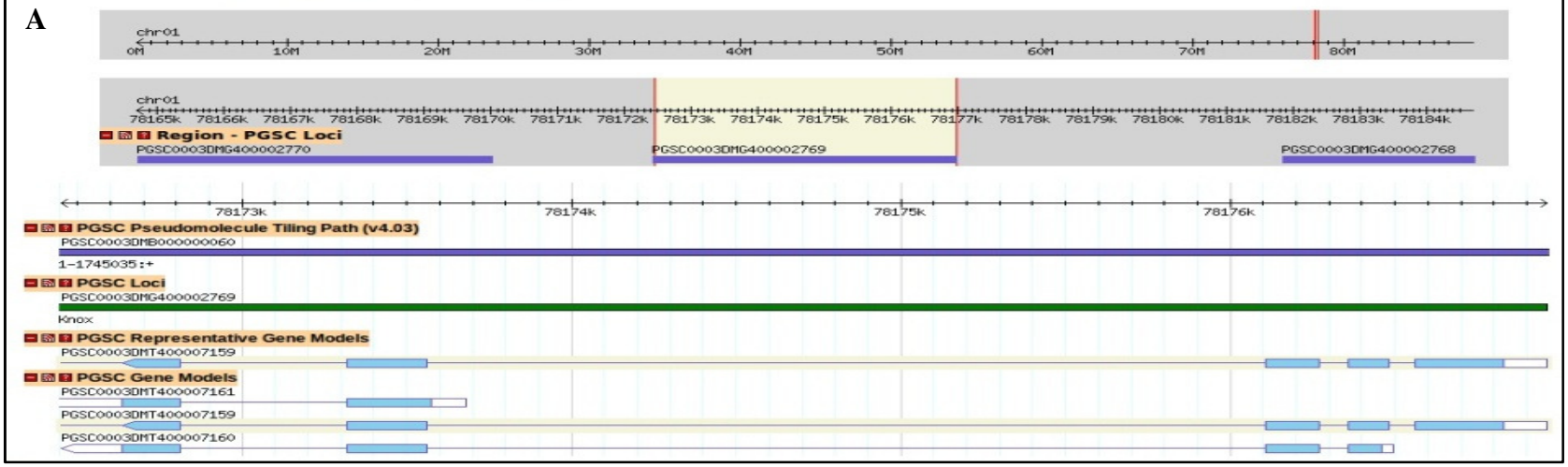
AACCTCCTGCTAGCTAACACGTAGTGTATAGTATTATGACAAAGTATGTATTTTGAAGTTTATAAATCGACAATTGTTAAACCATCTATGTTCAAAATACAAAATTTCTGTAG

Fig. 2.6 Location of *StKn1* gene in potato genome (A) with sequence of *StKn1* transcript (B) and domains in StKn1 protein (C)

Solanum tuberosum Petroselinum (StPTS)

A



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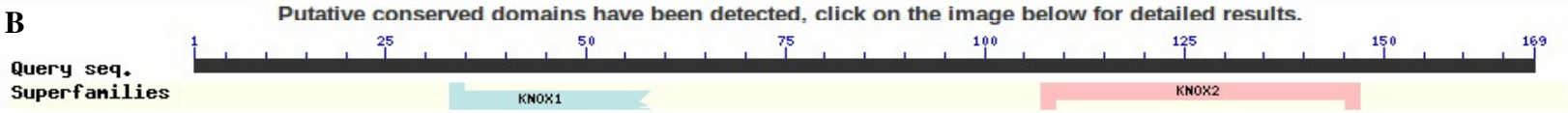

C >StPetroselinum [organism= *Solanum tuberosum*] ssp *andigena mini-KNOX* gene full length cDNA sequence

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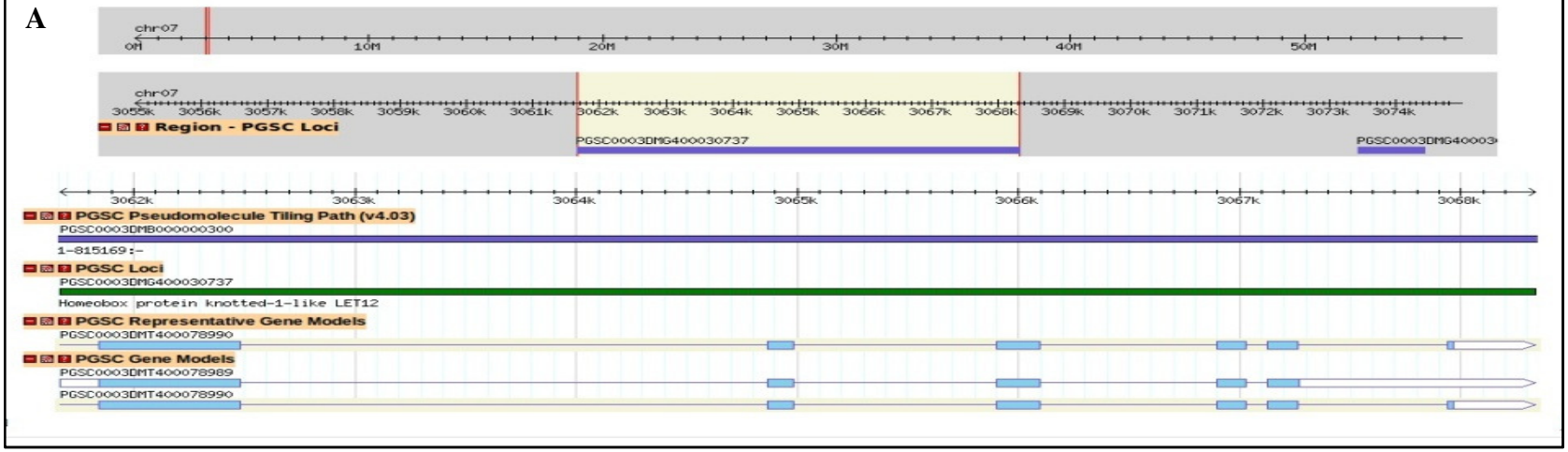
TGGAAGCATATTGTGTGGCTCTAAGCAAGCTAAAAGAGGCAATGGAGGAACCTCATTTGGAGTCCATAAAGTTCATCAATCATATGTATTCTCAGCTCAGTGAACTCATGG
AACTTCCTACTTCTACTTCCACACCATCTAATTTTGATGGGATGAAGGCCCGTGGCAACAAATGA

Fig. 2.7 Location of *StPTS* gene in potato genome with sequence of *StPTS* transcript (B) and domains in StPTS protein (C)

Solanum tuberosum Homeobox1 (StHox1)

A



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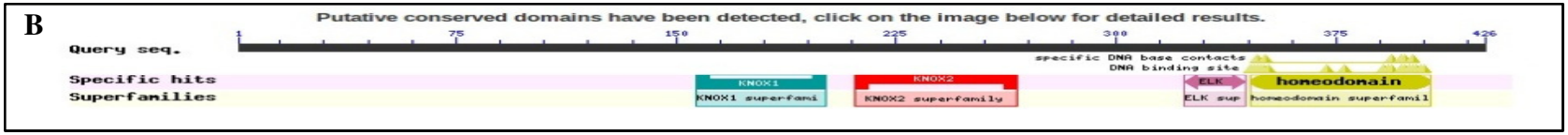

C

>StHOX1[organism= *Solanum tuberosum*] ssp *andigena* Class-II *KNOX* gene full length cDNA sequence

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CGCCTCTAGATGATAAAGACCTCGATCAATTTATGACACATTATGTTCTGTTGCTCTCTTCCTTTAAAGAACAACTGCAACAACATGTCCGTGTCCATGCAATGGAAGCTGTC
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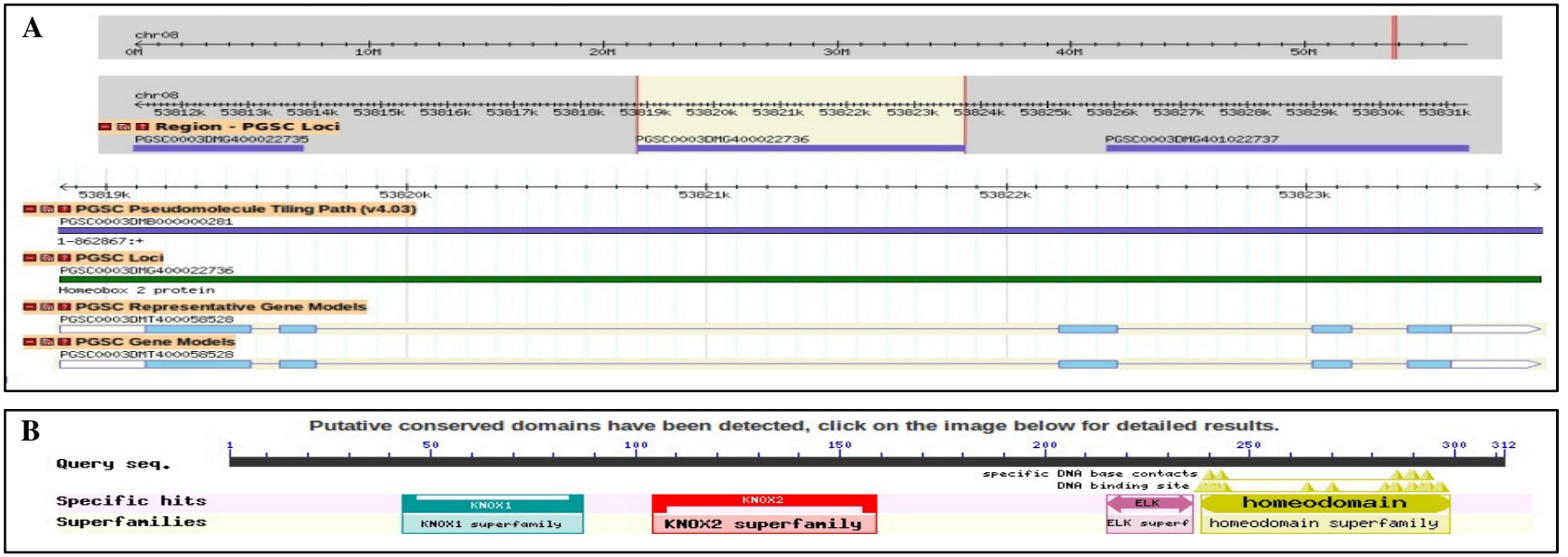
ATAGAAGTTGTAGATTATAATTACTTGTTCCACTG

Fig. 2.8 Location of *StHox1* gene in potato genome with sequence of *StHox1* transcript (B) and domains in StHox1 protein (C)

Solanum tuberosum Homeobox2 (StHox2)



C >StHOX2[organism= *Solanum tuberosum*] ssp *andigena* Class-II *KNOX* gene full length cDNA sequence

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TGTAATATATGGTACTCAGAATTGGTATACATATATGGTAGATTTGTGTTTTCAGTATACAAACTAACAGTACTTTTGAGCTCTTGAGTATTGACACTTTTTCTTGTAATGCTG
GTAATTGCTTAGTATTTCAATGAAT

Fig. 2.9 Location of *StHox2* gene in potato genome with sequence of *StHox2* transcript (B) and domains in StHox2 protein (C)

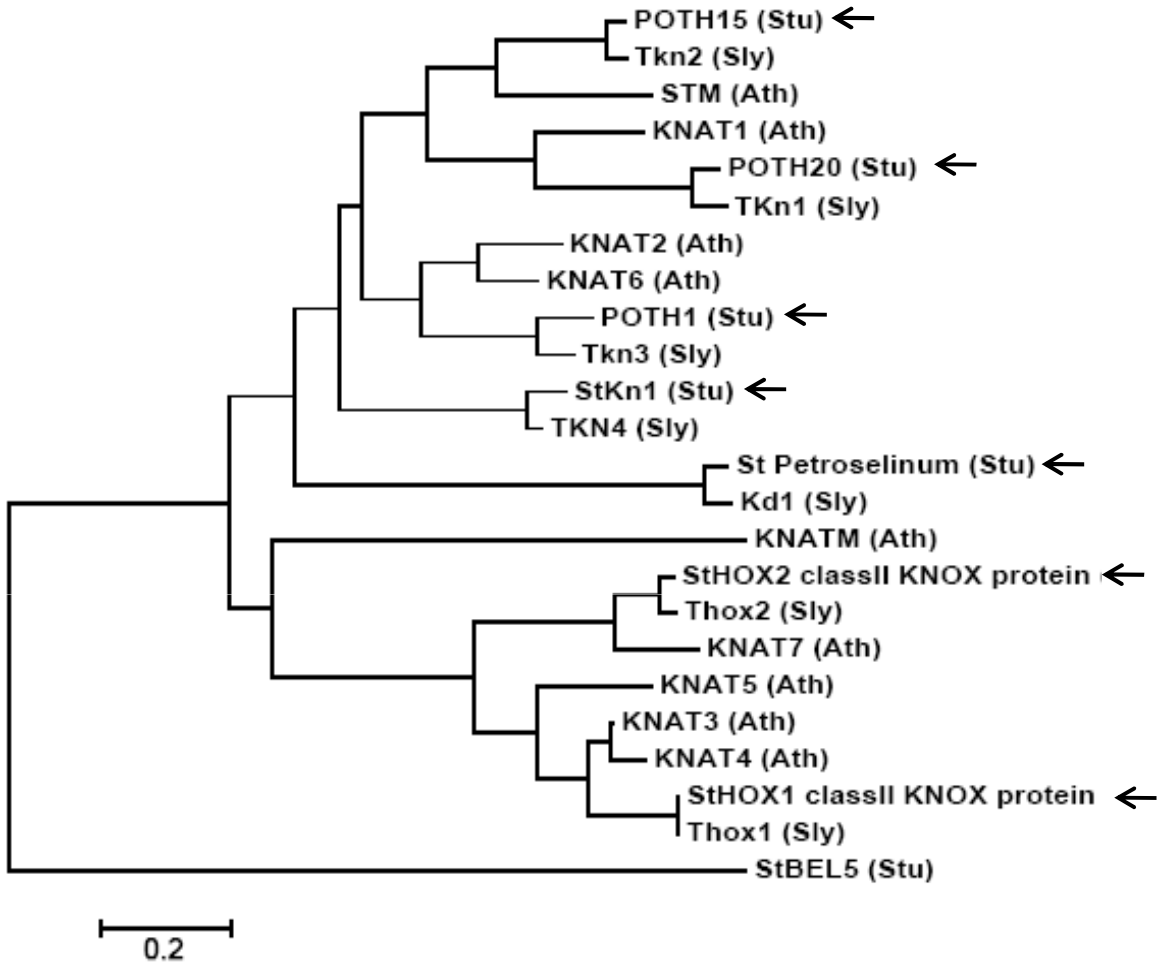

Figure 2.10 Phylogenetic tree for KNOX proteins from potato, tomato, and *Arabidopsis* was generated by the Neighbor-Joining method using MEGA6 (Tamura et al. 2013). The tree is rooted to *StBEL5* sequence. The branch length indicates number of amino acid substitution per site. Arrows indicate KNOX members in potato.

Chapter 3
POTH1: Studying the regulation and transcript mobility

3.1. Introduction

Earlier, *POTH1* was isolated from an early-stage tuber cDNA library of potato and *POTH1* mRNA was shown to have a widespread expression pattern (Rosin et al., 2003). Further, authors demonstrated that over-expression of *POTH1* in potato results in dwarf plants with altered leaf morphology. The levels of the bioactive GA were significantly reduced in the *POTH1* over-expression lines and they exhibited enhanced *in vitro* tuberization under both short-day and long-day photoperiods. Rosin et al. concluded that *POTH1* mediates the development of potato by acting as a negative regulator of GA biosynthesis. Later, Chen et al. (2004) demonstrated that POTH1, and its BEL1 partner, *St*BEL5, form a tandem dimer that binds to a specific TTGAC motif in the *ga20ox1* promoter and downregulates its activity (Chen et al. 2003, 2004). DNA-binding and transcription assays confirmed that the POTH1/*St*BEL5 interaction is necessary to affect *ga20ox1* promoter activity (Chen et al. 2004).

Using RNA movement assays and heterografting experiments Banerjee et al (2006) showed that the mRNA of *StBEL5* is mobile and that accumulation of this mobile RNA correlates with enhanced tuber formation (Banerjee et al. 2006a). The source of this RNA was demonstrated to be the veins of leaves and petioles and promoter activity of *StBEL5* was observed in companion cells and phloem parenchyma of petioles (Banerjee et al. 2006a). Interestingly, POTH1 RNA was also detected in phloem cells of potato through in situ hybridization and its presence in phloem was later verified using laser capture microdissection and RT-PCR (Yu et al. 2007; Campbell et al. 2008), but the movement of this RNA was not been confirmed. Using the dominant mutant *Me* of tomato, the transcript for *LeT6* (a tomato *KNOTTED1*-like homeobox gene) was demonstrated to be graft transmissible (Kim et al. 2001). The *LeT6* fusion transcript was shown to act as a systemic signal that altered leaf development in the scions of heteografts. Long-distance movement for the *GIBBERELLIC ACID INSENSITIVE* (*GAI*) transcript was verified in *Cucurbita maxima* and Arabidopsis (Haywood et al. 2005). *GAI* mRNA mobility was shown to be regulated by sequence in the 3´ end of its transcript (Huang and Yu 2009). Similarly, Banerjee et al. (2009) showed that the untranslated regions (UTRs) of *StBEL5* mediated its long-distance transport, stability and translation. In another interesting report, the RNA for a STM-like ortholog of pumpkin was found to be present in a phloem mobile ribonucleoprotein complex. Using the gel-mobility shift assays, the authors demonstrated that the specific polypyrimidine tract-binding protein, CmRBP50, plays a

key role in binding to RNAs and facilitating their long-distance transport (Ham et al. 2009). Several BELL and KNOX family transcripts were also observed to be present in phloem cells and in phloem-enriched sap of solanaceae plants (Yu et al. 2007; Campbell et al. 2008). Despite such evidence, reports of any long-distance transport remains restricted to *StBEL5* in potato and *LeT6* in the *Me* mutant of tomato. It is interesting to note that, transcripts encoding for interacting proteins (KNOX and BELL) are concurrently detected in the phloem of potato. To investigate the significance of this observation following experiments were undertaken.

(i) To study the POTH1 expression pattern, isolation and characterization of POTH1 promoter was carried out.

(ii) The long distance mobility of *POTH1* transcript was tested by performing grafting experiments with POTH1 over-expression lines and wild-type plants.

(iii) The role of POTH1-UTRs in the transcript mobility was studied by evaluating the effect of UTRs on the translation and by testing their ability to bind to trafficking proteins.

3.2. Materials and Methods

3.2.1. Plant material

The photoperiod-responsive potato (*Solanum tuberosum* ssp. *Andigena)* was used throughout this study. Plants of this subspecies tuberize only under short-day (< 12-h-light) conditions whereas under long days no tubers are formed. Tobacco (*Nicotiana tabacum* var Petite Havana) plants were also used in the study. *In vitro* cultures were grown at 25°C with 16- h-light and 8-h-dark photoperiod in a growth incubator (Percival Scientific). Soil grown plants were grown at 24°C with 16-h-light and 8-h-dark photoperiod in an environmental chamber (Percival scientific). For short days, the plants were shifted to 8-h-light and 16-h-dark photoperiod. Micrografts were maintained under a long-day photoperiod (LD) for 10 days and then transferred to short days or incubation was continued under LD.

3.2.2. Construct design and transformation

Upstream sequence of 1,627 nt from the *POTH1* start codon was isolated using GenomeWalker^{TM} Universal kit (Clontech Cat. No. 638904) following the manufacturer's instructions. The amplified sequence was subcloned into pCR2.1

(Invitrogen TopoTA cloning kit) and sequenced. Upstream sequence of *POTH1* was then amplified using primer pair 5´-CCCAAGCTTCTCTTGAGATGATATAAATATC-3´ and 5´-TTCTCTAGACAAAGTAGAGTAAATTACTTAG-3´ from pCR2.1. This amplicon was inserted upstream of β-glucuronidase in the binary vector pBI121. Full-length *POTH1* cDNA was cloned into the binary vector pBI121 to generate the 35S::POTH1 construct. A 10X histidine tag was added to the C-terminus of POTH1 by cloning a CAC decamer just before stop codon of POTH1 coding sequence. *Agrobacterium*-mediated transformation of the potato, *Solanum tuberosum* ssp. *andigena*, was done using the method described by Banerjee et al. (2006b). Tobacco, *Nicotiana tabacum* var Petite Havana, was transformed using the method described by Horsch et al. (1985). Kanamycin resistant transgenic plants were selected for further analysis.

3.2.3. Light inducibility of $_{prom}$POTH1

For the light induction experiment, sets of in vitro plants ($_{prom}$POTH1::GUS; 35S::GUS and $_{prom}$*St*PTB6::GUS) were incubated under following six conditions such as (i) long day (LD); (ii) dark for 8days; (iii) dark for 7 days followed by 24-h-light; (iv) dark for 8 days with transfer to MS + 6% sucrose 7 days post inoculation; (v) dark for 7 days followed by transfer to MS + 6% sucrose and under light for 24-h; and (vi) dark for 7 days followed by transfer to light for 24-h and returned back to dark for 3 days. The plant tissues were harvested for the assays at the end of respective incubation periods. 35S::GUS and $_{prom}$*St*PTB6::GUS lines served as negative and positive controls, respectively. Dark conditions were maintained in a dark room at 25°C. Light intensity in the growth chamber was 100 µmol m^{-2} s^{-1}. To check the effect of photoperiod on POTH1 promoter activity, 8-weeks old $_{prom}$POTH1::GUS transgenic plants were incubated under long day (16h light, 8h dark) or short day (8h light, 16h dark) photoperiods for four weeks. The GUS activity was quantified from leaves of these plants by MUG assay (Jefferson;1987).

For determining the effect of light on expression of *POTH1* transcript, a detached leaf experiment was conducted. Six to seven terminal leaflets of 8-week old soil grown wild type potato plants were collected and placed in petri-dishes containing liquid medium (MS basal salts without sucrose). The terminal leaflets were incubated in dark for 24-h followed by a transfer to light for 0 or 24-h respectively. The control set of leaves was incubated under long day conditions. Light and dark conditions were maintained as described above. After respective incubations, the leaves were harvested and total RNA was isolated using Qiagen RNeasy mini kit (Cat.No. 74104). One microgram of RNA was subjected to RT-PCR for POTH1 transcripts using Invitrogen

one step RT-PCR kit (Cat. No. 10928). 18S rRNA served as a control. Relative transcript levels were analyzed by Image J software (Abramoff et al. 2004).

3.2.4. Micrografting

In vitro cultures of *Solanum tuberosum* ssp. *andigena* line 7540 and 35S::POTH1 lines were used for grafting studies. Micrografting was done following the procedure explained by Banerjee et al. (2006a). The stock plants were grown for 3 to 4 weeks in growth incubators as described. Wild type stock plants were decapitated and used as rootstocks. 1.0 cm deep longitudinal slits were made at the cut end of rootstock plants. For scions, 2 to 3 cm long shoot tips of 35S::POTH1 line plants were taken and a V-shaped cut was made at their cut ends. Transgenic scions were then inserted in to the slits of wild type rootstock plants. Reverse grafts were also made with the 35S::POTH1 line as stock and wild type plantlets as scions. Grafts were cultured on MS basal medium with 2% sucrose and 0.2% phytagel and incubated in growth incubators under long-day conditions for 10 days. After 10 days stock and scion tissue samples were harvested, RNA was extracted, and RT-PCR analyses were performed.

3.2.5. Soil-grown heterografts

Wild type and transgenic tobacco (*N. tabacum* var Petite Havana) lines were maintained in a growth chamber until grafting was performed. Grafts were made with wild type and 35S::POTH1 transgenic tobacco. For grafting, scions were made by cutting off the shoot approximately 10 cm below the shoot tip and making a V-shaped notch at its cut end. Stocks were prepared by decapitating the plants approximately 10 cm above the soil and making a 1.0 cm deep slit at the cut end. Scions were inserted into the slits of the rootstock and pots were covered with a transparent polythene bag. Fifteen grafts were made and maintained in a growth chamber for hardening and samples were harvested from scions and stocks of heterografts at the end of 4 weeks of incubation period. Nested RT-PCR was performed with transgenic gene-specfic primers to detect the transgenic *POTH1* RNA. Specifically, samples collected from stocks for the RT-PCR analyses were stem sections 1.0 cm above the soil line, new leaves arising from axillary shoots, and root tips approximately 2.0 cm in length.

3.2.6. Translation assays

The pBI221 vector (Clontech) containing a 35S::GUS cassette was used for translation analyses. The 5´ UTR was amplified by primer pair 5´-CATCTAGAGAGTTTCTCTCCCTTTTAA-3´ and 5´-CGGGATCCATATATATCTAAAAAAAAACAAACA-3´ and 3´ UTR was amplified by primer pair 5´-ACTGAGCTCGTTTGAATGGAAATTGTGAA-3´ and 5´-GTCGAGCTCTTAGGTAAGCATTAATTTTGACA-3´. The amplicons were ligated in pBI221 to generate the POTH1-UTR-GUS fusion constructs. A tandem UTR construct was also generated (Fig. 3.9A). Plasmid containing 35S::Luciferase was used as an internal control.

For protoplast isolations, fully expanded leaves of 3-week old tobacco (*Nicotiana tabacum* var Petite Havana) cultures were used. The leaves were excised and painted with sterile K3 medium containing 10% Celite (C8656, Sigma), washed several times and finally placed in sterile K3 medium containing 0.5% cellulase and 0.1% macerase (Calbiochem) and incubated for 16 h at 28°C in dark. The protoplasts were filtered through sterile cheese cloth into a flask with bottleneck and centrifuged at 100g for 10 min. Protoplasts were collected from the bottleneck area and washed once with K3 medium with 0.4 M sucrose and resuspended in K3 medium with 0.4 M glucose to a final concentration of 4 x 10^6/ml.

700 µl of the protoplast suspension was taken in a 0.4 cm electroporation cuvette and mixed with 30 µl of 2 M KCl and the specified plasmid (5 µg of GUS-UTR construct plus 0.5 µg of 35S::Luc). Protoplasts were electroporated at 170 V, 125 µF in Gene Pulser X-Cell (BioRad). After electroporation, 4 ml of Murashige and Skoog (1962) basal medium was added and the protoplasts were incubated at 25°C for 48 h in dark. GUS and LUC activity assays were performed as explained below.

3.2.7. Histochemical, fluorometric and luminometric assays

GUS staining was done by incubating the samples in GUS staining buffer containing 1 M $NaPO_4$, pH 7; 0.25 M EDTA, pH 8; 10% TritonX 100; 1 mM 5-bromo-4-chloro-3-indolyl-β-D-GlcUA, 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide. Samples were cleared with 100% ethanol and photographed on Leica stereomicroscope (S8AP0).

Fluorometric analyses from tissue samples were performed as described by Jefferson (1987). Frozen samples were homogenized in 100 µl of GUS extraction buffer. Samples were then centrifuged at 17000g for 5 min on a benchtop centrifuge. Clear supernatant was removed and used for protein estimation by Bradford's method (Bradford 1976). Aliquots of extracts with approximately 10 µg of protein were made and their volume was made up to 50 µl with extraction buffer. 5 µl of GUS assay buffer was added to each sample and the samples were incubated at 37°C for 16 h. Reaction was stopped by adding 50 µl of stop buffer. Fluorescence of the samples was measured using multimode plate reader (Varioskan flash, Thermo Scientific) with excitation wavelength 355 nm and emission wavelength 465 nm.

At the end of 48 hr incubation, protoplasts were centrifuged at 8000g for 10 min at 25 °C. The protoplast pellet was flash frozen in liquid nitrogen. For flurometric measurements, frozen protoplast pellets were homogenized in 100 µl GUS extraction buffer and centrifuged at 17000g. Clear supernatant was removed and used for fluorometric measurements as explained by Jefferson (1987). Fluorescence of the samples was measured using multimode plate reader (Varioskan Flash, Thermo Scientific) with excitation wavelength 355 nm and emission wavelength 465 nm. For luminometric measurements, frozen protoplast pellets were homogenized in Cell Culture Lysis Reagent (CCLR, Cat.# E1531, Promega), centrifuged at 17000g and clear supernatant was collected. 100 µl of luciferase substrate (Promega) was injected to the 20 µl of clear supernatant and luminescence was measured for 15 sec in a multimode plate reader (Varioskan Flash, Thermo Scientific)

3.2.8. Yeast three-hybrid analysis

The host yeast strain YBZ-1 was transformed with RNA hybrid plasmids by the standard yeast transformation protocol using lithium acetate. Transformed cells were grown on plates containing synthetic medium lacking uracil (SD/-ura). Each of the protein plasmids were then transformed into the yeast strain YBZ-1 containing the target RNA hybrid plasmid and transformants were grown on SD/-leu/-ura plates. To assay RNA-protein interaction, induction of the *lacZ* reporter was measured with a Yeast β-galactosidase Assay Kit (Pierce Biotechnology). β-galactosidase activity was determined by following the equation; [1,000 x OD_{420}]/[time of color reaction (min) x volume of cells used (ml) x OD_{660}]. Each assay was replicated in three independent experiments. The YBZ-1 strains, plasmids *pIIIA/MS2-1* and *pACTII* were generous

gifts of Dr. Marvin Wickens, University of Wisconsin, Madison. The Yeast Three Hybrid work was carried out in collaboration with Prof. DJ Hannapel's laboratory at Iowa State University, Ames, Iowa.

3.3. Results

3.3.1. Characterization of the upstream sequence of *POTH1*

To study the pattern of *POTH1* expression, a 1.63 kb upstream sequence fragment (from the start codon) of *POTH1* was isolated and verified by sequencing (Fig. 3.1A). This upstream sequence was analysed for *cis*-regulatory elements using the promoter analysis software plantPAN (Chang et al. 2008) and PLACE (Higo et al. 1999). Several light regulatory elements (LREs) such as GATA, GT-1 and I-boxes (Vorst et al. 1990; Vauterin et al. 1999; Waksman et al. 1987) were observed in the *POTH1* promoter (Fig. 3.1B, Table 3.1). Additionally, the software analysis also identified the presence of a CAAT box and MYBST1 motifs that drive tissue-specific expression (Baranowskij et al. 1994; Xu et al. 2010).

Table 3.1 - Summary of light motifs identified in the POTH1 promoter

Sequence	Number of sequences	Light motif
GATAGGA	16	GATA
GGTTAA	6	GT-1
GATAAG	4	I-box

To determine the location of *POTH1* transcription, the *POTH1* promoter sequence was used to drive β-glucuronidase (GUS) expression in the binary vector pBI121 (Fig. 3.2A). The $_{prom}$POTH1::GUS construct was transformed to *S. tuberosum* ssp *andigena* line 7540 using *Agrobacterium*-mediated transformation (Banerjee et al. 2006b). GUS staining of the transgenic lines demonstrated that the *POTH1* promoter was active in a wide range of organs, including leaves, stamens, shoot tips, axial nodes, roots, stolons, and tubers (Fig. 3.2B to 3.2M). In leaves, the strongest expression was observed in veins (Fig. 3.2B to 3.2E, arrows). Transverse sections of both stems and

petioles showed promoter activity associated with phloem cells in both of these organs (Fig. 3.2J to 3.2K, arrows).

3.3.2. *POTH1* promoter is light inducible

As POTH1 promoter was observed to contain several light regulatory motifs, the effect of light on promoter activity was evaluated. The promoter of potato polypyrimidine tract binding protein *StPTB6* was previously demonstrated to be light inducible and hence $_{prom}$*St*PTB6::GUS lines were used as a positive control in these experiments. 35S::GUS lines were used as negative controls. *In vitro* plantlets of $_{prom}$POTH1::GUS; 35S::GUS and $_{prom}$*St*PTB6::GUS were incubated under six different conditions as described in methods. For testing light induction, plants were incubated in (i) long day (LD); (ii) dark for 8 days; and (iii) dark for 7 days followed by 24-h-light conditions. To differentiate between light induction and induction of gene expressions due to higher metabolism following illumination, sets of plants were also incubated in (iv) dark for 8 days with transfer to MS + 6% sucrose 7 days post inoculation or (v) dark for 7 days followed by transfer to MS + 6% sucrose and under light for 24-h. To check the effect of dark/light/dark cycles on GUS activity another set of plants were incubated in (vi) dark for 7 days followed by transfer to light for 24-h and was returned back to dark for 3 days.

No significant difference in relative GUS activity was detected in 35S::GUS lines under any of these conditions tested (Fig. 3.3A). $_{prom}$*St*PTB6::GUS lines (positive control) showed approximately 50% reduction in GUS activity under dark conditions whereas GUS activity was increased to approximately 80% in condition (iii) dark+light; 75% in condition (iv) dark+6% sucrose; and 88% in condition (v) dark+ light+6% sucrose respectively. A reduction of GUS activity was observed in plant grown under condition of (vi) dark+light+dark cycle (Fig. 3.3B). The $_{prom}$POTH1::GUS lines showed approximately threefold reduction in GUS activity (32%) when incubated under dark conditions (Fig. 3.3C). Compared to plants grown under dark conditions, the GUS activity increased approximately two fold (60%) in plants incubated in the dark for 7 days followed by 24 h illumination under white light (condition iii, dark + light). Incubation in 6% sucrose (condition iv, dark+sucrose) had marginal increase in GUS activity (41%). An increase of GUS activity to 71% was detected in plants incubated under conditions (v) dark+light+6% sucrose. Compared to plants grown under

conditions (iii) a reduction in GUS activity was observed in plants incubated under condition (vi) dark+light+dark (Fig. 3.3C). These results indicate that $_{prom}$POTH1 is induced by light and sucrose has a slight additive effect on the promoter activity.

In another experiment, the effect of photoperiod on POTH1 promoter was evaluated. The GUS activity was evaluated from the leaves of $_{prom}$POTH1::GUS plants incubated under either short day or long-day photoperiod. The Relative GUS activity from the leaves of SD and LD incubated plants did not show a significant difference (Fig. 3.3D).

