A tissue culture system facilitates examination of gene expression during breakage of vegetative dormancy in the potato tuber shoot apical meristem

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Abstract

While the shoot apical meristem's role during dormancy emergence in seeds is relatively well-understood, molecular factors governing vegetative endodormancy and sprouting in tuberous plants have not been well studied. A microtuber culture system was developed in order to study dormancy emergence in the shoot apical meristem of the potato (*Solanum tuberosum*). Microtubers were induced from subcultured shoot internodes, grown for 9 weeks, then harvested. The microtubers were hardened at 20° for 2 weeks then stored in the dark at 4°C. Subsequently, at 4-week intervals, microtubers were transferred to 20°C in the dark for 7 days. Meristem length was measured prior to RNA extraction. Two genes, *SHOOTMERISTEMLESS (STM)* and *WUSCHEL (WUS)*, are known to regulate the development of the shoot apical meristem in *Arabidopsis thaliana*. Primers for quantitative PCR (qPCR) were designed to amplifty putative potato *STM* and *WUS* homologs using expressed sequence tag (EST) sequences obtained during a basic local alignment search tool (BLAST) search of known petunia (*Petunia hybrida*) and tomato (*S. Lycopersicum*) *STM* and *WUS* sequences. Primer pairs for reference genes were obtained from the literature. Using qPCR we measured the expression level of *STM* normalized to two potato meristem reference genes, *L2* and *ef1-α*. *L2* expression increased 1.5 to 6 fold during the post harvest period while that of *ef1-α* expression was between 1 and 1.4 fold the levels found in dormant meristems immediately after harvest. By contrast, *STM* expression increased from 0.17 to 0.75 fold of week 2

values 7 weeks after harvest from 17.4 to 42.5 fold 11 weeks after harvest and increased further thereafter. The meristem length showed a statistically significant increase from 0.23 (+/- 0.01) mm to 0.33 (+/- 0.07) mm (mean +/- Standard Error) length between 7 and 11 weeks after harvest. Taken together, this data suggests dormancy break of shoot apical meristems in this microtuber culture system occurs between 7 and 11 weeks after harvest. This system will be used in the study of the molecular events occurring during dormancy break in the potato shoot apical meristem.

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Statement of Work Ownership

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2. Microtuber media and subculturing: Dr. David Law, Lauren Sinnemaki, Marlena Tassone, Christopher Edmunds.
3. Microtuber Measurements and Storage: Lauren Sinnemaki, Marlena Tassone, Christopher Edmunds
4. Primer Design: Christopher Edmunds
5. RNA isolation trouble shooting: Lauren Sinnemaki, Marlena Tassone (results not included), Christopher Edmunds
6. Endpoint PCR Troubleshooting: Lauren Sinnemaki, Marlena Tassone (results not included), Christopher Edmunds
7. RNA isolation: Christopher Edmunds
8. RNA quantification and Reverse Transcription: Christopher Edmunds
9. qPCR: Lauren Sinnemaki for the *L2* gene, Christopher Edmunds for all other genes.

Table of Abbreviations

CLV: CLAVATA

STM: SHOOTMERISTEMLESS

WUS: WUSCHEL

HAN: HANABA TARANU

qPCR: Quantitative Polymerase Chain Reaction

RT: Reverse Transcription

BLAST: Basic Local Alignment Search Tool

EST: Expressed Sequence Tag

ANOVA: Analysis of Variance

cDNA: Complementary DNA

mRNA: Messenger RNA

ABA: Abscisic Acid

SAM: Shoot Apical Meristem

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Chapter 1: Literature Review

1.0.0 Vegetative Dormancy and Agriculture

Agriculture is critical to growth, security, and economic development both in developed countries and in the poorest agriculture-based countries (http://www.potato2008.org/en/world/index.html). In agricultural crops, dormancy is an important life stage. Because of the economic importance of crop species, considerable effort has gone into the study of dormancy mechanisms that control plant growth (Anderson *et al.* 2001). Dormancy is defined here as the physiological state when a plant and its organs are incapable of visible growth under conditions which would otherwise be favourable to growth (Korableva & Ladyshenskaya, 1995). Vegetative dormancy can occur in propagules of plants such as stems, rhizomes, tubers, bulbs, stolons, creeping roots, etc (Anderson *et al.*, 2001). While dormancy in seeds is similar, seeds are a product of sexual reproduction and may thus be regulated by distinct mechanisms (Finch-Savage & Leubner-Metzger, 2006). Vegetative dormancy has also been studied in the axillary buds of perennial woody crop species such as apples (*Pyrus malus* L.) (Anderson *et al.*, 2001). In addition, much work has focused on vegetative dormancy in woody, fruit producing species (Anderson *et al.* 2001). Vegetative dormancy has been studied in the potato (*Solanum tuberosum* L.) because of its importance as a major food crop (Anderson *et al.*, 2001).

1.1.0 The Potato as a Major Food Crop

As world food prices of wheat, rice, and maize increase, the potato is being increasingly relied upon for food security (FAO). Since it is not traded on international markets as much as other staples, its price is determined by local economies rather than by world markets (FAO). This is particularly important as demand for food will increase greatly in the developing world over the next fifty years (Coleman, 2000). It is already the leading non grain food produced in the world and is the highest yielding crop per hectare (FAO; Coleman 2000). The potato is readily grown in the developing world, where consumption has increased from 10 kg per capita in 1963 to 22 kg per capita in 2003 (FAO). Unlike many other food crops, potatoes can be grown under environmental conditions that inhibit the growth of other crops including infertile or saline soils. Furthermore, 85% of the potato tuber total plant biomass (dry weight) is edible compared with cereals which are 50% edible (FAO). The aforementioned factors will force potato producers to optimize growth through differing genotype and management strategies, with a goal of optimizing yield when growing the crop in different geographical areas (Coleman, 2000). Sprouting of potato tubers diminishes the commercial and nutrional value of the organ. Increasing the understanding of the post-harvest physiology of the potato will become an important factor in maintaining local and global food security (Coleman, 2000). Despite these global concerns, study of the molecular mechanisms regulating the growth and development of this important crop is not as advanced in comparison

to other crop species such as corn and rice. There is a need to understand the physiology of potato tubers after harvest.

1.1.1 Postharvest Physiology and Agriculture

Maintenance of food shelf life is a major concern to all food producers as it impacts the commercial value of their product. The potato tubers remain physiologically dormant for a period of time after harvest (Suttle, 2004a). Depending on the cultivar, potatoes are dormant for one to fifteen weeks after harvest (Wiltshire & Cobb, 1996). As with other crop species, the nutritional and commercial value of potato tubers decreases over time (Suttle, 2004a). This is due to the hydrolysis of starch and the increase in reducing sugars which provide carbon and energy for the growing potato shoot (Trindade *et al.* 2004). However, unlike crops such as *Brassicaceae* (broccoli, cauliflower), or the closely related tomato (*Solanum lycopersicum*), potato tubers are dormant after harvest. Climacteric fruits such as tomatoes immediately increase ethylene production and respiration rates once harvested, which in turn gives them a short shelf life (Wang et al. 2008).

Potato shelf-life is maintained through temperature, chemical and light control (Wiltshire & Cobb, 1996). Sprouting generally increases at higher temperatures (Wiltshire & Cobb, 1996). However, at temperatures below 3°C the reducing sugar content increases, which decreases the value of potatoes as they darken when made into french fries (Wiltshire & Cobb, 1996). The tubers are normally stored temperatures of 4 to 8°C, in a dark, well-ventilated environment with high relative humidity (85 to 90 %)

(http://www.potato2008.org/en/potato/cultivation.html). Storage aims at preventing "greening" (the build up of chlorophyll beneath the peel, which is associated with solanine, a potentially toxic alkaloid) and losses in weight and quality (http://www.potato2008.org/en/potato/cultivation.html). Poor storage conditions results in a loss of quality and tuber size (http://www.potato2008.org/en/potato/cultivation.html). Further understanding of potato biology will aid in understanding tuber physiology. The difference in life strategies between tuber forming plants and crops such as tomatoes have implications to human food security in the future.

1.2.0 Potato Physiology

The potato is an annual, herbaceous, and dicotyledonous plant (Sarkar, 2008; Figure 1). New plants are generally propagated vegetatively by planting a piece of tuber containing an apical meristem (Figure 1). The vast majority of commercial potato agriculture uses the vegetative propagation technique (Sarkar, 2008; Jefferies and Lawson, 1991). Potato plants undergo several stages in their life cycle, including seed germination and emergence, tuber dormancy, tuber sprouting, emergence and shoot expansion, flowering, tuber development, and senescence of the shoots, flowers and leaves (Jefferies and Lawson, 1991). New potato plants can arise via sexual reproduction in the floral organs (Jefferies and Lawson, 1991). The tuber is a modified stem structure which accumulates reserve material and arises from the stolon (Coleman, 1987; Figure 1).

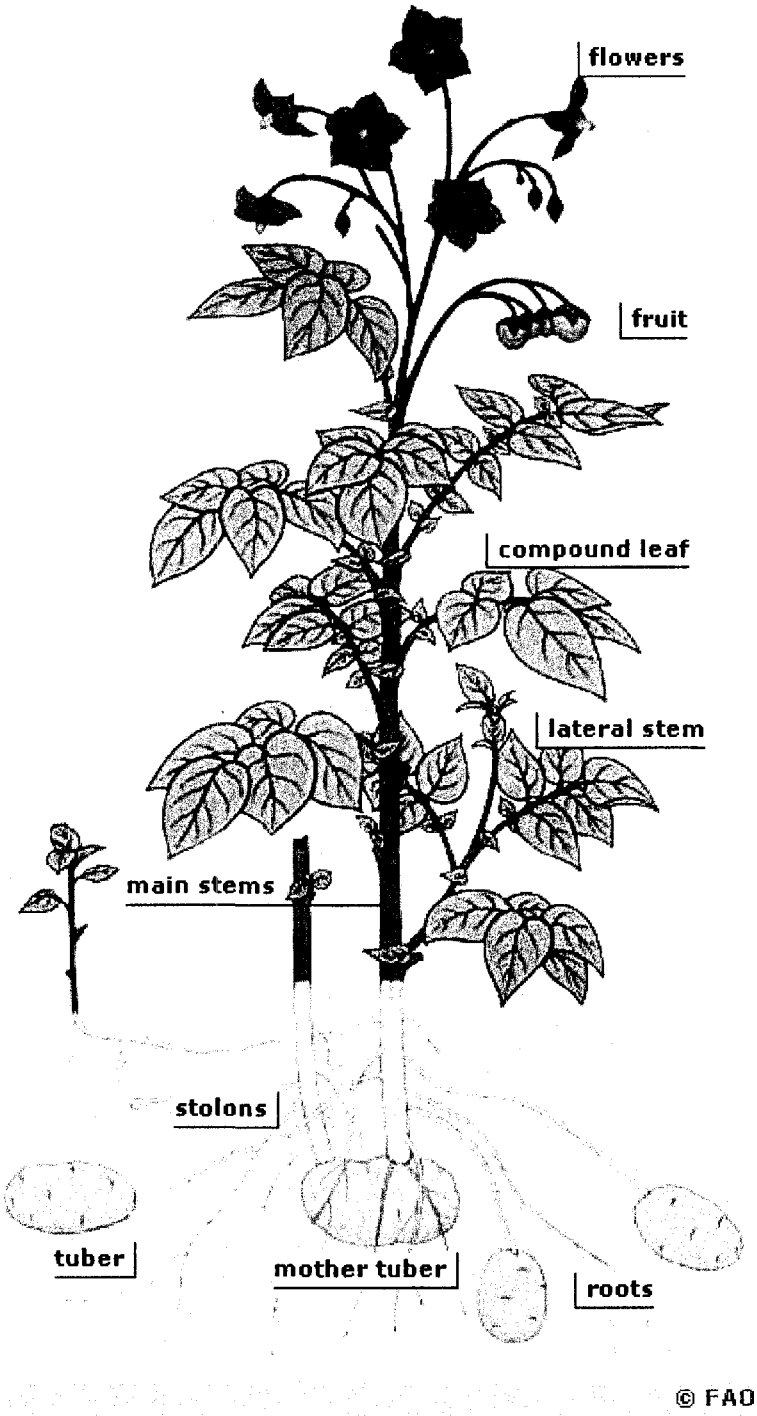

Figure 1: An image showing major functional organs within a potato plant which has been propagated vegetatively. The mother tuber contains shoot apical meristems which give rise to the main stems after dormancy ceases. The main stem then can produce underground modified stems known as stolons which may produce additional tubers (http://www.potato2008.org/en/potato/index.html).

1.2.1 Tuber Physiology in Solanum tuberosum

The specialized nutrient sink organ known as the tuber evolved as distinct mechanism for survival through vegetative dormancy, as it is more resistant to biotic and abiotic stresses than other structures within the plant (Suttle, 2004b). The potato has become a model for studying underground sink organs (Kloosterman *et al.* 2005). Potato tubers are a storage organ formed from the induction, initiation and growth of underground shoots called stolons (Sarkar, 2008). The stolon is a modified stem with a shortened broadened axis that grows from an internode beneath the soil, and elongates until tuber formation begins (Peterson *et al.* 1985; Claassens & Vreugdenhil, 2000). Favorable conditions for tuber formation and development are a short photoperiod, low soil nitrogen availability, low temperature, high sucrose and high levels of cytokinins (Claassens & Vreugdenhil, 2000; Tanaka *et al.* 2005). The tip of the stolon begins to grow radially and then cell expansion and division in the pith and the cortex begins (Wiltshire & Cobb 1996). A major biochemical change in the beginning of dormancy is the accumulation of starch (Claassens & Vreugdenhil, 2000). The amyloplasts of potato parenchyma cells are the location of starch synthesis (Claassens & Vreugdenhil, 2000; Figure 2).