Additionally, to evaluate the effect of light on *POTH1* mRNA accumulation pattern, detached leaflets from wild type potato plants were incubated under different light conditions such as dark for 24-h followed by a transfer to light for 0 or 24 h respectively and the control set of leaves was incubated under long-day conditions, as explained in methods. A semi-quantitative RT-PCR was carried out and the gel was analyzed by Image J software (Abramoff et al. 2004). Incubation of leaflets under dark conditions resulted in reduction of *POTH1* transcript to approximately 70% in comparison to leaftlets incubated under long-day conditions. Exposure of dark-incubated leaflets to light for 24-h returned the *POTH1* RNA accumulation to levels of control (Fig. 3.3E).

These results suggest that *POTH1* promoter activity and RNA accumulation are regulated by white light illumination but photoperiod exerts no significant effect on activity of the *POTH1* promoter.

3.3.3. *POTH1* mRNA is graft transmissible

POTH1 RNA was previously shown to be present in phloem cells of potato but its long distance transport was not reported (Yu et al. 2007; Campbell et al. 2008). To check the long distance mobility of *POTH1* transcript grafting technique was used (Fig. 3.4).

Because POTH1 over-expression (OE) lines are very slow growing, initial grafting experiments were performed with tissue culture plants *in vitro*. *In vitro* plantlets of wild type *S. tuberosum* ssp. *andigena* line 7540 and POTH1-OE line #11 were used and alternated as stock and scion. POTH1 OE line #11 was selected for its extreme phenotype (Rosin et al. 2003). After four weeks of long-day conditions, grafted plants were transferred to short day conditions for 10 days and samples were harvested. RNA was isolated from the lower stem of stocks and the upper stem of scions (Fig.

3.5A). Using gene-specific primers and the stem RNA as template, RT-PCR was performed to assess movement of *POTH1* RNA. A primer specific for a non-plant sequence tag present only in the transgenic *POTH1* RNA (Nopaline Synthase terminator sequence) was used to distinguish the transgenic transcript from the native RNA. Transgenic *POTH1* RNA was detected in wild type stocks harvested from the stems of the POTH1 line #11/WT heterografts and was confirmed by Southern hybridization (Fig. 3.5B, #11 as scion). No signal was detected in stems of wild type scions from the WT/POTH1 line #11 grafts. In these experiments, *POTH1* RNA demonstrated a unidirectional, downward movement in micrografts from scion to stock.

POTH1 RNA movement was also tested in heterografts of soil-grown plants. Because POTH1 OE potato lines are dwarf, have malformed leaves, and grow slowly, grafting onto wild type soil-grown potato was not possible. To overcome this problem, full-length *POTH1* sequence driven by the CaMV35S promoter was transformed into tobacco (*N. tabacum* var Petite Havana) and these transgenic lines were used in soil-grown heterografts. Over-expression of POTH1 in tobacco produced a phenotype similar to *KN1* OE lines observed in several species, including potato and tobacco (Sinha et al. 1993; Parnis et al. 1997; Tamaoki et al. 1997; Rosin et al. 2003). Transgenic tobacco lines exhibited a reduction in height, smaller, mouse-ear type leaves, and aberrant leaf venation (Fig. 3.6A to 3.6C). Similar to a recent study on KNOX gene over-expression (Box et al. 2011), flowers of these lines were more pale, had dissected petals, and an elongate style (Fig. 3.6D and 3.6E, arrows). Despite this abnormal phenotype, the tobacco lines were healthy and robust. Heterografts were made with the 35S:POTH1 lines of tobacco as scions and wild type lines as stocks (Fig. 3.7). The grafts were hardened and scion and stock samples were harvested 4 weeks after grafting. This time frame allowed for the induction of axillary shoot growth from the stock stem. RNA was extracted from newly emerged leaves of these axillary shoots, lower stems, and roots and RT-PCR was performed with transgene-specific primers (Fig. 3.8A). Leaves that were produced from these branching axillary shoots and used for RNA extraction were smaller in size compared to the WT/WT grafted lines (Fig. 3.8B). Transgenic *POTH1* RNA was detected in leaves from axillary shoots, lower stems, and roots (Fig. 3.8A) of the wild type stock of the 35S:POTH1/WT heterografts.

3.3.4. *POTH1/UTRs* are rich in polypyrimidine tracts

It has been demonstrated that RNAs travel through phloem as ribonucleoprotein complex. The transport proteins were shown to bind at the specific sites in RNA sequence such as polypyrimidine tracts. Analysis of *POTH1* sequence revealed presence of 32 polypyrimidine tracts dispersed through the sequence but the 5' and 3' UTRs were especially rich with seven PTs in each of them (Fig. 3.9A). Further, the secondary structure of POTH1 mRNA as predicted by m-fold indicated that 5′ and 3′ UTRs of *POTH1* mRNA formed stem-loop structures (Fig. 3.9B) where transport proteins could bind.

3.3.5. *POTH1/UTRs* repress translation

The translation of mobile RNAs is usually repressed at the site of their synthesis and this repression of translation is shown to be mediated by their UTRs (Wilhelm et al. 2000; Gu et al. 2004, Banerjee et al. 2009). Thus, the effect of the UTRs of *POTH1* RNA on its translation was studied. Constructs of 35S::GUS with or without the *POTH1* UTRs were cloned into pBI221 (Fig. 3.10A) and GUS activity of each construct was assayed by transient expression in tobacco protoplasts (Fig. 3.10B). Compared to GUS activity without any UTRs, activity was reduced, 30 to 35 % for the three constructs having at least one of the *POTH1* UTRs (Fig. 3.10B). Whereas for construct with tandem *POTH1-UTRs* (Fig. 3.10A, GUS + tandem UTRs), the GUS activity was further reduced to approximately 50% of the control (Fig. 3.10B). These results suggest that the UTRs of *POTH1* have a repressive effect on translation.

3.3.6. *POTH1 UTRs* interact with proteins

Protein chaperones were demonstrated to interact with mobile transcripts and mediate their translation and mobility (Ham et al., 2009). To assess the potential interaction of the UTRs of *POTH1* RNA with several RNA-binding proteins of potato, the yeast three-hybrid system was used. An initial screening indicated that the 5′ and 3′ UTRs of *POTH1* could interact with StPTB7, an alba-domain protein from potato (Fig. 3.11 A). Yeast colonies containing the *POTH1* UTR bait plasmid plus the alba-domain plasmid (*St*RBP7) grew robustly up to a concentration of 50 mM 3-AT in the selection media indicating a strong interaction (Fig. 3.10A). The Y3H results also indicated that

POTH1-UTRs interact with other PTB proteins of potato viz. StPTB3, StPTB5 and StPTB6.

Further, to the strength of interaction was evaluated by quantifying the expression of the marker gene β-galactosidase (Fig. 3.11B and 3.11C). The bait RNAs used for the β-galactosidase assay were, the CU-rich *T1* fragment of the 3′ UTR of *StBEL5* with *St*PTB6 as a positive control, and full-length *POTH1* 5′ UTR (135 nt) and *POTH1* 3′ UTR (202 nt) with StPTB6 and StPTB7 (alba-domain containing) proteins as test. As a negative control, a coding sequence (cds) fragment (*StBEL5 N*) of *StBEL5* was used. As shown by induction of β-galactosidase, both the 5′ and 3′ UTRs of *POTH1* interacted strongly with the alba-domain protein StPTB7, whereas only the 3′ UTR interacted with *St*PTB6 (Fig. 3.11B and 3.11C). Based on β-galactosidase activity, the strongest interaction occurred with the 5′ UTR of *POTH1* and StPTB7 with a 15-fold induction, whereas the 3′ UTR exhibited a 4.5-fold induction (Fig. 11C). On the other hand, the relatively strong interaction of *St*PTB6 with *T1* produced a 5.5-fold induction of β-galactosidase activity (Fig. 3.11B).

3.4. Discussion

3.4.1. *POTH1* transcript is mobile

Several studies have demonstrated the presence of different RNA populations in phloem cells or phloem exudate (Asano et al. 2002; Vilaine et al. 2003; Kehr et al., 2007). Despite of this, very little is known about the mechanisms that control long-distance transport of full-length mRNAs. Lucas et al. (1995) demonstrated that maize homeodomain protein, *KN1*and its mRNA can traffic cell-to-cell. Authors used microinjection studies of fluorescently labeled KN1 protein and showed that KN1has the ability to traffic between mesophyll cells, to increase the size exclusion limit of plasmodesmata and to specifically transport its mRNA. Later, a GFP-tagged *KN1* fusion was shown to be able to traffic in the leaf and SAM in *Arabidopsis* (Kim et al., 2002). *KNOX* homeodomain (HD) was demonstrated to be necessary and sufficient for intercellular trafficking, and it was also shown to promote the trafficking of the *KN1* mRNA (Kim et al., 2005). A specific group of chaperons was found to be essential for cell-to-cell trafficking of KN1 (Xu et al., 2011).

Kim et al. (2001) used grafting experiments with the mouse-ear (*Me*) leaf mutant in tomato to demonstrate long-distance movement of a chimeric *KNOX* mRNA

into wild type scions. The *Me* mutant of tomato produces a chimeric transcript produced by a fusion between PYROPHOSPHATE-DEPENDENTPHOSPHOFRUCTOKINASE (PFP) and *KNOX* gene *LeT6*. The authors demonstrated that chimeric *PFP-LeT6* fusion RNA transpors up from the *Me* stocks into the wild type heterografted scions and causes a change in leaf morphology in the wild type scion. The *Me* mRNA was detected in phloem cells by *in situ* reverse-transcriptase PCR indicating that it may be transported via phloem (Kim et al., 2001). In the current study, heterografting experiments confirmed that *POTH1*, a class I KNOX RNA of potato, moves through the phloem in a rootward direction. Similar to the *LeT6* fusion, *POTH1* RNA was detected in phloem cells and was graft-transmissable. Like *LeT6*, accumulation of the mobile RNA was correlated with a change in leaf morphology. Consistent with its capacity for mobility, the *POTH1* promoter was active in phloem cells of both stems and petioles.

Two other well-documented examples of RNA movement are the transport of *GAI* (*Gibberellic Acid Insensitive*) and *StBEL5*. *GAI* was shown to move up to shoot apices and whereas *StBEL5* was reported to move down towards roots and stolons (Haywood et al. 2005; Banerjee et al., 2006). Both *GAI* and *StBEL5* were found to have CU motif containing sequences present in their 3´ UTRs that mediate interactions with polypyrimidine tract binding (PTB) proteins (Banerjee et al. 2009; Ham et al. 2009; Huang and Yu 2009). In the case of *CmGAI,* this PTB protein is CmRBP50, the core protein of the phloem-mobile RNA/protein complex in pumpkin (Ham et al. 2009). Similarly, the UTRs of POTH1 were found to be rich in motifs where RBPs bind and were demonstrated to interact with such proteins.

3.4.2. RNA-binding proteins interact with both UTRs of *POTH1*

Proteins that bind to specific motifs in the sequence of mobile RNAs have been demonstrated to facilitate movement, stability, and translation (Bailey-Serres et al. 2009). Mobility of mRNA is often linked to its translational repression in non-plant RNAs (Ferrandon et al. 1994; Gu et al. 2004; Colegrove-Otero et al. 2005). The Staufen protein of *Drosophila* binds to *bicoid* and *oskar* mRNAs to determine their location in the egg cell and to repress translation until this occurs (St. Johnston et al. 1991; Ferrandon et al. 1994). Banerjee et al. (2006) reported a similar phenomenon where translation of the mobile transcript *StBEL5* was repressed by its UTRs. In the

present study, the UTRs of *POTH1* repressed translation of the β-glucuronidase marker and this effect is likely mediated by a RNA-binding protein. A yeast three-hybrid screen of the UTRs of *POTH1* against a catalog of RNA-binding proteins of potato revealed a strong interaction with an alba-domain protein StPTB7. A related RNA-binding protein (AT1G29250) was identified in phloem sap of pumpkin (Lin et al. 2009). This robust and specific interaction (Fig. 3.11A and 3.11C) is significant for several reasons. Alba-domain proteins function to regulate translation, process tRNA, and bind DNA (Aravind et al. 2003; Mani et al. 2011). Alba-type RNA-binding proteins have been characterized in *Trypanosoma brucei*, and they have been shown to bind specifically to a glycerol-responsive element (GRE) of 25 nucleotides present in the 3´ UTRs of RNAs encoding glycoproteins known as EP and GPEET procyclins. It was observed that both the UTRs of *POTH1* contain a putative conserved binding motif for the alba protein (Fig. 9C) and RNA-protein binding assays showed that both UTRs bind to the alba-domain protein StPTB7 (Fig. 3.11C). Because both the UTRs affected translation (Fig. 3.10), it is feasible that interaction of the alba protein with either of the UTRs influences translation of *POTH1*. Mfold-predicted secondary structures of the full-length *POTH1* transcript form distinct loop structures in both UTRs (Fig. 3.9B) similar to the model of the *GPEET* UTR (Vassella et al. 2004).

Ham et al. (2009) demonstrated that all six RNAs (including a *Knotted1* type, *CmSTM*) associated with the CmRBP50 RNA/protein complex contain CU motifs in their RNA sequence (Ham et al. 2009). Consistent with target motifs for proteins like CmRBP50, both of the UTRs of POTH1 were found to be rich in CU motifs. These motifs are recognized by PTB proteins to form ribonucleoprotein complexes that stabilize the RNA and facilitate long-distance transport. Binding assays showed that the 3´ UTR of *POTH1* and the *T1* region (from 3´ UTR sequence) of *StBEL5* also interacted with the CmRBP50-like protein of potato, designated *St*PTB6 (Fig. 3.11B). That the same PTB protein of potato binds 3´ UTR sequences from both *POTH1* and *StBEL5* (Fig. 3.11B) suggests a common mechanism for delivery and organ-specific localization of RNAs that encode proteins that function in a tandem transcriptional complex (Chen et al. 2004).

In summary, the results of the current study led us to conclude that

(i) The promoter of *POTH1* is rich in light regulatory motifs and the *POTH1* expression is affected by light.

(ii) Micrografts and heterografts with soil-grown plants confirmed that the mRNA of *POTH1* is graft transmissible and moves in a rootward direction.

(iii) *POTH1-UTRs* have repressing effect on the translation of marker gene *GUS* in tobacco protoplasts.

(iv) Polypyrimidine tracts as well as putative binding sites for an alba-domain protein were identified in both the 5´ and 3´ UTRs of the *POTH1* transcript. Protein/RNA binding assays confirmed interaction of both UTRs with the alba-domain protein and of the 3´ UTR with a polypyrimidine tract-binding protein.

Collectively, these results suggest that the full-length mRNA of *POTH1* is functional in long-distance trafficking.

Figures
Chapter 3

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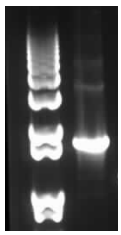
A



B

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-1577   CCGACTCTTGAGATGATATAAATATCCTTTTTTTTAAAACTCTCTTAGAA
-1527   AGTTTTTAAGTAAGTGATTAGACATGTAATATGAAATCATGATTATAAAT
-1477   TAGTATCTGGACAGTGCTATTTGAAATTATGATTTTAAATTATTTAAAAA
-1427   GTAGGATTTGAAATTTTAAATAGTGATTCCAATTTCTTTTTTAAATGTAAA
-1377   ACCCGGCTCATAAATTAATGTTTGTAAGAAAAAAAAACTCATCAAAAGAA
-1327   TACTAATGTTCCACATTAAGAGATTGTTCGTATGCGTATCATATAAAATA
-1277   ATTATATTAAATAATAACTAGTAAAGAGTGTTGGAATTGACATATTAAAG
-1227   TTAGATTATAAGTTGAAAACAACTTATGAAATAAGTTCTTATTTCACTTT
-1177   TATCTTATTTTTATAGTTTTGACCTAAAAATAAGTTCTTAAATCACTTTT
 1127   TATTTTTTCAAACACTACAAAAGGGTACAAAAAAACTTTTAAAGCCAAAA
-1077   TCATTTAAAATAACTCAATCCAAGCCTAATCAGAACTTGGGACGCCCAAC
-1027   AAGATATGAGGTGCTTGAAAATGGTCTAAATCTCTTGTTACTTCTATTGT
-977    TGATTAGTAAATCTTTATATAATTATGTTTATTTGAAAGTTTGTAATGAA
-927    AAAAGATATGTGGATTTATCGATATGATTCCATAAAATATTTCAATATCT
-877    TGTTTATGAATTATGGTTTATTCATTTGATAAGATTATATAAGAATTTAT
-827    TGTATATCCAAACAAAACTTTAATTTCAAACGGATCTGATTTCAAATCAT
-777    GATTTCATAGGACAAGAGTATATTACTACATTATATACTACTATTACATT
-727    TTTAACCCAGTATAATAAGCCGCAAGCATCTACAAAAAAATAAAAAATGA
-677    AAATTACTAAATAATTTAATTATTTTTTAATTTGCACACTATAAATTTGA
-627    ATCCGGAATCAAAAGGGCAACAAAAATTTTCAAAAATTCCAAAAGGGCAA
-577    CTCAATTAATTATGAAACCAAAAGGACAACAATTGTGTAACGGCGTCTAAA
-527    TCAGGGTTTCAAAAATGAGGCGCAAAAATCCATGGGCCTTACAAGGCTCA
-477    ATAACTTTTAGCCTTGTAAGGCTCAGCCGCCATGAGCCTTACAAGGCTCA
-427    AGGCCGTTAGCGCCTGGCAGATGATTTTTTTGTTTTTTTTTTTAAATTCG
-377    TTTTATTTTTCTTGAGCTATTAATAATTTATTTGTCCCTAATTATTTTCAT
-327    TCCCTTGCAAGCAAATATTTAATGTAAGTTGTGGTCTACTTTTTAGAATT
-277    TTGTACCACTAATTATATGATAAGTTAGAAACTTCTTGCTAGATACATGA
-227    GAACAAACGAGAGAATTAACAAGAAAAAGAAAAATAAAAAAATAGTTAAA
-177    AAATACTATTATTTATCAACCATTATATTTATGCATACTTATTACAAGTG
-127    TATTTTTAATTAATATATATAAACCAACGAAATATTAGATTCTTTTATTT
-77     TATCTCTAAGTAATGGGACCTCTCTTCCTCTTCACCTTCCCTTCTTTTCC
-27     TCTTCTCCTTTAATTTATTTTTCTCTC[CCTTTTGAAAAAGAAAAAAGAAA
+23     AACACAACACCCACTTCAAATATCAAACAAATTTCTCATTTGATTATTTC
+73     TAAGTAATTTACTCTACTTTGTATTTTTGTTTTTTTTTAGATATATATATG
+123    GATGATGAAATGT]ATGGTTTTCATTCAACAAGAGACGATTACGCGG
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CAAT BOX GT BOX ELEMENTS

Fig. 3.1 The POTH1 promoter. ~ 1.8 Kb sequence upstream POTH1 CDS was amplified from potato genomic DNA **(A)**. In silico analysis of POTH1 promoter indicated presence of several regulatory motifs **(B)**.

Fig. 3.2 Promoter activity of upstream sequence of *POTH1* . The promPOTH1::GUS construct **(A)**, a leaflet showing expression in the in mature leaves and mid-vein (**B to E**, arrow), in mature stamens (**F**, arrow), Shoot tip **(G)**, nodes **(H)**, roots **(I)**, a transverse section of the stem and petiole **(J and K)** a stolon tip **(L)**, sub-apical region of swollen stolon **(M)**, and the mini-tuber **(N)**. For the stem and petiole **(J and K)**, GUS staining is most intense along the epidermal layer and in the phloem side of vascular bundles (arrows).

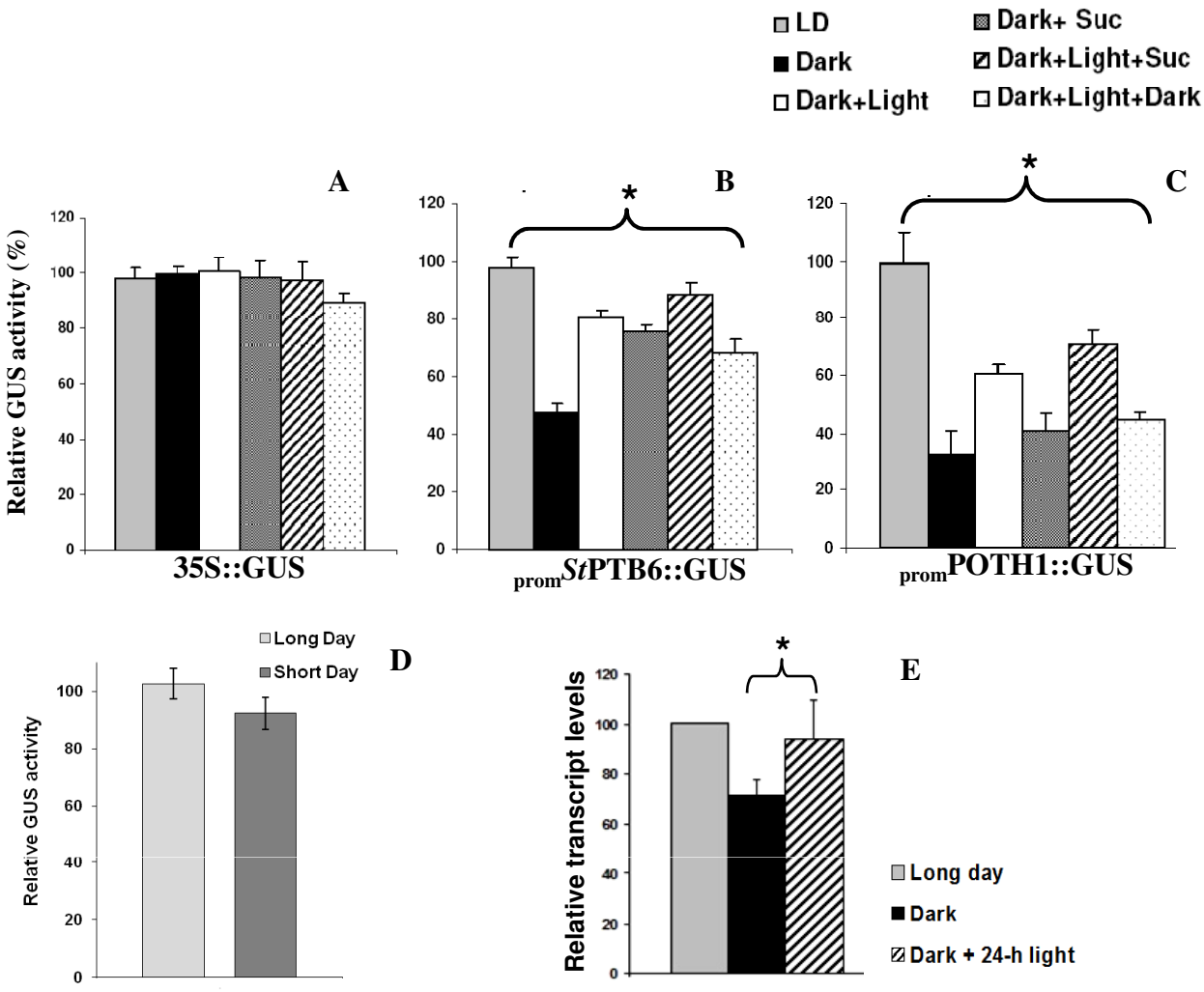


Fig. 3.3 Light induction of the *POTH1* promoter.

35S::GUS **(A)**, $_{prom}$*St*PTB6::GUS **(B)** and $_{prom}$POTH1::GUS **(C)** transgenic potato plants were incubated under following six conditions (i) long day (LD); (ii) dark for 8 days; (iii) dark for 7 days followed by 24-h-light; (iv) dark for 8 days with transfer to MS + 6% sucrose 7 days post inoculation; (v) dark for 7 days followed by transfer to MS + 6% sucrose and 24-h light; and (vi) dark for 7 days followed by transfer to light for 24-h and returned back to dark for 3 days. At the end of respective incubation periods, GUS activity was measured by 4-methylumbelliferyl-β-D-glucuronide (MUG) fluorescence assay. Data were analyzed by ANOVA. The mean ± SD of relative GUS activity for three replicates is plotted. Asterisk indicates significant difference due to treatments (p=0.01). *prom*POTH1::GUS transgenic plants were incubated under long day (16h light, 8h dark) and short day (8h light, 16h dark) photoperiods for four weeks and GUS activity was quantified by MUG assay **(D).** Effect of light on POTH1 mRNA accumulation **(E)**. Six to seven terminal leaflets were incubated in dark for 24-h followed by transfer to light for 0 or 24-h respectively. The control set was incubated under long day. Relative transcript levels for *POTH1* were analyzed by semi-quantitative RT-PCR using Image J (Abramoff et al. 2004) software. The data is mean ± SD of four replicates. Asterisk indicates significant difference due to treatment (p=0.05) **(E)**.

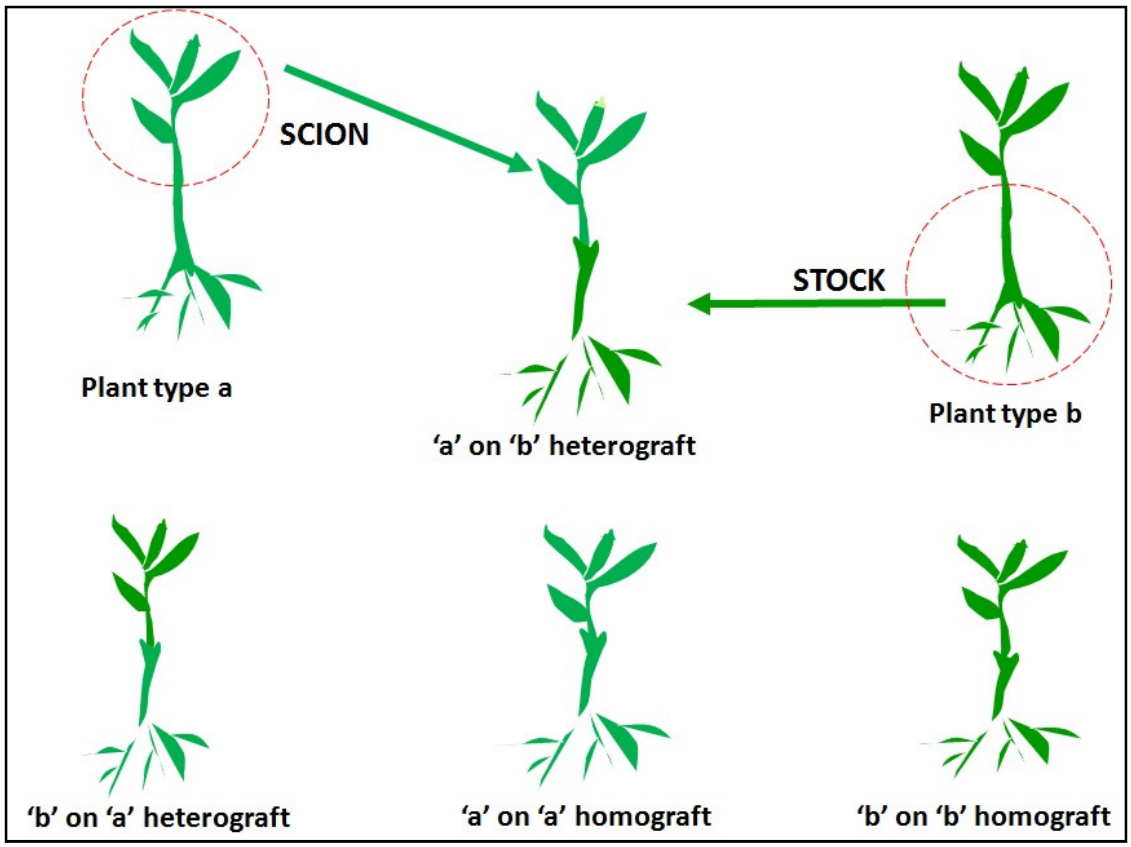


Fig. 3.4 Grafting : a tool to study mobility

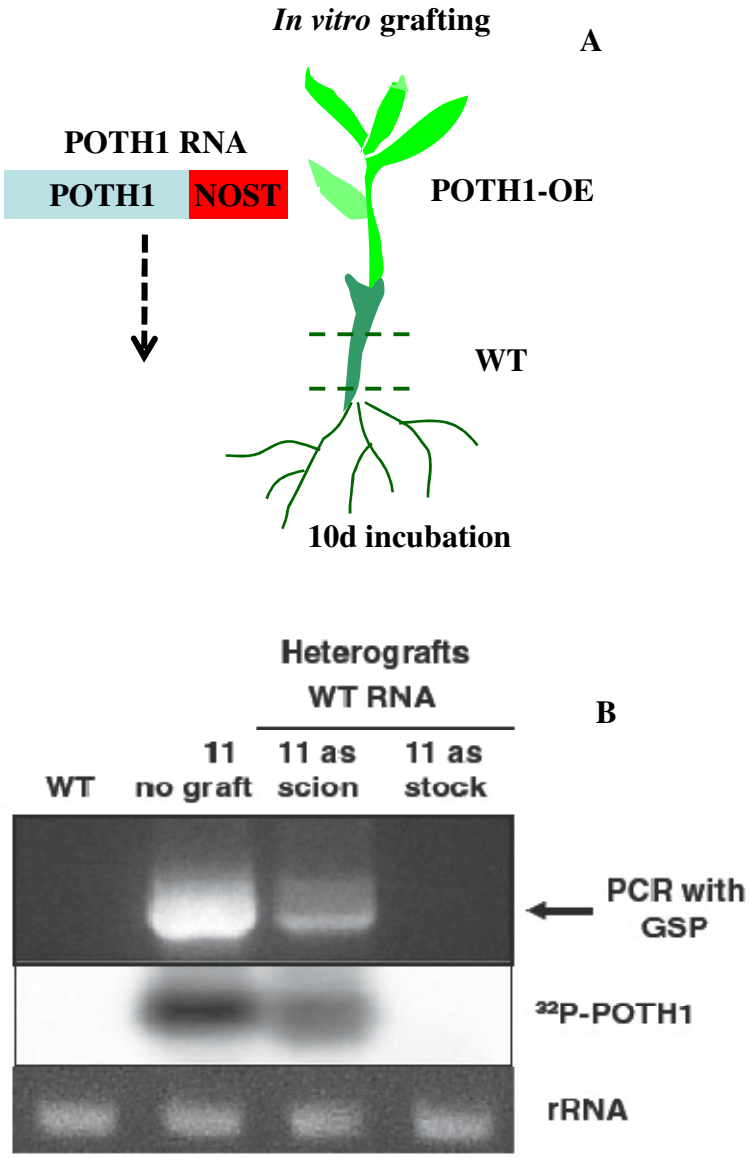


Fig. 3.5 POTH1 mRNA moves unidirectionally. Using grafts with tissue culture plants and RT-PCR with gene-specific primers (GSP), we demonstrate that RNA for POTH1 (the protein partner of *St*BEL5) moves across a graft union in one direction, towards the base of the plant (line 11 as scion). The same RNA is not detected moving upward towards the apex (#11 as stock). Line 11 is a transgenic potato line that overexpresses POTH1 mRNA (Rosin et al 2003). The no graft sample is a positive control. WT RNA was sampled for both heterografts. PCR was performed twice off cDNA template made from RNA and reverse transcriptase (RT). Two different GSPs for POTH1 were used with a non-plant sequence tag specific for the transgenic RNA of line 11. The two white bands **(A)** indicate the presence of the transgenic RNA. The dark bands **(B)** are PCR products that hybridized to a ^{32}P-labeled POTH1 probe.

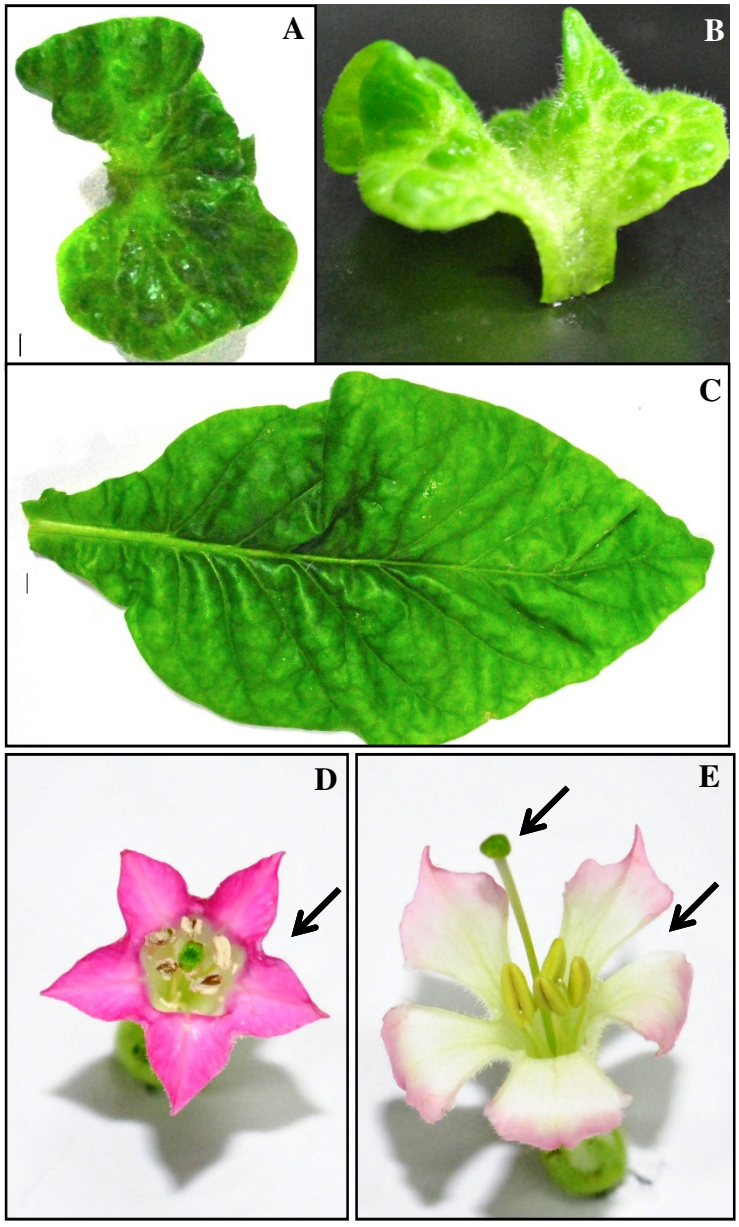


Fig. 3.6 Phenotype of OE lines of POTH1 in tobacco. Leaves of soil-grown OE lines **(A-B)** are reduced in size and curled with aberrant venation relative to control leaves **(C)**. Flowers of the OE lines exhibit an exaggerated cleft in the petal (arrows, **D-E**) and an elongate style (upper arrow, **E**). 35S:POTH1 OE lines, **(A), (B)**, and **(E)**; WT, **(C)** and **(D)**. Bars in **(A)** and **(C)** are 5 mm each.

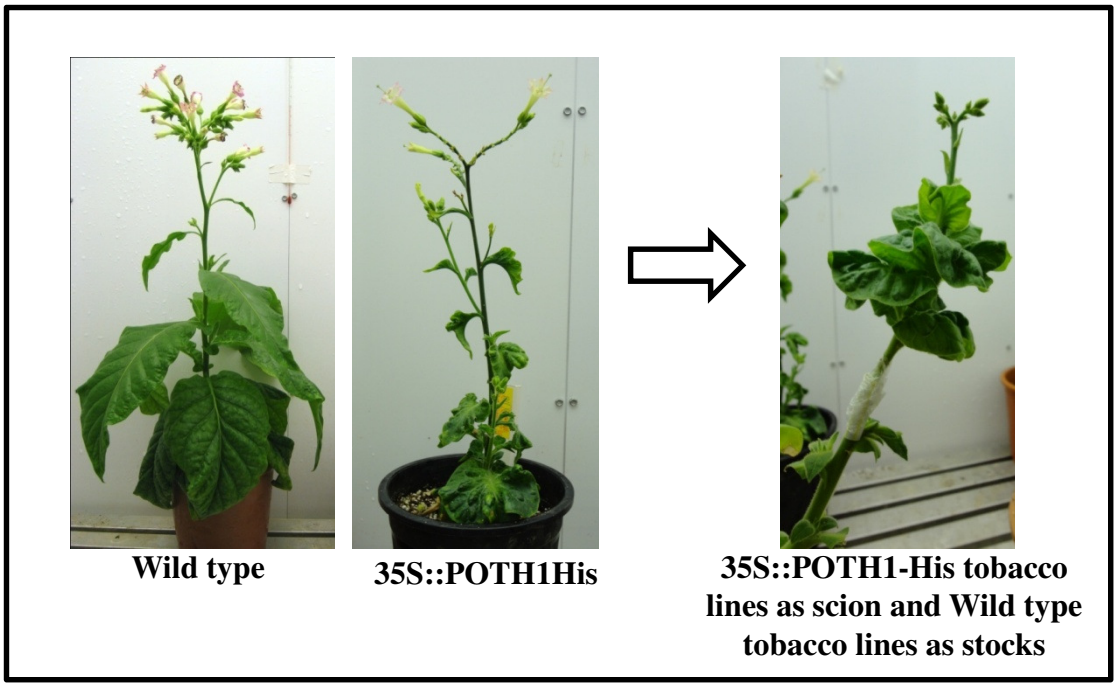


Fig. 3.7 Grafting of soil-grown tobacco lines.

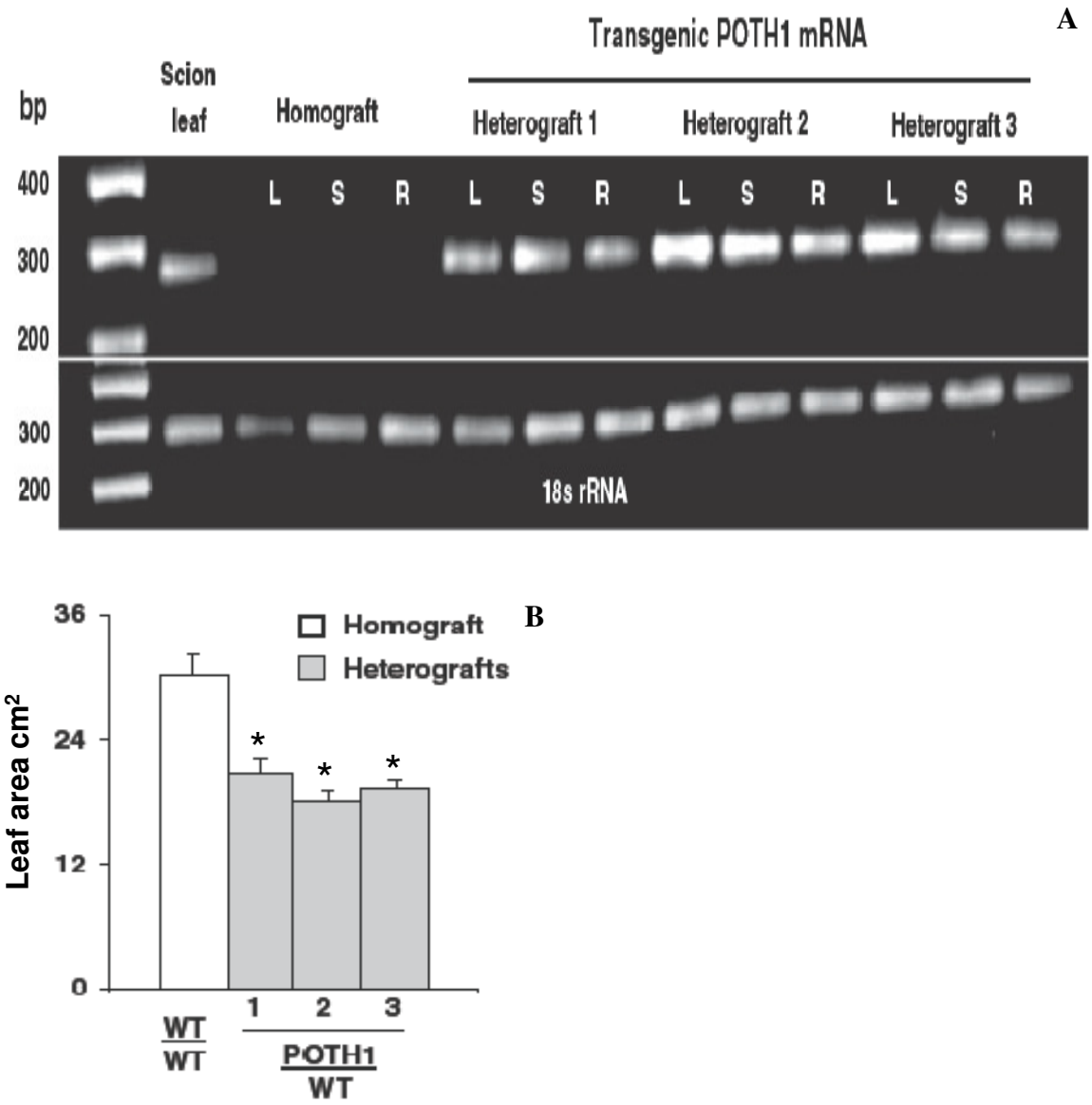


Fig. 3.8 Detection of transgenic *POTH1* RNA moving across a graft union (A). Grafts were generated with 35S::POTH1-His tobacco lines as scion and wild type tobacco lines as stocks. WT/WT tobacco homografts were used as a graft control. Leaf (L), stem (S), and root (R) samples were harvested 30 days post-grafting from wild type stock material from three heterografts. Samples collected from stocks for the RT-PCR analyses were stem sections 1.0 cm above the soil line, new leaves arising from axillary shoots, and root tips approximately 2.0 cm in length. RNA was extracted and a non-plant sequence tag fused to the transgenic construct was used to detect transgenic-specific transcripts. PCR reactions were performed twice with nested transgene-specific primers. Scion leaf RNA was used as a positive control with wild type gene-specific primers. 18s rRNA was used as a PCR control. Axillary shoot branches grew from the wild type stocks of both 35S:POTH1/WT and WT/WT grafts and leaves from these branches were scored for size **(B)**. Leaf area data from panel **(B)** are the means of three replicates ± SE.

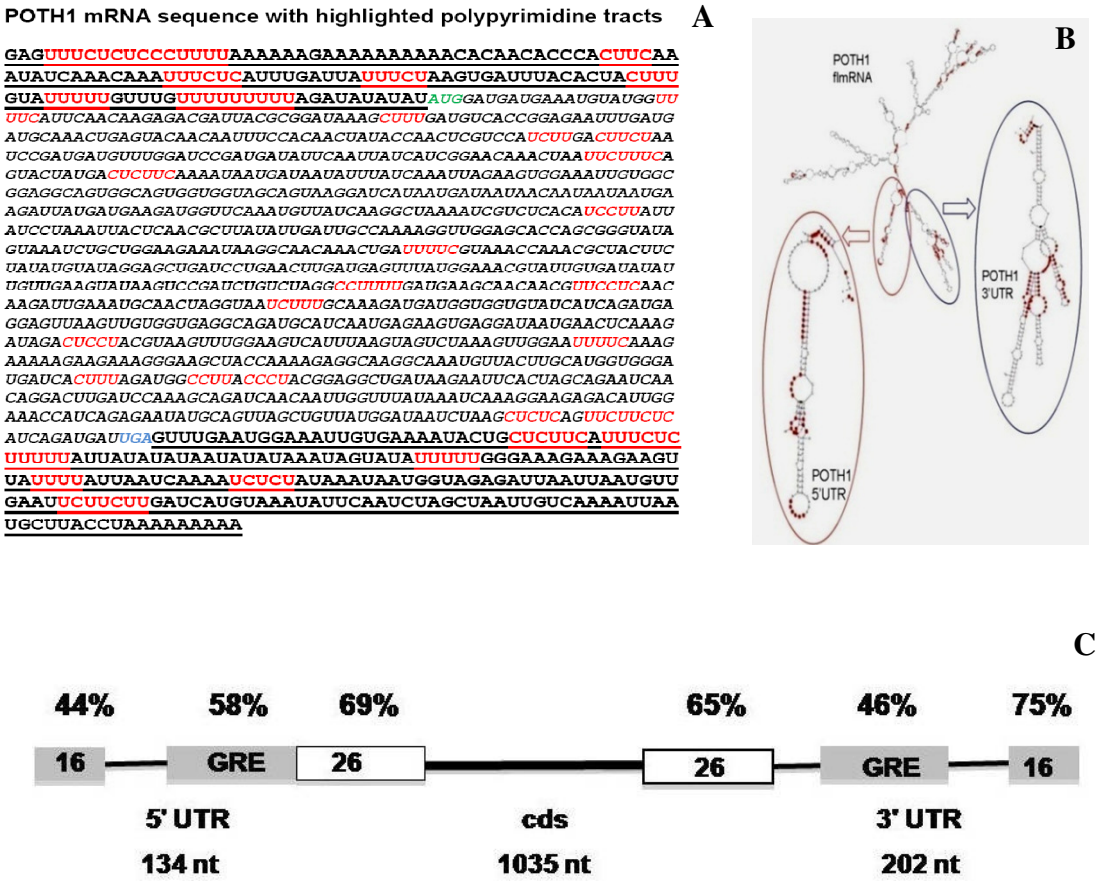


Fig. 3.9 Sequence and structure of POTH1 UTRs. Polypyrimidine tract rich UTRs of POTH1 mRNA (A). Secondary structure for POTH1 mRNA predicted by MFOLD. Polypyrimidine tracts highlighted in red (B). The glycerol-responsive elements (GRE) of *POTH1* 5′ UTR and 3′ UTRs match the conserved sequence from the *GPEET* 3′ UTR (Vassella et al. 2004) 58, and 46%, respectively. GRE is the sequence element on RNAs recognized by alba-domain proteins (Mani et al. 2011). POTH1 mRNA with conserved motifs in the untranslated regions. The regions designated 16, GRE, and 26 represent the three conserved regulatory elements present in the secondary structure of the *GPEET* 3′ untranslated region. The percent numbers above the conserved elements in panel **C** represent the overall sequence match to the *GPEET* elements

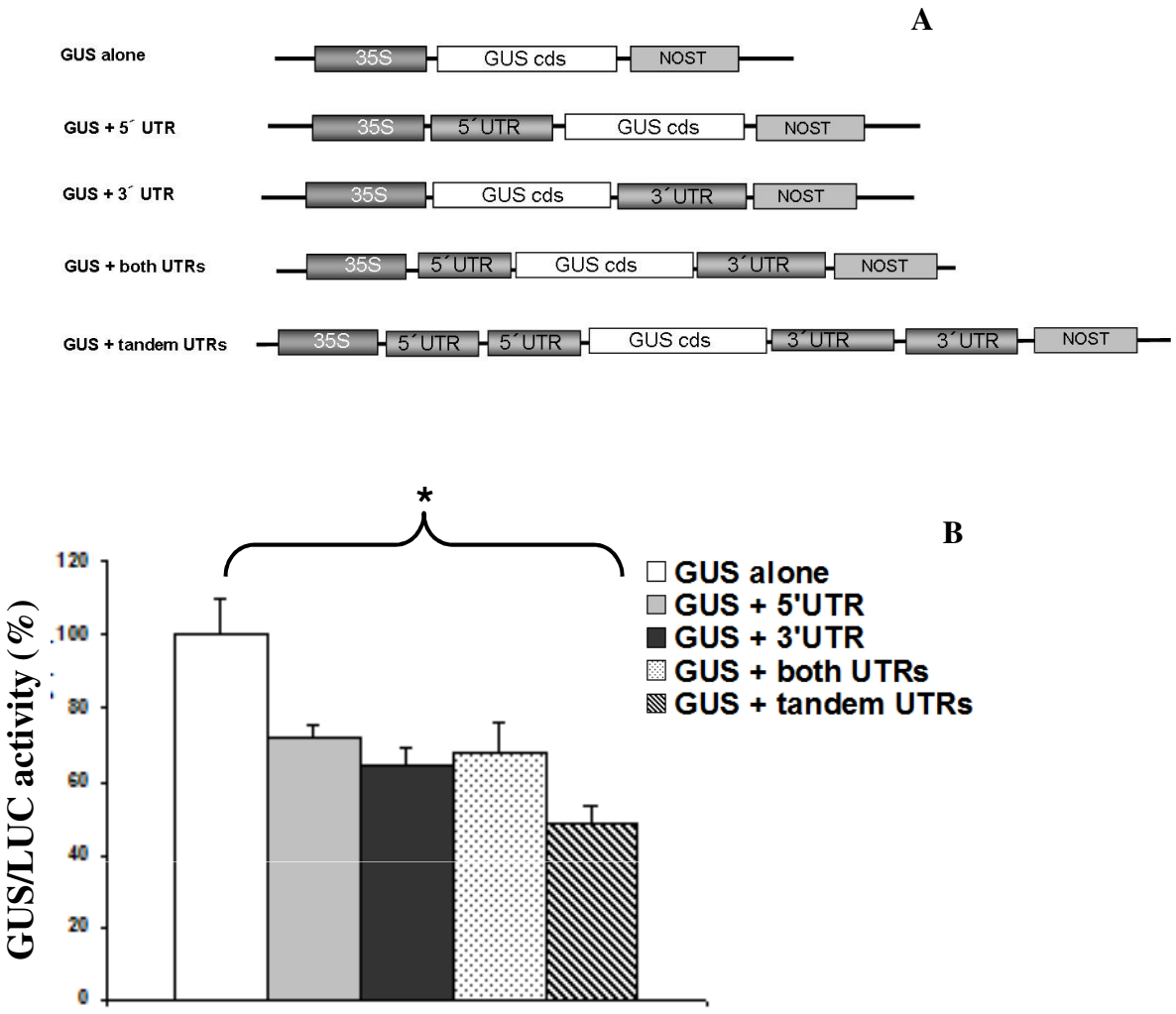
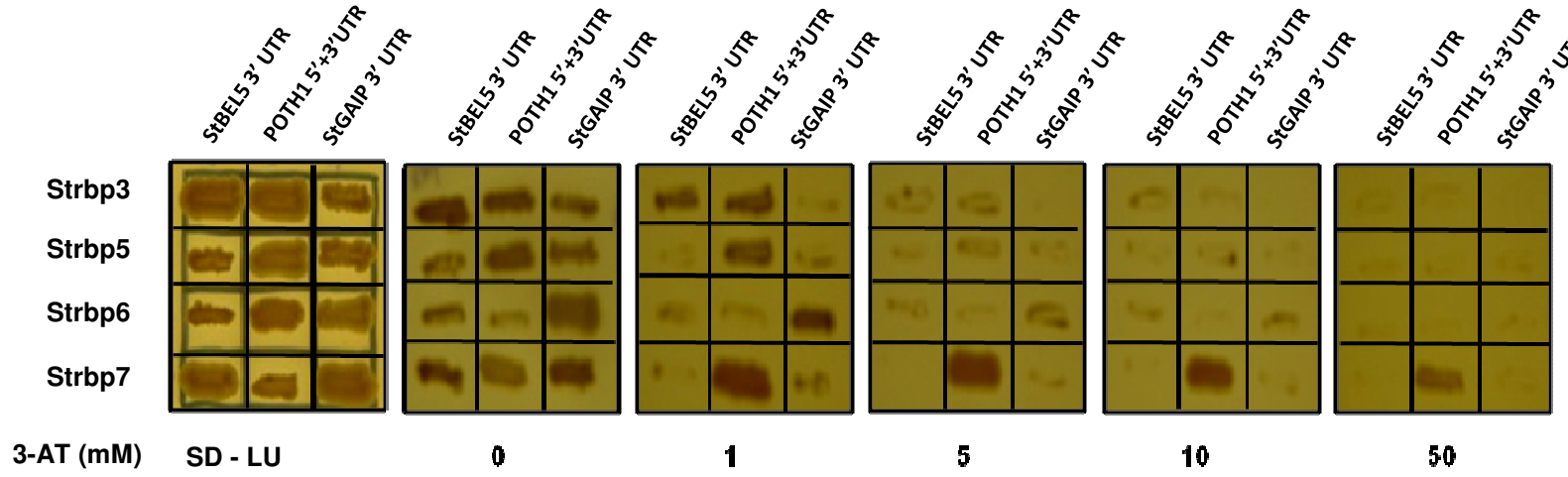


Fig. 3.10 The effect of the UTRs of *POTH1* on the translation efficiency of ß-glucuronidase (GUS) in tobacco protoplasts. Four constructs driven by the 35S promoter in pBI221 were analyzed; the *GUS* coding sequence alone, the 5' UTR of *POTH1* + *GUS*, *GUS* + the 3' UTR of *POTH1*, *GUS* plus both UTRs attached and GUS plus both UTRs in tandem. **(A)**. The luciferase gene in pBI221 under the control of the CaMV35S promoter was included as an internal control. A relative value for GUS expression **(B)** was obtained by dividing the GUS activity by the specific Luciferase activity. Each transfection was performed three times. Data were analyzed by ANOVA. The means ± SD of three replicates is plotted. Asterisk indicates significant difference due to treatments (p=0.01) NOST, nopaline synthase terminator; cds, coding sequence.



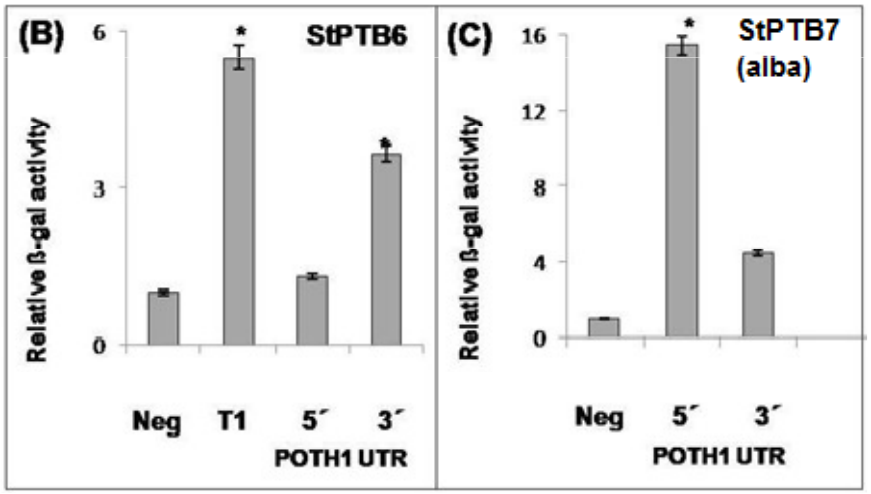


Fig. 3.11 Yeast three-hybrid growth assays for *HIS3* expression on triple selective medium (SD/-HLU) containing a gradient of 3-aminotrizole (3-AT) between 0 and 50 mM **(A)**. Three RNA baits were used: the 3´UTR of *StBEL5,* the combined 5' and 3' UTRs of *POTH1,* and the 3´ UTR of *StGAI*. Four phloem RNA-binding proteins of potato were tested: StRBP3 coding for StGRP7 (DFCI #TC20200); StRBP5 (AT1G11650); StRBP6 (AT5G40490); and StRBP7 (AT1G29250). Yeast three-hybrid assays were performed for the RNA-binding proteins StPTB6 **(B)** and an alba-domain protein **(C)** against RNA bait sequence for a negative control, Neg; for a CU-rich region of *StBEL5* 3′ UTR, T1; and *POTH1* 5′ or 3′ UTRs, 134 and 202 nt, respectively. Relative β-galactosidase activity is shown on the y-axis. The results shown are representative of at least three independent experiments. The asterisk indicates a significant difference relative to the negative control at a 95% confidence level.

Chapter 4

POTH15: Regulation, over-expression and target gene identification

4.1. Introduction

POTATO HOMEOBOX 15 (*POTH15*) is one of the *KNOX*-I gene that has been identified and cloned from potato genome (Chapter 2). A number of over-expression and null mutant studies have shown that class-I *KNOX* genes regulate various vegetative and reproductive developmental processes such as SAM maintenance, leaf development, floral development, tuber formation, bulbil formation and so on (Rosin *et al*., 2003; Abraham-Juarez *et al.*, 2010; Hay and Tsiantis, 2010). Our analysis has revealed that *POTH15* is a potato ortholog of *SHOOT MERISTEMLESS* (*STM*) gene of Arabidopsis. *STM* has been shown to be essential for initiation and maintenance of shoot apical meristem (Barton & Poethig 1993). *STM* orthologs from a number of species such as rice, maize, tobacco, tomato are studied (Vollbrecht et al. 1991; Matsuoka et al. 1993; Janssen et al. 1998; Tanaka-ueguchi et al. 1998; Bolduc et al. 2012) and these studies show that *STM* orthologs are important for development of various plant tissues like apical and axillary meristems, leaves, inflorescence, and fruit (Matsuoka et al. 1993; Parnis et al. 1997; Nishimura et al. 2000; Sakamoto et al. 2001; Takacs et al. 2012).

KNOX genes act as Transcription Factors (TFs) and they are hypothesized to regulate a complex network of hormones and transcription factors. In spite of such a notion, the knowledge of target genes regulated by KNOX is limited. Previous studies have demonstrated that KNOX genes down-regulate *GA20-OXIDASE* (GA anabolism enzyme) expression and up-regulate *GA2-OXIDASE* (GA catabolic enzyme) expression thus resulting in low GA levels in the tissue. Apart from GA, KNOX genes have been shown to regulate levels of cytokinin and auxin. An interesting study by Bolduc *et al.* (2012) have identified several direct targets of *Kn1* including other homeobox genes and hormone metabolism genes in maize. Recently, Tsuda *et al.* (2014) showed that a rice *KNOX-I* gene *OSH1* represses the brassinosteroid (BR) phytohormone pathway through activation of BR catabolism genes and concluded that local control of BR levels by *KNOX* genes could be a key regulatory step in SAM function. In summary, these studies establish the importance of *KNOX* in plant development and reproduction.

The focus of the current work has been to understand the role of *KNOX* genes in potato development and tuberization. Previously, *POTH1* was shown to regulate vegetative development and tuberization in potato (Rosin *et al*., 2003) and its mRNA was demonstrated to be phloem mobile (Chapter 3,

Mahajan *et al*., 2012). As a part of the current study, six novel *KNOX* genes in potato were identified and validated (*Solanum tuberosum* ssp. *Andigena* 7540). Of them, *POTATO HOMEOBOX 15* (*POTH15*; a class-I *KNOX*) is the interest of the current study. To comprehend the role of POTH15 in potato development following experiments were undertaken.

(i) The photoperiodic regulation of *POTH15* abundance was studied by tissue specific qPCRs under LD/SD photoperiod.

(ii) To study the *POTH15* expression pattern, isolation and characterization of *POTH15* promoter was carried out.

(iii) The effect of *POTH15* over-expression in potato and tobacco was studied.

(iv) Putative target genes of POTH15 were identified by performing comparative RNA-seq of POTH15 over-expression and wild-type lines.

4.2. Materials and Methods

4.2.1. Plant material and growth conditions

Potato (*Solanum tuberosum* ssp. *andigena* 7540 and *Solanum tuberosum* cult Desiree) and tobacco (*Nicotiana tabacum* var *Petite Havana*) were used as model systems in this study. *In vitro* cultures of all the plants were maintained at 22±1°C with Long Day (LD; 16h light - 8h dark) or Short Day (SD; 8h light - 16 h dark) photoperiod in a growth incubator (Percival Scientific, USA). Soil grown plants of potato and tobacco were maintained at 22±1°C in an environmental chamber (Percival Scientific, USA) under LD or SD photoperiod as per experimental conditions.

4.2.2. Construct design and plant transformations

The full-length *POTH15* sequence was amplified using primers P15FL-FP-XbaI and P15FL-RP-KpnI and cloned downstream of CaMV35*S* promoter in the binary vector pCAMBIA1300 to generate *35S*::*POTH15* construct. The DNA sequence upstream of *POTH15* CDS (Coding DNA Sequence) was obtained from the PGSC database. *POTH15* promoter (1645 bp upstream *POTH15* coding sequence) was PCR amplified from potato genomic DNA using primers Pr15RE-F2 and pr15RE-FLR2 (Table 4.1) and was cloned in to binary vector pBI121 to generate $_{\text{prom}}$*POTH15*::*GUS* construct. The constructs were transformed to *Agrobacterium tumefaciens* strain GV2260.

Agrobacterium-mediated transformation of *Solanum tuberosum* ssp. *andigena and* Desiree was carried out by the method described in Banerjee *et al*. (2006) and tobacco by Horsch *et al*. (1985).

4.2.3. Expression analysis of POTH15 by qRT-PCR

For tissue-specific expression analysis of *POTH15*, potato (*Solanum tuberosum* ssp. *andigena* 7540) plants were used. Sixteen plants were transferred to soil and maintained under LD photoperiod for eight weeks. Later, half of the plants were transferred to tuber inducing SD conditions, whereas remaining plants were maintained under LD conditions. Different tissues (leaf, petiole, shoot tip, stem, stolon, and swollen stolon) were harvested 15 days post SD/LD induction. Tissues were frozen in liquid nitrogen immediately after harvest and stored at -80°C till further use. Total RNA was isolated from the frozen tissue using TRizol (Invitrogen) method as per the manufacturer's instructions. One microgram of total RNA was reverse transcribed using MMLV-RT (Promega) and gene specific primers POTH15-qR2 for POTH15 and 18S-rRNA-RP for 18S-rRNA. qPCRs were performed on Mastercylcer ep Realplex using primers POTH15-qF2 and POTH15-qR2 for *POTH15*, and 18S-rRNA-FP2 and 18S-rRNA-RP for *18S rRNA*. The reactions were carried out using KAPA SYBR green master mix (Kapa Biosystems) and incubated at 95°C for 2 min followed by 40 cycles of 95°C for 15s and 60°C for 30s. 18S-rRNA was used for normalization for all the reactions. PCR specificity was checked by melting curve analysis, and data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.2.4. Morphometric analysis

Wild-type and *35S*::*POTH15* lines of potato (*andigena*) were transferred to soil and maintained in an environmental chamber for ten weeks under LD conditions. Plant height, number of nodes and leaves per plant, internodal distance, and leaflet number per leaf were measured for all the plants. The data were plotted using the software 'Graph – Version 4.4.2' (www.padowan.dk).

4.2.5. *In vitro* tuberization

For *in vitro* tuberization experiment, shoot tips (3-4 cm) of wild-type and 35*S*::*POTH15* lines were cultured on MS + 6% sucrose (w/v) and incubated under LD conditions as previously described (Rosin *et al*., 2003). Plants were

examined for tuber activity and the number of tubers was counted over a period of six weeks. The data obtained were plotted using the software 'Graph – Version 4.4.2' (www.padowan.dk).

4.2.6. Histology

For histology, leaves and stems of ten weeks old plants of both wild-type and 35*S*::*POTH15* lines were fixed using 4% paraformaldehyde in phosphate buffered saline (0.1M, pH 7). The blocks of fixed tissues were prepared in 4% agarose in PBS (w/v) and then sectioned on vibratome (Leica).

4.2.7. Analysis of β-glucoronidase (GUS) activity

GUS assay (Jefferson, 1987) was done by incubating the tissue samples of prom*POTH15*::*GUS* potato lines in assay buffer containing 1 M $NaPO_4$, pH 7; 0.25 M EDTA, pH 8; 10% TritonX 100; 1 mM 5-bromo-4-chloro-3-indolyl-β-D-GlcUA, 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide. Samples were cleared with 100% ethanol and photographed under Leica stereo microscope S8AP0 or Zeiss compound microscope.

4.2.8. RNA isolation, library preparation and RNA sequencing

Shoot apex (4-5 cm) were harvested and pooled from six plants each of the wild-type and 35*S*::*POTH15* lines (G8 and E2-13). Total RNA was isolated using Trizol (Invitrogen). RNA samples were quantified on Qubit using Qubit RNA HS kit (Invitrogen). Ten micrograms of RNA was taken and the poly (A) RNA was enriched using Dynabeads® mRNA direct microkit (Ambion) following the manufacturer's instructions. The libraries were prepared using Ion Total RNA-Seq V2 kit (Life technologies). Quality and concentrations of the libraries was determined using DNA1000 chip on a Bioanalyzer (Agilent). Libraries were then sequenced on Ion Proton^{TM} platform. The reads were obtained in FastQ format and quality was checked using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). RNA-seq data analyses were performed using the Tuxedo suite. The reads were aligned to potato reference genome sequence (PGSC_DM_v3.4_gene.fasta.zip) using Bowtie 2.0 (Langmead *et al*., 2009) and TopHat-Version 2.0.13 (Kim *et al*., 2011) software with default parameters. The reads that aligned to the genome were quantified by Cuffquant and Cufflinks program (Trapnell *et al*., 2013), which provided relative abundance values by calculating fragments per kilobase

of exon per million fragments mapped (FPKM) (Mortazavi *et al*., 2008, Mizrachi *et al*., 2010). Cufflinks was also used to find isoforms, promoters, Translation Start Site (TSS) and sites of alternative splicing. The differential expression analysis of genes was performed using Cuffdiff package-2.2.1 (Trapnell *et al*., 2013). The Cuffdiff results were compiled and visualized using the R package *CummeRbund*-Version 2.0 (Goff *et al*., 2011). To functionally classify differentially expressed genes, Gene Ontology (GO) analyses were performed. Since potato genome is not well annotated, we retrieved *Arabidopsis* orthologs for respective potato genes. TAIR database (https://www.arabidopsis.org) was used to obtain GO terms. Using GO analysis tools at TAIR database, the GO terms were further clustered into GOslim categories for easier comparison.

4.2.9. Validation of *POTH15* targets by qRT–PCR

Wild-type and 35*S*::*POTH15* lines of potato were transferred to soil and maintained in a green house for ten weeks. Total RNA was isolated from the shoot apex (4-5 cm) of the wild-type and *POTH15* over-expression lines using Trizol (Invitrogen). Three microgram of total RNA was used for cDNA synthesis using oligo(dT) primer and SuperScript-III reverse transcriptase (Invitrogen). qPCRs were performed on Mastercylcer ep Realplex using gene specific primers (Table 4.1). The reactions were carried out using KAPA SYBR green master mix (Kapa Biosystems) and incubated at 95°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 30s. *StActin* was used for normalization for all the reactions. PCR specificity was checked by melting curve analysis, and data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 4.1 - List of primers used.

Gene name	Primer name	Sequence
POTH15	POTH15-RTF	GGCTCATCCTCACTACCCTCG
	POTH15-RTR	CAGCATCTCACAATAAGCCTCCA
POTH15	c-P15-5'RACE	CCATTTCCATTTCCATTACCATTTCC
	n-P15-5'RACE	CAAGATGTACTAGTATTTCCACTAG
POTH15	POTH15-FLF	AGGAATATTGGGTGTTTGCTT
	POTH15-FLR	ACGTACAAACTAATAAATTTTATAAGA
$_{prom}$POTH15	Pr15RE-F2	CACCAAGCTTTGAATGGAAGGGATTTAGCATCAC
	Pr15RE-FLR2	TAGACCCGGGCACACTTTCTTCTTGAGTCTAAATTTTGTTCC
POTH15	P15FL-FP-XbaI	TTCATCTAGAAGGAATATTGGGTGTTTGCTT
	P15FL-RP-KpnI	CCTGGTACCACGTACAAACTAATAAATTTTATAAGAG
POTH15	P15-qF2	GGCTCATCCTCACTACCCTCG
	P15-qR2	CAGCATCTCACAATAAGCCTCCA
18S-rRNA	18S-rRNA-FP2	GGGCATTCGTATTTCATAGTCAGAG
	18S-rRNA-RP	CGGTTCTTGATTAATGAAAACATCCT
StTCP	StTCPqFP	CTGAGGAAGGAGACGATGGTGG
	StTCPqRP	CTGATTGAGAGGAGTGATTATTC
StNAC2	StNAC2 qFP	TGTCAGGCCAAATAGAGCAGC
	StNAC2 qRP	AACCTCATAGATCCACTTTGCT
StNAC TF2	StNAC TF2 qFP	GAGCGTTCGATCGGAGACCAAT
	StNAC TF2 qRP	ACCCTCTTGTTGTTGTGTGAGTTGA
StAintegumenta	StAintegqFP	AAGCTCATCGGATGAAAGTACT
	StAintegqRP	CTTGTGCATTCATAGTTGTACCCT
StLipoxygenase	StLipo 158 qFP	GCAAGACGATGGAACAATGAAG
	StLipo 158 qRP	GGCTGCGTGTGTATTCAACCA
StCytochrome P450	StCytP450 qFP2	TCGAGTACTAGTCAATGCATG
	StCytP450 qRP2	AACTGAGCTAAAGGATGTATAAC
StGA2ox	StGA2ox qFP2	AACAACACTTCCGGCCTTCAA
	StGA2ox qRP2	CTATGCTTCACACTCTTAAACC
StAS2	StAS2 qFP2	TGGCTTCATCTTCATCACCTTCAT
	StAS2 qRP3	ACAGCGTCTTCCCTTTGGTGAG
StSUPERMAN	StSupermanAqFP	TGATGATGTCTCCAAGTACTCCA
	StSupermanAqRP	TGAATCCCTCTTGTTGCTACC
StLOB	StLOB qFP2	GACCATCTCCACCAACATCAC
	StLOB qRP2	ATGTCCAAAGAGTCTCCAGGGTC
StSAUR	StSAURqFP	CGAGAGGATGCGTTACGGTGTTG
	StSAURqRP	GATGATGGTGGTGGTGATGAACG
StActin	StActin F	GCTTCCCGATGGTCAAGTCA
	StActin R2	CAGGTCCTTTCTGATATCAACGT

4.3. Results

4.3.1. Transcript abundance of *POTH15* is photoperiod regulated

To investigate if photoperiod has any effect on abundance of *POTH15* transcript, the relative levels of *POTH15* mRNA in photoperiod responsive wild-type potato (*Andigena*) plants was examined. . *POTH15* levels were evaluated in shoot tips, leaves, petioles, stems, roots and stolons of potato plants grown under both SD and LD conditions through qRT-PCR analysis (Fig. 4.1). Among all the tissue types evaluated, shoot tips, stolons and stems showed higher abundance of *POTH15* mRNA under tuber inducing SD conditions. Petiole and roots exhibited high accumulation of *POTH15* mRNA under LD conditions compared to SD conditions. However, mRNA abundance in leaves did not change in both in SD and LD photoperiod tested (Fig. 4.1).