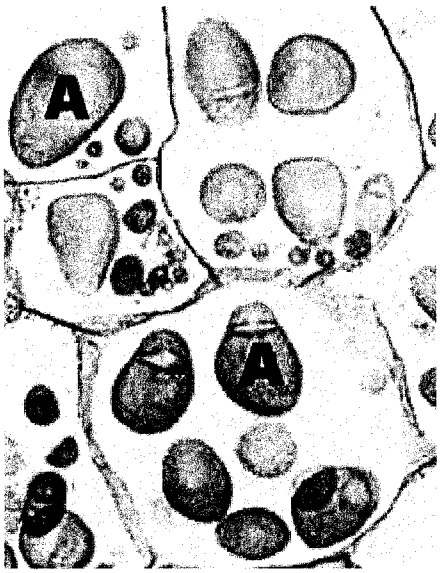

Figure 2: Parenchyma cells with amyloplasts (A) from a potato tuber (Commercial Slide).

Starch accumulates in the developing tuber and an increase in patatin, a storage glycoprotein, is also observed (Wiltshire & Cobb, 1996). The tuber is described as a stem with nodes, internodes, and axillary buds. During the growth of the tuber, the shoot apical meristems in the eyes of the swelling stolon become dormant (Claassens & Vreugdenhil, 2000). The meristems are totipotent and dormant for a variable period of time after harvest (Wiltshire & Cobb, 1996).

The molecular events surrounding tuber formation have been the subject of some research. Gene expression, enzyme activity, carbohydrate transport and chemistry, as well as protein accumulation have all been extensively researched (Appeldoorn *et al.* 1997; Bachem *et al.* 1996). However, the molecular processes regulating meristem sprouting following dormancy emergence are poorly understood.

1.2.2 Tuber Dormancy and Sprouting

There are several definitions of tuber dormancy. Korableva and Ladyshenskaya (1995) define dormancy as the physiological state in which a plant and its organs are incapable of visible growth under apparently optimal conditions. Wiltshire and Cobb (1996) consider dormancy to begin at tuber initiation and end when the meristem is capable of extension growth. Dormancy is also considered the physiological state of the tuber in which autonomous sprout growth will not occur even placed under ideal conditions for sprouting e.g. (darkness, temperature 15-20°C, 90% relative humidity) (Reust, 1986). A more specific definition was proposed by Jefferies and Lawson (1991), who

differentiated innate dormancy (the postharvest period when meristem growth is suppressed from enforced dormancy). Furthermore, dormancy can be subdivided into three distinct definitions depending on its control (Campbell *et al.* 1996). Ecodormancy is defined as growth arrest in meristems due to external environmental factors such as photoperiod or temperature. Paradormancy is defined as growth arrest in meristems due to external physiological factors. For example a dominant bud can arrest the growth of other buds (Vreugdenhil, 2007). Endodormancy is growth arrest in meristems due to internal physiological factors (Campbell *et al.* 1996). The role of endodormancy can be thought of as inhibiting the growth of structures such as tubers, bulbs, and seeds if conditions are temporarily favourable yet likely will get worse, for example in late autumn (Vreugdenhil, 2007).

The biochemical changes leading to sprouting at the end of endodormancy have begun to be investigated (Law & Suttle, 2004; Agrimonti *et al.* 2000; Coleman & Coleman, 2000). A breakdown of starch in the tuber has been observed during sprouting (Davies & Viola, 1988; Davies & Ross 1987). Starch phosphorylase (STP) catalyzes a reversible reaction between starch $_n$ and starch $_{n-1}$ plus glucose -1-phosphate (Claassens & Vreugdenhil, 2000). However, its activity is more often observed during starch degradation at the end of dormancy (Claassens & Vreugdenhil, 2000). α-amylase activity is also increased at the end of dormancy (Claassens & Vreugdenhil, 2000). The function of the starch degradation is to provide substrates for glycolysis and thus C-skeletons for anabolism and ATP synthesis that power heterotrophic plant

growth. Several studies have investigated the role of plant growth regulators in these processes.

1.2.3 Plant Growth Regulators and Potato Dormancy

Much research has focused on the hormonal and environmental control of tuber dormancy (Suttle, 2004b; Wiltshire & Cobb, 1996; Coleman, 1987). Dormant tubers are still metabolically active, although at a lower rate than rapidly growing plant organs (Wiltshire & Cobb, 1996). Suttle (1998) showed that ethylene is produced during tuber development, and tubers exposed to ethylene production inhibitors such as silver nitrate or 2,5-norbornadiene resulted in premature sprouting. While abscisic acid (ABA) does not appear to play a role in tuber formation (Xu *et al.* 1998), Suttle & Hultstrand (1994) showed that it is important in dormancy maintenance. While these results suggest that ABA is directly involved in the initiation and possible maintenance of dormancy, there appears to be no relationship between tuber ABA levels and dormancy break (Suttle & Hultstrand, 1994). The application of gibberellins in the very early stages of tuber development results in stolon formation at the apical meristem of the tuber and the reversion of tuber development to a stolon-like development (Claassens & Vreugdenhil, 2000). The addition of gibberellins at a later stage of development results in sprouting of the shoot apical meristem (Claassens & Vreugdenhil, 2000). During tuber development, the concentration of gibberellins needed to break dormancy increases and then decreases near the end of endo-dormancy (Claassens & Vreugdenhil, 2000). Endogenous gibberellins decrease before tuber formation and the level remains low during tuber growth. These

findings have led Claassens & Vreugdenhil (2000) to suggest that endo-dormancy breaking is the reverse of dormancy initiation. In summary, auxins have not been implicated in dormancy break, however are needed for shoot growth after dormancy break (Suttle, 2004b). Similarily, gibberellins are not thought to directly break dormancy, however, they are involved in shoot elongation afterwards. ABA is needed for tuber formation and dormancy, however has not been shown to regulate dormancy break (Suttle, 2004b). Ethylene has been shown to prevent dormancy break, however, its possible interaction with ABA has not been characterized in potato tubers (Suttle, 2004b). Cytokinins are likely candidates for being directly involved in dormancy break. As they stimulate cells in a G-1 cell cycle block, and bud meristems are in the G-1 phase of the cell cycle, they are expected to break dormancy (Campbell *et al.* 1996) and have been shown to do so (Suttle, 2004b). The molecular control of sprouting has also been investigated but has not yet been definitively linked to the signal transduction chains controlled by plant growth regulators.

1.2.4 Molecular Control of Dormancy and Sprouting

Several studies have been conducted to determine the molecular events surrounding tuber induction, dormancy, and sprouting. A common theme of these studies is that a coordinated regulation of many metabolic pathways is required for all three processes (Kloosterman *et al.* 2005). Synthesis of DNA, RNA and proteins continuously occurs, although these activities increase when dormancy break occurs (Law and Suttle, 2003; McDonald & Osbourne, 1988). Sprouting is likely to be controlled by interacting genes expressed throughout the

tuber in addition to genes that are expressed in the meristem (Trindade *et al.*, 2004). It has also been shown that more genes are expressed during tuber formation and tuber sprouting than are expressed during dormancy (Trindade *et al.* 2004; Bachem *et al.* 2000). However, the biochemical and molecular events that control sprouting are largely unknown (Trindade *et al.* 2004; Agrimonti *et al.* 2000).

Some of the work that has been done has characterized epigenetic changes in potato meristems during dormancy break (Law & Suttle, 2005). When cytosine is methylated to 5-methylcytosine in a promoter region, gene silencing can occur (Law & Suttle, 2005). Changes in 5-methylcytosine content in potato tuber meristem DNA have been shown to occur during dormancy break (Law & Suttle, 2003). Furthermore, histone acetylation has been shown to occur during natural or chemically induced dormancy break in the meristems of tubers (Law & Suttle, 2004). Promoter regions are hypothesized to require histone multiacetylation in order to allow transcriptional activators to bind to promoter regions (Struhl, 1998). In fact, Law and Suttle (2004) found that histone H3.1 and H3.2 showed increased histone multi-acetylation during dormancy emergence in potato meristems. There is a definite difference between dormancy and sprouting in both meristems and tubers, which implies that transcription of a distinct set of genes is tightly regulated during this developmental transition (Agrimonti *et al.* 2000). The identification of such genes would increase understanding of dormancy break (Agrimonti *et al.* 2000). Agrimonti *et al.* (2000) recently used differential display reverse transcriptase

polymerase chain reaction (DDRT-PCR) to analyze two cDNAs during dormancy break. These two cDNAs, putatively named *G1-1* and *A2-1*, were up-and down-regulated, respectively. *A2-1 in silico* analysis showed this protein coded for a putative ATPase, however it is not known why this gene was down regulated (Agrimonti *et al.* 2000). This study focused on whole tuber tissue samples.

1.3.0 Microtuber Model Systems

For molecular studies, using a microtuber culture system possesses several advantages over the study of field grown potatoes. Since it is impossible to predict when stolons begin to tuberize, field potatoes grown in an agricultural setting will be at disparate and unknown physiological states following harvest and in storage (Appeldoorn *et al.* 1997). Furthermore, studies by Sung *et al.* (1989) have shown that field tubers of the same size show considerable variation in their rates of growth and development. A more controlled *in vitro* system may be used in place of field tubers, as every potato internode has the capability of forming a tuber (Appeldoorn, *et al.* 1997). Microtubers are easily produced by transplanting single nodes of shoot tissue into sterile high sucrose, low nitrogen media and cultivating them in an environment with a short photoperiod and cool temperatures (Claassens & Vreugdenhil 2000; Coleman & Coleman, 2000). When using an *in vitro* system, sprouting has been shown to be far more synchronized (Claassens & Vreugdenhil, 2000). Microtuber formation is therefore more simultaneous than field tuber formation (Claassens & Vreugdenhil, 2000). In stored tubers, potato sprouting occurs over a long period (Claassens & Vreugdenhil, 2000). To shorten the sprouting period, either Rindite

(7:3:1 anhydrous ethylene chlorohydrin ; ethylene dichloride ; carbon tetrachloride) or carbon disulphide may be applied to tubers exiting endo-dormancy, however these chemicals are highly toxic (Claassens & Vreugdenhil, 2000). Gibberellic acid application in conjunction with wounding is a possible method to study coordinated tuber sprouting in the future (Claassens & Vreugdenhil, 2000). The simplest way to study dormancy break in tubers is a time course study conducted over a series of months while they naturally emerge from endodormancy.

Microtubers appear reasonably analogous to field grown tubers. For example, Pathirana *et al.* (2008) showed that the formation of hexose sugars during cold storage in microtubers is analogous to the process in field tubers. Addition of charcoal to nutrient media greatly enhances microtuber growth, as it may absorb inhibitory metabolites such as phenylacetic acid and p-OH-benzoic acid (Bizzari *et al.*, 1995). Microtubers have shown correlation with field grown potatoes in their development and behaviour (Leclerc *et al.* 1995). Gopal *et al.* (1998) showed that in addition to the length of the photoperiod, the amount of available sucrose, and temperature, genotype had an important influence on microtuber induction. All of these factors control tuber shoot apical meristem development and contribute to the timeframe of tuber sprouting.

1.4.0 Shoot Apical Meristems

The shoot apical meristem is a highly organized structure (Sussex, 1989). Cells derived from this meristem form all the above-ground portions of the plant (Bowman & Eshed, 2000). The meristem is represented by three distinct zones

each with its own cell division rate (Bowman & Eshed, 2000). The eventual fate of each cell depends on its position in the meristem. The peripheral zone produces the lateral organs of the plant (Bowman & Eshed, 2000). The rib zone, located basal to the central zone, produces the stem tissue (Bowman & Eshed, 2000; figure 3). The apical central zone maintains a reservoir of self-renewing stem cells which continuously replenish the other two zones (Bowman & Eshed, 2000; Williams & Fletcher, 2005). Stem cells are capable of generating differentiated daughter cells while they have unlimited self-renewing capacity. Despite this, they themselves divide only infrequently (Stahl & Simon, 2005). The development of plants, from potatoes to trees, is dependent on the fine maintenance of both the stem cell pool and the meristem (Bhalla & Singh, 2005). The stem cells produce daughter cells which have two types of fate (Williams & Fletcher, 2005). Daughter cells that remain in the center continue to function as stem cells and those that leave the central zone begin dividing more rapidly and become the tissues of the plant. The organization and timing of embryogenesis in *A. thaliana* is controlled by shoot apical meristem stem cells (Weigel & Jurgens, 2002). Many *Arabidopsis* mutants have been screened and the functions of several genes important in embryogenesis have been deduced.

Bowman and Eshed, 2000

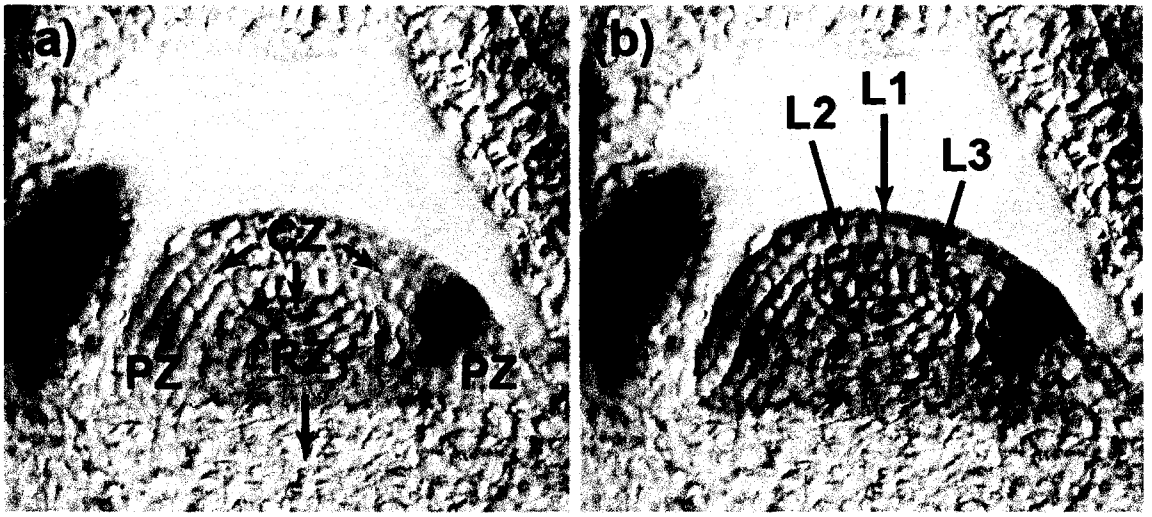

Figure 3: The shoot apical meristem of *A. thaliana.* The central zone (CZ), rib zone (RZ) and peripheral zones (PZ) are shown in (A). Layers one (L1), two (L2) and three (L3) are shown in (B). In the apex or central zone, there is a stem cell reservoir. The fine maintenance of the stem cell reservoir and the shoot apical meristem is crucial to the development of plants.

Directly underneath the stem cells, a group of cells called the organizing center expresses a transcription factor encoded by the gene *WUSCHEL (WUS)* (Weigel & Jurgens, 2002). *WUS* is the earliest gene expressed during embryonic plant development (Bowman & Eshed, 2000). *WUS* was originally discovered as a mutation which specifically disturbed shoot and floral meristem development in *Arabidopsis* (Laux *et al.* 1996). Another gene, *SHOOTMERISTEMLESS (STM)*, prevents the differentiation of cells (Weigel & Jurgens, 2002). In *Arabidopsis*, *STM* expression begins in the late 16 cell globular embryo (Long and Barton, 1998). In mutants lacking *STM*, the maintenance of the meristem fails, and it disappears because the stem cell pool is not maintained (Weigel & Jurgens, 2002). In *Arabidopsis*, the earliest acting genes *WUS* and *STM* are required for establishment or maintenance of stem cell fate, whereas genes expressed later are involved in regulating the size of the central zone (Bowman & Eshed, 2000). After the initial globular stage, the embryo of *Arabidopsis* enters the heart stage, where the size of the meristem increases dramatically, and adjacent leaf primordia become visible (Bowman & Eshed, 2000). During the heart stage, the expression of *STM* spreads throughout most of the meristem but not into the emerging leaf primordia (Weigel & Jurgens, 2002; Bowman & Eshed, 2000). During the heart stage, a transcription factor encoded by *WUS* stimulates the central zone cells to remain as stem cells and they in turn express *CLAVATA3* (*CLV3*). The mechanism by which *WUS* stimulates *CLV3* is unclear (Weigel & Jurgens, 2002). *CLV3* encodes a small secreted polypeptide (Rojo *et al.* 2002; Lenhard & Laux, 2003) that is hypothesized to interact with a *CLAVATA1* (*CLV1*)

- *CLAVATA2* (*CLV2*) receptor complex in the organizing center. Trotochaud *et al.* (1999) proposed a model of *CLV1-CLV3* interaction leading to the phosphorylation of downstream transcription factors which in turn inhibits transcription of *WUS*. *CLV1* was originally cloned by Clark *et al.* (1997), who hypothesized that its function was a signal transduction component acting in communication of cell division and/or differentiation. *CLV1* was originally discovered in *Arabidopsis* as a mutation that caused extra floral organs and progressive enlargement of the shoot and floral meristems (Leyser & Furner, 1992). Jeong *et* al. (1999) showed that *CLV2* encodes a receptor like protein and demonstrated that it is required for *CLV1* protein accumulation. A study by Lenhard & Laux (2003) caused the complete failure of the shoot apical meristem by using an *ATML1* promoter to express one copy of *CLV3*. When five copies of *CLV3* were expressed using *pCLV3::(CLV3)5* expressing plants, the meristem was merely reduced in size (Lenhard & Laux, 2003). Since the high level of *CLV3* led to a downregulation of *WUS* expression, which in turn reduced transgene expression level, a new balance in the *WUS-CLV3* feedback loop was established (Lenhard & Laux, 2003). This study indicated that a small amount of *CLV3* transcript copies is needed to maintain the meristem. Many Solanaceous species have had their *STM*, *WUS* and *CLV3* homologs sequenced and characterized, however at this time *S. tuberosum* has not (Gerco *et al.* 2005). The analysis of these genes would be beneficial to the understanding of potato tuber shoot apical meristem regulation and development.

1.5.0 Gene Expression Analysis

Cells in a multicellular organism alter the expression of genes in response to certain extracellular cues or conditions. This expression can be regulated at the DNA, RNA and protein levels (Alberts *et al.* 2002). More specifically, an organism can control the expression of a given gene by altering (a) how often that gene is transcribed into RNA, (b) splicing or processing of the mRNA or (c) the transport of the mRNAs from the nucleus to the cytosol (Alberts *et al.* 2002). Additionally, in the cytosol the mRNA can be turned over. Finally, protein products can be activated, deactivated, degraded or compartmentalized to increase or reduce their activity in the cell (Alberts *et al.* 2002). As genomes are sequenced, the problem becomes analyzing expression of genes and their protein products and relating this information to changes in metabolism in the cell (Storey *et al.* 2004). Since RNA is a relatively easily accessible molecule in the cell, it is a commonly assayed level of gene expression and is readily amplified using RT-PCR (Farrell, 2005). RT-PCR is therefore an extremely widely used technique to study RNA expression in an organism (Farrell, 2005).

1.5.1 Predicting Metabolic Changes Using Gene Expression

Expression analysis of genes and their products can be used to further understand metabolism and its control (Storey *et al.* 2004). This is a significant challenge, because despite the large number of techniques available for analyzing gene expression, the properties of a functional protein or the kinetic or regulatory properties of an enzyme cannot be deduced solely from genetic analysis (Storey *et al.* 2004). Many organisms that have had their genomes

sequenced have not had their metabolism studied, although the prediction of metabolic phenotype can sometimes be made from gene sequences. The total amount of a given enzyme is dependent upon the relative rates of synthesis and degradation of mRNA (Storey *et al.* 2004). Since most genes encode enzymes, the level of expression of DNA, RNA and proteins can be seen as "coarse" metabolic control (Storey *et al.* 2004). Consequently, the expression of any enzyme encoding gene elucidated by qPCR can be used to predict coarse metabolism. Since metabolic activity increases with dormancy break in potato tuber meristems, it is expected genes encoding metabolic enzymes will increase their expression during dormancy break.

1.6.0 Quantitative Reverse Transcription Polymerase Chain Reaction (qPCR)

The implementation of PCR in life science has fundamentally influenced all aspects of basic research. Since small quantities of template material can be amplified in a few hours, this technique's enormous potential for advancing research has become apparent over the past several decades (Farrell, 2005). qPCR has become the method of choice for the quantification of mRNA (Nolan *et al.* 2006). A qPCR assay consists of a combination of three steps. These steps are the reverse transcriptase dependent conversion of RNA into complementary DNA (cDNA), the amplification of cDNA using PCR, and the detection and quantification of amplification products in real time (Nolan *et al.* 2006). The reverse transcription (RT) of RNA into cDNA followed by PCR is presently the most sensitive method for the detection of specific RNA molecules and have

largely supplanted less sensitive and more cumbersome techniques such as northern blotting (Cikos *et al.* 2007).

1.6.1 Reverse Transcription

Correct reverse transcription of RNA into cDNA is a crucial step in any qPCR assay. cDNA is the product of an enzymatic *in vitro* synthesis using RNA as template material (Farrell, 2005). The first requirement of successful reverse transcription is the presence of an intact mRNA molecule in the reaction mixture (Farrell, 2005). As polynucleotides are assembled in the 5'->3' direction, the primer molecule that provides the necessary 3'-OH group must be close to the 3' end of the mRNA template if the reaction is to be successful (Farrell, 2005). As mRNA cannot be amplified with PCR directly, it is reverse transcribed to double-stranded cDNA using the enzyme reverse transcriptase (Nagaraj *et al.* 2007). Complementary DNA is synthesized stepwise, one strand at a time, from an aliquot of total RNA or poly (A)+ mRNA (Farrell, 2005). Heat denaturation to disrupt RNA secondary structures is followed by annealing of a short poly (T) primer to the poly (A) tail of the template mRNA (Farrell, 2005). The 3'OH group associated with the primer provides the means to support the 5'->3' synthesis of cDNA (Farrell, 2005). Alternatively, random hexamer primers or mixtures can be added to the reactions and result in internal priming of first strand cDNA rather than priming from the 3' end as with poly T primers (Farrell, 2005). Complementary DNA is synthesized from RNA templates and in total represents the RNA complexity present in the cells at the moment of total RNA isolation

(Farrell, 2005). The amount of cDNA produced in the reaction must accurately represent the amount of RNA template (Bustin, 2002).

1.6.2 qPCR

qPCR uses a fluorescent signal to measure PCR product accumulation (Cikos *et al.* 2007). This fluorescent signal is generally a compound which is planar and fits itself (intercalates) between the bases within the DNA strand. qPCR's simplicity and ease of use have made it the most widely used *in vitro* mRNA quantification assay (Bustin, 2002). With qPCR, it is not necessary to know the exact amount of mRNA molecules present in a sample, as target mRNA quantities can be compared to those of one or more internal control mRNAs (Cikos *et al.* 2007). There are a number of commercially available fluorescent molecules that can be used to measure amplification of a target sequence. SYBR green is among the least expensive and works by intercalating with double-stranded DNA molecules (Farrell, 2005). When bound to double-stranded DNA this molecule absorbs blue light and fluoresces green light which is detected by the qPCR instrument. A certain threshold fluorescence value is set for every PCR reaction, which represents a point where the fluorescence of the PCR product is greater than the background fluorescence (Cikos *et al.* 2007). Since the threshold fluorescence is the same for every reaction, the amount of PCR product at the threshold for any reaction is the same and the fluorescence generated above this threshold is proportional to the amount of DNA in the reaction (Cikos *et al.* 2007; Nolan *et al.* 2006). The number of PCR cycles needed to reach the threshold for a sample is known as the cycle threshold (Ct)

value. In general, the higher the quantity of target mRNA template present at the beginning of PCR, the fewer PCR cycles are needed to reach the Ct (Cikos *et al.* 2007). The success of this assay is highly dependent on the quality and quantity of starting template and optimal assay design, as detailed below (Nolan *et al.* 2006).

1.6.3 Primer Design

A primer is defined as a short single stranded DNA sequence which is artificially synthesized in order to complement and anneal to the gene of interest. In order for PCR to function properly, the investigator must have some knowledge of primer sequences. The sequences, which flank the fragment of DNA to be amplified, must be known because site directed complementarity is necessary to anneal and amplify the correct region of DNA. The primers must be designed so that they will base-pair to opposite strands of the heat denatured template, such that their 3' ends face one another. The primers must be equimolar in the reaction mixture as each strand of template must be amplified symmetrically. Primers 20-25 base pairs long are generally specific enough to amplify the gene of interest. The primers must not have a mismatch at the 3'-OH terminus, as the 3'-OH base (or ultimate base) absolutely must be complementary to the sequence of interest (Farrell, 2005).

1.6.4 Expressed Sequence Tags (ESTs)

EST sequencing provides clues as to genes whose expression varies under different environmental or developmental conditions (Dembinsky et al, 2007). ESTs are short 100-800 base pair sequences of complementary DNA

derived from mRNA that is expressed in an organism. Messenger RNA in the cell is reverse transcribed to form a large number of cDNA molecules. cDNA molecules are then cloned to make libraries representing a set of transcribed genes of the original cell. The clones are sequenced randomly in both directions to obtain 5' and 3' ESTs (Nagaraj *et al.* 2007). ESTs enable gene discovery, aid genome sequencing, and are used in proteome analysis (Nagaraj *et al.* 2007; Rudd 2003). The cDNA libraries from which ESTs are derived are subject to sampling bias and may not represent low abundance transcripts. In addition, they may account for only 60% of an organism's genes (Bonaldo *et al.* 1996). Despite these concerns, ESTs are an effective tool for organisms whose genome is not completely sequenced (Nagaraj *et al.* 2007). They are used extensively when attempting to analyze expression levels of developmentally important genes in non-model species such as the potato.