4.3.2. Promoter activity of *POTH15*

GUS assay for the $_{prom}$*POTH15*::*GUS* transgenic lines of potato (cult Desiree) in a tissue-specific manner showed that the *POTH15* promoter is active in shoot apex, axillary nodes, petiole, stolons, tuber pith, and tuber eyes (Fig. 4.2). The promoter sequence was analysed for the presence of *cis* regulatory elements using the promoter analysis software plantPAN (Chang *et al.*, 2008) and PLACE (Higo *et al.*, 1999). Several light regulatory elements such as GATA, GT-1 and I-boxes were detected in the *POTH15* promoter. The plantPAN software also identified presence of binding sites for several other transcription factors such as MYB, AthB1, AthB5, AtHB9, AGL15 and AGL3 (Table 4.2) in the *POTH15* promoter.

4.3.3. Over-expression of *POTH15* alters multiple morphological traits

In order to investigate the role of *POTH15* in development, 35*S*::*POTH15* transgenic lines were generated and five independent lines (G8, G9, G11, E2-13 and E2-18) were selected for further analysis. These lines showed ten to fifteen fold increase of *POTH15* transcripts compared to wild-type plants (Fig. 4.3) tested under *in vitro* conditions. Over-expression lines exhibited severe morphological changes in plant architecture (Fig. 4.4). The 35*S*::*POTH15* lines were shorter and slender compared to wild-type plants (Fig.4.4 B to D and 4.4 G) and these lines exhibited marked difference in leaf shape and size.

Table 4.2. List of regulatory motifs in *POTH15* promoter predicted by PlantPan (Chang et al., 2008).

Motif	**Number**
AGL-3	2
AG	10
Athb-1	30
ANT	1
Athb-5	28
Athb-9	6
CDC-5	2
RAV1	12
ACGTATERD1	2
ANAERO1CONSENSUS	2
AP1	4
ARR10	7
ARR1AT	34
AtMYC2	1
Bellringer	7
AGL-15	20
CCA1	2
ATHB33	74
DPBF-1	1
GARE	2
GATABOX	23
GBF5	3
GT1CONSENSUS	45
IBOX	2
L1BOX	1
MYB1	13
MYCATERD1	1
MYCATRD22	1
MYCCONSENSUSAT	8
POLASIG1	12
PREATPRODH	3
SORLIP1	1
SRE	3
SURE	2
SURECOREATSULTR11	3
TAAAGSTKST1	11
TBOX	1
WBOX	2

Wild-type potato plants had ovate leaflets (Fig. 4.4 E, F), whereas *POTH15* over-expression lines developed curved, mouse ear shaped leaflets (Fig. 4.4 H, I). The petiole, petiolule and rachis of *POTH15* over-expression lines were also severely shortened. The venation was changed to palmate from pinnate. Compared with wild-type plants, the leaves on *POTH15* over-expression lines were clustered closer to the stem as they had short petioles (Fig.4.4 G). Thus, the leaves of *POTH15* over-expression lines were significantly smaller with a bouquet of leaflets arranged on the petiole (Fig. 4.4 G to J) in contrast to spaciously arranged leaflets of wild-type plants (Fig. 4.4 E, F). Again when the individual leaflets of *POTH15* over-expression lines were examined, multiple small secondary leaflets were also observed on each of the primary leaflets (Fig. 4.4 H to J).

All the over-expression lines showed significant reduction in plant height compared to wild-type (Fig. 4.5A). *POTH15* over-expression lines also had significantly more number of nodes per plant compared to wild-type plants (Fig. 4.5C). Interestingly, *POTH15* over-expression lines were found to retain most of the leaves in the stem in contrast to the wild-type plants, which abscessed 7-8 basal leaves (Fig.4.5B). The internodal distances for *POTH15* over-expression lines were not significantly different compared to wild-type plants; however, the variation in the internodal distance was higher in wild-type plants (Fig. 4.5D).

The number of leaflets per leaf for both wild-type and *POTH15* over-expression plants were also compared. Interestingly, *POTH15* over-expression plants had fewer leaflets per leaf than wild-type plants. Majority of the wild-type leaves had 5 or 7 leaflets (34% or 53% of total leaves respectively), on the other hand, 60 to 80 % of the leaves of *POTH15* over-expression lines had either 1 or 3 leaflets (Fig. 4.5E). Wild-type plants almost always had odd number of leaflets on a given leaf but leaves with 2, 4, and 6 leaflets were more common in *POTH15* over-expression lines (Fig. 4.5E).

Cross sections of the stem from *POTH15* over-expression lines showed a lteration in cellular architecture compared to wild-type stem. Vascular bundles were uniformly distributed throughout the wild-type stem, whereas, they were clustered in *POTH15* over-expression lines (4.6 A to F). Cross sections of wild-type leaves displayed well organized vertically packed palisade cells and parenchymatous tissue (Fig. 4.6 G, H), whereas cellular organization in *POTH15* over-expression lines was found to be severely hampered with lobbing

of leaflets on the abaxial side (Fig. 4.6 I to O). Leaf cross section also revealed that *POTH15* over-expression lines had deformed mid-vein. Remarkably, leaves of *POTH15* over-expression lines developed centres of meristem-like cells on adaxial side (Fig. 4.6 M, N). Overall, leaf development was drastically affected in *POTH15* over-expression lines. Similarly, 35*S*::*POTH15* tobacco lines showed marked defects in plant development with curled, mouse-ear shaped leaves and altered leaf and stem cell architecture (Fig. 4.7, 4.8, 4.9).

4.3.4. *In vitro* tuberization

To check the effect of *POTH15* over-expression on tuberization, wild-type and 35*S*::*POTH15* lines were cultured on MS + 6% sucrose medium and incubated under LD conditions for six weeks. Wild-type plants showed tuber activity after two weeks in culture conditions, whereas *POTH15* over-expression lines exhibited tuber activity in first week of incubation itself. Although *POTH15* over-expression lines exhibited earliness in tuberization, from four weeks onwards, their tuber numbers did not differ from the wild-type lines (Fig. 4.10). Thus over-expression of *POTH15* resulted in earliness of tuberization.

4.3.5. *POTH15* regulates key developmental genes

To detect genes and pathways that are regulated by *POTH15*, we performed a transcriptome analysis using eight weeks old soil grown plants of wild-type and 35*S*::*POTH15* (two independent lines; G8 and E2-13). The details of the reads obtained and aligned to the genome for each sample are given in Table 4.3.

Table 4.3. Number of reads obtained and aligned to the potato genome in the RNA-seq analysis.

Sample	Total number of reads	Overall alignment rate
Wild-type (WT)	3,035,538	36.34%
POTH15 over-expression line G8	36,481,430	52.07%
*POTH15*over-expression line E2-13	15,442,365	44.59%

Over-expression of *POTH15* in *Solanum tuberosum* spp. *andigena* 7540 resulted in expression changes of 270 genes in G8 line, while 299 genes in E2-13 line compared to wild-type plants. Out of 270 genes in G8 line, 257 genes were significantly up-regulated (adjusted $p<0.05$), whereas 13 genes were found to be down-regulated (adjusted $p<0.05$). Similarly, the number of genes up-regulated and down-regulated in E2-13 over-expression line was 285 and 14 respectively. Altogether, 137 genes were found to be differentially expressed in both *POTH15* over-expression lines, of which only four genes had down-regulation. The RNA-seq data analysis also revealed 79 differentially expressed novel genes (Table 4.4).

To functionally classify differentially expressed genes, gene ontology analysis was performed. Since potato genome is not well annotated, *Arabidopsis* orthologs for respective potato genes were retrieved. Out of our set of 356 known differentially expressed genes, *Arabidopsis* orthologs for 297 genes were retrieved and remaining 51 genes were classified as genes of unknown function. TAIR database (https://www.arabidopsis.org) was used to obtain Gene Ontology (GO) terms. GO analysis categorized up-regulated genes in 2044 biological processes, 647 molecular functions and 510 cellular components GO terms. Likewise, down-regulated genes were categorized in 138 biological processes, 32 molecular functions and 49 cellular components GO terms (Fig. 4.11). Using GO analysis tools at TAIR database, the GO categories were further clustered into GOslim categories for easier comparison. Our GOslim term analysis performed on differentially expressed genes set revealed enrichment in terms associated with cellular and metabolic processes, response to abiotic and biotic stimuli, response to stress and developmental processes (Fig. 4.11 A to F). The enriched categories comprise genes that are involved in hormone metabolism and hormone responses such as ethylene responsive nuclear protein, Small Auxin Up-regulated RNA (SAUR) family protein, jasmonic acid induced WRKY protein, caboxy-lyase, cytokinin riboside 5'-monophosphate phosphoribohydrolase (*LOG1*), *LOG3* etc. Additionally, they included genes involved in signal transduction and cell cycle regulation such as MAD2, serine/threonine protein kinase, MAP kinases, calmodulin binding protein etc. Several TFs such as Homeobox, NAC domain protein, TCP, MADS box, Myb-domain DNA binding protein, AP2/ERF domain-containing protein

were also differentially expressed (Table 4.4). About 44% of up-regulated genes were categorized for enzyme activity like transferases, hydrolases, kinases and others. Genes with enzyme activity were also over-represented in the list of down-regulated genes. Apart from this, 33% of up-regulated genes had molecular function as 'binding', including binding to DNA, RNA and protein (Fig. 4.11 B, E). Under cellular component category, nucleus, plasma membrane and other membranes sub-categories were over-represented (Fig. 4.11 C). Likewise, most of the down-regulated genes were predominantly involved in stress response, cell organization and biogenesis and they had extracellular localization (Fig. 4.11 D, F). Functions for the differentially expressed genes in our analysis are listed in Table 4.4

4.3.6. Validation of *POTH15* targets

To validate RNA-seq results, we examined expression of 11 candidate genes in wild-type and *POTH15* over-expression lines. Out of these 11 genes, nine were differentially regulated in our RNA-seq analysis, while two genes Viz. *StGA2ox* and *StAS2* were selected for qPCR validation, as they were previously shown to be regulated by KNOX (Bolduc and Hake, 2009; Semiarti *et al*., 2001). As shown in Fig. 4.12, the transcript levels of *StLipoxygenase*, *StNAC-TF2*, *StCyt-P450*, *StTCP5*, *StGA2ox*, *StLOB*, *StAintegumenta* were significantly higher in both the G8 and E2-13 lines, when compared to wild-type plants. *StNAC2* transcript levels were found to be significantly increased in over-expression line E2-13, whereas *StSAUR* transcript had significantly higher expression in over-expression line G8 only. Interestingly, transcript levels of *StSUPERMAN* and *StAS2*, the genes that are involved in regulation of cell proliferation and boundary specification, were significantly down-regulated in both the over-expression lines. These results are in accordance with our RNA-seq analysis indicating the reliability of the analysis.

4.4. Discussion

Apart from *POTH1* (Rosin *et al*., 2003, Mahajan *et al*., 2012), no other *KNOX* gene in potato has yet been characterized. Here we report, six novel *KNOX* genes from potato including *POTH15* (a *STM* ortholog in potato). This study describes the photoperiodic regulation of *POTH15*, characterisation of its

over-expression phenotype, and identification and validation of *POTH15* target genes in potato.

4.4.1 *POTH15* regulation

In simple leaf species, the expression of class-I *KNOX* genes is usually confined to meristems and stems, whereas in compound leaf species, they are expressed in leaf primordia as well (Bharathan *et al*., 2002; Uchida *et al*., 2007). Earlier, Rosin *et al*. (2003) have detected the *POTH1* mRNA in the SAM, leaf primordia, leaf lamina, developing leaflets, stolon, and stem vascular tissue in potato. Although a number of studies (Kerstetter *et al*., 1994; Bharathan *et al*., 2002; Uchida *et al*., 2007) have described the *KNOX* expression patterns, photoperiod mediated regulation of *KNOX-I* transcript abundance at the tissue-specific level is not yet reported. The potato line used in this study (*S. tuberosum* ssp. *andigena*) is photoperiod dependent for tuberization, which produces tubers only under SD conditions. Therefore, we studied the effect of photoperiod on the abundance of *POTH15* mRNA in a tissue-specific manner. Under LD conditions, petiole and roots exhibited higher abundance of *POTH15* mRNA compared to SD conditions, whereas under tuber inducting SD conditions, *POTH15* mRNA was accumulated at high levels in shoot tips, followed by stolons and stems (Fig. 4.1). Moreover, the *POTH15* promoter was found to be active in shoot apex, axillary nodes, petioles, stolons, tuber pith, and tuber eyes (Fig. 4.2). Previously, the promoter of *POTH1*, another Class-I *KNOX* gene in potato, was shown to be active in a wide range of organs, including shoot tips, axial nodes, stolons, leaves, stamens and roots (Mahajan *et al*., 2012). In contrast to *POTH1* promoter activity, *POTH15* promoter was active in tuber pith and tuber eyes (Fig. 4.2). Similar to *POTH1* promoter, the analysis of the *POTH15* promoter sequence showed the presence of several light regulatory motifs such as GATA, GT-1, GBF5, SORLIP1, I-boxes (Table 4.2; Terzaghi and Cashmore, 1995; Hudson and Quail, 2003; Chatterjee *et al.*, 2007). Overall, the photoperiod dependent expression pattern of *POTH15* (Fig. 4.1), activity of its promoter in tuber specific tissues (Fig. 4.2), presence of several light regulatory motifs in its promoter sequence (Table 4.2), and earliness in tuberization in *POTH15* over-expression lines under *in vitro* conditions (Fig. 4.10), suggest that *POTH15* might be involved in regulation of

tuber development in potato. Previously, transcripts of *TKN2* (Kim *et al*., 2001), *STMP* (Ham *et al*., 2009), and *POTH1* (Mahajan *et al*., 2012) were demonstrated to be phloem mobile. Interestingly, a truncated sequence of *POTH15* (361 bp) was also detected in potato phloem by Campbell *et al.* (2008). It would be interesting to investigate if *POTH15* also acts as a phloem mobile signal, and plays a role in potato development.

4.4.2. *KNOX* over-expression phenotype

Several studies have previously demonstrated the effect of *KNOX* over-expression in diverse plant species such as *Arabidopsis*, tomato, potato, tobacco, lettuce, maize, rice, agaves and populus (Rosin *et al.*, 2003; Hake *et al.*, 2004; Du *et al*., 2009; Abraham-Juarez *et al*., 2010; Tsuda *et al.*, 2014). These studies have established that *KNOX-1* over-expression results in drastic developmental alterations in plants including defects in SAM maintenance and leaf development. Similarly, in the present report, over-expression of *POTH15* in potato resulted in severe defects in plant architecture (Fig. 4.4; Fig. 4.5, Fig. 4.6). The 35*S*::*POTH15* potato lines were shorter and slender than wild-type plants and they had more number of nodes and leaves per plant (Fig. 4.5). Their leaves were significantly smaller with leaflets characterized by a curved, mouse ear shape phenotype (Fig. 4.4). Further, the cellular organization in leaves and stems of *POTH15* over-expression lines was altered and some leaves exhibited centres of meristem-like cells on their adaxial side (Fig. 4.6). Similar to potato over-expression lines, 35*S*::*POTH15* tobacco lines showed a drastically altered plant development with curled, mouse-ear shaped leaves with meristem-like structures on their adaxial sides. Occasionally, we also observed development of ectopic leaf on the adaxial side of the older leaf (Fig. 4.7, Fig. 4.8, Fig. 4.9). All these phenotypes of potato and tobacco 35*S*::*POTH15* lines are consistent with earlier studies of *KNOX-I* over-expression (Muller *et al*., 1995; Janssen *et al*., 1998; Tamaoki *et al*., 1997; Hake *et al.*, 2004). Rosin *et al.* (2003) have demonstrated that *POTH1* over-expression lines in potato produced more tubers at a faster rate under *in vitro* conditions. In the current study, although over-expression of *POTH15* accelerated *in vitro* tuberization, the total number of tubers did not differ significantly between the wild-type and 35*S*::*POTH15* lines after six weeks of incubation (Fig. 4.10). Ectopic expression of *KNOX-I* genes

was previously shown to either enhance or block leaflet formation depending on the developmental stage of the leaf and competency of the cells to respond to leaflet promoting signals (Shani *et al.*, 2009; Hay and Tsiantis, 2010). For example, when *Kn1* was over-expressed in tomato; the normally dissected leaves having 8 to 16 leaflets became severely dissected with up to 1000 leaflets (Hareven *et al*., 1996, Janssen *et al*., 1998). In contrast to this, we observed that *POTH15* over-expression lines in potato showed a decrease in the number of leaflets per leaf but the leaflets were lobed and they produced multiple secondary leaflets (Fig. 4.4; Fig. 4.5). Thus, over-expression of *POTH15* in potato and tobacco alters multiple morphological traits.

4.4.3. *POTH15* targets

As *KNOX* proteins act as transcription factors, identifying their target genes is imperative to understand how *KNOX* genes can regulate diverse developmental processes in plants. Earlier, *KNOX-I* TFs have been shown to regulate the levels of GA and cytokinin (Sakamoto *et al.*, 2001; Hay *et al.*, 2002; Chen *et al*., 2004; Yanai *et al.*, 2005; Bolduc and Hake, 2009) and the biosynthesis of lignin (Mele *et al.*, 2003; Hay and Tsiantis, 2010). A screen for *STM* targets revealed CUC1 (CUP SHAPED COTYLEDON 1) is a direct target of *STM* in *Arabidopsis* (Spinelli *et al*., 2011). Another interesting study by Bolduc *et al.* (2012) demonstrated that direct targets of *Kn1* include other homeobox and hormone metabolism genes. Recently, Tsuda *et al.* (2014) also showed that a rice *KNOX-I* gene *OSH1* targets BR catabolism genes and regulate SAM functions. In spite of their significance in plant development, no screen for *KNOX* target genes in potato is reported yet.

In this report, we have performed RNA-seq analysis of wild-type and *POTH15* over-expression potato lines to identify the putative targets of *POTH15*. Our analysis revealed 435 differentially expressed genes including 79 novel genes. Functional analysis of these genes categorized them in different cellular and metabolic processes, response to abiotic and biotic stimuli, response to stress, and developmental processes (Fig. 4.11 A to F), suggesting *POTH15* function in various developmental processes in potato. As over-expression lines of *POTH15* were used for RNA-seq analysis, the list of differentially expressed genes could include both direct as well as indirect targets of *POTH15*.

Validation of 11 candidate target genes by qRT-PCR further confirmed the reliability of our RNA-sequencing results.

In this study, *POTH15* over-expression lines showed a significant increase in *StGA2ox* transcript levels compared to wild-type (Fig. 4.12), which is consistent with the previous observations of Jasinski *et al*. (2005) and Bolduc and Hake (2009). Hormone-like compounds (e.g. jasmonic acid, methyl jasmonate and tuberonic acid) derived from lipoxygenases (LOXs) are known to have strong tuber inducing activity *in vitro* (Siedow, 1991; Koda, 1992). Suppressor mutants of a tuber specific *LOX* (*StLOX1*) exhibited a significant reduction in StLOX1 activity in stolons and tubers, which was associated with the reduced tuber yield and disrupted tuber formation (Hannapel *et al*., 2004). Here, *POTH15* over-expression lines also showed an earliness in tuberisation *in vitro* (Fig. 4.12), which could be correlated with the increased *StLipoxygenase* transcript levels detected in these over-expression lines (Fig. 4.12), indicating a possible role for lipoxygenase in tuber development. *KNOX* genes are known to positively regulate the expression of NAC-domain transcription factors such as CUC 1-3 which are involved in organ boundary maintenance (Aida *et al*., 1997, 1999; Vroemen *et al*., 2003; Hibara *et al*., 2006). Consistent with the previous findings (Takada *et al*., 2001; Hake *et al*., 2004; Blein *et al*., 2008; Hay and Tsiantis, 2010), transcript abundance of NAC-domain transcription factors such as *StNAC2* and *StNACTF2* was significantly increased in *POTH15* over-expression line (E2-13) (Fig. 4.12). SAUR genes are anticipated to be involved in auxin signaling and stress defence response (Jain and Khurana, 2009; Kant *et al*., 2009; Wu *et al*., 2012). Our results show that *POTH15* over-expression lines had an increased abundance of *StSAUR* transcript (Fig. 4.12), suggesting a possible role of *POTH15* in auxin signalling pathway. Earlier, it was demonstrated that *KNOX-I* class genes up-regulated *CytP450* genes associated in BR catabolism, and are involved in cell elongation and cell wall modification (Donaldson and Luster, 1991; Sun *et al.*, 2010; Tsuda *et al*., 2014). Similar to the observation of Tsuda *et al.* (2014), *StCytP450* transcript levels were significantly higher in *POTH15* over-expression lines compared to wild-type (Fig. 4.12), indicating the positive effect of *KNOX* genes on *CytP450* expression.

A number of epigenetic regulators of *KNOX-I* expression such as AS1, AS2, PRCs, SUPERMAN (SUP) have been identified in *Arabidopsis* (Brewer *et al.*, 2004; Xu and Shen, 2008; Ori *et al.*, 2002; Kim *et al.*, 2012). In our study, the transcript levels of *StAS2* and *StSUP* in *POTH15* over-expression lines were found to be significantly reduced (Fig. 4.12). It would be interesting to understand if *KNOX* genes have any feedback mechanism with these *StAS2* and *StSUP genes.* Previously, it was shown that the heteromer of AS2 and CINCINNATA-LIKE TEOSINTE BRANCHED 1-CYCLOIDEA-PCF (TCP) transcription factors binds to the promoters of *KNOX* genes and represses their expression to regulate leaf development (Martin-Trillo and Cubas, 2010; Kieffer *et al.*, 2011; Li *et al.*, 2012). Here, qRT-PCR validation showed that the *StTCP5* transcript levels were significantly increased in *POTH15* over-expression line E2-13 (Fig. 4.12), suggesting that *POTH15* may directly or indirectly upregulate *StTCP* expression. In conlcusion, *POTH15* appears to regulate a range of target genes involved in diverse developmental processes in potato.

The results of the present study can be summarised as:

(i) Tissue-specific qRT-PCR analysis exhibited a higher abundance of *POTH15* mRNA in shoot tips and stolons under tuber inducing short day conditions.

(ii) Upstream sequence of *POTH15* drives β-glucuronidase expression in apical and axillary meristems, petiole, tuber eyes and tuber pith indicating its role in meristem maintenance, leaf development and tuberization.

(iii) In accordance with this, over-expression of *POTH15* in potato altered multiple morphological traits including leaf development and exhibited early tuberization under *in vitro* conditions.

(iv) Comparative transcriptomic analysis of wild-type and *POTH15* over-expression lines identified 435 differentially expressed genes including 79 novel target genes with unknown functions. Functional analysis of these genes revealed their involvement in key biological processes such as metabolism, biotic and abiotic stress responses, regulation of transcription, and signal transduction.

(v) qRT-PCR of selected candidate genes validated their differential expression in wild-type and *POTH15* over-expression lines.

Figures

Chapter 4

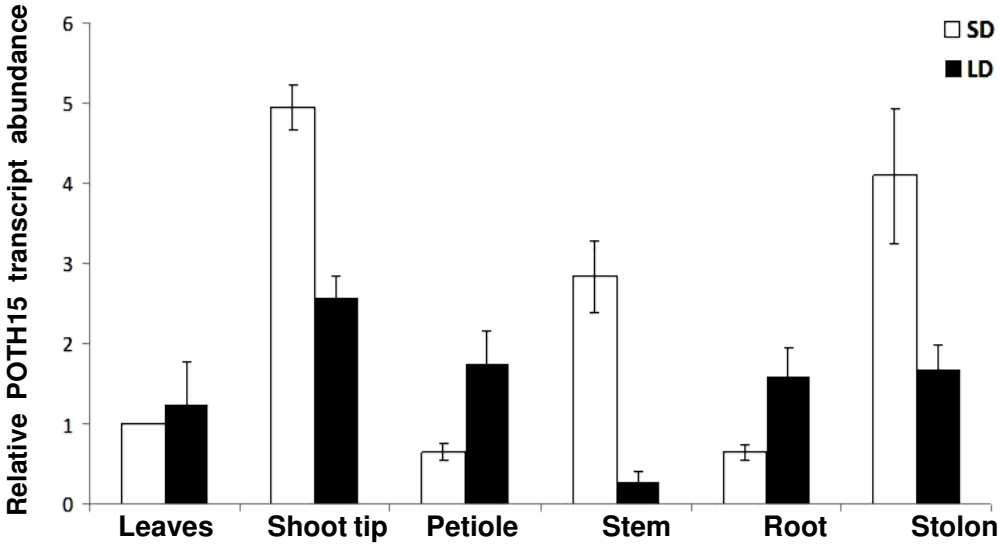


Fig. 4.1. **Tissue-specific abundance of POTH15.** POTH15 abundance in leaves, shoot tip, petiole, stem, root and stolon of wild-type *S. tuberosum* ssp. *Andigena* plants grown under SD and LD conditions for 15 days. Data are mean +/- SD for three biological replicates.

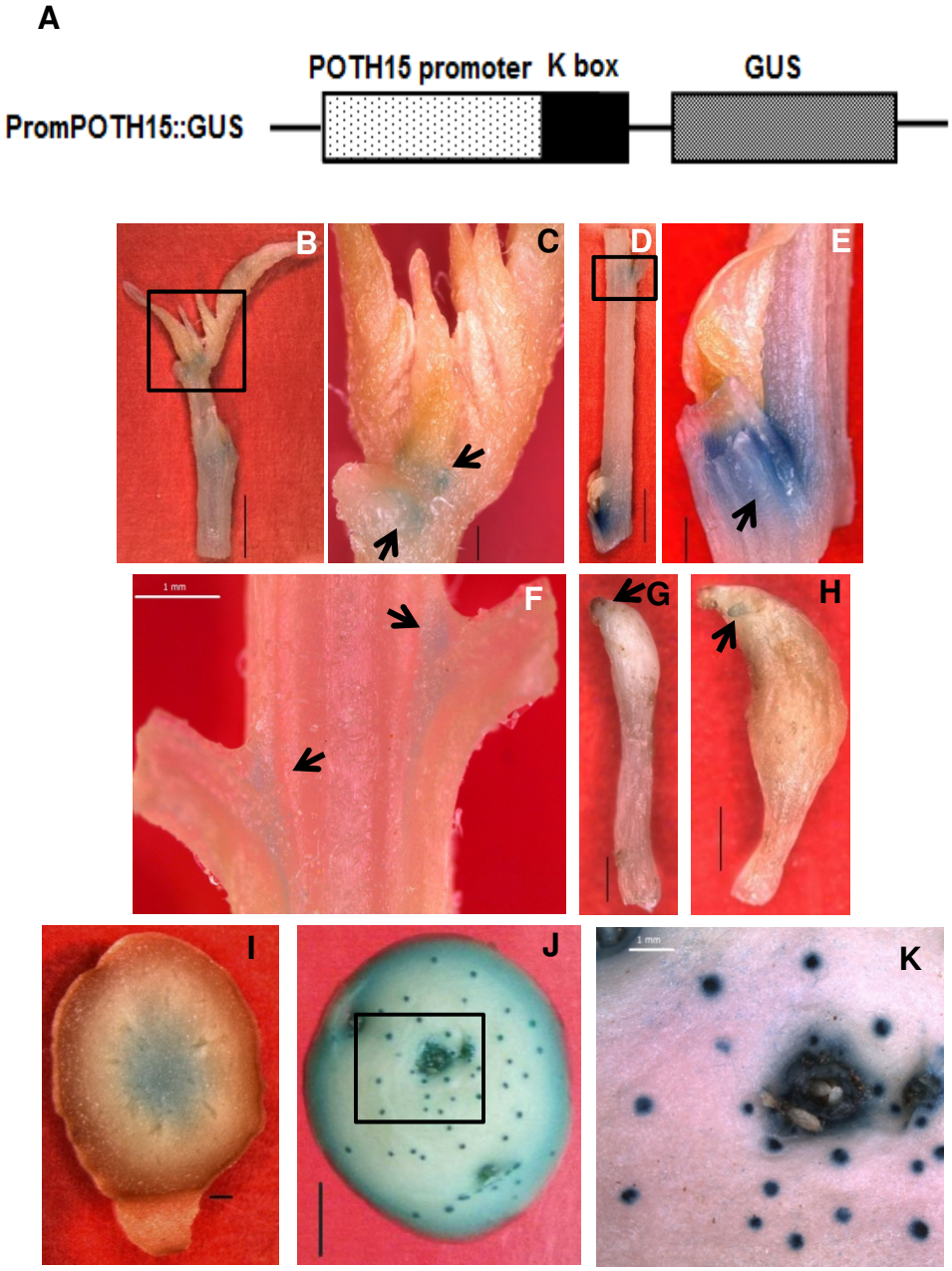


Fig. 4.2. The POTH15 promoter expression. The $_{prom}$POTH15::GUS construct (A). Promoter activity of POTH15 upstream sequence in shoot tip (B), axillary nodes (D), petiole (F), swollen stolon (G, arrow), minituber (H, arrow), pith (I) and eyes of tuber (J) in *S. tuberosum* cult Desiree. C, E and K are magnified images of B, D and J, respectively (highlighted regions). Scale bars in B, D, G, H and J are 5 mm and in C, E, I and K are 1 mm respectively.

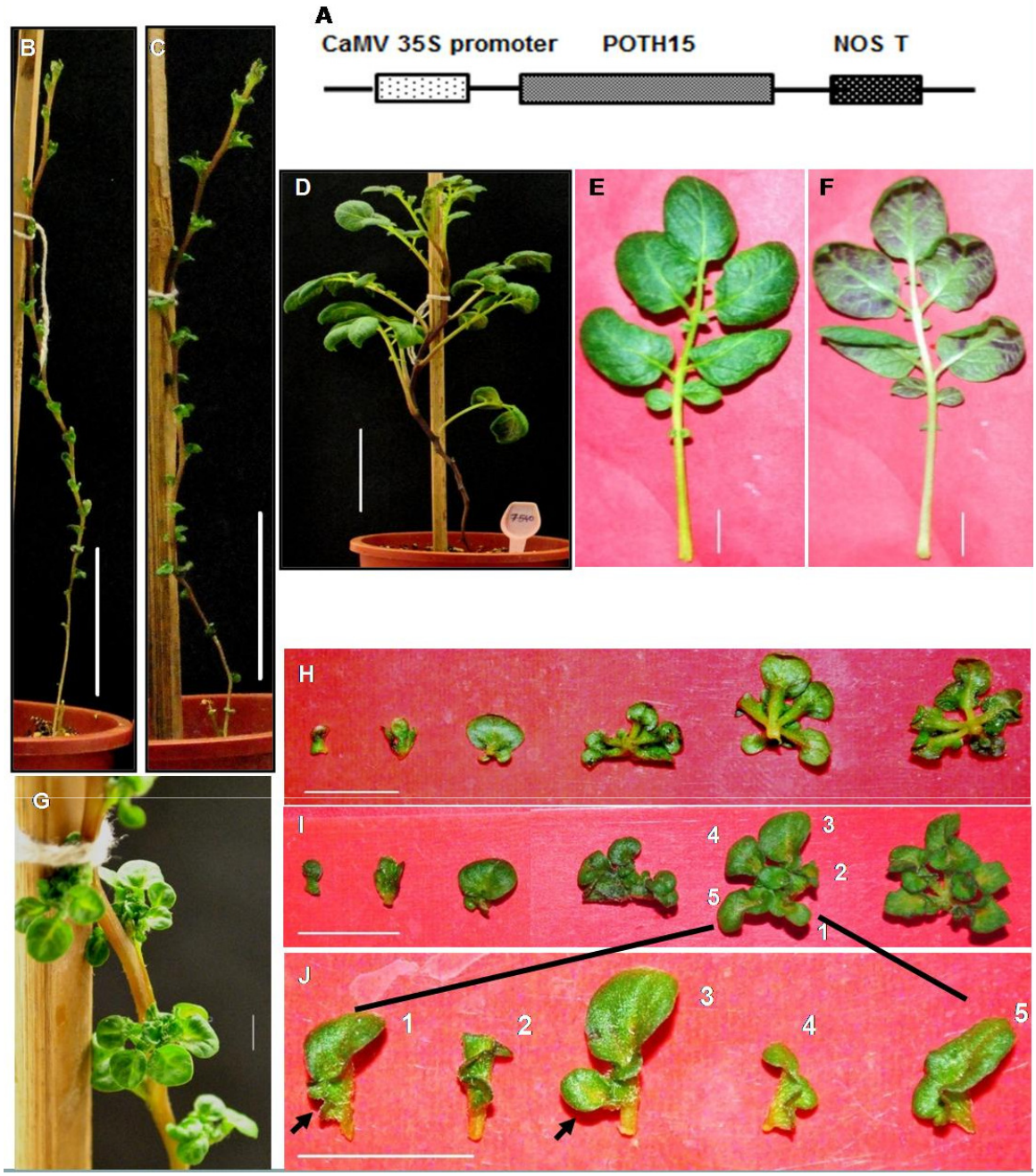


Fig. 4.3. POTH15 over-expression drastically changes the plant architecture. The *35S*::POTH15 construct (A). Eight weeks old potato plants over-expressing POTH15 (B and C). Wild-type potato lines (D). The dorsal and ventral view of the leaves from wild-type (E and F) and POTH15 over-expression (H and I) lines. The leaflets on leaves of POTH15 over-expression lines were dissected out to visualize the secondary leaflets (J, arrows). The scale bars in B, C and D are 5 cm and scale bars in E through J are 1 cm.