1.6.5 Melt Curve Analysis During qPCR

During qPCR, competitive side reactions can occur because Taq polymerase may amplify from double stranded primer-primer interactions (primer dimmers), single primer hairpins, or other small amplification artifacts (Bustin, 2002). Melt curve analysis is a powerful way to distinguish the desired amplification products from other undesired small products and is used to optimize real time PCR conditions. DNA will loosen its double stranded helical structure at a certain temperature determined by the length and GC content of the amplified DNA. The melting temperature (Tm) of a PCR product is defined as the temperature at which half of the DNA structure has melted. Since the

desirable result of PCR is a large amount of a single DNA fragment, the melting of this product occurs at a given temperature. As the real time PCR instrument heats the products in the tube, the DNA steadily unwinds releasing fluorescent molecules (e.g. SYBR green) bound to the double stranded DNA. The declining fluorescence is continuously monitored as the two strands of DNA unwind and melt peaks are calculated by taking the first negative derivative (-dF/dT) of the curve. As the derivative curve shows peaks upon the melting of each PCR product, a single peak indicates a single PCR product while multiple peaks represent multiple products. Since the desired target PCR products are longer than the undesired products, the desired products produce a higher Tm than the undesired products (Nolan *et al.* 2006).

1.6.6 qPCR assay in Solanum tuberosum

Our objective in conducting gene expression assays in potato using qPCR is to determine which genes increase their expression during dormancy break and normalize these values to suitable internal controls. Nicot *et al.* (2005) studied several genes in potato tubers under biotic and abiotic stress. A suitable internal control gene's mRNA transcript levels should remain reasonably consistent under different types of environmental and developmental conditions. Typically, the internal control is known as a housekeeping gene or reference gene and often encodes a protein that functions in routine cell processes, such as the enzymes involved in glycolysis (Nicot *et al.* 2005). Quantifying reference gene expression in each sample provides a baseline for simple correction of the expression of genes of interest between experiments. Though the assay of a

housekeeping gene is essential in qPCR, there appears to be no reference transcript which will be suitable in all circumstances. For this reason, it is essential that the housekeeping genes to be used as references are tested in the experimental system to ensure their expression changes are minimal (Farrell, 2005). In potato tubers some work has been done to identify suitable reference genes. Nicot *et al.* (2005) found that out of 9 genes whose transcript levels were measured under salt, cold, and late blight exposure, the genes *L2*, adenosine phosphoribosyl transferase (*aprt*) and elongation factor 1-α (*ef1-α*) were among those that varied the least under all conditions. *L2* encodes a ribosomal protein which constantly functions to synthesize new protein (Nicot *et al.* 2005). The *aprt* gene encodes an enzyme which converts adenosine to adenosine monophosphate in plants (Itai *et al.* 2000). The protein synthesis factor elongation factor 1-α has been shown to bundle and sever microtubules (Durso and Cyr, 1994; Shiina *et al.* 1994). Given that Trindade *et al.* (2004) showed that more genes were expressed during sprouting than during dormancy, we anticipate therefore that gene expression analysis can be used to probe the expression of genes specific to dormancy break. The expression levels of these genes are expected to change significantly during the transition of the meristem out of vegetative dormancy.

1.6.7 Calculation of Fold Induction

The calculation of fold induction of a gene using the "delta-delta-Ct" method is a relative quantification of the amount by which a target gene is expressed at a given experimental point while normalizing it to an endogenous

reference. The mathematical formula is $2^{-\Delta\Delta ct}$ (Applied Biosystems, 1997). The derivation of the equation comes from the formula which describes exponential amplification during PCR. The fold induction calculation of a target transcript normalizes its expression to a reference transcript for both control, and experimental points. It then compares these two values and gives a relative quantification of target expression while assigning the control a value of 1.

1.7.0 Research Objective

The first objective of this research was to develop a microtuber model system that provides microtubers that are analogous to field-grown tubers. The second objective was to develop primers for *WUS* and *STM* homologues in potato meristems in order to successfully amplify and successfully quantify these genes. While the sequences of these genes is known for many other Solanaceous species, they have not yet been characterized in *S. tuberosum*. The third objective was to use meristem length data and qPCR to determine when dormancy break occurs microtubers generated under our laboratory conditions. Taken together, this initial characterization of potato meristem dormancy break will allow us to further investigate the molecular events that accompany this developmental shift.

Chapter 2: Materials & Methods

2.0.0 Microtuber & Potato Shoot Culture and Treatment

2.0.1 Plant Material

Locally-grown potato (*Solanum tuberosum* cv. "White") tubers were obtained commercially in late September, hardened at room temperature for 2 weeks and subsequently stored in the dark in a cold room at 3-4°C. The following January, after the tubers had exited endodormancy, they were brought to room temperature and left in the dark for 4 weeks. After this period, the meristems had grown to ~2 cm long. They were excised, surface sterilized by a 1-min immersion in a 1% (w/v) sodium hypochlorite solution (e.g., 20% (v/v) commercial bleach diluted in deionized water), then rinsed in 5 changes sterile distilled water. The excised meristems were surface dried on sterile paper towels, then placed in 16 x 100 mm tissue culture tubes (Fisher) that contained potato shoot medium (see below) and grown at 20°C using a 16-h photoperiod. The etiolated shoots rapidly greened (within 1 week) when grown under these conditions.

2.0.2 Potato Shoot Media

Potato shoot media was made as detailed in Suttle & Hultstrand (1994). The volume of the media was generally 500 ml per subculture to allow 100 tubes for single node explants at 5 ml per tube.

2.0.3 Microtuber Induction Media

The microtuber media recipe was obtained from Suttle & Hultstrand (1994). Microtuber media (Suttle & Hultstrand, 1994) was made for 20 magenta

boxes (Fisher), each containing 100 ml of media. The total volume of media was 2000 ml.

2.0.4 Potato Shoot Subculture and Microtuber culture

Potato shoots, suitable for microtuber induction, were subcultured approximately every four weeks (Figure 4).

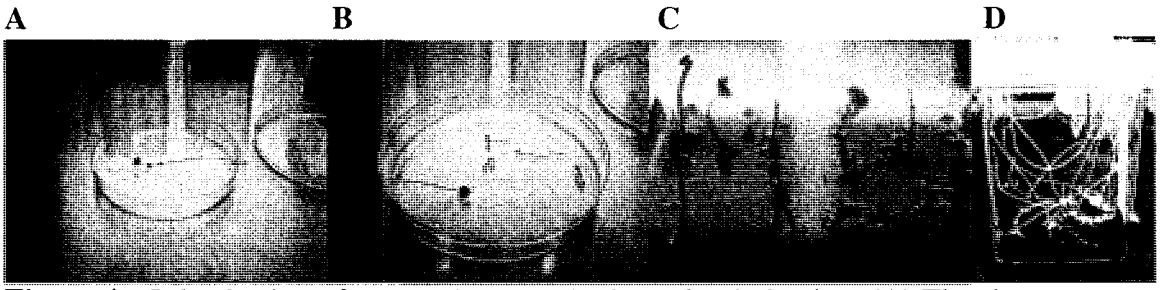

Figure 4: Subculturing of potato shoots and microtuber induction. (A) The shoots are removed and placed on a sterile Petri dish (Fisher). (B) A sterile scalpel is used to cut above each node on the the lower 1/3rd of shoot stems. (C) The explants from the lower 1/3rd are placed in microtuber induction media. The upper 2/3rds of the shoot is cut above each node and placed into new shoot media. (D) 9 weeks after culturing the shoots, microtubers have grown and are ready for harvest.

Microtubers were also cultured at the same time, as they were made using shoot tissue. The maximum suitable time for subculture of shoot tissue was nine weeks as the explants begin to outgrow the culture tube and run out of available media. The shoots were removed from previous single shoot culture tubes under sterile conditions. The shoot tissue was cut with a scalpel above every node in a biological safety hood. Under sterile conditions, a single node explant (each explant contains a node, a leaf, and the internode below the node) from the bottom third of the shoot was placed into the microtuber induction media. The single node explants for microtubers were planted in a 5 x 5 grid in each magenta box for a total of 25 explants per box. Single node explants from the top 2/3rd of the shoot were individually placed into sterile culture tubes containing 5 ml media per tube. The shoots were cultured as for the initial shoot cultures in 2.0.1.

2.0.5 Microtuber Growth, Harvest, and Treatment

The microtubers were produced in a growth cabinet over 9 weeks at 20°C in continuous darkness. At the end of the 9 week period, the microtubers were harvested under sterile conditions. Residual stems and new stolon growth were trimmed off the tuber with a sterile scalpel and about 15 microtubers were placed in each of 6 sterile Petri dishes. The harvested tubers were placed for 2 weeks at 20°C in continuous darkness in order to let their periderm harden and to allow them to enter dormancy. At the end of 2 weeks, 1 petri dish of microtubers was removed from the growth cabinet. The remaining microtubers were placed at 4°C in continuous darkness and were removed every 4 weeks afterwards.

2.0.6 Microtuber Measurements and Storage

At four-week intervals after harvest, the microtubers were brought to 20°C in darkness for one week, then observed under a dissecting microscope. Dead or shriveled microtubers were discarded. Shoot apical meristems were removed from healthy microtubers by thinly slicing them with a sterile scalpel on either side of a meristem such to create a disc containing an intact meristem. The disc was then cut on either side to create a rectangle which again retains the meristem. The meristem was measured using a microruler (Carl Zeiss) mounted in a dissecting microscope and noting the length from the surface of the periderm to the tip of the meristem. The meristem proper was then removed and placed into a sterile 1.5 ml Eppendorf tube on dry ice. Pooled meristems were subsequently stored at -80°C. Each tube contained 5 to 15 meristems. The means and standard deviations of length measurements were calculated across all batches of microtubers.

2.1.0 Gene Expression Analysis

Before gene expression analysis could be conducted, primers for all four genes investigated (*STM, WUS, L2, and ef1-α*) were tested using endpoint PCR. Gene expression analysis consisted of isolating RNA from pooled meristems for every time point, quantifying the RNA concentration, conducting expression assays using qPCR, and normalizing the results to the expression of a suitable reference gene.

2.1.1 Total RNA Isolation

The isolation of total RNA was performed by following the TRIzol™ manufacturer's protocol (Invitrogen) with some additional details and additional reagents. Prior to the isolation of total RNA, mortars and pestles were baked in aluminum foil at 200°C for at least 4 hours, then stored at -20°C. Meristem samples were removed from the -80°C freezer and immediately placed on dry ice. The mortars and pestles were chilled using liquid nitrogen and tissue samples added and ground to a fine powder. The starting fresh weight of tissue for extraction was approximately 30 mg. 500 µl of TRIzol™ reagent (Fisher) was added while grinding continued. This was pipetted into an RNAse free tube and the mortar was washed with another 500 µl of TRIzol™ reagent. The sample was incubated for 5 minutes at room temperature. 200 µl of chloroform was added and the tubes were shaken vigorously by hand for 15 seconds. The sample was then incubated again at room temperature for 3 minutes followed by centrifugation at 12,000 x *g* for 15 minutes at 4°C. The upper aqueous phase containing the total RNA was collected and transferred to a fresh tube. 250 µl of isopropanol and 250 µl of high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl) was added to the tubes and mixed by inversion in order to precipitate total RNA. The samples were incubated at room temperature for 10 minutes. The samples were then centrifuged at 12,000 x *g* for 10 minutes at 4°C. The supernatant was gently discarded and the RNA pellet was allowed to air dry for a few minutes. One ml of cold 75% (w/v) ethanol (stored at -20°C) was added to the tubes, which were then vortexed to release the pellet. The tubes were

then incubated at -20°C for at least 1 hour. Following incubation, the tubes were centrifuged at 7,500 x *g* for 5 minutes. The ethanol was carefully decanted and the RNA pellet was allowed to air dry. Finally the RNA was dissolved in 20 µl of DEPC ddH_2O by pipetting the solution a few times. The dissolved RNA was aliquoted by placing 2 µl into each tube in preparation for the next procedure.

2.1.2 Quantification of Total RNA

Total RNA quality and quantity were analyzed using the BIO-RAD Experion Automated Electrophoresis Station. The analysis provides a chromatogram showing 28S, 18S and 5S ribosomal RNA subunit fluorescence which is used to analyze RNA quality and concentration. The Experion system uses microfluidic technology to automate electrophoresis for protein and nucleic acid analysis. The system uses a microfluidic chip with a series of plastic wells bonded over a glass plate which is etched with microchannels (Figure 5). Once these channels are primed with gel matrix and samples are pipetted into the wells, the station directs the samples through the microchannels by controlling precise voltages and currents. The analysis was performed by the manufacturer's instructions (BIO-RAD). The station electrodes were cleaned with DEPC treated water after each analysis.