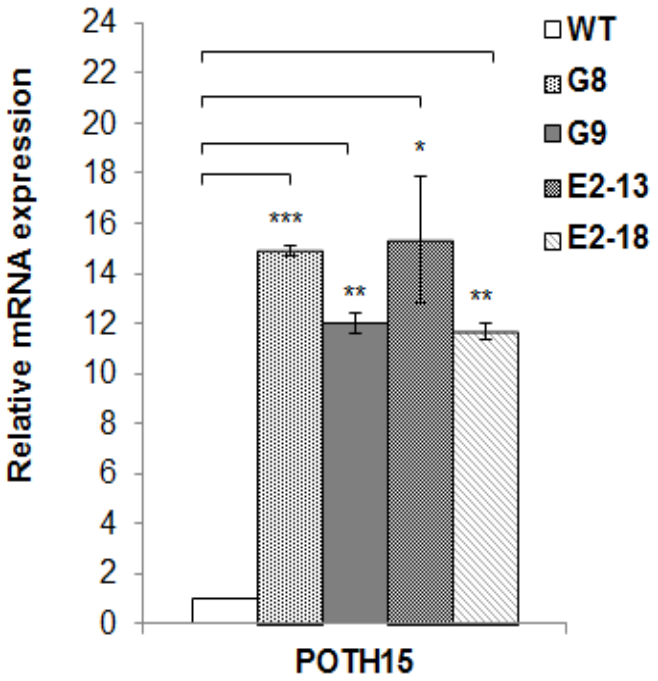


Fig. 4.4. The POTH15 transcript levels in over-expression lines. Relative mRNA levels of POTH15 in over-expression lines (G8, G9, E2-13 and E2-18) is shown with respect to wild-type (WT) from four weeks old *in vitro* grown plants. Data represent mean +/- SD for three biological replicates. Data were analysed by t-test separately for WT and each over-expression line. Asterisk *, ** and *** represents significant difference at P≤0.05 P≤0.01 and P≤0.001, respectively.

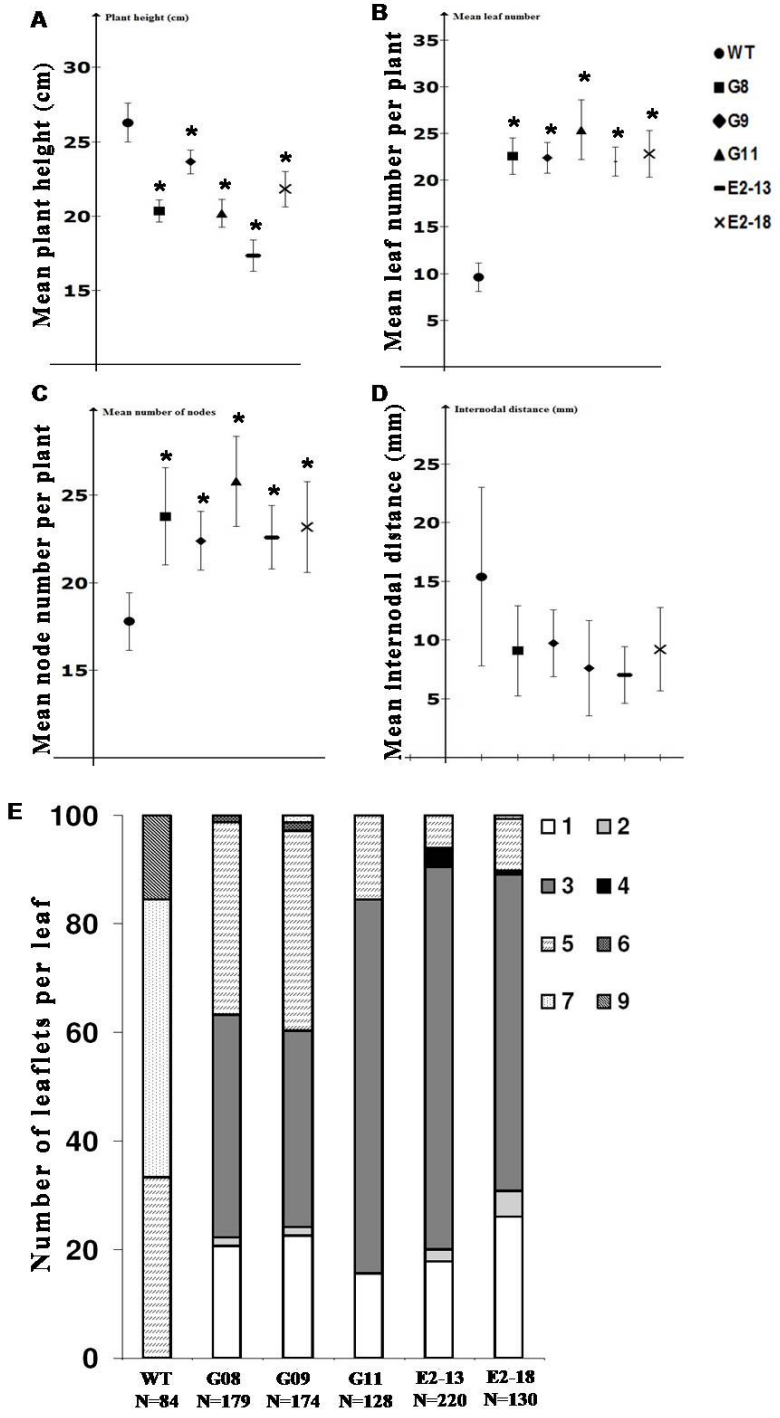


Fig. 4.5. POTH15 over-expression alters multiple morphological traits. Wild-type and POTH15 over-expression lines (G8, G9, G11, E2-13 and E2-18) were grown for ten weeks in LD conditions and plant height (A), number of leaves per plant (B), number of nodes per plant (C), and internodal distance (D) were measured for six individual plants per line and means +/- SD were plotted. Leaflet number per leaf was also determined for these plants (E).

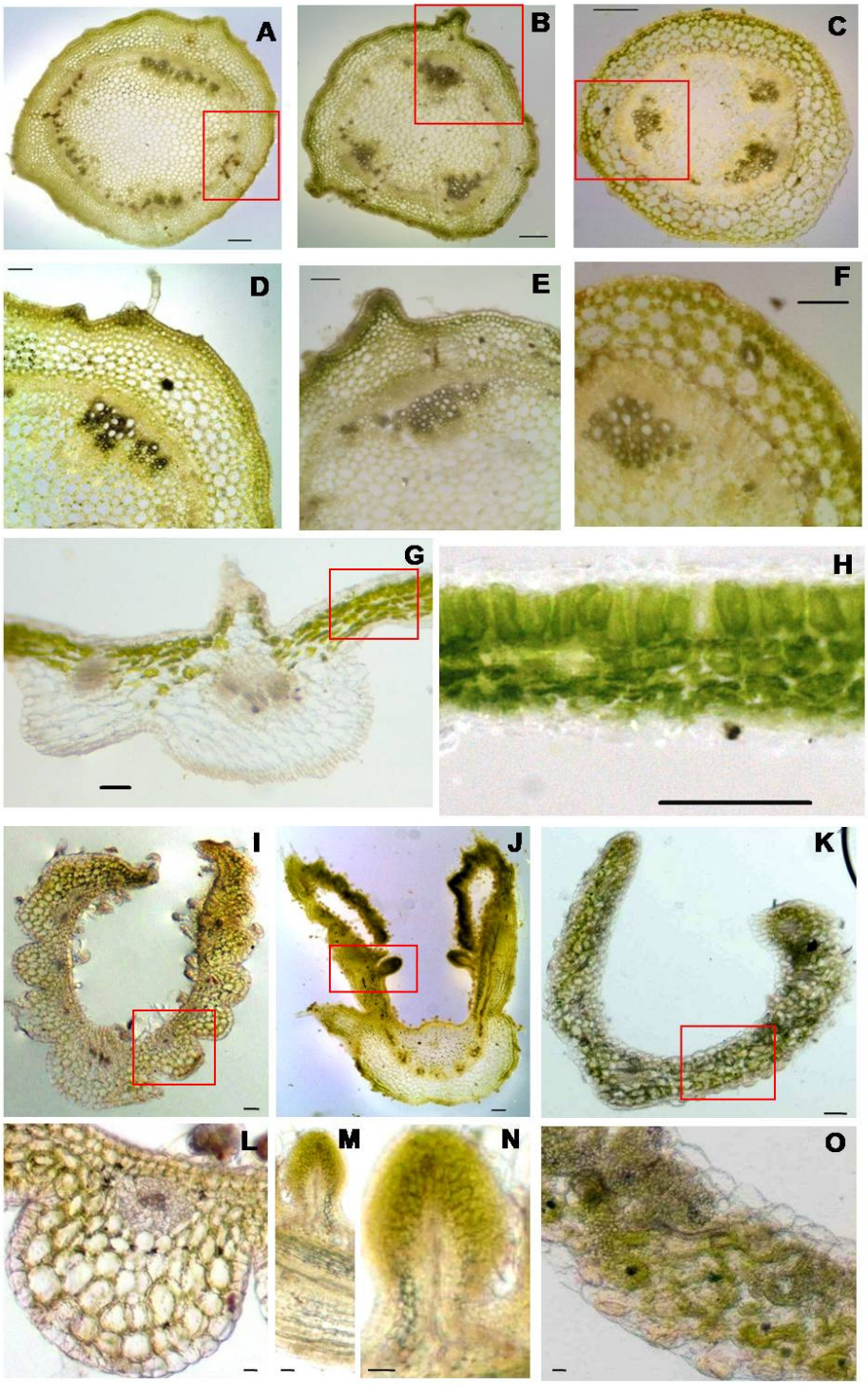


Fig. 4.6. POTH15 over-expression changes cell arrangements in stem and leaves. Cross sections of the stem from wild type (A and D where D is a magnified view of A) and POTH15 over-expression lines (B,C, E and F where E and F are magnified views of B and C respectively). Leaf cross sections of the wild type (G and H where H is a magnified view of G) and POTH15 over-expression lines (I to O where L, M+N and O are magnified view of I, J and K, respectively). Scale bars in A through K are 100 microns. Scale bars in L through O are 20 microns.

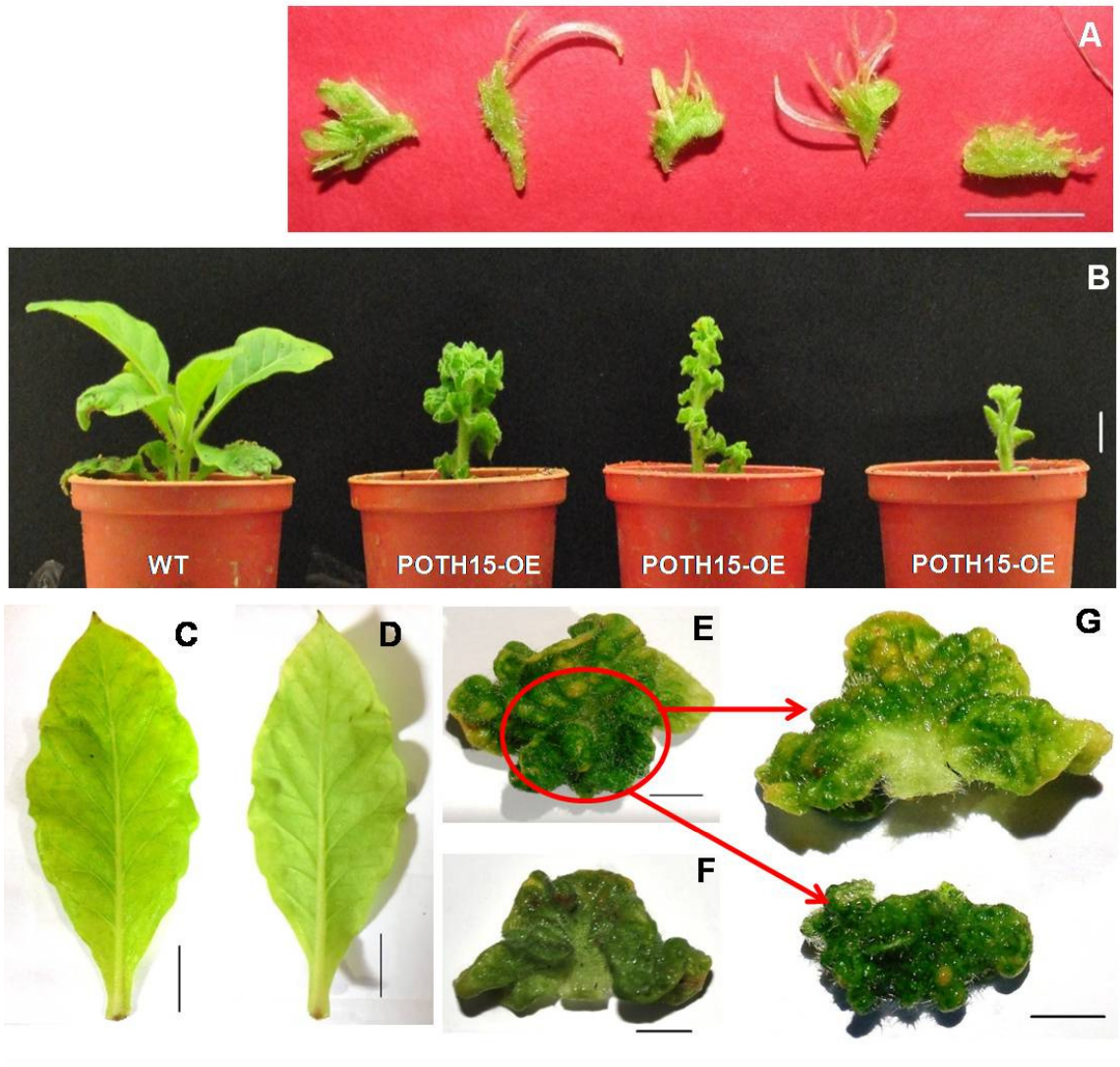


Fig. 4.7. POTH15 over-expression drastically changes the plant architecture in tobacco (*Nicotiana tabacum* var *Petite Havana*). Leaves of *in vitro* grown POTH15 over-expression lines of tobacco (A). Eight weeks old plants of wild-type (WT) and POTH15 over-expression (B) tobacco lines. The dorsal and ventral view of the leaves from wild-type (C and D) and POTH15 over-expression lines (E to G where G is a magnified view of E with two fused leaves separated). The mature leaves of POTH15 over-expression lines of tobacco showed leaf-on-leaf phenotype. The scale bars in A, B, C and D are 1 cm and scale bars in E, F and G are 5 mm.

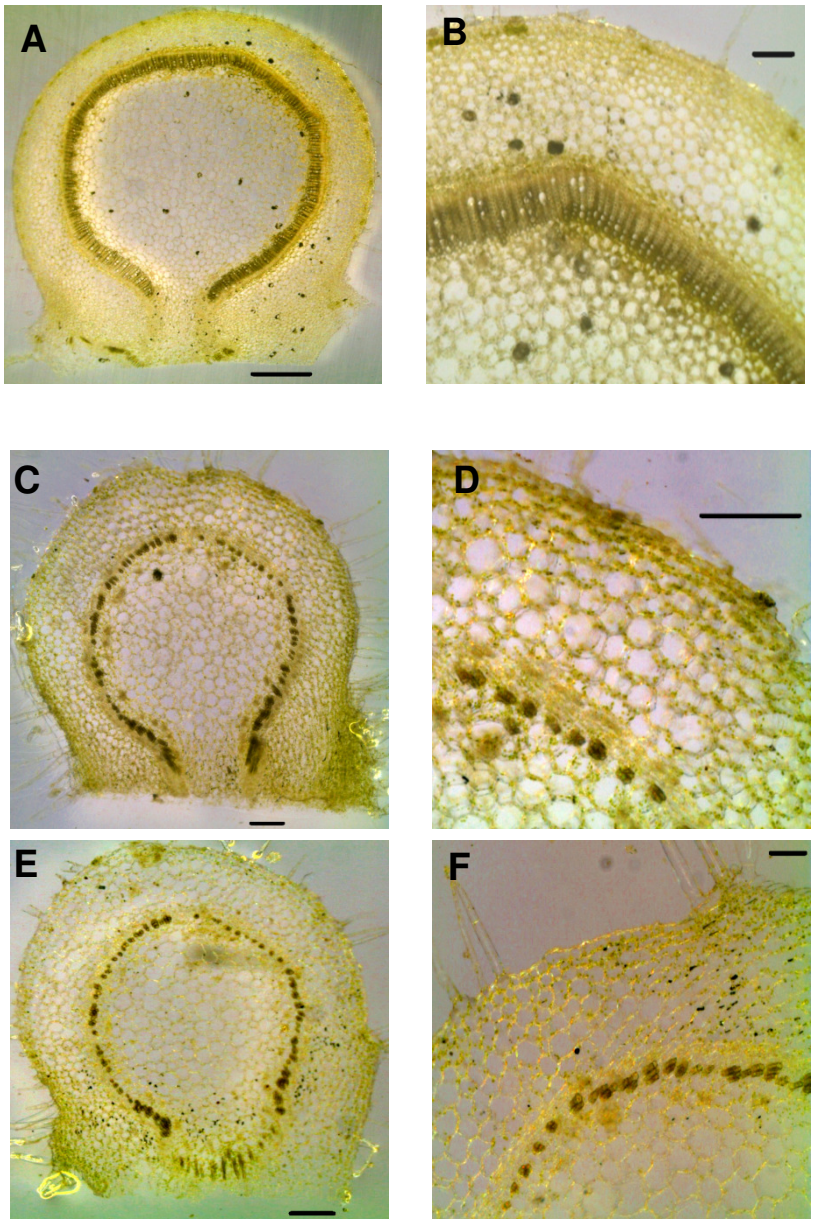


Fig. 4.8 POTH15 over-expression in tobacco changes cell arrangements in stem. Cross sections of stem of the wild-type (A) and POTH15 over-expression (C and E) lines of tobacco. B, D and F are magnified views of A, C and E, respectively. Scale bars in A, C, D and E are 50 microns, in B and F are 10 microns.

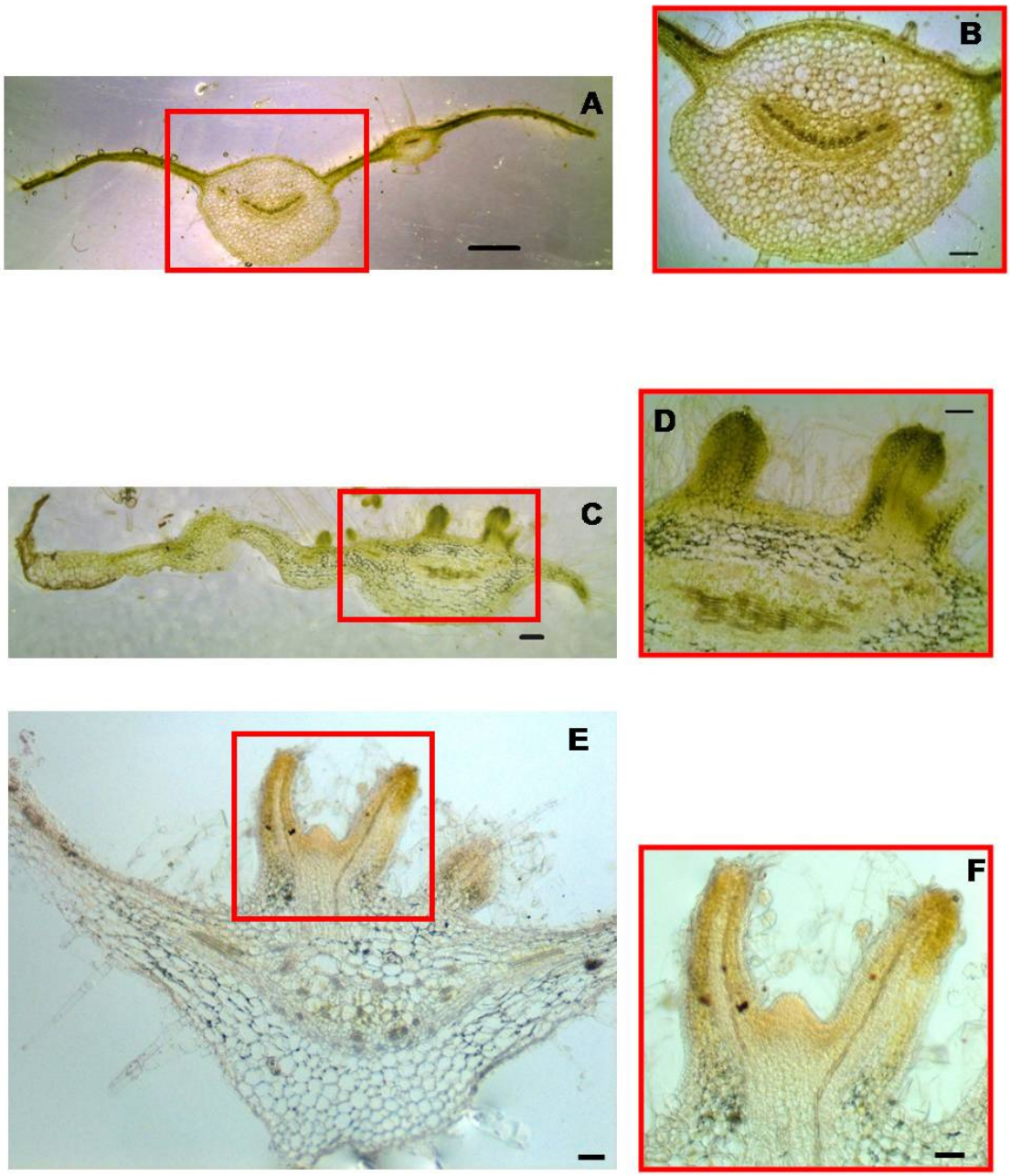


Fig. 4.9. POTH15 over-expression in tobacco changes cell arrangements in leaves. Cross sections of the leaves of the wild type (A) and POTH15 over-expression (C and E) lines of tobacco. B, D and F are magnified views of A, C and E, respectively. Meristem-like structures (D and F) were observed on POTH15 over-expression leaves.

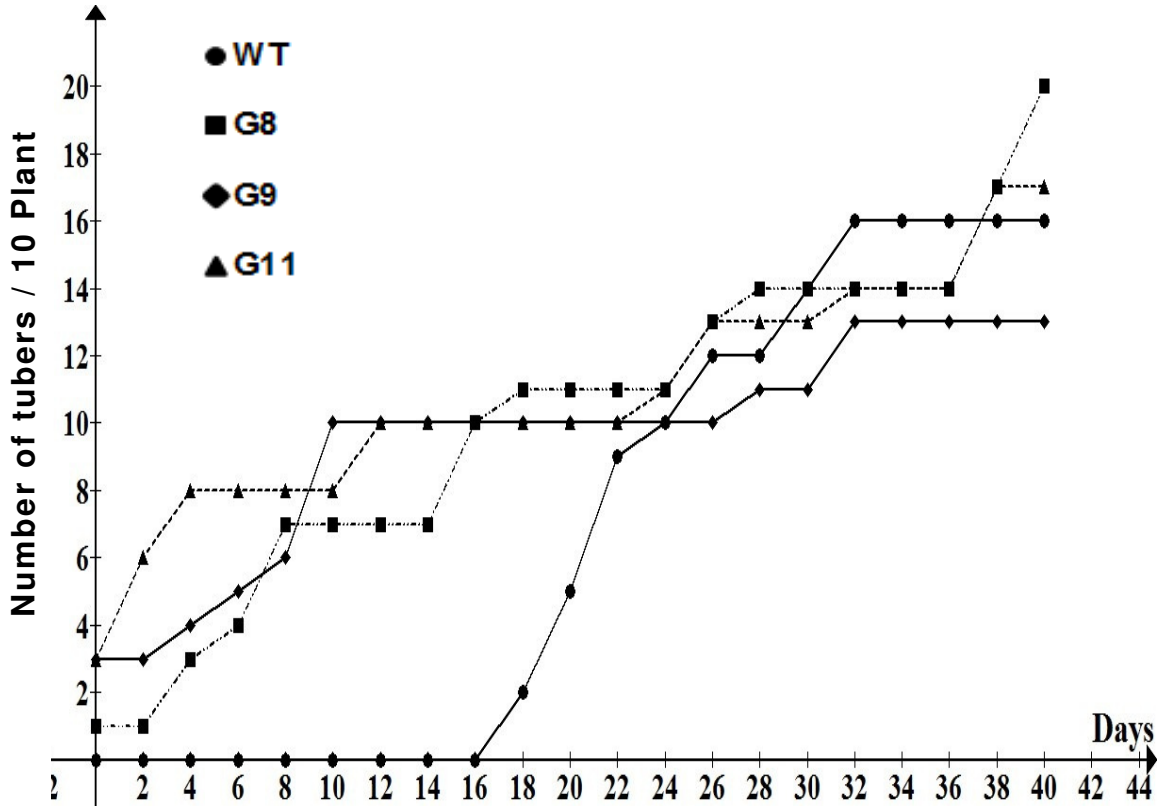
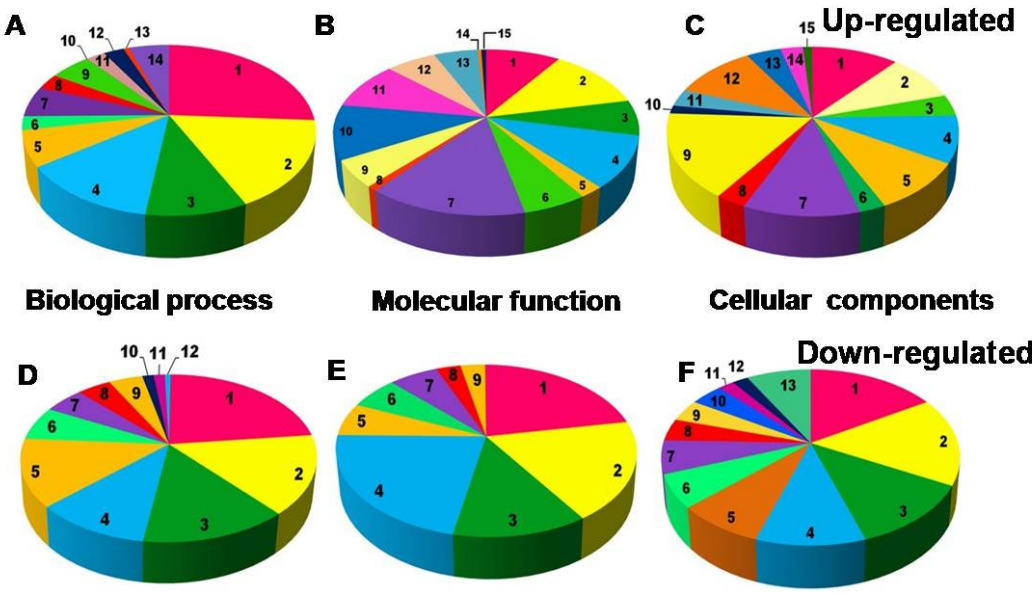


Fig. 4.10. *POTH15* over-expression accelerates *in vitro* tuberization. Rate of *in vitro* tuberization for POTH15 over-expression lines (G8, G9 and G11) and wild-type (WT) was measured by incubating the plantlets on MS medium containing 6% sucrose for 42 days under LD condition.



S.No	Biological Processes	A (%)	(D) %
1	Other cellular processes	26	23
2	Other metabolic processes	17	15
3	Response to abiotic or biotic stimulus	9.5	14
4	Response to stress	13	11
5	Other biological processes	6.7	13
6	Cell organization and biogenesis	2.6	6
7	Developmental processes	5.9	5
8	Protein metabolism	3.1	4
9	Transport	5.3	4
10	Electron transport or energy pathways	0.05	1.5
11	Transcription,DNA-dependent	2.5	1.5
12	Unknown biological processes	2.7	0.7
13	DNA or RNA metabolism	0.7	-
14	Signal transduction	5.3	-

S.No	Molecular Function	B (%)	E (%)
1	Hydrolase activity	9.4	22
2	Other binding	12	19
3	Protein binding	7	12.5
4	Unknown molecular functions	10	22
5	Other molecular functions	2.2	6.3
6	Transporter activity	5.7	6.3
7	Transferase activity	14	6.3
8	Nucleic acid binding	0.9	3.1
9	Transcription factor activity	5	3.1
10	Other enzyme activity	10.6	-
11	Kinase activity	9.4	-
12	Protein binding	6.3	-
13	Nucleotide binding	5.6	-
14	DNA or RNA binding	0.5	-
15	Receptor binding or activity	0.6	-
16	Structural molecule activity	-	-

S.No	Cellular Components	C (%)	F (%)
1	Other cytoplasmic components	11	16
2	Extracellular	9	16
3	Cell wall	4	12
4	Chloroplast	9	10
5	Other intracellular components	9.6	8
6	Plastid	2.5	6
7	Plasma membrane	11	6
8	Mitochondria	2.9	4
9	Nucleus	16	4
10	Other cellular components	1.6	4
11	Cytosol	3.3	2
12	Other membranes	11	2
13	Unknown cellular components	4.5	8
14	Golgi apparatus	2.9	-
15	ER	1.2	-
16	Ribosome	-	-

Fig. 4.11. Functional classification of genes differentially expressed between POTH15 over-expression and wild-type plants. Genes were classified by functional categories under following Gene Ontology (GO) terms - biological process (A and D), molecular function (B and E) and cellular components (C and F) and by up-regulated (A-C) and down-regulated (D-F) genes. The number of genes assigned to each functional category are represented as percentage.

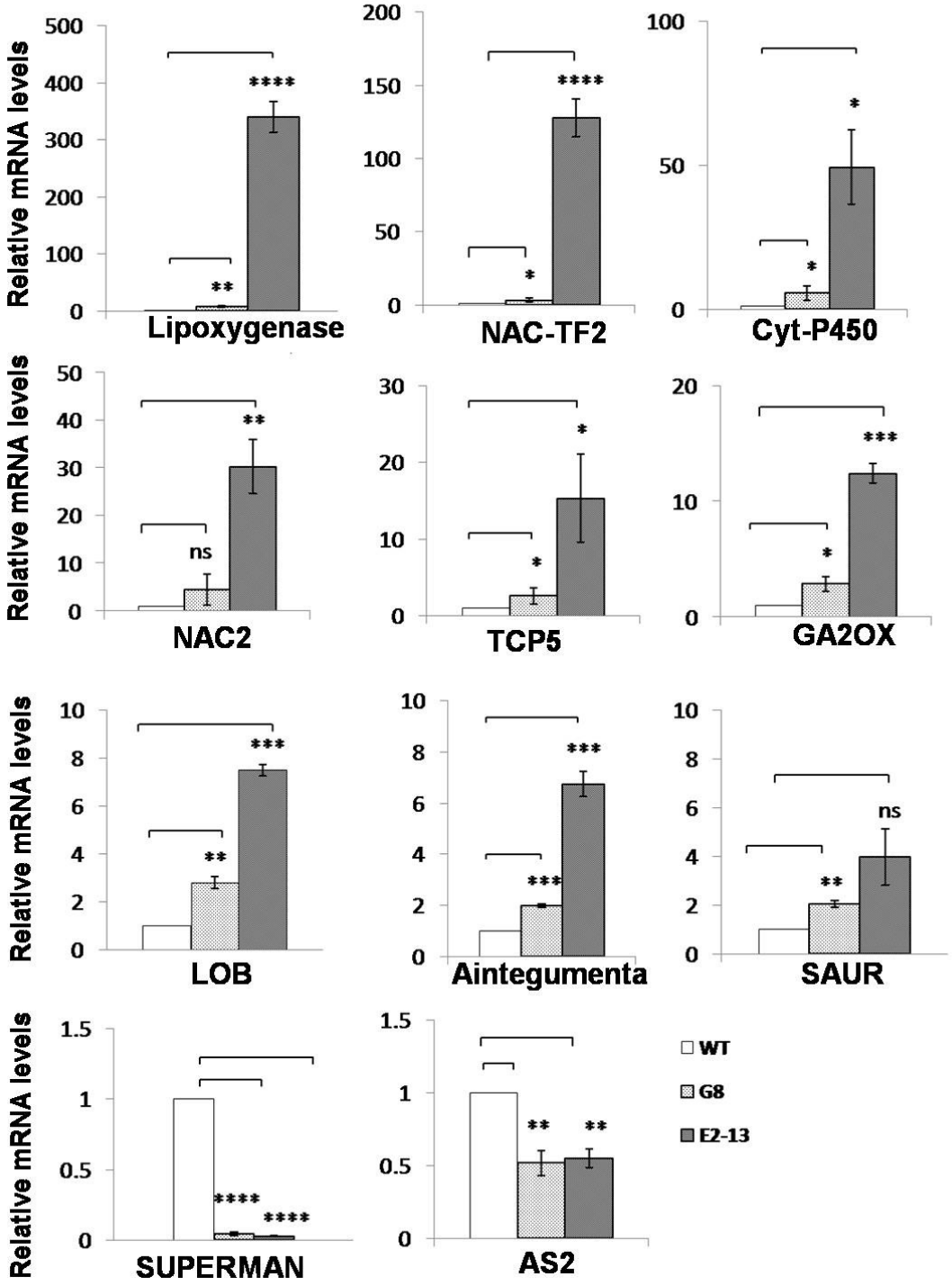


Fig. 4.12 Validation of POTH15 target genes by qRT-PCR. Selective candidate genes were chosen from RNA sequencing analysis for their validations. Relative mRNA levels for candidate genes in POTH15 over-expression lines (G8 and E2-13) is shown with respect to wild-type (WT). Data from three biological replicates for each gene were analysed by t-test separately for WT and G8, and also for WT and E2-13 lines. Asterisk * represents significant difference at P≤0.05, ** P≤0.01, *** at P≤0.001 or **** at P≤0.0001. ns=not significant (P>0.05).