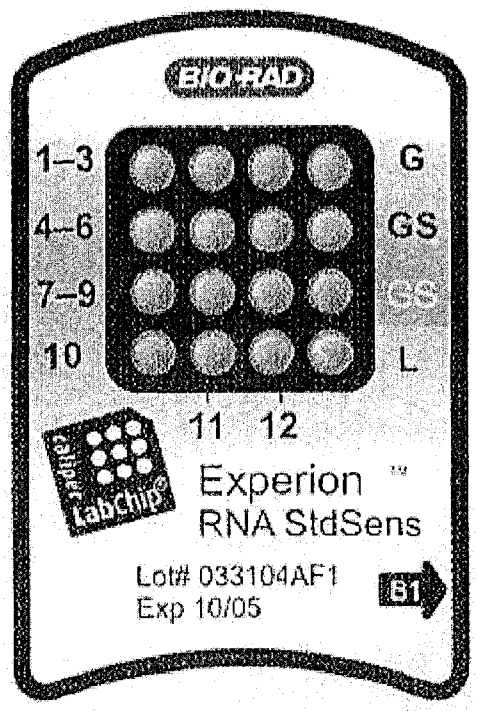

Figure 5: Image of an ExperionTM Stdsens LabChip used for total RNA quantification and quality analysis. Image was taken from the Experion™ RNA Stdsens Analysis Kit instruction manual (BIO-RAD).

2.1.3 Reverse Transcription of messenger RNA to complementary DNA

The reverse transcription protocol was modified from Farrell (2005). First, the amount of starting RNA was normalized using the concentration determined in section 2.1.2. The volume of sample needed to provide 100 ng of RNA was determined. A priming mastermix was prepared containing 1 µl dNTPs (10 mM) (Fermentas), 1.2 µl Oligo dT primer (0.5 mg/ml) (Fermentas), and 2 µl of random hexamer primers (0.2 µg/µl) (Fermentas) per sample. RNA and DEPC H_2O were added to each tube which were then heated to 70°C for 5 minutes then cooled on ice for 2 minutes. This allowed the RNA to denature and the primers to anneal to complementary sequences. A second reverse transcription master mix was prepared containing 4 µl DEPC H_2O, 4 µl 5x RT buffer (Fermentas), 1 µl RNAse inhibitor (20 U/µl), and 1 µl M-MLV reverse transcriptase (20 U/µl) per sample. RT Master mix (10 µl) was added to each primed sample. The samples were incubated in a Techne TC-312 thermocycler at 25°C for 5 minutes, 37°C for one hour and then denatured at 99°C for 5 minutes to inactivate the reverse transcriptase. The cDNA samples were then stored at -20°C prior to PCR.

2.1.4 Primer Design

When conducting a BLAST search, the S-Score and E-Value are given with the results. The S-score is a measure of the similarity of the query to the sequence shown. The E–value is the probability due to chance, that there is another alignment with a similarity greater than the given S-score. A typical threshold for an E-value is 10^{-15} or lower (http://mcdermott.chem.columbia.edu/biophys/evalue.shtml.pdf).

Primers were designed for several genes of interest and sequences were obtained from the literature for several housekeeping genes. Primer pairs for the housekeeping genes cytoplasmic ribosomal protein *L2*, elongation factor alpha (*ef1-α*), and adenine phosphoribosyltransferase (*aprt*) were obtained from Nicot et al.. (2005). Primer pairs for a putative *SHOOTMERISTEMLESS* homolog and a putative *WUSCHEL* homolog were designed from EST sequences obtained during a basic alignment search tool (BLAST) search of known petunia (*Petunia hybrida*) and tomato (*Solanum lycopersicum*) *STM* and *WUS* sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi; see appendix 2 for sequence alignments). When the tomato (AJ538329.1) *WUS* sequence was BLASTed against an unknown EST database, three *S. tuberosum* EST clones (gi|53700754|gb|CV47, DR034003.2, CV475978.1) were found, each with an E value of 8×10^{-14}. When a BLAST search of the known *P. hybrida STM* sequence was performed, four *S. tuberosum* EST clones (DV624511.1, DR037062.1, DR037061.1, DN587987.1) were found with E values of 0 for the first, and 9 x 10-109 for the next three. These *WUS* and *STM* sequences were aligned and primers designed and analyzed with DNAMAN (Lynnon Biosoft) using standard primer design parameters. Minimizing the complementarity of each pair lessened self hybridization during PCR, and similar annealing temperatures for each primer pair were chosen. The primer sequences used are shown in Table 1.

Table 1: Primer sequences used for qPCR analysis of potato meristem gene expression. Primers for *L2*, *ef1-α*, and *aprt* were all taken from Nicot *et al.*, 2005. Primers for the genes *STM* and *WUS* were designed using DNAMAN™ from *Solanum tuberosum* EST sequences obtained from a BLAST search of known *STM* and *WUS* sequences from petunia and tomato respectively.

Gene	Primer Name	Primer Sequence	Melting Temperature (Tm) (50mM NaCl)
STM	*STM* F1	5'-GCAAGCTTCTGGGATTCCG-3'	56.5°C
STM	*STM* R1	5'-CAGTTTCATCGACCCTCAGGC-3'	58.0°C
WUS	*WUS* F	5'-TGCATCAATTCACACGTGG-3'	53.6°C
WUS	*WUS* R1	5'-TCCTCCTGGGTGAGATTCCAC-3'	56.0°C
ef1-α	*ef1 alpha* fwd	5'-ATTGGAAACGGATATGCTCCA-3'	54.2°C
ef1-α	*ef1 alpha* rev	5'-TCCTTACCTGAACGCCTGTCA-3'	58.1°C
aprt	*aprt* fwd	5'-GAACCGGAGCAGGTGAAGAA-3'	57.2°C
aprt	*aprt* rev	5'-GAAGCAATCCCAGCGATACG-3'	56.1°C
L2	*L2* fwd	5'-GGCGAAATGGGTCGTGTTAT-3'	55.6°C
L2	*L2* rev	5'-CATTTCTCTCGCCGAAATCG-3'	54.0°C

2.1.5 Primer Analysis by PCR and Gel Electrophoresis

In order to test the suitability of the primers, endpoint PCR was performed, followed by gel electrophoresis. This allowed a comparison of expected fragment size to experimental fragment size, as well as optimization of reaction conditions. The PCR was performed using the AccessQuick™ RT PCR system and the manufacturer's instructions (Promega). The amount of cDNA and water varied depending on the primer pair being tested. The PCR was run in a Techne TC-512 gradient thermocycler. Reaction conditions were an initial 94°C denaturation step for 5 minutes, followed by 30-60 cycles of an annealing step for 30 seconds at variable temperature depending on primers, extension step for 30 seconds at 72°C, and denaturation step for 30 seconds at 94°C. The final extension step was 5 minutes at 72°C. To avoid primer hairpin formation or dimerization, every test of primer pair specificity included a control reaction in

which no cDNA template was added in order to ensure that no bands were formed under this condition. Exact optimization details are provided in chapter 3.

Products were analyzed on a 1% (w/v) agarose gel in TAE buffer (Sambrook & Russell, 2001). Visualization of products used either a Chemi-Genius Bio Imaging System (Fisher), or a Pharos FX Molecular Imager (BIO-RAD). The observation of expected product sizes and the qualitative analysis of primer specificity by comparison of band brightness between samples were performed after imaging the PCR products.

2.1.6 Gene Expression Analysis using qPCR

Gene expression analysis was performed using a Cepheid Smartcycler (Fisher). A 2X master mix was prepared with 15.5 µl DEPC H_2O, 2.5 µl 10x Hot Start PCR buffer (Fermentas), 0.5 µl dNTPs (10mM) (Fermentas), 0.375 µl forward primer, 0.375 µl reverse primer, 3 µl $MgCl_2$ (25 mM) (Fermentas), 0.25 µl hot start Taq DNA polymerase, and 1 µl SYBR Green (Sigma; diluted 1000 fold in DEPC-H_2O), per sample. One microliter of cDNA was then added. Reaction conditions were an initial 95°C denaturation step for 150 seconds, followed by 90 cycles of an annealing step for 30 seconds at variable temperature depending on primers (see table 1), extension step for 30 seconds at 72°C, and denaturation step for 15 seconds at 95°C. The final extension step was 5 minutes at 72°C. The Cepheid Smartcycler was programmed to continue cycles until 7 cycles after the fluorescence in all samples was above that of the background. Melt curve analysis was then performed by increasing the temperature from 60-95°C at a rate of 0.2°C/second. The Cepheid Smartcycler software program took the first

derivative of this curve and samples that did not generate a melt curve of sufficient quality were not included.

Each qPCR sample was run in triplicate. The mean and standard deviations were calculated for each set of triplicate samples. Individual sample sets with a standard deviation greater than 0.6 were not included in the calculations. When one of the three replicates in each sample showed a Ct value that was significantly (SD > 0.6) different from the other two, they were discarded as outliers. *STM* was normalized to both reference genes, and then to the original calibrator value of the expression of *STM* normalized to both reference genes at 2 weeks after harvest. This was given the value 1 (Applied Biosystems, 1997)

Chapter 3: Results

3.0.0 Primer Validation and RT-PCR Optimization

In order to ensure that gene expression may be reliably determined with our primers, endpoint PCR was first conducted using sets and products visualized using gel electrophoresis to ensure that they were of the correct size.

3.0.1 Endpoint PCR Primer Validation and Optimization

Endpoint PCR was conducted for each gene described in Chapter 2. Nicot *et al.* (2005) used an annealing temperature of 58.0°C during PCR amplification of the gene *aprt*. The expected fragment length was 101 base pairs (bp) (Nicot *et al.*, 2005). When annealing temperatures of 57.6°C and 58.2°C were used in this procedure one band of this approximate size was observed at each annealing temperature (Figure 6).

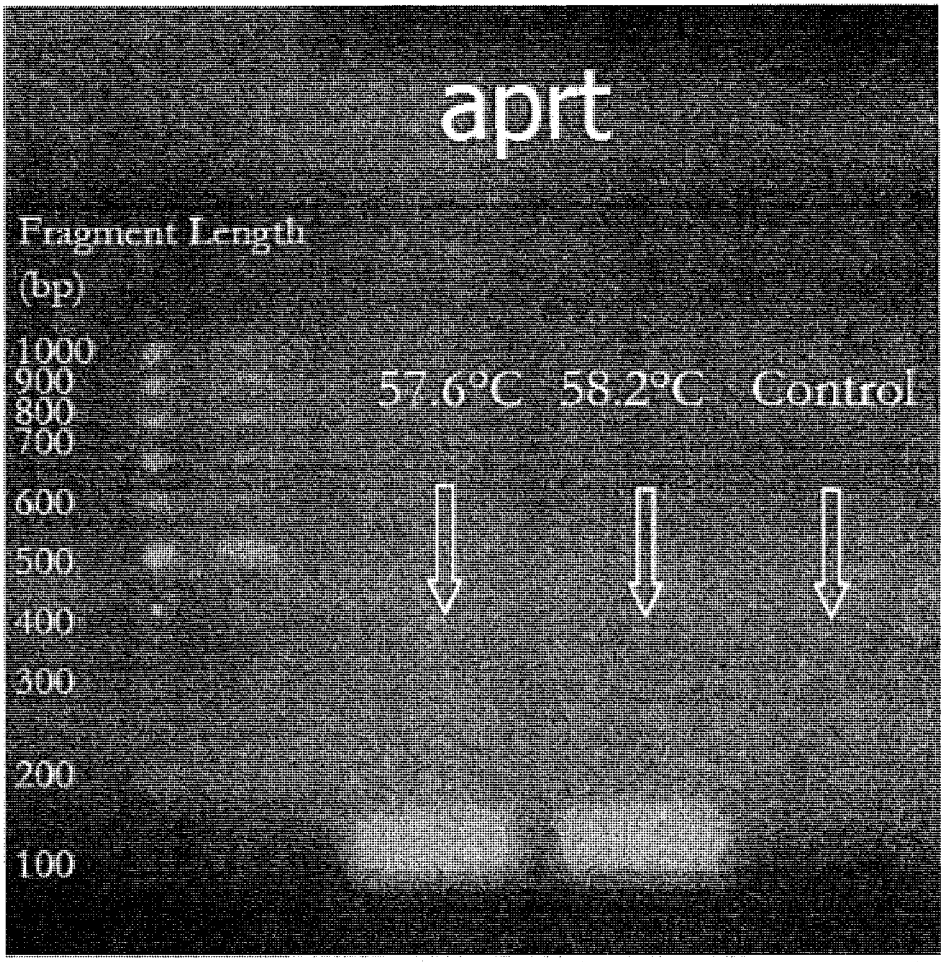

Figure 6: A 1% (w/v) agarose gel showing PCR products of the *aprt* primer pair using annealing temperatures of 57.6°C and 58.2°C. The expected fragment size for *aprt* was 101 base pairs. A control lane in which PCR was performed without a cDNA template and a DNA ladder standard was also included. The image was acquired with a Chemi Genius Bio Imaging System.

Similarily, PCR was performed on the genes *L2* and *ef1-α*. Although Nicot *et al.* (2005) used 58°C as an annealing temperature for *L2*, it was found that only lower annealing temperatures of approximately 51°C could yield visible bands on an agarose gel (Figure 7). Bands were observed using a 58°C annealing temperature for *ef1-α* which is the temperature specified in Nicot *et al.* 2005 (Figure 7). The non-template controls for *L2* and *ef1-α* did not produce any bands (Figure 7). In each case, one band of 121 bp is expected. Figure 8 shows PCR products that lie between the 100 and 200 base pair standards.