Table 4.4: List of putative POTH15 target genes.

Common targets		
X_LOC ID	**Gene ID**	**Function**
XLOC_002470	PGSC0003DMT400051310	Plus agglutinin
XLOC_002603	PGSC0003DMT400029420	Epoxide hydrolase 1
XLOC_002727	PGSC0003DMT400023183	Xyloglucanase inhibitor
XLOC_002837	PGSC0003DMT400017574	Gene of unknown function
XLOC_003207	PGSC0003DMT400000043	Enoyl-CoA-hydratase
XLOC_003669	PGSC0003DMT400053410	Glycine/D-amino acid oxidases
XLOC_004015	Novel	
XLOC_004038	PGSC0003DMT400079481	Conserved gene of unknown function
XLOC_004219	PGSC0003DMT400051423	Oxidoreductase
XLOC_004534	Novel	
XLOC_005124	PGSC0003DMT400085237	Extensin class 1 protein
XLOC_005125	PGSC0003DMT400046319	Extensin class 1 protein
XLOC_006038	PGSC0003DMT400048873	Kinesin-3
XLOC_006293	PGSC0003DMT400076498	Nodulin
XLOC_006319	PGSC0003DMT400032698	Caspase
XLOC_006483	PGSC0003DMT400066669	Methylketone synthase Ib
XLOC_006486	PGSC0003DMT400066685	Methylketone synthase Ib
XLOC_006765	Novel	
XLOC_008004	Novel	
XLOC_008075	PGSC0003DMT400009365	Conserved gene of unknown function
XLOC_008262	PGSC0003DMT400055196	Transcription cofactor
XLOC_008355	Novel	
XLOC_008896	PGSC0003DMT400055257	NHL25 (NDR1/HIN1-LIKE 25)
XLOC_008971	Novel	
XLOC_009109	PGSC0003DMT400003000	ACI19
XLOC_009130	PGSC0003DMT400068332	Transcription factor
XLOC_009347	PGSC0003DMT400054399	Ethylene-responsive nuclear protein
XLOC_009529	PGSC0003DMT400081251	Phospholipase A1
XLOC_009871	Novel	
XLOC_009876	PGSC0003DMT400009069	Abscisic acid and environmental stress-inducible protein TAS14
XLOC_010438	Novel	
XLOC_010589	PGSC0003DMT400034915	Zinc finger protein
XLOC_010594	Novel	
XLOC_010629	PGSC0003DMT400012918	ARF GAP-like zinc finger-containing protein ZIGA3
XLOC_010855	PGSC0003DMT400048994	RRNA intron-encoded homing endonuclease
XLOC_011150	PGSC0003DMT400008751	Amino acid transporter
XLOC_011587	PGSC0003DMT400008365	SAUR family protein
XLOC_011718	PGSC0003DMT400079728	Sucrose synthase
XLOC_012531	PGSC0003DMT400011185	GDSL esterase/lipase
XLOC_012683	PGSC0003DMT400056024	Nonspecific lipid-transfer protein AKCS9
XLOC_013444	PGSC0003DMT400051443	Pectinacetylesterase
XLOC_013701	PGSC0003DMT400026293	Conserved gene of unknown function
XLOC_014074	PGSC0003DMT400051046	JA-induced WRKY protein
XLOC_014080	PGSC0003DMT400051072	Conserved gene of unknown function
XLOC_014664	PGSC0003DMT400083501	Sn-1 protein

Table 4.4: List of putative POTH15 target genes.

X_LOC ID	Gene ID	Function
XLOC_015969	PGSC0003DMT400056524	Laccase
XLOC_016389	PGSC0003DMT400009552	Carboxy-lyase
XLOC_016565	PGSC0003DMT400015287	Auxin-induced protein 5NG4
XLOC_017859	PGSC0003DMT400024517	Sulfate transporter
XLOC_017913	PGSC0003DMT400079239	Conserved gene of unknown function
XLOC_018265	PGSC0003DMT400025854	Cyclin B
XLOC_018396	Novel	
XLOC_018582	PGSC0003DMT400033749	Lipase
XLOC_018608	PGSC0003DMT400037720	Zinc finger protein
XLOC_018910	PGSC0003DMT400047882	Gene of unknown function
XLOC_019647	PGSC0003DMT400069792	ESC
XLOC_019914	PGSC0003DMT400050578	GMP synthase
XLOC_020106	PGSC0003DMT400047438	NAC domain protein NAC2
XLOC_020362	PGSC0003DMT400045024	Plant mitotic spindle assembly checkpoint protein mad2
XLOC_021117	PGSC0003DMT400034124	Pleiotropic drug resistance protein 1
XLOC_021139	Novel	
XLOC_021278	PGSC0003DMT400059946	Aspartic proteinase nepenthesin-1
XLOC_021661	PGSC0003DMT400077488	MLO1
XLOC_021895	PGSC0003DMT400072143	Serine/threonine-protein kinase CCR3
XLOC_022391	PGSC0003DMT400067224	Gene of unknown function
XLOC_023374	PGSC0003DMT400065334	Phosphatidylinositol transfer protein
XLOC_024008	PGSC0003DMT400050906	Cytochrome P450
XLOC_024010	PGSC0003DMT400050911	Sesquiterpene synthase 2
XLOC_024592	PGSC0003DMT400015111	Regulator of gene silencing
XLOC_025181	Novel	
XLOC_025848	Novel	
XLOC_025933	Novel	
XLOC_026178	Novel	
XLOC_026879	PGSC0003DMT400035807	Transcription factor
XLOC_027274	PGSC0003DMT400035264	Sucrose sythase
XLOC_027437	PGSC0003DMT400038867	Sesquiterpene synthase
XLOC_027543	PGSC0003DMT400082813	Serine-threonine protein kinase, plant-type
XLOC_027712	PGSC0003DMT400018295	Conserved gene of unknown function
XLOC_027974	Novel	
XLOC_028468	PGSC0003DMT400028158	Lipoxygenase
XLOC_028995	PGSC0003DMT400048698	F-box/kelch-repeat protein SKIP25
XLOC_029537	Novel	
XLOC_029834	PGSC0003DMT400077533	Conserved gene of unknown function
XLOC_030192	Novel	
XLOC_030526	PGSC0003DMT400004952	ABA 8'-hydroxylase CYP707A1
XLOC_030671	PGSC0003DMT400004660	Major pollen allergen Ory s 1
XLOC_030966	PGSC0003DMT400031571	NAC domain protein
XLOC_031034	Novel	
XLOC_031137	PGSC0003DMT400052384	Axi 1 protein from Nicotiana tabacum
XLOC_031428	Novel	
XLOC_031491	PGSC0003DMT400016625	Conserved gene of unknown function
XLOC_032289	Novel	

Table 4.4: List of putative POTH15 target genes.

X_LOC ID	Gene ID	Function
XLOC_032481	PGSC0003DMT400030271	Disease resistance-responsive family protein
XLOC_032540	PGSC0003DMT400080377	Purple acid phosphatase
XLOC_032544	PGSC0003DMT400091740	Conserved gene of unknown function
XLOC_032554	Novel	
XLOC_032714	PGSC0003DMT400021904	FAD binding domain containing protein
XLOC_033569	PGSC0003DMT400052292	Dopamine beta-monooxygenase
XLOC_033762	PGSC0003DMT400092910	Circadian clock coupling factor ZGT
XLOC_033778	Novel	
XLOC_033983	Novel	
XLOC_034011	PGSC0003DMT400081462	Conserved gene of unknown function
XLOC_034029	PGSC0003DMT400082079	Non-specific lipid-transfer protein
XLOC_034044	PGSC0003DMT400019863	Sulfate/bicarbonate/oxalate exchanger and transporter sat-1
XLOC_034122	PGSC0003DMT400044231	Rhicadhesin receptor
XLOC_034793	PGSC0003DMT400033417	Non-specific lipid-transfer protein
XLOC_035345	PGSC0003DMT400011342	Non-imprinted in Prader-Willi/Angelman syndrome region protein
XLOC_035507	Novel	
XLOC_035597	Novel	
XLOC_035820	PGSC0003DMT400061011	Gene of unknown function
XLOC_036179	PGSC0003DMT400034192	Ripening induced protein
XLOC_036261	PGSC0003DMT400049951	Conserved gene of unknown function
XLOC_036999	PGSC0003DMT400049398	Conserved gene of unknown function
XLOC_037209	PGSC0003DMT400072374	Transcription factor
XLOC_037342	PGSC0003DMT400076781	Gene of unknown function
XLOC_037380	PGSC0003DMT400021122	Conserved gene of unknown function
XLOC_037998	PGSC0003DMT400023891	Chorimate mutase
XLOC_038463	Novel	
XLOC_038713	PGSC0003DMT400020884	UDP-glucuronic acid/UDP-N-acetylgalactosamine transporter
XLOC_038898	PGSC0003DMT400040153	Ptpla domain protein
XLOC_039775	Novel	
XLOC_039827	PGSC0003DMT400082375	Conserved gene of unknown function
XLOC_039946	Novel	
XLOC_040037	PGSC0003DMT400020834	Fasciclin-like AGP 13
XLOC_040394	PGSC0003DMT400022096	Integral membrane single C2 domain protein
XLOC_040536	PGSC0003DMT400000801	Senescence-associated protein
XLOC_040719	PGSC0003DMT400071670	Gene of unknown function
XLOC_041026	PGSC0003DMT400056839	Conserved gene of unknown function
XLOC_041202	PGSC0003DMT400013766	Basic helix-loop-helix protein BHLH5
XLOC_041653	Novel	
XLOC_041725	PGSC0003DMT400011633	Gene of unknown function
XLOC_041881	Novel	
XLOC_041977	PGSC0003DMT400020405	Glycerol-3-phosphate transporter / glycerol 3-phosphate permease, putative
XLOC_042289	PGSC0003DMT400033427	Pirin
XLOC_042875	PGSC0003DMT400037469	Glucan endo-1,3-beta-glucosidase
XLOC_043210	PGSC0003DMT400075385	Conserved gene of unknown function
XLOC_043301	PGSC0003DMT400043447	Chalcone synthase J

Table 4.4: List of putative POTH15 target genes.

G8 Specific targets		
X_LOC ID	**Gene ID**	**Function**
XLOC_002471	PGSC0003DMT400051311	Extensin class 1 protein
XLOC_003227	PGSC0003DMT400000098	Conserved gene of unknown function
XLOC_003238	Novel	
XLOC_004277	Novel	
XLOC_004877	PGSC0003DMT400038769	Gene of unknown function
XLOC_005739	PGSC0003DMT400033191	ATP binding protein
XLOC_005965	Novel	
XLOC_006085	PGSC0003DMT400063738	Conserved gene of unknown function
XLOC_006347	PGSC0003DMT400083793	Periaxin
XLOC_006545	PGSC0003DMT400001917	EMB2219
XLOC_006706	Novel	
XLOC_007256	PGSC0003DMT400026994	Peptide methionine sulfoxide reductase msrA
XLOC_007900	PGSC0003DMT400043083	Homeobox protein knotted-1-like LET6
XLOC_008005	Novel	
XLOC_008114	PGSC0003DMT400009298	Gene of unknown function
XLOC_008177	PGSC0003DMT400067896	Neoxanthin synthase
XLOC_008204	PGSC0003DMT400078627	Methionyl-tRNA formyltransferase
XLOC_008545	PGSC0003DMT400032859	NOI
XLOC_008780	PGSC0003DMT400025396	Gene of unknown function
XLOC_009043	PGSC0003DMT400026937	BHLH transcription factor
XLOC_009285	PGSC0003DMT400026864	Replication factor A protein
XLOC_010197	Novel	
XLOC_010411	Novel	
XLOC_010602	PGSC0003DMT400068775	Conserved gene of unknown function
XLOC_010702	Novel	
XLOC_011230	PGSC0003DMT400036349	TMV induced protein 1-2
XLOC_011272	PGSC0003DMT400053116	MLO1
XLOC_011296	PGSC0003DMT400021511	Alpha-xylosidase
XLOC_011353	PGSC0003DMT400048507	Beta expansin 2
XLOC_011473	PGSC0003DMT400037083	Low-temperature-induced 65 kDa protein
XLOC_011604	PGSC0003DMT400001575	Cucumisin
XLOC_011636	PGSC0003DMT400008151	Conserved gene of unknown function
XLOC_011687	PGSC0003DMT400051118	Uncharacterized mitochondrial protein
XLOC_011739	PGSC0003DMT400026364	Miraculin
XLOC_011769	PGSC0003DMT400039492	Kunitz-type protease inhibitor
XLOC_011922	PGSC0003DMT400062840	Axonemal dynein light chain
XLOC_012026	PGSC0003DMT400063081	Basic 7S globulin 2 small subunit
XLOC_012100	PGSC0003DMT400001652	Conserved gene of unknown function
XLOC_012218	PGSC0003DMT400014741	RAB11F
XLOC_012390	PGSC0003DMT400058973	Conserved gene of unknown function
XLOC_012573	PGSC0003DMT400013025	Gaba(A) receptor-associated protein
XLOC_012726	PGSC0003DMT400038502	Gene of unknown function
XLOC_012819	PGSC0003DMT400096705	Conserved gene of unknown function
XLOC_013346	PGSC0003DMT400059235	Conserved gene of unknown function
XLOC_013377	PGSC0003DMT400062320	MADS-box transcription factor FBP22
XLOC_013386	PGSC0003DMT400037108	Conserved gene of unknown function
X_LOC ID	**Gene ID**	**Function**

Table 4.4: List of putative POTH15 target genes.

XLOC_013728	PGSC0003DMT400039353	Miraculin
XLOC_014165	PGSC0003DMT400036981	Acyl carrier protein
XLOC_014496	Novel	
XLOC_014621	PGSC0003DMT400041040	NBS-LRR protein
XLOC_014660	PGSC0003DMT400015347	Conserved gene of unknown function
XLOC_014797	PGSC0003DMT400082464	Transcription factor NF-Y CCAAT-binding
XLOC_015816	Novel	
XLOC_015881	PGSC0003DMT400002036	Histidine-rich glycoprotein
XLOC_015887	PGSC0003DMT400002054	Extensin
XLOC_016124	Novel	
XLOC_016235	PGSC0003DMT400020526	N-hydroxycinnamoyl/benzoyltransferase 6
XLOC_016252	PGSC0003DMT400020576	Glutamate-gated kainate-type ion channel receptor subunit GluR5
XLOC_016454	PGSC0003DMT400025674	Conserved gene of unknown function
XLOC_017633	PGSC0003DMT400054060	Gene of unknown function
XLOC_017711	PGSC0003DMT400063852	Nodulin MtN3 family protein
XLOC_017993	PGSC0003DMT400012617	Serine esterase family protein
XLOC_018060	PGSC0003DMT400020647	L-asparaginase
XLOC_018333	Novel	
XLOC_018724	PGSC0003DMT400065796	Myb-like DNA-binding protein
XLOC_019668	PGSC0003DMT400069841	R2R3 transcription factor MYB108 1
XLOC_020365	PGSC0003DMT400045028	Ketoacyl-ACP Reductase (KAR)
XLOC_020978	PGSC0003DMT400037826	Ethylene response factor
XLOC_021672	PGSC0003DMT400062174	Major intrinsic protein 2
XLOC_021700	PGSC0003DMT400036420	Xyloglucan endotransglucosylase-hydrolase XTH6
XLOC_022307	PGSC0003DMT400030994	Membrane channel protein
XLOC_022329	Novel	
XLOC_022986	PGSC0003DMT400078006	17.6 kD class I small heat shock protein
XLOC_023928	PGSC0003DMT400042155	Histidine-rich glycoprotein
XLOC_024372	PGSC0003DMT400074437	Phosphate abc transporter
XLOC_024425	PGSC0003DMT400083083	Nam 18
XLOC_024672	PGSC0003DMT400019410	LOB domain-containing protein
XLOC_025152	PGSC0003DMT400078980	Conserved gene of unknown function
XLOC_025313	PGSC0003DMT400077035	NBS-coding resistance protein
XLOC_025745	PGSC0003DMT400033858	24K germin
XLOC_026108	PGSC0003DMT400024275	Conserved gene of unknown function
XLOC_026218	PGSC0003DMT400071094	Gibberellin 2-oxidase
XLOC_026316	PGSC0003DMT400049666	Jasmonic acid 2
XLOC_026705	PGSC0003DMT400079424	Dicyanin
XLOC_026730	PGSC0003DMT400054213	Conserved gene of unknown function
XLOC_027917	PGSC0003DMT400056943	Gene of unknown function
XLOC_027929	PGSC0003DMT400056916	Salicylic acid-induced protein 19
XLOC_028234	PGSC0003DMT400014796	Calmodulin binding protein
XLOC_028273	PGSC0003DMT400014872	NAC domain protein
XLOC_028346	PGSC0003DMT400077567	Avr9/Cf-9 rapidly elicited protein 180
XLOC_028356	PGSC0003DMT400024968	K+ channel inward rectifying
XLOC_028531	PGSC0003DMT400040357	F-box domain containing protein

Table 4.4: List of putative POTH15 target genes.

X_LOC ID	Gene ID	Function
XLOC_028698	PGSC0003DMT400021142	Class II small heat shock protein Le-HSP17.6
XLOC_029013	PGSC0003DMT400040626	Receptor serine-threonine protein kinase
XLOC_029084	PGSC0003DMT400024109	Receptor protein kinase
XLOC_029218	PGSC0003DMT400012303	Oxidoreductase
XLOC_029396	Novel	
XLOC_029475	PGSC0003DMT400031928	Zn finger protein
XLOC_029749	PGSC0003DMT400015021	Gene of unknown function
XLOC_030346	Novel	
XLOC_030827	PGSC0003DMT400007905	Osmotin
XLOC_030903	PGSC0003DMT400031717	Na(+)/H(+) antiporter
XLOC_031130	PGSC0003DMT400052365	Kinase
XLOC_031185	PGSC0003DMT400022781	Osmotic avoidance abnormal protein
XLOC_031452	PGSC0003DMT400030779	Conserved gene of unknown function
XLOC_031456	PGSC0003DMT400065943	ATP binding protein
XLOC_031916	Novel	
XLOC_032482	PGSC0003DMT400030272	Transferase, transferring glycosyl groups
XLOC_032632	PGSC0003DMT400042877	PAKRP1L
XLOC_032770	Novel	
XLOC_032912	PGSC0003DMT400093315	ATP binding protein
XLOC_033003	PGSC0003DMT400053881	Plasma intrinsic protein 2,1
XLOC_033093	PGSC0003DMT400011361	Conserved gene of unknown function
XLOC_033465	PGSC0003DMT400045117	Conserved gene of unknown function
XLOC_033976	PGSC0003DMT400081388	Pectinesterase
XLOC_034065	PGSC0003DMT400089589	Autoinhibited calcium ATPase
XLOC_034509	PGSC0003DMT400037320	Glycosyltransferase, CAZy family GT8
XLOC_034698	PGSC0003DMT400049895	DNA binding protein
XLOC_035600	PGSC0003DMT400028828	Oxidoreductase
XLOC_035955	PGSC0003DMT400018497	P-coumaroyl quinate/shikimate 3'-hydroxylase
XLOC_036092	Novel	Novel
XLOC_037277	PGSC0003DMT400072217	Nodulin
XLOC_037937	PGSC0003DMT400010195	Citrate binding protein
XLOC_038884	PGSC0003DMT400040125	Serine-threonine protein kinase, plant-type
XLOC_039274	Novel	
XLOC_039732	PGSC0003DMT400059031	Aspartate aminotransferase
XLOC_039987	PGSC0003DMT400019126	Acyl CoA reductase
XLOC_040274	Novel	
XLOC_040646	PGSC0003DMT400022541	Conserved gene of unknown function
XLOC_041998	PGSC0003DMT400020450	Actin binding protein
XLOC_043021	PGSC0003DMT400074224	Cell division control protein
XLOC_043139	PGSC0003DMT400030203	RCG54054
XLOC_043315	PGSC0003DMT400043424	Membrane protein
E2-13 specific targets		
X_LOC ID	**Gene ID**	**Function**
XLOC_001553	PGSC0003DMT400058621	CMPG1b
XLOC_001660	PGSC0003DMT400055049	U-box protein
XLOC_001767	PGSC0003DMT400097155	F-box family protein

Table 4.4: List of putative POTH15 target genes.

X_LOC ID	Gene ID	Function
XLOC_002756	PGSC0003DMT400023246	Long cell-linked locus protein
XLOC_003049	PGSC0003DMT400004254	Rac gtpase
XLOC_003175	PGSC0003DMT400071403	Conserved gene of unknown function
XLOC_003483	PGSC0003DMT400073563	Verticillium wilt disease resistance protein
XLOC_003638	Novel	
XLOC_003708	Novel	
XLOC_003977	PGSC0003DMT400066753	Conserved gene of unknown function
XLOC_004151	PGSC0003DMT400048002	Amino acid transporter
XLOC_004229	PGSC0003DMT400025186	Conserved gene of unknown function
XLOC_004320	PGSC0003DMT400054949	U-box protein
XLOC_004383	PGSC0003DMT400080854	Conserved gene of unknown function
XLOC_004428	PGSC0003DMT400022478	Hsp20/alpha crystallin family protein
XLOC_004878	Novel	
XLOC_005273	PGSC0003DMT400058335	Zinc finger family protein
XLOC_005346	PGSC0003DMT400011129	Plasma membrane associated protein
XLOC_006095	PGSC0003DMT400063765	Exonuclease
XLOC_006264	PGSC0003DMT400061044	PAP fibrillin
XLOC_006327	PGSC0003DMT400032669	White-brown-complex ABC transporter family
XLOC_006400	PGSC0003DMT400066508	Conserved gene of unknown function
XLOC_006432	PGSC0003DMT400066559	C2 domain-containing protein
XLOC_006606	PGSC0003DMT400004046	Snakin-2
XLOC_006704	Novel	
XLOC_006835	Novel	
XLOC_007052	Novel	
XLOC_007280	PGSC0003DMT400033890	Calcium-transporting ATPase 2, plasma membrane-type
XLOC_007391	PGSC0003DMT400017780	Gene of unknown function
XLOC_007740	PGSC0003DMT400076444	DNA binding protein
XLOC_007782	PGSC0003DMT400057819	Conserved gene of unknown function
XLOC_008313	PGSC0003DMT400010272	Triacylglycerol lipase
XLOC_008322	PGSC0003DMT400010296	AP2/ERF domain-containing transcription factor
XLOC_008380	PGSC0003DMT400026047	Transferase, transferring glycosyl groups
XLOC_008395	PGSC0003DMT400003740	Amino acid transporter
XLOC_008562	PGSC0003DMT400052233	Polcalcin Jun o
XLOC_009015	PGSC0003DMT400011464	Homeobox protein
XLOC_009524	PGSC0003DMT400027849	ERF transcription factor 4
XLOC_009568	PGSC0003DMT400076465	Lectin-receptor kinase 3
XLOC_009628	PGSC0003DMT400057523	FAD-linked oxidoreductase 1
XLOC_009981	PGSC0003DMT400078766	Conserved gene of unknown function
XLOC_010083	PGSC0003DMT400010342	ATP binding protein
XLOC_010434	Novel	
XLOC_010458	Novel	
XLOC_010477	Novel	
XLOC_010480	Novel	
XLOC_010491	Novel	
XLOC_010509	Novel	
XLOC_010663	PGSC0003DMT400048102	Conserved gene of unknown function
XLOC_011192	PGSC0003DMT400047469	Gene of unknown function

Table 4.4: List of putative POTH15 target genes.

X_LOC ID	Gene ID	Function
XLOC_011279	Novel	
XLOC_011549	PGSC0003DMT400023448	Receptor serine/threonine kinase
XLOC_011736	PGSC0003DMT400026369	Avr9/Cf-9 rapidly elicited protein 140
XLOC_011796	PGSC0003DMT400039412	Cytochrome P450
XLOC_012403	PGSC0003DMT400058933	Lipoxygenase
XLOC_012508	PGSC0003DMT400071509	Aquaporin NIP1.1
XLOC_012675	PGSC0003DMT400062634	Gamma-glutamyl-gamma-aminobutyrate hydrolase
XLOC_013714	PGSC0003DMT400026272	Cysteine protease inhibitor 1
XLOC_014205	PGSC0003DMT400014397	Calmodulin-binding protein
XLOC_014310	PGSC0003DMT400006485	Downstream target of agl15-4
XLOC_014430	Novel	
XLOC_014442	Novel	
XLOC_014491	Novel	
XLOC_014638	Novel	
XLOC_014648	Novel	
XLOC_014715	PGSC0003DMT400075839	Defensin J1-2
XLOC_015689	PGSC0003DMT400071761	Ascorbate oxidase
XLOC_016019	PGSC0003DMT400024511	Trehalose-6-phosphate synthase
XLOC_016062	PGSC0003DMT400079158	Phi-1 protein
XLOC_016496	PGSC0003DMT400007419	UPF0497 membrane protein 1
XLOC_016554	PGSC0003DMT400015323	Wall-associated kinase
XLOC_016599	PGSC0003DMT400075778	Avr9/Cf-9 rapidly elicited protein 74
XLOC_016706	PGSC0003DMT400065446	Gene of unknown function
XLOC_017017	PGSC0003DMT400008416	Conserved gene of unknown function
XLOC_017896	PGSC0003DMT400079206	Phi-1 protein
XLOC_018064	PGSC0003DMT400020769	Gene of unknown function
XLOC_018395	Novel	
XLOC_020429	PGSC0003DMT400056352	WRKY-type DNA binding protein
XLOC_020605	PGSC0003DMT400005987	Avr9/Cf-9 induced kinase 1
XLOC_020895	PGSC0003DMT400036326	Gene of unknown function
XLOC_021407	PGSC0003DMT400037957	Conserved gene of unknown function
XLOC_021614	PGSC0003DMT400077273	MAP kinase
XLOC_021407	PGSC0003DMT400037957	Conserved gene of unknown function
XLOC_022034	PGSC0003DMT400070942	Gene of unknown function
XLOC_022525	PGSC0003DMT400042528	Stem 28 kDa glycoprotein
XLOC_022555	PGSC0003DMT400042663	Gene of unknown function
XLOC_022693	PGSC0003DMT400083142	Conserved gene of unknown function
XLOC_022811	PGSC0003DMT400069515	UP-9A
XLOC_022945	PGSC0003DMT400077934	ZPT2-12
XLOC_023140	PGSC0003DMT400061736	Histone H2A
XLOC_024016	PGSC0003DMT400080724	Neryl diphosphate synthase 1
XLOC_024078	PGSC0003DMT400062084	Phospholipase A1
XLOC_024334	PGSC0003DMT400073388	Conserved gene of unknown function
XLOC_024413	PGSC0003DMT400010449	Conserved gene of unknown function
XLOC_024661	Novel	
XLOC_024696	PGSC0003DMT400078148	Conserved gene of unknown function
XLOC_024720	PGSC0003DMT400078312	Gene of unknown function

Table 4.4: List of putative POTH15 target genes.

X_LOC ID	Gene ID	Function
XLOC_025055	PGSC0003DMT400028958	Cellulose synthase 3
XLOC_025119	PGSC0003DMT400091248	Gene of unknown function
XLOC_025706	PGSC0003DMT400082749	Calmodulin-binding protein
XLOC_026153	PGSC0003DMT400044522	Receptor-like kinase
XLOC_027242	PGSC0003DMT400033834	OBP3-responsive gene 4
XLOC_027485	PGSC0003DMT400067274	Ccd1
XLOC_028028	Novel	
XLOC_028113	Novel	
XLOC_028325	PGSC0003DMT400064933	6,7-dimethyl-8-ribityllumazine synthase
XLOC_028345	PGSC0003DMT400077568	Avr9/Cf-9 rapidly elicited protein 180
XLOC_028389	Novel	
XLOC_028846	PGSC0003DMT400019058	MRNA, 1346 bp sequence
XLOC_028935	PGSC0003DMT400074719	Aromatic amino acid decarboxylase 1B
XLOC_029186	PGSC0003DMT400067458	Major pollen allergen Ory s 1
XLOC_029386	PGSC0003DMT400032105	SP1L
XLOC_029472	PGSC0003DMT400031935	Trehalase
XLOC_029721	PGSC0003DMT400014950	WRKY transcription factor-30
XLOC_029729	PGSC0003DMT400014976	B2 protein
XLOC_030265	PGSC0003DMT400035924	Vacoular processing enzyme 1
XLOC_030348	PGSC0003DMT400019032	Ubiquitin-protein ligase
XLOC_030420	PGSC0003DMT400074709	Aromatic amino acid decarboxylase 1B
XLOC_030423	PGSC0003DMT400038293	Tyramine hydroxycinnamoyl transferase
XLOC_030595	PGSC0003DMT400024052	Calcium-dependent protein kinase substrate protein
XLOC_030652	PGSC0003DMT400045148	Transcriptional regulator SUPERMAN
XLOC_031153	PGSC0003DMT400005560	Glutathione S-transferase
XLOC_031349	PGSC0003DMT400055525	Cytokinin riboside 5'-monophosphate phosphoribohydrolase LOG3
XLOC_031426	PGSC0003DMT400077751	Transcription factor
XLOC_032213	PGSC0003DMT400009749	Ankyrin repeat-containing protein
XLOC_032585	Novel	
XLOC_033981	PGSC0003DMT400081405	Heat shock protein binding protein
XLOC_034075	PGSC0003DMT400015630	Restricted tev movement 1
XLOC_034250	PGSC0003DMT400067977	Tir-nbs-lrr resistance protein
XLOC_034606	PGSC0003DMT400056253	Glutaredoxin
XLOC_034903	PGSC0003DMT400016636	Receptor-kinase
XLOC_035158	PGSC0003DMT400061975	Xyloglucan endotransglycosylase
XLOC_035279	PGSC0003DMT400021274	Wound-responsive AP2 like factor 2
XLOC_035318	PGSC0003DMT400043534	Gene of unknown function
XLOC_035604	PGSC0003DMT400028812	Zinc finger family protein
XLOC_035780	PGSC0003DMT400072481	VQ
XLOC_035996	PGSC0003DMT400029256	F-box family protein
XLOC_036195	PGSC0003DMT400068470	Conserved gene of unknown function
XLOC_036230	PGSC0003DMT400048612	Zinc finger protein
XLOC_036801	PGSC0003DMT400021314	Wound-responsive AP2 like factor 2
XLOC_037005	PGSC0003DMT400049412	Conserved gene of unknown function
XLOC_037333	PGSC0003DMT400061000	Glycosyltransferase
XLOC_037494	Novel	

Table 4.4: List of putative POTH15 target genes.

X_LOC ID	Gene ID	Function
XLOC_037553	PGSC0003DMT400034766	Conserved gene of unknown function
XLOC_037706	PGSC0003DMT400041671	Serine/threonine-protein kinase SAPK1
XLOC_037957	PGSC0003DMT400015528	Prolyl 4-hydroxylase alpha subunit
XLOC_038753	PGSC0003DMT400050688	Gene of unknown function
XLOC_038890	PGSC0003DMT400090011	ZPT2-9
XLOC_038953	PGSC0003DMT400034517	EH-domain-containing protein 1
XLOC_039344	PGSC0003DMT400084332	F-box family protein
XLOC_039806	Novel	
XLOC_040007	PGSC0003DMT400067708	NAC-domain protein
XLOC_040108	PGSC0003DMT400090967	Regulator of gene silencing
XLOC_040109	PGSC0003DMT400096032	Regulator of gene silencing
XLOC_040111	PGSC0003DMT400007736	Regulator of gene silencing
XLOC_040439	PGSC0003DMT400020229	Stress induced protein
XLOC_040479	PGSC0003DMT400007479	Serine-threonine protein kinase, plant-type
XLOC_040559	Novel	
XLOC_041724	PGSC0003DMT400011631	Gene of unknown function
XLOC_042077	PGSC0003DMT400000882	Calmodulin binding protein
XLOC_043064	PGSC0003DMT400010859	Conserved gene of unknown function
XLOC_043411	PGSC0003DMT400014122	Hcr2-0A

SUMMARY

KNOX genes are ubiquitous to green plants and they are known to play vital role in plant development and patterning. *KNOX* genes belong to a Three Amino acid Loop Extension (TALE) superclass of transcription factors (Burglin et al., 1997). KNOX TFs form a dimer with another family of TFs from TALE superclass, viz. The BELL-TFs and a complex network of KNOX-BELL heterodimers regulate the target genes in a tissue specific manner (Bellaoui et al., 2001; Smith et al., 2002; Hay and Tsiantis, 2010). In lower plants, *KNOX* genes regulate gamete identity and zygotic development (Lee et al., 2008; Sakakibara et al., 2013). In angiosperms, *KNOX-I* genes are shown to regulate diverse developmental programs such as meristem maintenance, leaf development, floral development, tuberization and so on (Hay and Tsiantis, 2010; Ragni et al, 2007; Uchida et al, 2010). In spite of an important role KNOX genes play in plant development, only a handful of KNOX target genes are identified so far (Giacomo et al., 2013). To have a deeper understanding about how the KNOX genes function, it is crucial to identify their targets from various plant species. Another important research question is how KNOX gene expression is regulated? Our knowledge about regulation of KNOX gene expression is limited to a few studies, which have shown that KNOX gene expression is repressed outside its expression domain by different epigenetic regulators (Hay and Tsiantis, 2010). It would be interesting to understand how KNOX gene expression is regulated across different developmental pathways to generate diverse plant forms.

To investigate the KNOX gene function, the model system chosen in the current study is potato (*Solanum tuberosum*). The focus of this study is to understand the role of *KNOX* genes in potato development and tuberization. Previous to the present study, only one potato *KNOX* gene (*Potato Homeobox1*, *POTH1*) was studied and it was demonstrated to regulate vegetative development in potato by modulating GA and cytokinin levels (Rosin et al., 2003; Chen et al., 2004). Notably, the *POTH1* transcript was found to be present in phloem sap and phloem cells of potato (Yu et al., 2007; Campbell et al., 2008). Other than the mentioned studies, there were no reports of *KNOX* gene functions from potato. Hence, the objectives of the current study were:

1. To carry out a thorough literature survey on *KNOX* genes in plants
2. To identify, validate and obtain full-length sequence for potato KNOX genes

3. To study the regulation of POTH1 expression and to investigate the mobility of *POTH1* mRNA
4. To understand the regulation, over-expression and to identify the target genes of *POTATO HOMEOBOX15 (POTH15).*

Chapter 1: Introduction

A through literature survey was carried out on KNOX genes discovery, regulation and their functions across plant species. Although, there are numerous studies carried out of KNOX genes in plants, for the purpose of the present study, a short list of 70 reports has been provided as a table (Table 1.1). This literature survey suggested that role of KNOX genes in regulation of tuberization as well as other developmental pathways in potato is not clearly understood.

Chapter 2: *KNOX* genes in potato (*Solanum tuberosum* ssp *Andigena*)

Previous to current study only one potato *KNOX* gene, *POTH1*, was identified from potato (Rosin et al., 2012). To explore the existence and functions of additional KNOX genes in potato, the Potato Genome Sequence Database was mined. In this part of the work, experiments were carried out to identify, validate and clone potato *KNOX*genes.

The results of these experiments are summarized as follows:

(i) Six novel KNOX genes from potato genome were identified, validated and cloned. Thus including POTH1, the number of KNOX genes identified from potato is now seven.

(ii) The sequences for all these genes have been deposited to NCBI and their accession numbers are *POTH20* (KP335125), *POTH15* (KJ477687), *StPetroselinum* (KJ477688), *StKn1* (KJ477691), *StHOX1* (KJ477689), and *StHox2* (KJ477690).

(iii) Further, a phylogenetic analysis of potato KNOX proteins along with tomato and *Arabidopsis* KNOX proteins revealed that four of them belong to Class-I (*POTH1, POTH15, POTH20,* and *StKn1*), two belongs to Class-II (*StHox1* and *StHox2*) and one (*StPTS*) is a mini-KNOX.

Considering the previous reports and the importance of KNOX genes in potato, we choose to continue our work with POTH1 and POTH15, which are summarized in Chapter 3 and 4.

Chapter 3: POTH1: Studying the regulation and transcript mobility

Before the present study,POTH1 was shown to regulate vegetative development in potato and its transcript was found to be present in phloem. In this part of the work, experiments were conducted to understand the regulation of POTH1 expression and to investigate the mobility of POTH1 transcript across graft-unions. The findings obtained based on these experiments are summarized as follows:

(i) The promoter of *POTH1* is rich in light regulatory motifs and the *POTH1* expression is affected by light.

(ii) Micrografts and heterografts with soil-grown plants confirmed that the mRNA of *POTH1* is graft transmissible and moves in a rootward direction.

(iii) *POTH1-UTRs* have repressing effect on the translation of marker gene *GUS* in tobacco protoplasts.

(iv) Polypyrimidine tracts as well as putative binding sites for an alba-domain protein were identified in both the 5´ and 3´ UTRs of the *POTH1* transcript.

(v) Protein/RNA binding assays confirmed interaction of both UTRs with the alba-domain protein and of the 3´ UTR with a polypyrimidine tract-binding protein.

Collectively, these results suggest that the full-length mRNA of *POTH1* is functional in long-distance trafficking in potato. These findings are published as a paper (Mahajan et al. 2012)

Chapter 4: POTH15: Regulation, over-expression and target gene identification.

POTATO HOMEOBOX 15 (*POTH15*) is one of the *KNOX*-I gene that has been identified and cloned from potato genome. To comprehend the role of POTH15 in potato

development, experiments pertaining to regulation, over-expression and target gene identification of POTH15 were conducted. The findings obtained as a part of the *POTH15* studies are summarized as follows:

(i) Tissue-specific qRT-PCR analysis exhibited a higher abundance of *POTH15* mRNA in shoot tips and stolons under tuber inducing short day conditions.

(ii) Upstream sequence of *POTH15* drives β-glucuronidase expression in apical and axillary meristems, petiole, tuber eyes and tuber pith indicating its role in meristem maintenance, leaf development and tuberization.

(iii) In accordance with this, over-expression of *POTH15* in potato altered multiple morphological traits including leaf development and exhibited early tuberization under *in vitro* conditions.

(iv) Comparative transcriptomic analysis of wild-type and *POTH15* over-expression lines identified 435 differentially expressed genes including 79 novel target genes with unknown functions.

(v) Functional analysis of these genes revealed their involvement in key biological processes such as metabolism, biotic and abiotic stress responses, regulation of transcription, and signal transduction.

(vi) qRT-PCR of selected candidate genes validated their differential expression in wild-type and *POTH15* over-expression lines.

In conclusion, this is the first report of a tissue-specific photoperiodic regulation of *POTH15* and identification of its potential target genes in potato. This study provides new insights into the role of *POTH15* and its target genes in regulating plant development and tuberization in potato. The above findings are submitted for a publication (Mahajan et al. 2015).

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The mRNA of a Knotted1-like transcription factor of potato is phloem mobile

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Abstract Potato Homeobox1 (POTH1) is a Knotted1-like transcription factor from the Three Amino Acid Loop Extension (TALE) superfamily that is involved in numerous aspects of development in potato (*Solanum tuberosum* L). POTH1 interacts with its protein partner, *St*BEL5, to facilitate binding to specific target genes to modulate hormone levels, mediate leaf architecture, and enhance tuber formation. In this study, promoter analyses show that the upstream sequence of *POTH1* drives β-glucuronidase activity in response to light and in association with phloem cells in both petioles and stems. Because *POTH1* transcripts have previously been detected in phloem cells, long-distance movement of its mRNA was tested. Using RT-PCR and transgenic potato lines over-expressing *POTH1*, in vitro micrografts demonstrated unilateral movement of *POTH1* RNA in a rootward direction. Movement across a graft union into leaves from newly arising axillary shoots and roots of wild type stocks was verified using soil-grown tobacco heterografts. Leaves from the wild type stock containing the mobile *POTH1* RNA exhibited a reduction in leaf size relative to leaves from wild type grafts. Both untranslated regions of *POTH1* when fused to an expression marker β-glucuronidase, repressed its translation in tobacco protoplasts. RNA/protein binding assays demonstrated that the UTRs of *POTH1* bind to two RNA-binding proteins, a polypyrimidine tract-binding protein and an alba-domain type. Conserved glycerol-responsive elements (GRE), specific to alba-domain interaction, are duplicated in both the 5′ and 3′ untranslated regions of *POTH1*. These results suggest that *POTH1* functions as a mobile signal in regulating development.

Keywords Alba domain · Long-distance transport · Polypyrimidine tract-binding · POTH1 · *Solanum tuberosum* L · *StBEL5* · Tuberization

Introduction

Knotted1-like homeobox (KNOX) transcription factors are important regulators of plant development and are ubiquitous in the plant kingdom. KNOX genes belong to the TALE superclass of homeobox transcription factors (Bürglin 1997). The first KNOX gene to be reported, KNOTTED1 from maize, is involved in formation and maintenance of shoot apical meristem (Vollbrecht et al. 1991). Based on expression pattern and sequence divergence, KNOX genes are grouped into two subclasses; class I and II (Kerstetter et al. 1994). Class I KNOX genes are involved in diverse developmental processes like shoot apical meristem maintenance, compound leaf formation, floral development and tuberization (Rosin et al. 2003; Ragni et al. 2007; Hay and Tsiantis 2010; Uchida et al. 2010).

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KNOX proteins interact with BEL1-like homeobox proteins, another group of transcription factors from the TALE superclass (Smith et al. 2002; Chen et al. 2003; Hamant and Pautot 2010). The KNOX-BELL interactions are selective, thus different KNOX-BELL dimers regulate a unique set of downstream genes (Hay and Tsiantis 2010). For example in potato, the KNOX protein, POTH1, and its BELL partner, *St*BEL5, form a tandem dimer that binds to a specific TTGAC motif in the *ga20ox1* promoter and downregulates its activity (Chen et al. 2003, 2004). DNA-binding and transcription assays confirmed that the POTH1/*St*BEL5 interaction is necessary to affect *ga20ox1* promoter activity (Chen et al. 2004). Transgenic potato lines over-expressing POTH1 exhibited enhanced tuber formation in vitro and reduced levels of gibberellic acid in leaves. Similar to other KNOX over-expression lines, these potato transgenic lines were dwarf and had altered leaf morphology and venation, suggesting a role for POTH1 in vegetative pattern formation (Rosin et al. 2003).

RNA movement assays and heterografting experiments have previously shown that the mRNA of *StBEL5* is mobile. The source of this RNA is the veins of leaves and petioles and promoter activity of *StBEL5* has been observed in companion cells and phloem parenchyma of petioles (Banerjee et al. 2006a). Whereas, POTH1 RNA was identified in phloem cells through in situ hybridization and its presence was verified using laser capture microdissection and RT-PCR (Yu et al. 2007; Campbell et al. 2008), movement of this RNA has not yet been confirmed. Using the dominant mutant *Me* of tomato, it was demonstrated that the transcript for *LeT6* (a tomato KNOTTED1-like homeobox gene) to be graft transmissible (Kim et al. 2001). This *LeT6* fusion transcript was able to act as a systemic signal altering leaf development in the scions of heteografts. Long-distance movement for the *GIBBERELLIC ACID INSENSITIVE* (*GAI*) transcript was verified in *Cucurbita maxima* and Arabidopsis (Haywood et al. 2005). Similarly, grafting studies in potato demonstrated that *StBEL5* mRNA is transported from leaves to stolons and that accumulation of this mobile RNA was correlated with enhanced tuber formation (Banerjee et al. 2006a). Further studies showed that the untranslated regions (UTRs) of *StBEL5* mediated its long-distance transport, stability and translation (Banerjee et al. 2009). *GAI* mRNA mobility was regulated by sequence in the 3′ end of its transcript (Huang and Yu 2009). The RNA for a STM-like ortholog of pumpkin was identified as a component in a mobile ribonucleoprotein complex that moves through the phloem. In this system, gel-mobility shift assays demonstrated the role of a specific polypyrimidine tract-binding protein, CmRBP50, in binding to RNAs and facilitating their long-distance transport (Ham et al. 2009).

StBEL5 and several other BELL and KNOX family transcripts were also detected in phloem cells and in phloem-enriched sap of solanaceae plants (Yu et al. 2007; Campbell et al. 2008). Despite such evidence, reports of any long-distance transport remains restricted to *StBEL5* in potato and *LeT6* in the *Me* mutant of tomato. It is interesting that in potato, transcripts encoding for interacting proteins (KNOX and BEL1-like) are concurrently detected in the phloem. To investigate the significance of this observation, experiments focusing on *POTH1* expression and movement in potato and tobacco have been undertaken. Micrografts and heterografts with soil-grown plants confirm that the mRNA of *POTH1* is graft transmissible and moves in a rootward direction. Putative binding sites for an alba-domain protein were identified in both the 5′ and 3′ UTRs of the *POTH1* transcript. Protein/RNA binding assays confirmed interaction of both UTRs with the alba-domain protein and of the 3′ UTR with a polypyrimidine tract-binding protein. These results suggest that, in addition to *StBEL5*, the full-length mRNA of *POTH1* is also functional in long-distance trafficking.

Methods

Plant material

The photoperiod-responsive potato (*Solanum tuberosum* ssp. *andigena)* was used throughout this study. Plants of this subspecies tuberize only under short-day (<12-h-light) conditions whereas under long days no tubers are formed. Tobacco (*Nicotiana tabacum* var Petite Havana) plants were also used in the study. In vitro cultures were grown at 25 °C with 16-h-light and 8-h-dark photoperiod in a growth incubator (Percival Scientific). Soil grown plants were grown at 24 °C with 16-h-light and 8-h-dark photoperiod in an environmental chamber (Percival scientific). For short days, the plants were shifted to 8-h-light and 16-h-dark photoperiod. Micrografts were maintained under a long-day photoperiod (LD) for 10 days and then transferred to short days or incubation was continued under LD.

Construct design and transformation

Upstream sequence of 1,627 nt from the *POTH1* start codon was isolated using GenomeWalker™ Universal kit (Clontech Cat. No. 638904) following the manufacturer's instructions. The amplified sequence was subcloned into pCR2.1 (Invitrogen TopoTA cloning kit) and sequenced. Upstream sequence of *POTH1* was then amplified using primer pair 5′-CCCAAGCTTCTCTTGAGATGATATAAATATC-3′ and 5′-TTCTCTAGACAAAGTAGAGTAAATTACTTAG-3′ from pCR2.1. This amplicon was inserted

upstream of β-glucuronidase in the binary vector pBI121. Full-length *POTH1* cDNA was cloned into the binary vector pBI121 to generate the 35S::POTH1 construct. A 10X histidine tag was added to the C-terminus of POTH1 by cloning a CAC decamer just before stop codon of POTH1 coding sequence. *Agrobacterium*-mediated transformation of the potato, *Solanum tuberosum* ssp. *andigena*, was done using the method described by Banerjee et al. (2006b). Tobacco, *Nicotiana tabacum* var Petite Havana, was transformed using the method described by Horsch et al. (1985). Kanamycin resistant transgenic plants were selected for further analysis.

Light inducibility of $_{prom}$POTH1

For the light induction experiment, sets of in vitro plants ($_{prom}$POTH1::GUS; 35S::GUS and $_{prom}$*St*PTB6::GUS) were incubated under following six conditions such as (i) long day (LD); (ii) dark for 8 days; (iii) dark for 7 days followed by 24-h-light; (iv) dark for 8 days with transfer to MS + 6 % sucrose 7 days post inoculation; (v) dark for 7 days followed by transfer to MS + 6 % sucrose and under light for 24-h; and (vi) dark for 7 days followed by transfer to light for 24-h and returned back to dark for 3 days. The plant tissues were harvested for the assays at the end of respective incubation periods. 35S::GUS and $_{prom}$*St*PTB6::GUS lines served as negative and positive controls, respectively. Dark conditions were maintained in a dark room at 25 °C. Light intensity in the growth chamber was 100 µmol m^{-2} s^{-1}.

For determining the effect of light on expression of POTH1 transcript, a detached leaf experiment was conducted. Six to seven terminal leaflets of 8-week old soil grown wild type potato plants were collected and placed in petri-dishes containing liquid medium (MS basal salts without sucrose). The terminal leaflets were incubated in dark for 24-h followed by a transfer to light for 0 or 24-h respectively. The control set of leaves was incubated under long day conditions. Light and dark conditions were maintained as described above. After respective incubations, the leaves were harvested and total RNA was isolated using Qiagen RNeasy mini kit (Cat.No. 74104). One microgram of RNA was subjected to RT-PCR for POTH1 transcripts using Invitrogen one step RT-PCR kit (Cat. No. 10928). 18S rRNA served as a control. Relative transcript levels were analyzed by Image J software (Abramoff et al. 2004).

Micrografting

In vitro cultures of *Solanum tuberosum* ssp. *andigena* line 7540 and 35S::POTH1 lines were used for grafting studies. Micrografting was done following the procedure explained by Banerjee et al. (2006a). The stock plants were grown for 3–4 weeks in growth incubators as described. Wild type stock plants were decapitated and used as rootstocks. 1.0 cm deep longitudinal slits were made at the cut end of rootstock plants. For scions, 2–3 cm long shoot tips of 35S::POTH1 line plants were taken and a V-shaped cut was made at their cut ends. Transgenic scions were then inserted into the slits of wild type rootstock plants. Reverse grafts were also made with the 35S::POTH1 line as stock and wild type plantlets as scions. Grafts were cultured on MS basal medium with 2 % sucrose and 0.2 % phytagel and incubated in growth incubators under long-day conditions for 10 days. After 10 days stock and scion tissue samples were harvested, RNA was extracted, and RT-PCR analyses were performed.

Soil-grown heterografts

Wild type and transgenic tobacco (*N. tabacum* var Petite Havana) lines were maintained in a growth chamber until grafting was performed. Grafts were made with wild type and 35S::POTH1 transgenic tobacco. For grafting, scions were made by cutting off the shoot approximately 10 cm below the shoot tip and making a V-shaped notch at its cut end. Stocks were prepared by decapitating the plants approximately 10 cm above the soil and making a 1.0 cm deep slit at the cut end. Scions were inserted into the slits of the rootstock and pots were covered with a transparent polythene bag. Fifteen grafts were made and maintained in a growth chamber for hardening and samples were harvested from scions and stocks of heterografts at the end of 4 weeks of incubation period. Nested RT-PCR was performed with transgenic gene-specfic primers to detect the transgenic *POTH1* RNA. Specifically, samples collected from stocks for the RT-PCR analyses were stem sections 1.0 cm above the soil line, new leaves arising from axillary shoots, and root tips approximately 2.0 cm in length.

Translation assays

The pBI221 vector (Clontech) containing a 35S::GUS cassette was used for translation analyses. The 5′ UTR was amplified by primer pair 5′-CATCTAGAGAGTTTCTCT CCCTTTTAA-3′ and 5′-CGGGATCCATATATATCTAA AAAAAAACAAACA-3′ and 3′ UTR was amplified by primer pair 5′-ACTGAGCTCGTTTGAATGGAAATTGT GAA-3′ and 5′-GTCGAGCTCTTAGGTAAGCATTAATT TTGACA-3′. The amplicons were ligated in pBI221 to generate the POTH1-UTR-GUS fusion constructs. A tandem UTR construct was also generated (Fig. 6a). Plasmid containing 35S::Luciferase was used as an internal control.

For protoplast isolations, fully expanded leaves of 3-week old tobacco (*Nicotiana tabacum* var Petite Havana)

cultures were used. The leaves were excised and painted with sterile K3 medium containing 10 % Celite (C8656, Sigma), washed several times and finally placed in sterile K3 medium containing 0.5 % cellulase and 0.1 % macerase (Calbiochem) and incubated for 16 h at 28 °C in dark. The protoplasts were filtered through sterile cheese cloth into a flask with bottleneck and centrifuged at 100 *g* for 10 min. Protoplasts were collected from the bottleneck area and washed once with K3 medium with 0.4 M sucrose and resuspended in K3 medium with 0.4 M glucose to a final concentration of 4×10^6/ml.

700 µl of the protoplast suspension was taken in a 0.4 cm electroporation cuvette and mixed with 30 µl of 2 M KCl and the specified plasmid (5 µg of GUS-UTR construct plus 0.5 µg of 35S::Luc). Protoplasts were electroporated at 170 V, 125 µF in Gene Pulser X-Cell (Bio-Rad). After electroporation, 4 ml of Murashige and Skoog (1962) basal medium was added and the protoplasts were incubated at 25 °C for 48 h in dark. GUS and LUC activity assays were performed as explained below.

Histochemical, fluorometric and luminometric assays

GUS staining was done by incubating the samples in GUS staining buffer containing 1 M $NaPO_4$, pH 7; 0.25 M EDTA, pH 8; 10 % TritonX 100; 1 mM 5-bromo-4-chloro-3-indolyl-β-D-GlcUA, 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide. Samples were cleared with 100 % ethanol and photographed on Leica stereomicroscope (S8AP0).

Fluorometric analyses from tissue samples were performed as described by Jefferson (1987). Frozen samples were homogenized in 100 µl of GUS extraction buffer. Samples were then centrifuged at 17,000 *g* for 5 min on a benchtop centrifuge. Clear supernatant was removed and used for protein estimation by Bradford's method (Bradford 1976). Aliquots of extracts with approximately 10 µg of protein were made and their volume was made up to 50 µl with extraction buffer. 5 µl of GUS assay buffer was added to each sample and the samples were incubated at 37 °C for 16 h. Reaction was stopped by adding 50 µl of stop buffer. Fluorescence of the samples was measured using multimode plate reader (Varioskan flash, Thermo Scientific) with excitation wavelength 355 nm and emission wavelength 465 nm.

At the end of 48 h incubation, protoplasts were centrifuged at 8,000 *g* for 10 min at 25 °C. The protoplast pellet was flash frozen in liquid nitrogen. For flurometric measurements, frozen protoplast pellets were homogenized in 100 µl GUS extraction buffer and centrifuged at 17,000 *g*. Clear supernatant was removed and used for fluorometric measurements as explained by Jefferson (1987). Fluorescence of the samples was measured using multimode plate reader (Varioskan Flash, Thermo Scientific) with excitation wavelength 355 nm and emission wavelength 465 nm. For luminometric measurements, frozen protoplast pellets were homogenized in Cell Culture Lysis Reagent (CCLR, Cat.# E1531, Promega), centrifuged at 17,000 *g* and clear supernatant was collected. 100 µl of luciferase substrate (Promega) was injected to the 20 µl of clear supernatant and luminescence was measured for 15 s in a multimode plate reader (Varioskan Flash, Thermo Scientific).

Yeast three-hybrid analysis

The host yeast strain YBZ-1 was transformed with RNA hybrid plasmids by the standard yeast transformation protocol using lithium acetate. Transformed cells were grown on plates containing synthetic medium lacking uracil (SD/-ura). Each of the protein plasmids were then transformed into the yeast strain YBZ-1 containing the target RNA hybrid plasmid and transformants were grown on SD/-leu/-ura plates. To assay RNA–protein interaction, induction of the *lacZ* reporter was measured with a Yeast β-galactosidase Assay Kit (Pierce Biotechnology). β-galactosidase activity was determined by following the equation; $[1{,}000 \times OD_{420}]/[\text{time of color reaction (min)} \times \text{volume of cells used (ml)} \times OD_{660}]$. Each assay was replicated in three independent experiments. The YBZ-1 strains, plasmids *pIIIA/MS2-1* and *pACTII* were generous gifts of Dr. Marvin Wickens, University of Wisconsin, Madison.

Results

Characterization of the upstream sequence of *POTH1*

To study the pattern of *POTH1* expression, a 1.63 kb upstream sequence fragment (from the start codon) of *POTH1* was isolated and verified by comparison to potato genomic sequence (http://potatogenomics.plantbiology.msu.edu/index.html). To determine the location of *POTH1* transcription, the *POTH1* promoter sequence was used to drive β-glucuronidase (GUS) expression in the binary vector pBI121. The $_{prom}$POTH1::GUS construct was transformed to *S. tuberosum* ssp *andigena* line 7540 using *Agrobacterium*-mediated transformation (Banerjee et al. 2006b). Several transgenic lines were generated and three high-expressing lines with consistent patterns of expression were used for further analysis. GUS staining demonstrated that the *POTH1* promoter was active in a wide range of organs, including shoot tips, axial nodes, stolons, leaves, stamens and roots (Fig. 1, Fig. S1). In leaves, the strongest expression was localized to veins (Fig. 1a, b, arrows). Transverse sections of both stems and petioles showed promoter activity associated

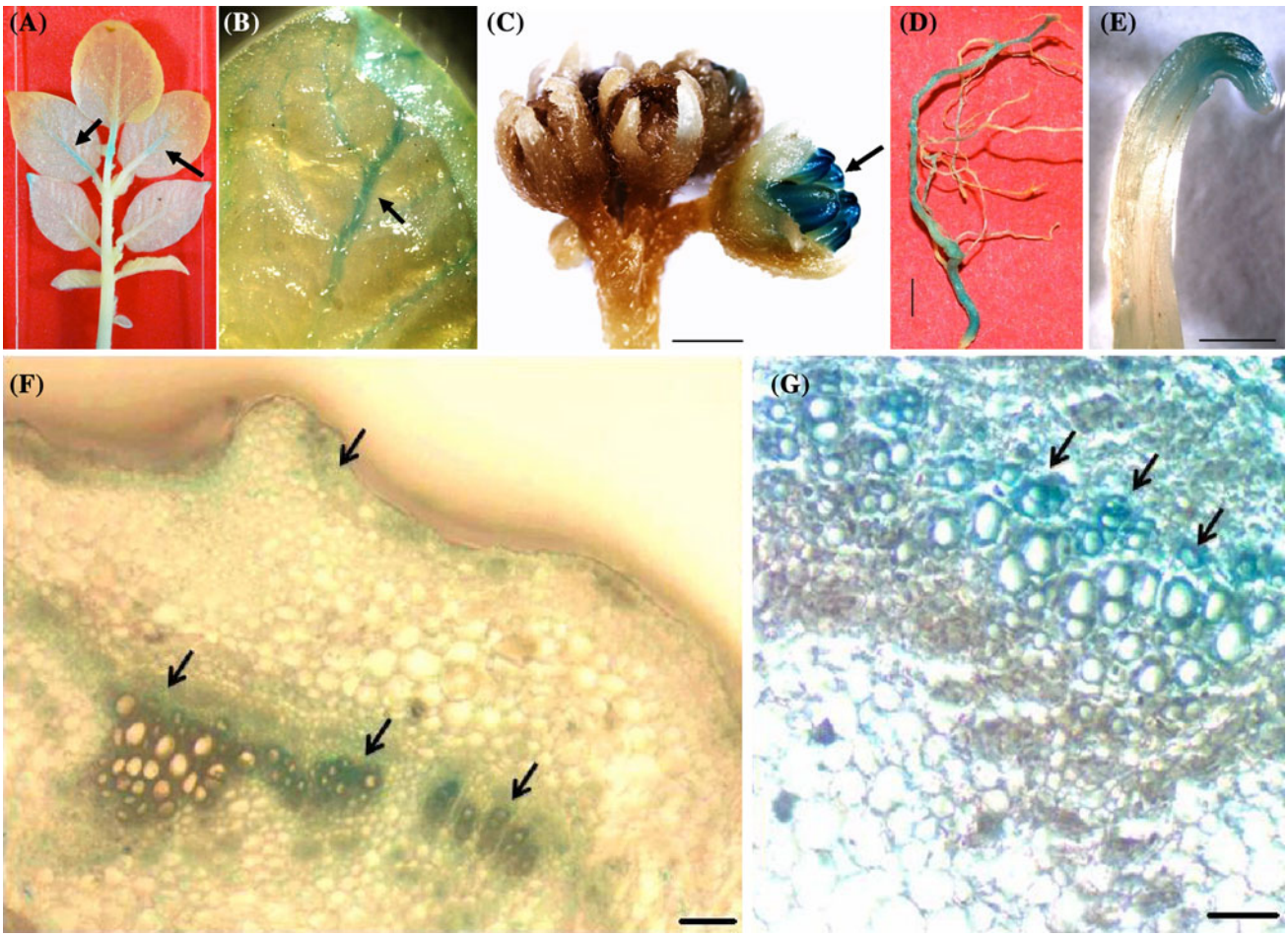

Fig. 1 Promoter activity of upstream sequence of *POTH1* in mature leaves (**a**), a leaflet showing expression in the mid-vein (**b**, *arrow*), in mature stamens (**c**, *arrow*), roots (**d**), a stolon tip (**e**), a transverse section of the stem (**f**), and a transverse section of a petiole (**g**). For the stem **f**, GUS staining is most intense along the epidermal layer and in the phloem side of vascular bundles (*arrows*). For petioles, GUS activity is associated with phloem cells (**g**, *arrows*). *Bars* in **c**, **d** and **e** are 5 mm each whereas *bars* in **f** and **g** are 100 µm each

with phloem cells in both of these organs (Fig. 1f, g, *arrows*).

This upstream sequence was analysed for *cis*-regulatory elements using the promoter analysis software plantPAN (Chang et al. 2008) and PLACE (Higo et al. 1999). Several light regulatory elements (LREs) such as GATA, GT-1 and I-boxes (Vorst et al. 1990; Vauterin et al. 1999; Waksman et al. 1987) were observed in the *POTH1* promoter (Table S1). Additionally, the software analysis also identified the presence of a CAAT box and MYBST1 motifs that drive tissue-specific expression (Baranowskij et al. 1994; Xu et al. 2010).

Light inducibility of POTH1

Because the promoter of POTH1 was active in leaf veins and shoot apices, the effect of light on promoter activity was evaluated. Sets of in vitro plants ($_{prom}$POTH1::GUS; 35S::GUS and $_{prom}$*St*PTB6::GUS) were incubated under six different conditions as described in methods. 35S::GUS and $_{prom}$*St*PTB6::GUS served as negative and positive controls, respectively. For testing light induction, plants were incubated in (i) long day (LD); (ii) dark for 8 days; and (iii) dark for 7 days followed by 24-h-light conditions. To differentiate between light induction and induction of gene expressions due to higher metabolism following illumination, sets of plants were also incubated in (iv) dark for 8 days with transfer to MS + 6 % sucrose 7 days post inoculation or (v) dark for 7 days followed by transfer to MS + 6 % sucrose and under light for 24-h. To check the effect of dark/light/dark cycles on GUS activity another set of plants were incubated in (vi) dark for 7 days followed by transfer to light for 24-h and was returned back to dark for 3 days. No significant difference in relative GUS activity was detected in 35S::GUS lines under any of these conditions tested (Fig. 2a).

$_{prom}$*St*PTB6::GUS lines (positive control) showed approximately 50 % reduction in GUS activity under dark

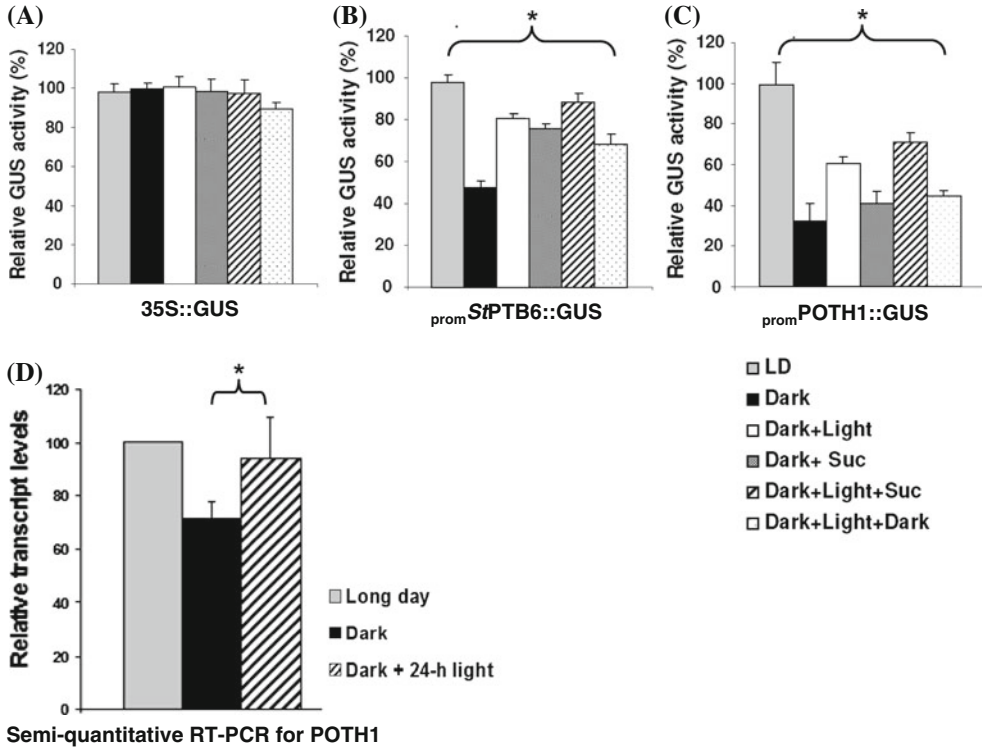

Fig. 2 Light induction of the *POTH1* promoter. Light induction of the *POTH1* promoter. 35S::GUS (**a**), $_{prom}$*St*PTB6::GUS (**b**) and $_{prom}$POTH1::GUS (**c**) transgenic potato plants were incubated under following six conditions (i) long day (LD); (ii) dark for 8 days; (iii) dark for 7 days followed by 24-h-light; (iv) dark for 8 days with transfer to MS + 6 % sucrose 7 days post inoculation; (v) dark for 7 days followed by transfer to MS + 6 % sucrose and 24-h light; and (vi) dark for 7 days followed by transfer to light for 24-h and returned back to dark for 3 days. At the end of respective incubation periods, GUS activity was measured by 4-methylumbelliferyl-β-D-glucuronide (MUG) fluorescence assay. Data were analyzed by ANOVA. The mean ± SD of relative GUS activity for three replicates is plotted. Asterisk indicates significant difference due to treatments ($p = 0.01$). Effect of light on POTH1 mRNA accumulation (**d**). Six to seven terminal leaflets were incubated in dark for 24-h followed by transfer to light for 0 or 24-h respectively. The control set was incubated under long day. The leaves were harvested and total RNA was isolated. One microgram of RNA was subjected to RT-PCR for POTH1 transcripts .18S rRNA served as a control. Relative transcript levels were analyzed by Image J (Abramoff et al. 2004) software. The data is mean ± SD of four replicates. Asterisk indicates significant difference due to treatment ($p = 0.05$) (**d**)

conditions whereas GUS activity was increased to approximately 80 % in condition (iii) dark + light; 75 % in condition (iv) dark + 6 % sucrose; and 88 % in condition (v) dark + light + 6 % sucrose respectively. A reduction of GUS activity was observed in plant grown under condition of (vi) dark + light + dark cycle (Fig. 2b). The $_{prom}$POTH1::GUS lines showed approximately threefold reduction in GUS activity (32 %) when incubated under dark conditions (Fig. 2c). Compared to plants grown under dark conditions, the GUS activity increased approximately twofold (60 %) in plants incubated in the dark for 7 days followed by 24 h illumination under white light (condition iii, dark + light). Incubation in 6 % sucrose (condition iv, dark + sucrose) had marginal increase in GUS activity (41 %). An increase of GUS activity to 71 % was detected in plants incubated under conditions (v) dark + light + 6 % sucrose. Compared to plants grown under conditions (iii) a reduction in GUS activity was observed in plants incubated under condition (vi) dark + light + dark (Fig. 2c). Our results indicate that $_{prom}$POTH1 is induced by light and sucrose has a slight additive effect on the promoter activity. In a separate experiment, photoperiod exerted no effect on activity of the *POTH1* promoter in leaves (Fig. S2).