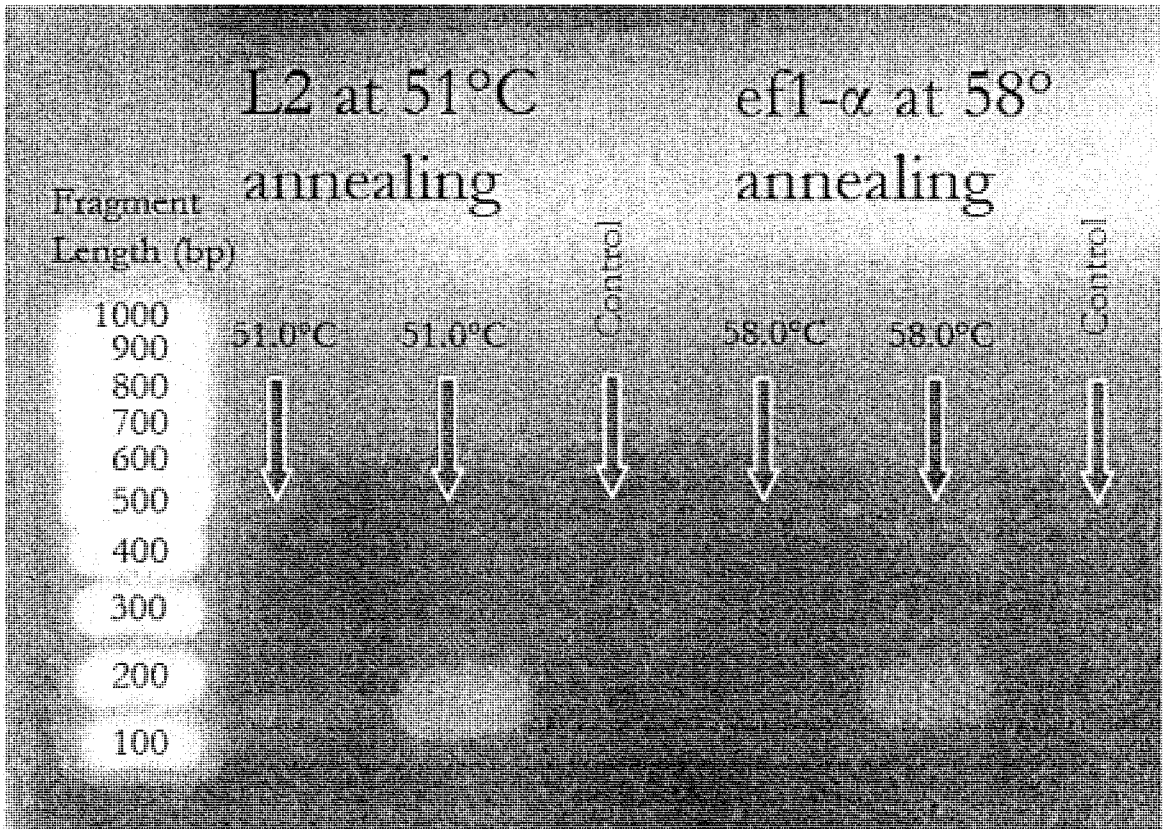

Figure 7: 1% agarose gel showing PCR products of the *L2* and *ef1-α* primer pairs, which were run at annealing temperatures of 51.0°C and 58.0°C. Two control lanes in which PCR was performed without a cDNA template as well as a DNA ladder standard were also included. Neither control lane showed bands. The expected DNA fragment size for both *L2* and *ef1-α* is 121 base pairs. The difference in brightness between the bands for both primer pairs is due to the quality of input RNA (left band 28S/18S ratio = 1.19, right band 28S/18S ratio = 1.50). The image was acquired with a Chemi Genius Bio Imaging System.

The *STM* F1 and *STM* R1 primers were tested using 30 cycles of PCR at annealing temperatures of 50.2°C, 52.2°C, 53.3°C, and 54.2°C. *STM* F1 and *STM* R1 were expected to produce a fragment of 213 bp. As previous PCR attempts using these primers with 1 µl of cDNA did not yield visible bands, input cDNA was increased to 2.5 µl. Bands on the gel were observed only when the exposure time was increased to the maximum of 5 seconds (Figure 8). With maximum exposure, a faint bands was observed from reactions with annealing temperatures of 52.2°C and 53.3°C. These bands were located between 200 and 300 bp (Figure 8). No bands were observed in the control lane (Figure 8). As the bands were faint, the PCR was repeated with 60 cycles of amplification at annealing temperatures of 49.6°C, 50.2°C, 51.6°C, 52.2°C, and 53.3°C, with a non-template control. The amount of template cDNA was increased from 2.5 µl to 5 µl. Figure 9 shows strong bands under these conditions, with clear bands of just over 200 bp in size in all lanes. The cleanest and darkest band occurred using an annealing temperature of 51.6°C. Again, non-template controls did not yield EtBr-staining bands.

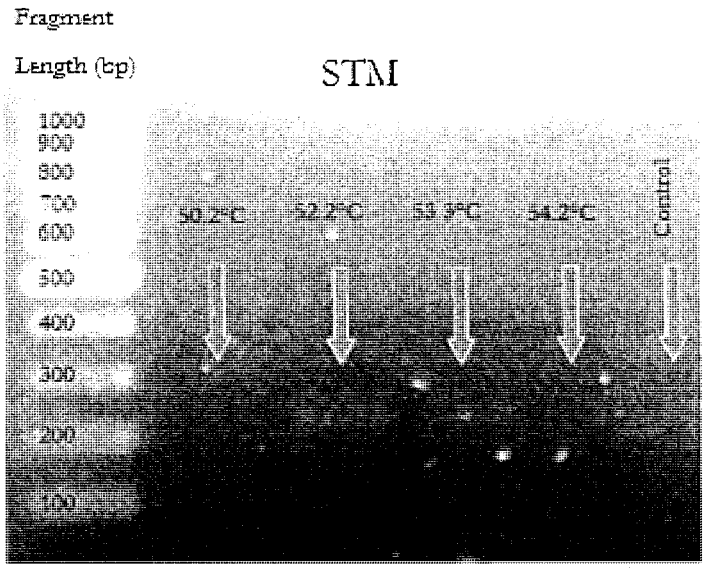

Figure 8: 1% (w/v) agarose gel showing PCR products observed when using the primer pair *STM* F1 and *STM* R1 using annealing temperatures of 50.2°C, 52.2°C, 53.3°C and 54.2°C. The expected fragment length for *STM* F1 and *STM* R1 was 213 base pairs. Two faint PCR products were seen between 200 and 300 base pairs at annealing temperatures of 52.2°C and 53.3°C. A control lane in which PCR was performed without a cDNA template and a DNA ladder standard were also included. The control lane showed no distinct bands. The image was acquired with a Chemi Genius Bio Imaging System.

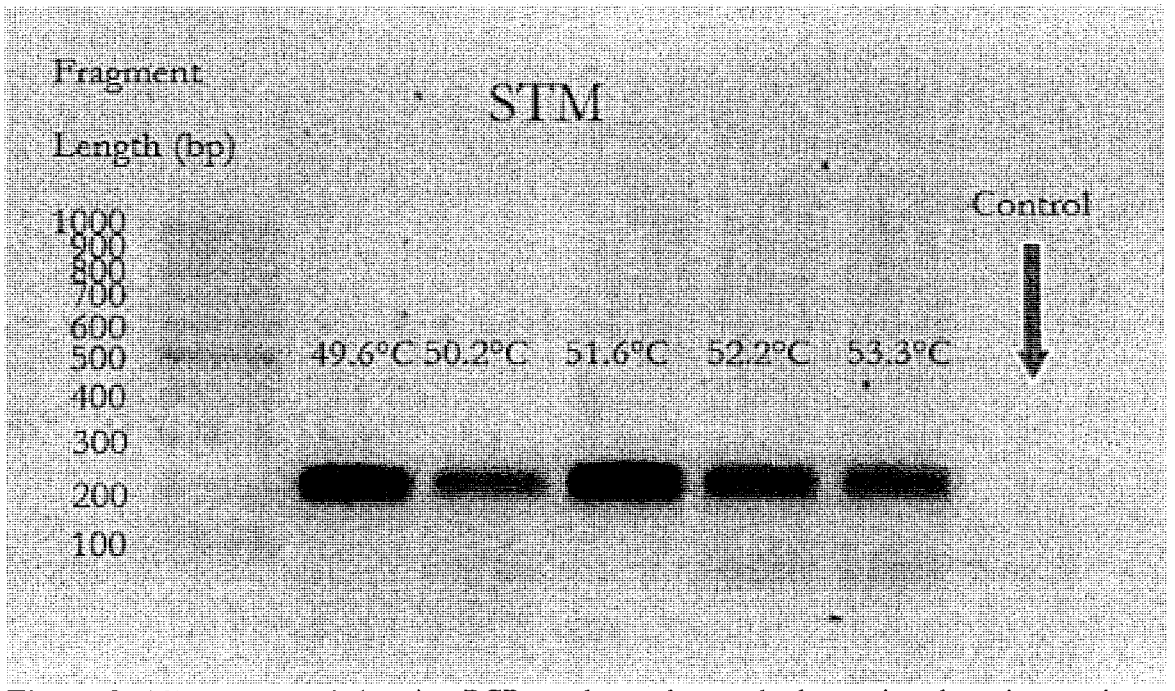

Figure 9: 1% agarose gel showing PCR products observed when using the primer pair *STM* F1 and *STM* R1 using annealing temperatures of 49.6°C, 50.2°C, 51.6°C, 52.2°C, and 53.3°C for 60 cycles of PCR. The expected fragment length for *STM* F1 and *STM* R1 was 213 base pairs. All bands appeared at just over 200 base pairs with the strongest at an annealing temperature of 51.6°C. A control lane in which PCR was performed without a cDNA template and a DNA ladder standard were also included. The control reaction showed no bands. The image was acquired with a Pharos FX Molecular Imager (BIO-RAD)

The *WUS* F and *WUS* R1 primers were optimized using 60 cycles of PCR at annealing temperatures between 45.7°C and 52.4°C (Figure 10). Using *WUS* F and *WUS* R1 was expected to produce a fragment of 250 base pairs. As it is expected that *WUS* is expressed in low copy numbers, the PCR cycles were doubled and the amount of template cDNA was increased to 5 µl. The final optimization is shown in Figure 10, with the strongest band located between the 200 and 300 bp ladder markers at an annealing temperature of 50.7°C. Unlike the previous primer pairs, *WUS* consistently showed an EtBr stained product at approximately 100 bp in all temperatures and gels run (Figure 10). No bands are present in the control lane except the 100 bp signal (Figure 10).

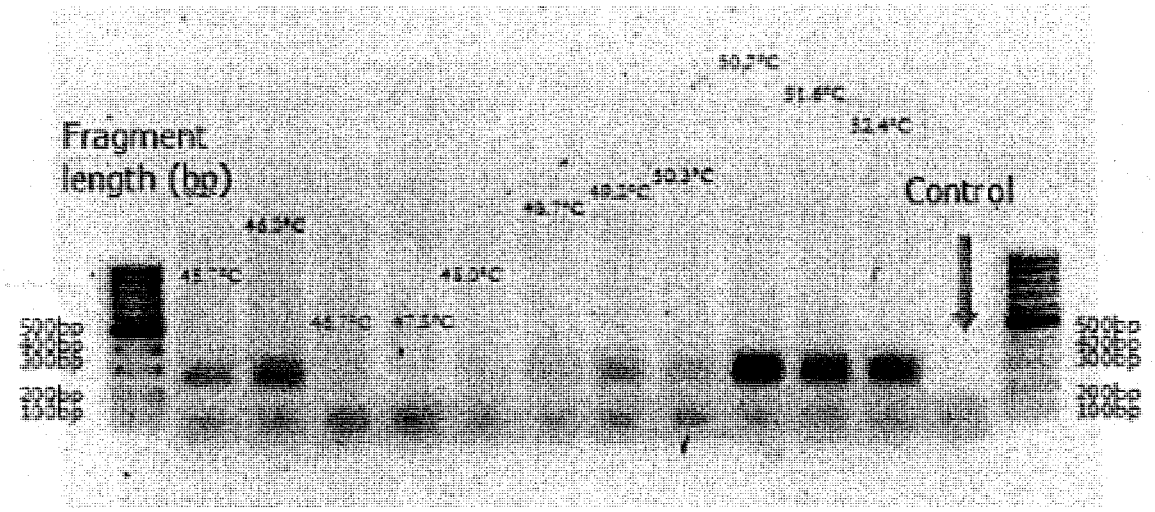

Figure 10: 1% (w/v) agarose gel showing PCR products observed when using the primer pair *WUS* F and *WUS* R1 and annealing temperatures between 45.7°C and 52.4°C. The expected fragment length for *WUS* F and *WUS* R1 was 250 base pairs. All bands appeared between 200 and 300 base pairs with the strongest at an annealing temperature of 50.7°C. A control lane in which PCR was performed without a cDNA template and a DNA ladder standard were also included. The image was acquired with a Pharos FX Molecular Imager (BIO-RAD)

3.0.2 qPCR Validation and Optimization

All primers which showed successful endpoint PCR results were optimized for qPCR using the same concentrations of reagents and the Cepheid Smartcycler (Fisher) with the addition of SYBR Green. The threshold of 30 fluorescence units was set by the manufacturer. The *aprt*, *L2* and *ef1-α* reference genes all amplified consistently when triplicate PCR was performed for a single biological sample (Figures 11, 12, 13). The first derivative of the melt curve also showed a single sharp peak for all optimization temperatures, which indicated that a single product was formed during each reaction (Figures 11, 12, 13).