Additionally, to evaluate the effect of light on *POTH1* mRNA accumulation pattern, detached leaflets from wild type potato plants were incubated under different light conditions (dark for 24-h followed by a transfer to light for 0 or 24 h respectively and the control set of leaves was incubated under long-day conditions) as explained in methods. A semi-quantitative RT-PCR was carried out and the gel was analyzed by Image J software (Abramoff et al. 2004). Incubation of leaflets under dark conditions resulted in reduction of POTH1 transcript to approximately 70 % in comparison to leaftlets incubated under long-day conditions. Exposure of dark-incubated leaflets to light for 24-h returned the *POTH1* RNA accumulation to levels of

control (Fig. 2d). These results suggest that *POTH1* promoter activity and RNA accumulation are regulated by white light illumination.

POTH1 mRNA is graft transmissible

Because of the evidence for the presence of *POTH1* RNA in phloem cells (Yu et al. 2007; Campbell et al. 2008), heterografts were utilized to determine if *POTH1* transcripts were graft transmissible. Because POTH1 overexpression (OE) lines are very slow growing, initial grafting experiments were performed with tissue culture plants in vitro. In vitro plantlets of wild type *S. tuberosum* ssp. *andigena* line 7540 and POTH1 OE line #11 were used and alternated as stock and scion. POTH1 OE line #11 was selected for its extreme phenotype (Rosin et al. 2003). After 4 weeks of long-day conditions, grafted plants were transferred to short day conditions for 10 days and samples were harvested. RNA was isolated from the lower stem of stocks and the upper stem of scions (Fig. 3). Using gene-specific primers and the stem RNA as template, RT-PCR was performed to assess movement of *POTH1* RNA. A primer specific for a non-plant sequence tag present only in the transgenic *POTH1* RNA was used to distinguish the transgenic transcript from the native RNA. Transgenic *POTH1* RNA was detected in wild type stocks harvested from the stems of the POTH1 line #11/WT heterografts and was confirmed by Southern hybridization (Fig. 3a, #11 as scion). No signal was detected in stems of wild type scions from the WT/POTH1 line #11 grafts. In these experiments, *POTH1* RNA demonstrated a unidirectional, downward movement in micrografts from scion to stock.

POTH1 RNA movement was also tested in heterografts of soil-grown plants. Because POTH1 OE potato lines are dwarf, have malformed leaves, and grow slowly, grafting onto wild type soil-grown potato was not possible. To overcome this problem, full-length *POTH1* sequence driven by the CaMV35S promoter was transformed into tobacco (*N. tabacum* var Petite Havana) and these transgenic lines were used in soil-grown heterografts. Twenty independent transgenic lines were screened and three high-expressers were used for further analysis. Overexpression of POTH1 in tobacco produced a phenotype similar to Knotted1 OE lines observed in several species, including potato and tobacco (Sinha et al. 1993; Parnis et al. 1997; Tamaoki et al. 1997; Rosin et al. 2003). Transgenic tobacco lines exhibited a reduction in height, smaller, mouse-ear type leaves, and aberrant leaf venation (Fig. 4a–c). Similar to a recent study on KNOX gene over-expression (Box et al. 2011), flowers of these lines were more pale, had dissected petals, and an elongate style (Fig. 4d, e, arrows). Despite this abnormal phenotype, the tobacco lines were healthy and robust. Heterografts were made with the 35S:POTH1 lines of tobacco as scions and wild type lines as stocks. The grafts were hardened and scion and stock samples were harvested 4 weeks after grafting. This time frame allowed for the induction of axillary shoot growth from the stock stem. RNA was extracted from newly emerged leaves of these axillary shoots, lower stems, and roots and RT-PCR was performed with transgene-specific primers (Fig. 5a). Leaves that were produced from these branching axillary shoots and used for RNA extraction were smaller in size compared to the WT/WT grafted lines (Fig. 5b). Transgenic *POTH1* RNA

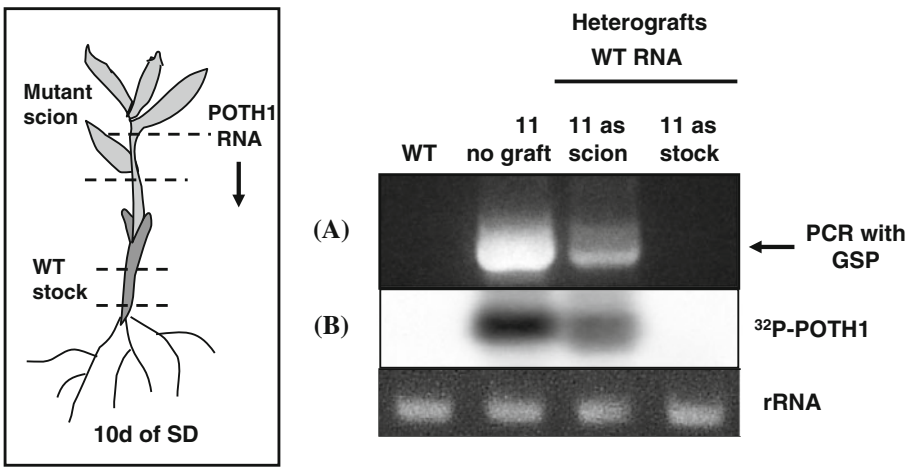

Fig. 3 POTH1 mRNA moves unidirectionally. Using grafts with tissue culture plants and RT-PCR with gene-specific primers (GSP), we demonstrate that RNA for POTH1 (the protein partner of *St*BEL5) moves across a graft union in one direction, towards the base of the plant (line 11 as scion). The same RNA is not detected moving upward towards the apex (#11 as stock). Line 11 is a transgenic potato line that overexpresses POTH1 mRNA (Rosin et al. 2003). The no graft sample is a positive control. WT RNA was sampled for both heterografts. PCR was performed twice off cDNA template made from RNA and reverse transcriptase (RT). Two different GSPs for POTH1 were used with a non-plant sequence tag specific for the transgenic RNA of line 11. The two white bands (**a**) indicate the presence of the transgenic RNA. The dark bands (**b**) are PCR products that hybridized to a ^{32}P-labeled POTH1 probe

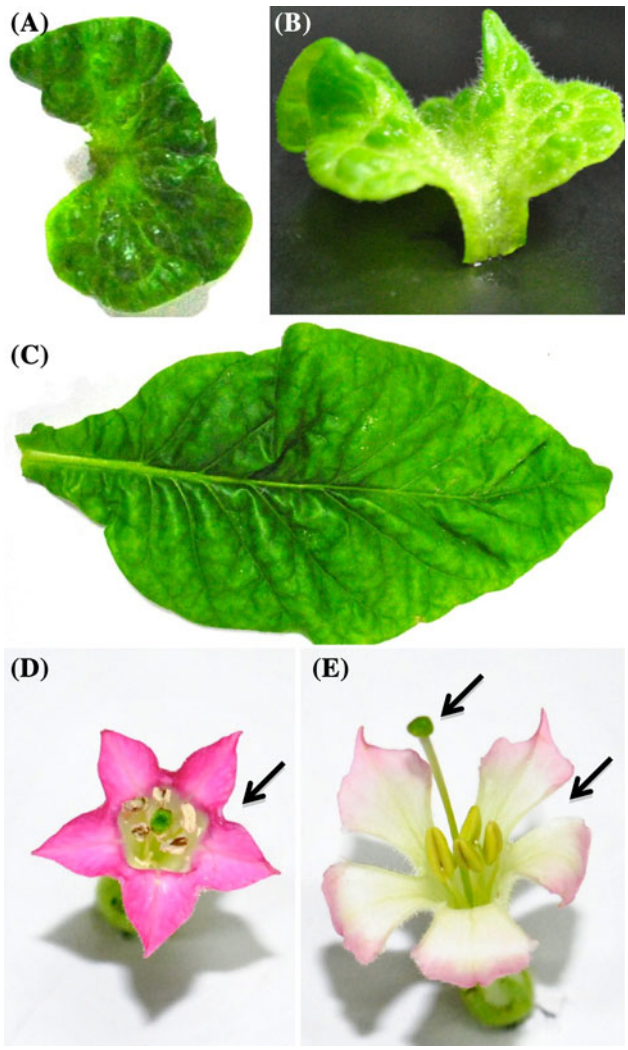

Fig. 4 Phenotype of OE lines of POTH1 in tobacco. Leaves of soil-grown OE lines (**a**–**b**) are reduced in size and curled with aberrant venation relative to control leaves (**c**). Flowers of the OE lines exhibit an exaggerated cleft in the petal (*arrows*, **d**–**e**) and an elongate style (upper arrow, **e**). 35S:POTH1 OE lines, (**a**, **b**), and (**e**); WT, (**c**) and (**d**). Bars in (**a**) and (**c**) are 5 mm each

was detected in leaves from axillary shoots, lower stems, and roots (Fig. 5a) of the wild type stock of the 35S:POTH1/WT heterografts.

POTH1/UTR fusions repress translation

Because numerous mobile RNAs also exhibit a mechanism for repression of translation that is mediated by their UTRs (Wilhelm et al. 2000; Gu et al. 2004), the effect of the UTRs of *POTH1* RNA on its translation was assessed. Constructs of 35S::GUS with or without the *POTH1* UTRs were cloned into pBI221 (Fig. 6a) and GUS activity of each construct was assayed by transient expression in tobacco protoplasts (Fig. 6b). Compared to GUS activity without any UTRs, activity was reduced, 30–35 % for the three constructs having at least one of the *POTH1* UTRs (Fig. 6b). Whereas for construct with tandem POTH1 UTRs (Fig. 6a, GUS + tandem UTRs), the GUS activity was further reduced to approximately 50 % of the control (Fig. 6b). RT-PCR confirmed that the levels of GUS transcripts in the transfected protoplasts were equivalent for all the constructs. These results suggest that the UTRs of *POTH1* have a repressive effect on translation.

Screening for proteins that bind the UTRs of *POTH1*

Because of the possibility that protein chaperones interact with *POTH1* transcripts in mediating translation and mobility, the yeast three-hybrid system was applied to assess the potential interaction of the UTRs of *POTH1* RNA with several RNA-binding proteins of potato. An initial screening indicated that the 5′ and 3′ UTRs of *POTH1* designed as a fusion RNA bait interacted with an alba-domain protein (Fig. 7a). In this assay, growth on the higher concentrations of 3-aminotrizole (3-AT) are an indication of a strong interaction. Yeast colonies containing the *POTH1* UTR bait plasmid plus the alba-domain plasmid (*St*RBP7) grew robustly up to a concentration of 50 mM 3-AT in the selection media (Fig. 7a). As a more stringent test for interaction, induction of the marker β-galactosidase was quantified (Fig. 7b, c). Because of the numerous CU motifs present in both the 5′ and 3′ UTRs of *POTH1*, a polypyrimidine tract RNA-binding (PTB) protein of potato was also selected to test for interaction. PTB proteins bind specifically to polypyrimidine tracts in RNA, most commonly present in the 3′ UTR (Auweter and Allain 2008). The bait RNAs used for the β-galactosidase assay were the CU-rich *T1* fragment of the 3′ UTR of *StBEL5* with *St*PTB6 as a positive control and full-length *POTH1* 5′ UTR (135 nt) and *POTH1* 3′ UTR (202 nt) with the same potato PTB protein and the alba-domain protein of potato. As a negative control, a coding sequence (cds) fragment (*StBEL5 N*) of *StBEL5* was used. As shown by induction of β-galactosidase, both the 5′ and 3′ UTRs of *POTH1* interacted strongly with the alba-domain protein, whereas only the 3′ UTR interacted with *St*PTB6 (Fig. 7b, c). Based on β-galactosidase activity, the strongest interaction occurred with the 5′ UTR of *POTH1* and the alba-domain protein with a 15-fold induction, whereas the 3′ UTR exhibited a 4.5-fold induction (Fig. 7c). As a comparison, the relatively strong interaction of *St*PTB6 with *T1* produced a 5.5-fold induction of β-galactosidase activity (Fig. 7b).

To explain the strong interaction of these UTRs with the alba-domain protein, a conserved glycerol-response element (GRE), specific for alba-domain interaction (Vassella et al. 2004), was detected in both UTRs of POTH1

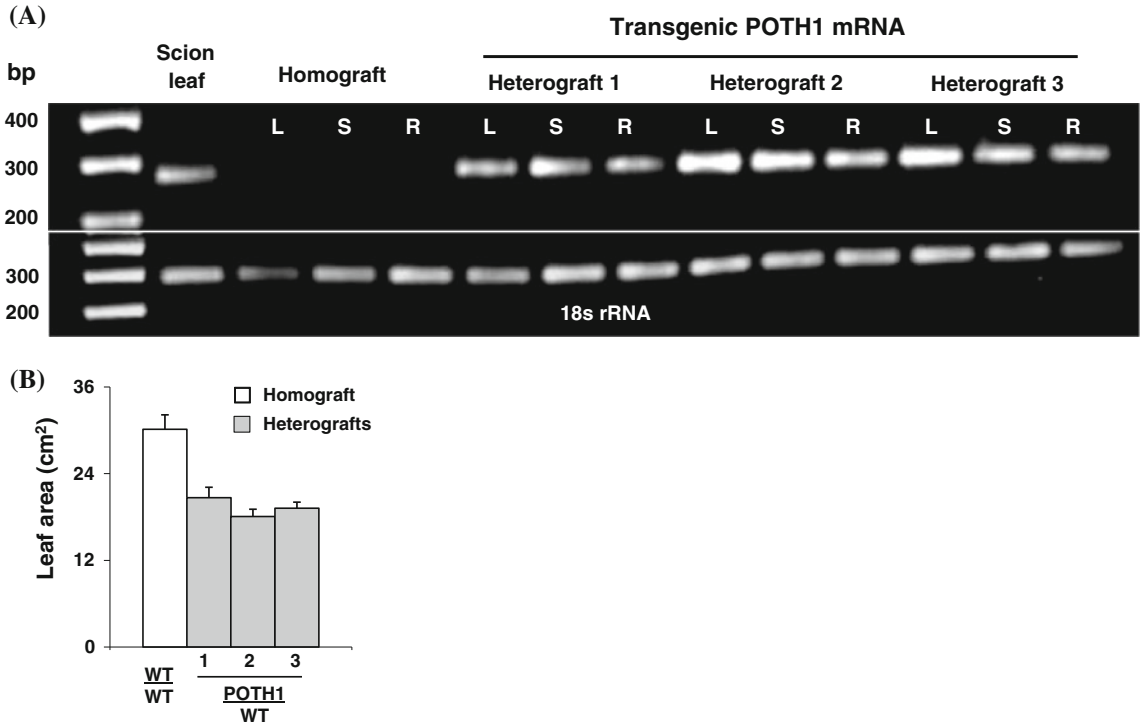

Fig. 5 RT-PCR detection of transgenic *POTH1* RNA moving across a graft union (**a**). Grafts were generated with 35S::POTH1-His tobacco lines as scion and wild type tobacco lines as stocks. WT/WT tobacco homografts were used as a graft control. Leaf (L), stem (S), and root (R) samples were harvested 30 days post-grafting from wild type stock material from three heterografts. Samples collected from stocks for the RT-PCR analyses were stem sections 1.0 cm above the soil line, new leaves arising from axillary shoots, and root tips approximately 2.0 cm in length. RNA was extracted and a non-plant sequence tag fused to the transgenic construct was used to detect transgenic-specific transcripts. PCR reactions were performed twice with nested transgene-specific primers. Scion leaf RNA was used as a positive control with wild type gene-specific primers. 18 s rRNA was used as a PCR control. Axillary shoot branches grew from the wild type stocks of both 35S:POTH1/WT and WT/WT grafts and leaves from these branches were scored for size (**b**). Leaf area data from panel (**b**) are the means of three replicates ± SE

(Fig. 8a). Both are located in loops of the secondary structure of a Mfold-generated model of *POTH1* (Fig. 8b, arrows). In addition to the GRE, two other conserved motifs have been identified in *POTH1* sequence (Fig. 8c, d) that are functional in the 3′ UTR of a procyclin RNA, designated *PGEET* (Fig. 8d), from *Trypanosoma brucei* that binds to alba-domain proteins (Mani et al. 2011). Both UTRs of *POTH1* contain conserved sequences similar to these additional motifs to make up a cassette of three motifs (designated 16-mer, GRE, and 26-mer) that are aligned in opposite directions in either UTR (Fig. 8c). Specific RNA-binding proteins bind to a fusion of the 26-mer and the GRE (Walrad et al. 2009). A similar arrangement of these elements is present in the 5′ UTR of *POTH1* (Fig. 8c) and this conserved motif pattern may explain the enhanced binding affinity of the 5′ UTR (>threefold more than the 3′ UTR) for the alba-domain protein (Fig. 7c). As a target for PTB proteins, the 3′ UTR of *POTH1* also contains a cluster of four CU motifs, made up of two pairs separated by an intervening sequence of 67 nt (Fig. 8c, arrows).

Discussion

A new mobile RNA

Although hundreds of RNAs have been identified in phloem cells or phloem exudate (Asano et al. 2002; Vilaine et al. 2003), very little is known about the mechanisms that control long-distance transport of full-length mRNAs. In this study, heterografting experiments coupled with RNA detection methods confirm that *POTH1*, a class I KNOX RNA of potato, moves through the phloem in a rootward direction. The maize KNOTTED1 (KN1) homeodomain protein was the first plant protein found to traffic cell-to-cell (Lucas et al. 1995; Kim et al. 2005). Subsequent studies have shown that class I KNOX proteins promote transport of the *KN1* mRNA and that this traffficking occurs through plasmodesmata (Lucas et al. 1995). Long-distance transport of Knotted1 mRNA has also been previously reported (Kim et al. 2001). Grafting experiments with the mouse-ear (Me) leaf mutant in tomato were used to demonstrate long-distance movement of a chimeric

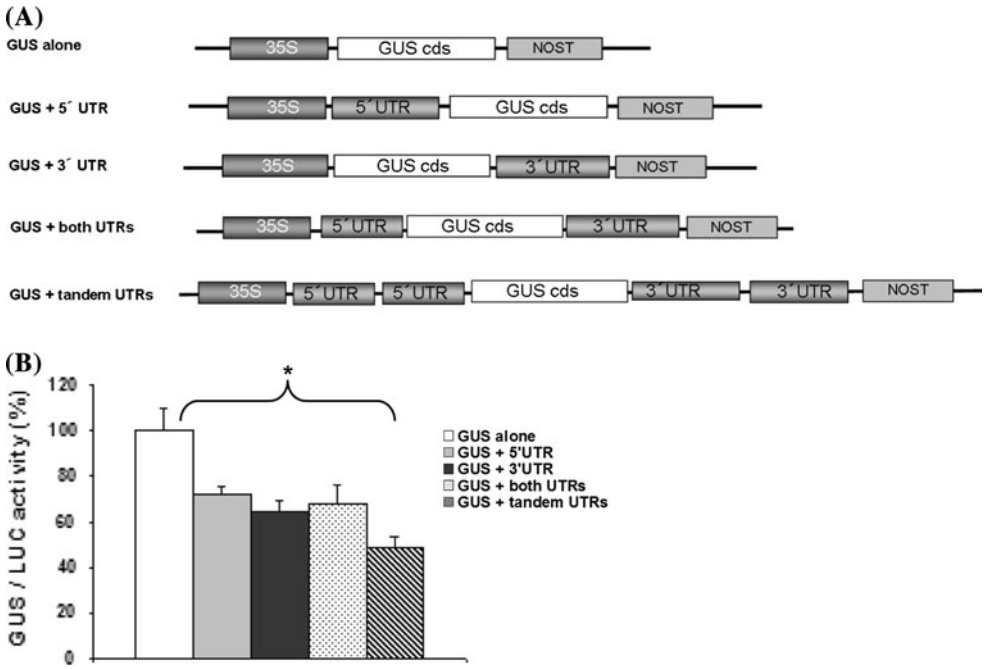

Fig. 6 The effect of the UTRs of *POTH1* on the translation efficiency of ß-glucuronidase (GUS) in tobacco protoplasts. Four constructs driven by the 35S promoter in pBI221 were analyzed; the *GUS* coding sequence alone, the 5′ UTR of *POTH1* + *GUS*, *GUS* + the 3′ UTR of *POTH1*, *GUS* plus both UTRs attached and GUS plus both UTRs in tandem. **a** The luciferase gene in pBI221 under the control of the CaMV35S promoter was included as an internal control. A relative value for GUS expression **b** was obtained by dividing the GUS activity by the specific luciferase activity. Each transfection was performed three times. Data were analyzed by ANOVA. The mean ± SD of three replicates is plotted. Asterisk indicates significant difference due to treatments ($p = 0.01$) NOST, nopaline synthase terminator; cds, coding sequence

Knotted1 mRNA into wild type scions. This fusion RNA was composed of sequence from a pyrophosphate-dependent phosphofructokinase (PFP) and LeT6, a class I KNOTTED1-like transcription factor of tomato. The chimeric *PFP-LeT6* fusion RNA was transported up from the Me stocks into the wild type heterografted scions and caused a change in leaf morphology in the wild type scion. This chimeric transcript was detected in phloem sieve tubes and associated companion cells and accumulated in the scion shoot apices and leaf primordia. Similar to the *LeT6* fusion, *POTH1* RNA was detected in phloem cells and was graft-transmissable. Like *LeT6*, accumulation of the mobile RNA was correlated with a change in leaf morphology.

Two other well-documented examples of RNA movement are the transport of *GAI* (*Gibberellic Acid Insensitive*) and *StBEL5*. Whereas *GAI* moves up to shoot apices and *StBEL5* moves down towards roots and stolons, both contain sequences present in their 3′ UTRs that mediate interactions with RNA-binding proteins (Banerjee et al. 2009; Ham et al. 2009; Huang and Yu 2009). Both are rich in CU motifs and both bind to polypyrimidine tract binding (PTB) proteins. In the case of *CmGAI,* this PTB protein is CmRBP50, the core protein of the recently discovered phloem-mobile RNA/protein complex in pumpkin (Ham et al. 2009). In addition to the polyprimidine motifs, the 3′ UTR of *StBEL5* contains clusters of UAUU (specific to select zinc finger proteins) and UAGUA motifs (Banerjee et al. 2009). These same motifs are conserved in the 3′ UTR of *BEL5* orthologs from tomato and tobacco as well as in *StBEL11* (AF406698) and *StBEL29* (AF406702). Remarkably, here we report on the long-distance phloem movement of *POTH1*, the transcription factor partner (Chen et al. 2004) of another mobile RNA, *StBEL5*. StBEL5 and POTH1 in a tandem complex were required to regulate transcription of the target promoter of *GA20 oxidase1* (Chen et al. 2004). In concert with BEL5, *POTH1* RNA is a likely candidate as a functional mobile signal for tuberization. Consistent with its capacity for mobility, the *POTH1* promoter was active in phloem cells of both stems and petioles.

RNA-binding proteins interact with both UTRs of *POTH1*

Proteins that bind to specific motifs in the sequence of mobile RNAs facilitate movement, stability, and translation (Bailey-Serres et al. 2009). In this study, the UTRs of *POTH1* repressed translation of the *β*-glucuronidase marker and this effect is likely mediated by a RNA-binding protein. Mobility of mRNA is often linked to its

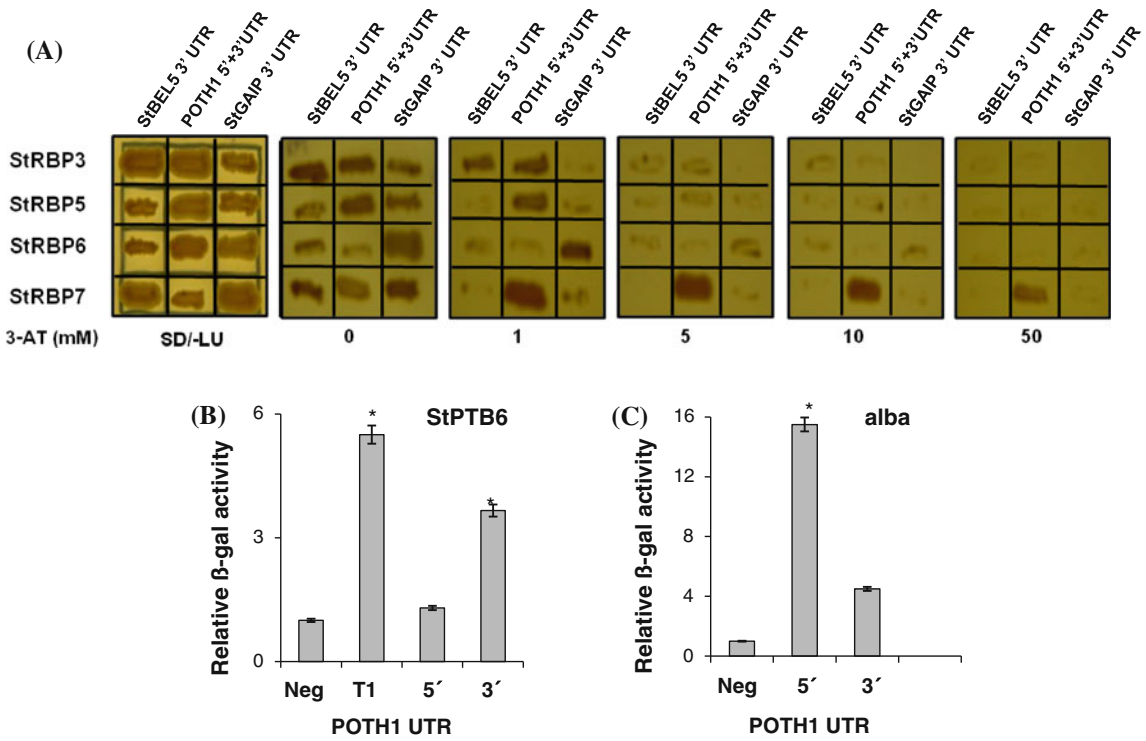

Fig. 7 Yeast three-hybrid growth assays for *HIS3* expression on triple selective medium (SD/-HLU) containing a gradient of 3-aminotrizole (3-AT) between 0 and 50 mM (**a**). Three RNA baits were used: the 3′ untranslated region (UTR) of *StBEL5,* the combined 5′ and 3′ UTRs of *POTH1,* and the 3′ UTR of *StGAI*. Four phloem RNA-binding proteins of potato were tested: *St*RBP3 coding for *St*GRP7 (DFCI #TC20200); *St*RBP5 coding for RNA-Binding Protein 45 (AT1G11650); StRBP6 coding for two-RRM protein (AT5G40490); and *St*RBP7 coding for an alba-domain protein (AT1G29250). Yeast three-hybrid assays were performed for the RNA-binding proteins StPTB6 (**b**) and an alba-domain protein (**c**) against RNA bait sequence for a negative control, Neg; for a CU-rich region of *StBEL5* 3′ UTR, T1; and *POTH1* 5′ or 3′ UTRs, 134 and 202 nt, respectively. Relative *β*-galactosidase activity is shown on the y-axis. The results shown are representative of at least three independent experiments. The asterisk indicates a significant difference relative to the negative control at a 95 % confidence level. The empty pMS2-1 vector showed no measurable *β*-galactosidase activity

translational repression in non-plant RNAs (Ferrandon et al. 1994; Gu et al. 2004; Colegrove-Otero et al. 2005). The Staufen protein of *Drosophila* binds to *bicoid* and *oskar* mRNAs to determine their location in the egg cell and to repress translation until this occurs (St Johnston et al. 1991; Ferrandon et al. 1994). A preliminary yeast three-hybrid screen of the UTRs of *POTH1* against a catalog of RNA-binding proteins of potato revealed a strong interaction with an alba-domain protein (Mani et al. 2011). This robust and specific interaction (Fig. 7a, c) is significant for several reasons. A related RNA-binding protein (AT1G29250) was identified in phloem sap of pumpkin (Lin et al. 2009). Alba-type RNA-binding proteins have been characterized in *Trypanosoma brucei*, the causal agent of African sleeping sickness, and bind specifically to a glycerol-responsive element (GRE) of 25 nucleotides present in the 3′ UTRs of RNAs encoding glycoproteins known as EP and GPEET procyclins. The surface of the trypanosome is covered by a dense coat of these glycoproteins as an adaptation to avoid the host's immune system. Alba-domain proteins function to regulate translation, process tRNA, and bind DNA (Aravind et al. 2003; Mani et al. 2011). RNA/protein binding assays show that both UTRs of *POTH1* bind to the alba-domain protein (Fig. 7c) and that both UTRs contain a putative conserved binding motif for the alba protein (Fig. 8c). Because both the UTRs affected translation (Fig. 6), it is feasible that interaction of the alba protein with either of the UTRs influences translation of *POTH1*. Conserved motifs identified in *POTH1* UTRs function to repress translation of procyclin mRNAs of *Trypanosoma brucei* (Furger et al. 1997; Hotz et al. 1997). Both PTB and alba-domain proteins function in this repression mechanism. Mfold-predicted secondary structures of the full-length *POTH1* transcript form distinct loop structures containing the GRE in both UTRs (Fig. 8b, arrows) similar to the model of the *GPEET* UTR (Fig. 8d, LII region).

Binding assays showed that the 3′ UTR of *POTH1* and the *T1* region (from 3′ UTR sequence) of *StBEL5* also interacted with the CmRBP50-like protein of potato, designated *St*PTB6 (Fig. 7b). Both of these 3′ UTR sequences are rich in CU motifs, consistent with target motifs for

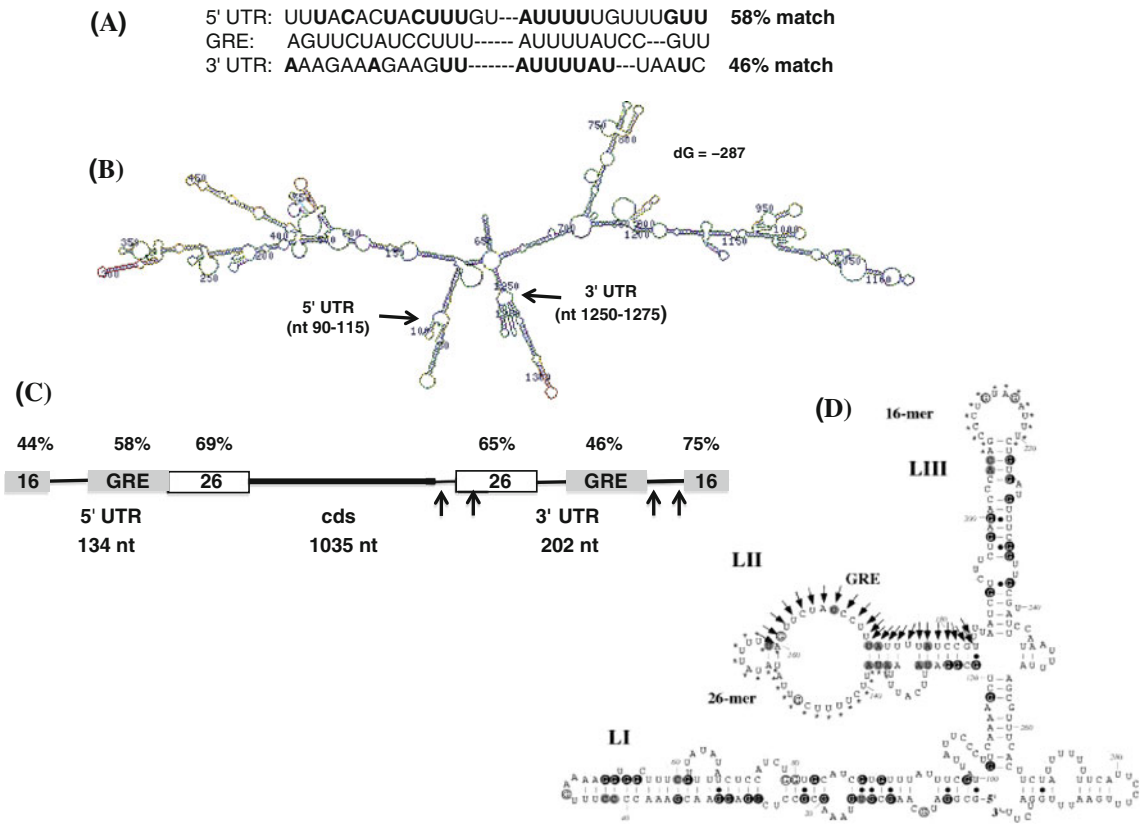

Fig. 8 The glycerol-responsive elements (GRE) of *POTH1* 5′ UTR and 3′ UTRs match the conserved sequence from the *GPEET* 3′ UTR (Vassella et al. 2004) 58 and 46 %, respectively (**a**). Location of GRE motifs (**b**, *arrows*) off Mfold model (Zuker 2003) of *POTH1* UTRs. GRE is the sequence element on RNAs recognized by alba-domain proteins (Mani et al. 2011). POTH1 mRNA with conserved motifs in the untranslated regions (**c**). The regions designated 16, GRE, and 26 represent the three conserved regulatory elements present in the secondary structure of the *GPEET* 3′ untranslated region (**d**, *arrows* and *asterisks*). The *arrows* (**c**) indicate the location of CU (polypyrimidine) motifs. The percent numbers above the conserved elements in panel **C** represent the overall sequence match to the *GPEET* elements. GRE is the glycerol-responsive element (Vassella et al. 2004). The Mfold model of the *GPEET* 3′ untranslated region (**d**) was used with permission of the author (I. Roditi)

proteins like CmRBP50. For example, all six RNAs (including a *Knotted1* type, *CmSTM*) associated with the CmRBP50 RNA/protein complex contain CU motifs in their RNA sequence (Ham et al. 2009). These motifs are recognized by PTB proteins to form ribonucleoprotein complexes that stabilize the RNA and facilitate long-distance transport. That the same PTB protein of potato binds 3′ UTR sequences from both *POTH1* and *StBEL5* (Fig. 7b) suggests a common mechanism for delivery and organ-specific localization of RNAs that encode proteins that function in a tandem transcriptional complex (Chen et al. 2004).

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