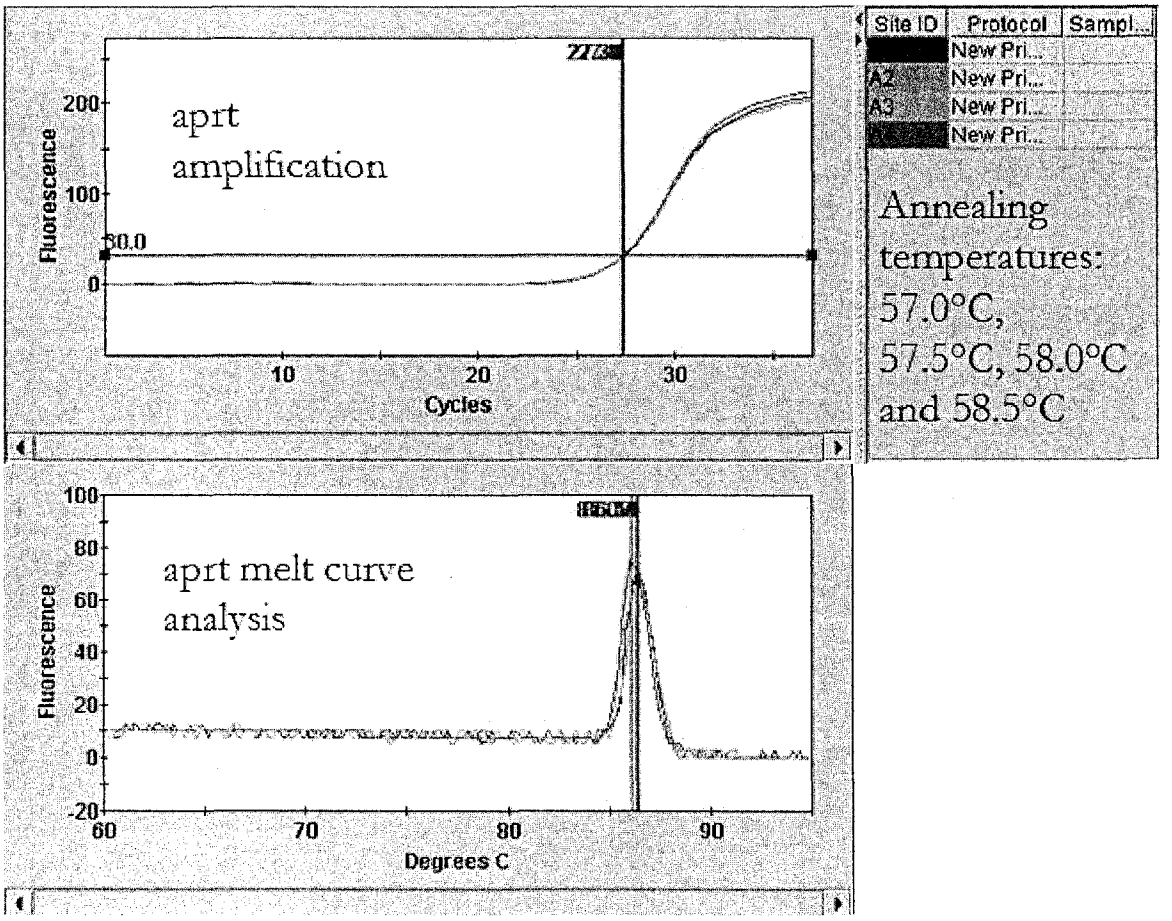

Figure 11: Amplification of PCR product using *aprt* forward and reverse primers was consistent within a biological sample. A single sample was run at annealing temperatures of 57.0°C, 57.5°C, 58.0°C, and 58.5°C. All 4 reactions using the same sample amplified at similar cycle threshold values and produced similar melt curve peaks. While 4 annealing temperatures were used, it was determined that 58.0°C was the most suitable temperature for experimentation as the variation between results at this temperature was very low.

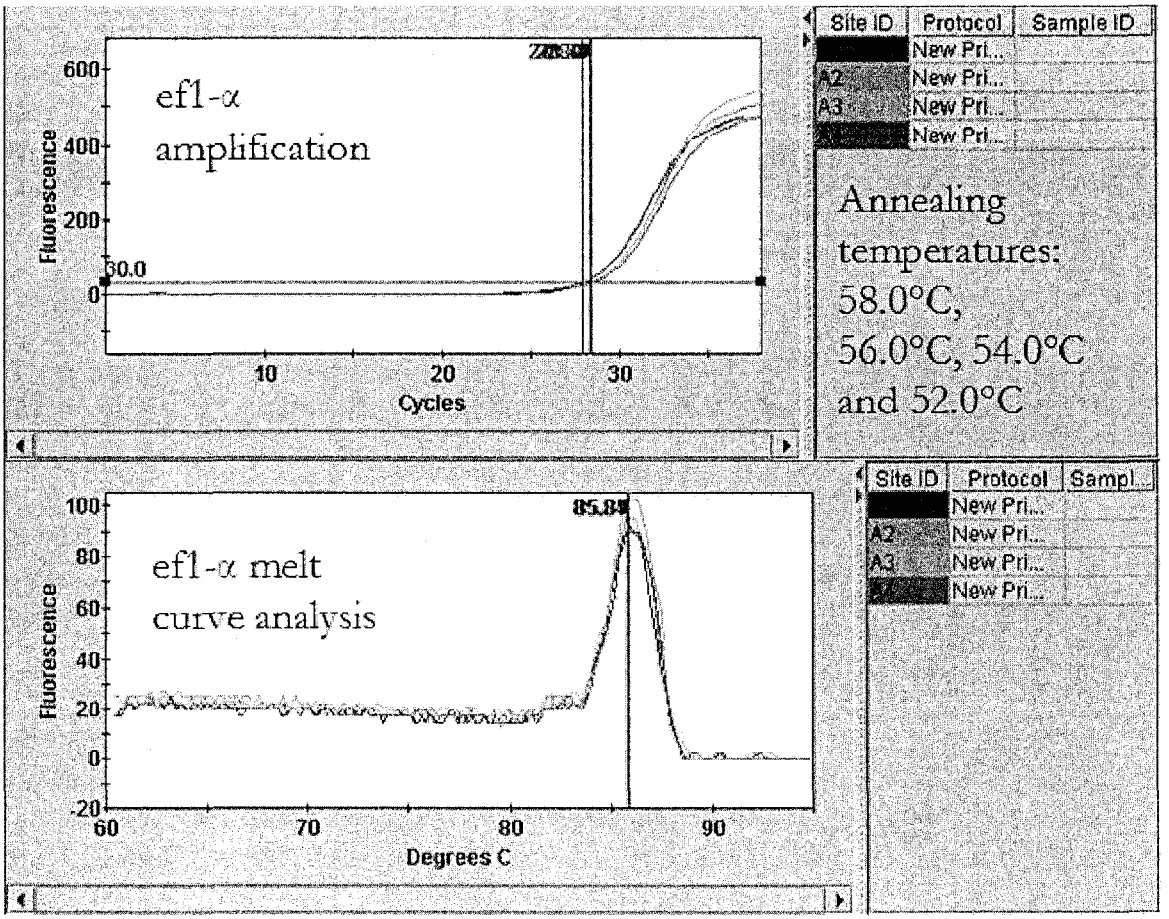

Figure 12: Amplification of PCR product using *ef1-α* forward and reverse primers was consistent within a biological sample. A single sample was run at annealing temperatures of 58.0°C, 56.0°C, 54.0°C, and 52.0°C. All 4 reactions using the same sample amplified at similar cycle threshold values and produced similar melt curve peaks. While 4 annealing temperatures were used, it was determined that 58.0°C was the most suitable temperature for experimentation as the variation between results was very low.

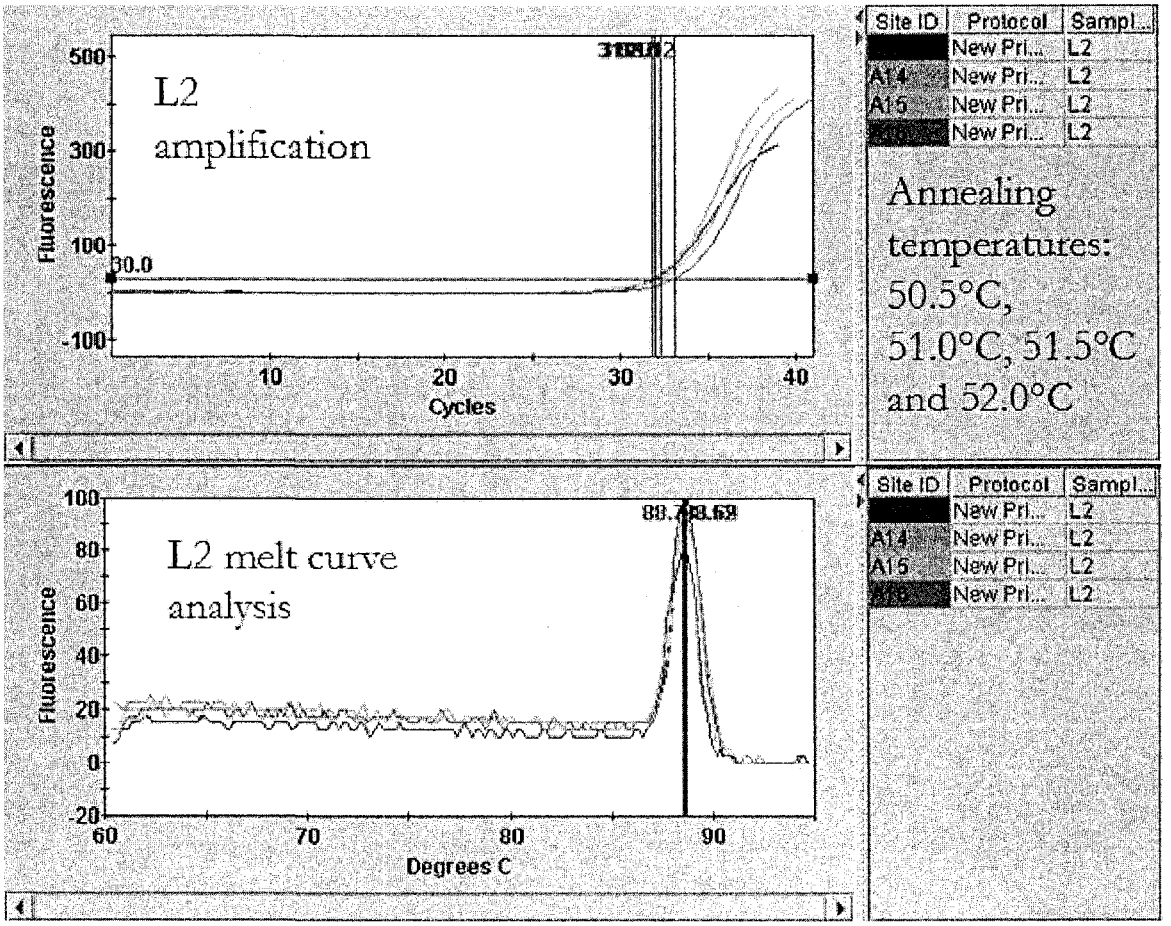

Figure 13: Amplification of PCR product using *L2* forward and reverse primers was consistent within a biological sample. A single sample was run at annealing temperatures of 50.5°C, 51.0°C, 51.5°C, and 52.0°C. All 4 reactions using the same sample amplified at a similar cycle threshold value and produced very similar melt curve peaks. While 4 annealing temperatures were used, it was determined that 51.0°C was the most suitable temperature for experimentation as the variation between results was very low.

Optimization results for *WUS* primers were inconsistent. Figure 14 shows a typical *WUS* amplification and melt curve. Within a single sample, the Ct values varied widely. The melt curve analysis showed a broad parabolic curve (Figure 14), rather than a more desirable sharp peak (Figures 11, 12, 13). Furthermore, the melting temperature at the *WUS* amplicon was approximately 77°C. Given that the melting temperatures of *L2*, *ef1-α* and *aprt* were approximately 85°C, 88°C, and 86°C for products of 121 bp, 121 bp, and 101 bp respectively (Figures 11, 12, 13), unfavorable small side products caused by primer dimerization or hairpins were likely formed in the *WUS* qPCR reactions.

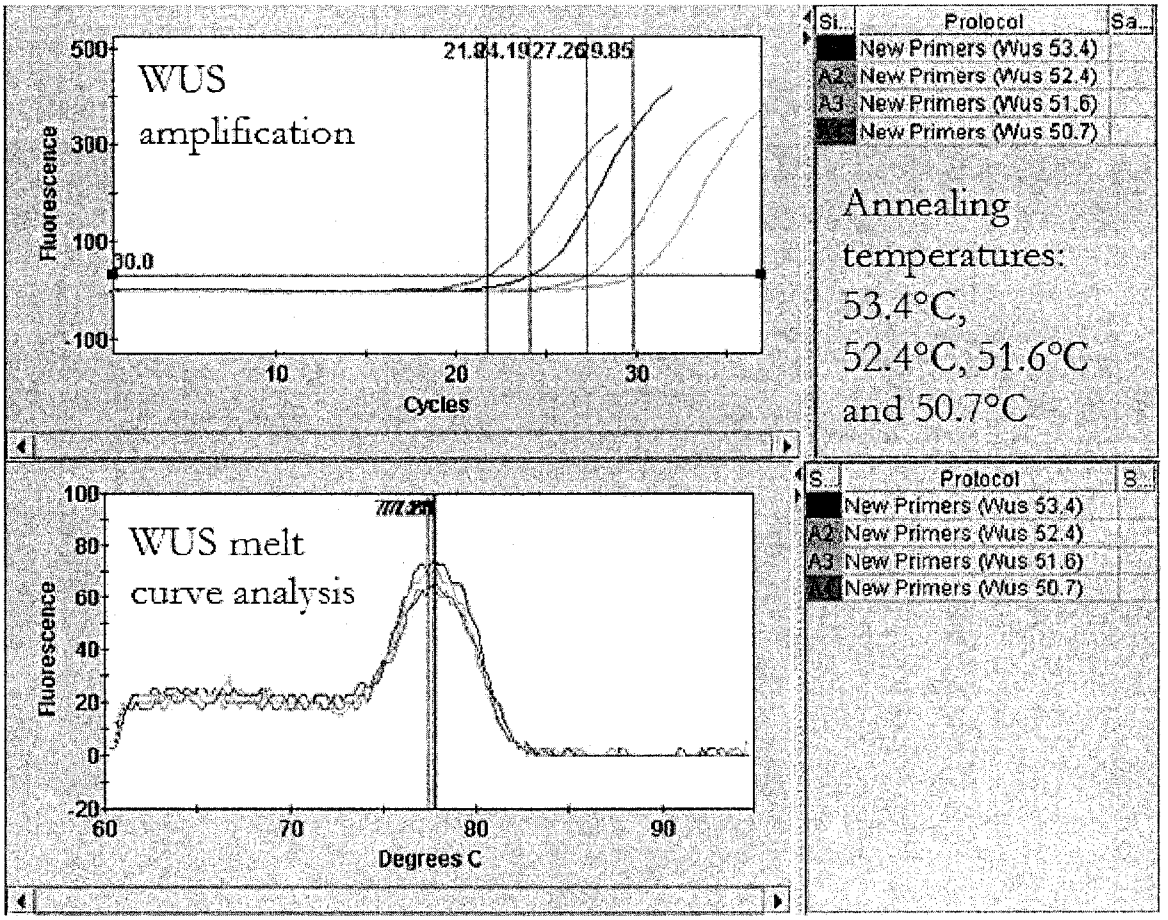

Figure 14: Amplification of PCR product using *WUS* F and *WUS* R1 primers was not consistent within a biological sample. A single sample was run at annealing temperatures of 53.4°C, 52.4°C, 51.6°C, and 50.7°C. All 4 reactions using the same sample amplified at different cycle threshold values and produced similar melt curve peaks. The melt curve analysis shows a consistent unfavorable peak at 77°C. Low product melt curve values indicate a small product is formed during PCR.

In contrast to the *WUS* results, *STM* amplification was mostly consistent between the triplicates in any given biological sample. However, this was not always the case. Figure 15 shows a typical *STM* amplification, with a higher Ct value than the housekeeping genes analyzed, and a sharp melt curve peak at approximately 86°C. The optimization of the annealing temperature for *STM* was based on data shown in Figure 9.

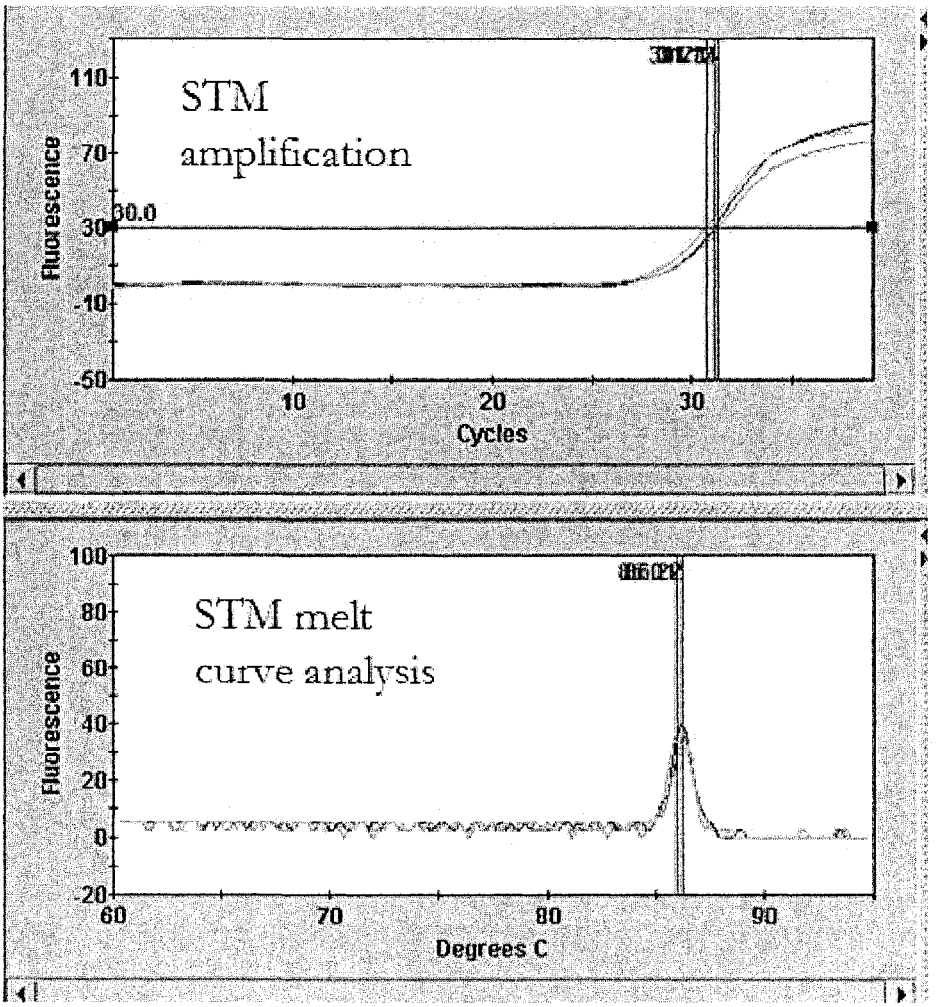

Figure 15: Amplification of PCR product using *STM* F1 and *STM* R1 primers was consistent within a biological sample. A single sample was run at an annealing temperature of 51.6°C. All 4 reactions using the same sample amplified at similar cycle threshold values and produced similar melt curve peaks.

3.1.0 Microtuber Meristem Length Measurements

Meristem length measurements were recorded in order to correlate changes in meristem morphology and growth to changes in gene expression during dormancy break.

3.1.1 Meristem Length Over Time Since Harvest

Meristem length measurements were taken for each batch of microtubers. All lengths for all microtubers were averaged at a given timepoint. The mean and standard error was calculated across all measurements at each timepoint, and the results are summarized in Figure 16. A two-way ANOVA ($p > 0.05$) test was performed for each time point against each other time point. The p-values are found in Table 2.

Table 2: p-values from a two-way ANOVA analysis of every time point in figure 12 against every other time point. These values represent the value for all microtubers pooled within a given time point. (* significantly different at the p = 0.05 level)

Time after Harvest (weeks)	2	7	11	15	19
2					
7	0.71				
11	0.04*	0.05*			
15	<0.001*	<0.001*	0.15		
19	<0.001*	<0.001*	0.06	0.25	

At 2 and 7 weeks after harvest, the meristem lengths were significantly lower than at 11, 15 and 19 weeks after harvest. However, there was no significant difference in length between 2 and 7 weeks after harvest. Therefore, a significant increase in meristem length occurred between 7 and 11 weeks after harvest (Figure 16).

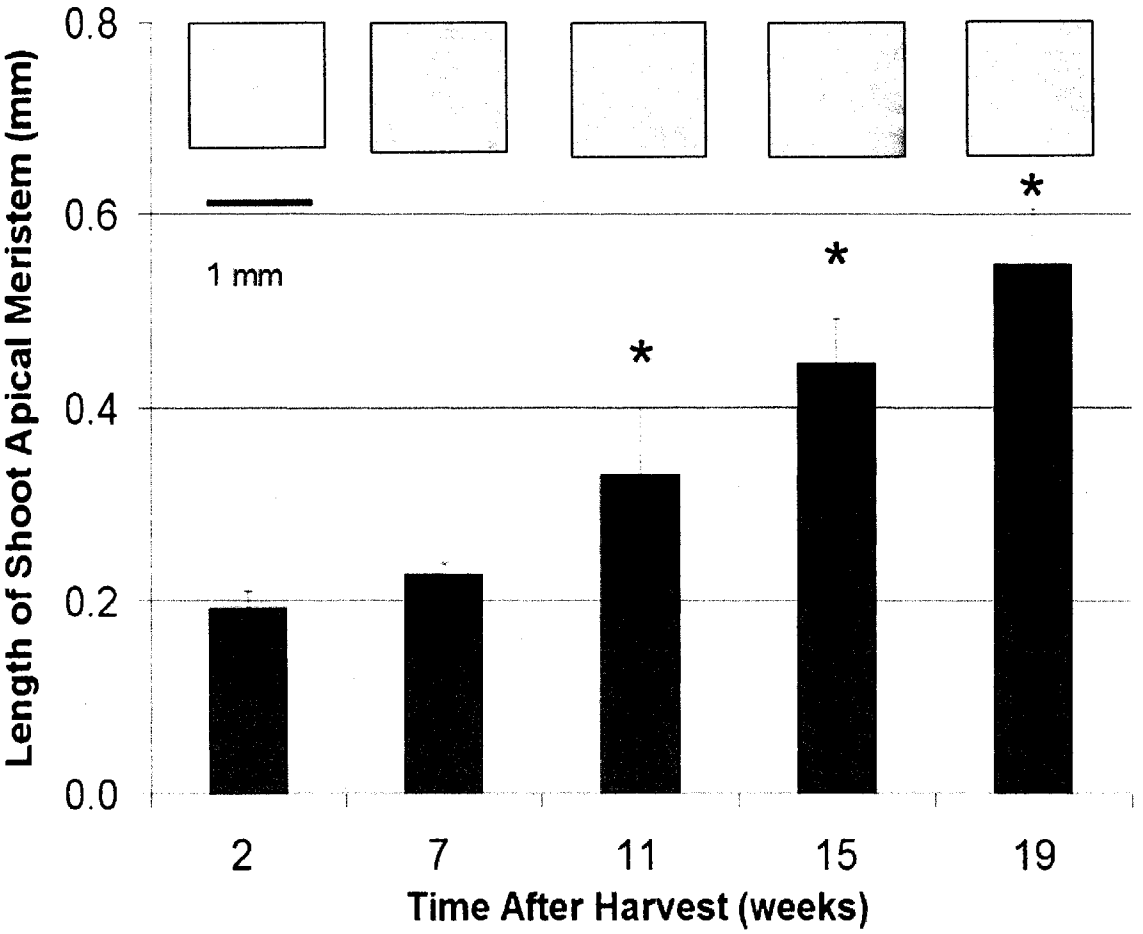

Figure 16: Meristem length (mean +/- standard error, n >25) across all microtubers measured at every time point analyzed after harvest. Significant increases in length compared to dormant controls at t = 2 weeks are marked with an asterisk and first arose between 7 and 11 weeks after harvest. Several representative photographs of meristems at each corresponding time point are shown.

3.2.0 qPCR Results

qPCR results are presented for *STM* as fold induction of the *STM* gene when compared to both *L2* and *ef1-α*. All data given are expressed as the fold induction of each gene compared to dormant meristem controls from 2 weeks after harvest. *WUS* could not be included in the results as its amplification was inconsistent, with poor melt curves in all samples. The housekeeping gene *aprt* did not amplify consistently when processing individual RNA samples and therefore could not be included.

3.2.1 Housekeeping Gene Validation of L2 and ef1-α

Housekeeping gene validation was performed to show that the fold induction of *L2* and *ef1-α* did not vary significantly when compared to dormant expression levels in control meristems sampled from microtubers 2 weeks after harvest. While fold induction of the housekeeping genes was generally within the recommended +/- 0.4 fold of the control (Filby & Tyler, 2007), *L2* was induced between 1.5 and 6 fold (figure 17). The fold differences between samples suggest that this variation is significant but more replicate samples need to be run in order give this conclusion statistical weight (figure 17). In contrast, *ef1-α* showed much less variation than *L2* as the fold induction compared to expression levels 2 weeks after harvest varied between 0.47 and 1.38 fold (figure 18).

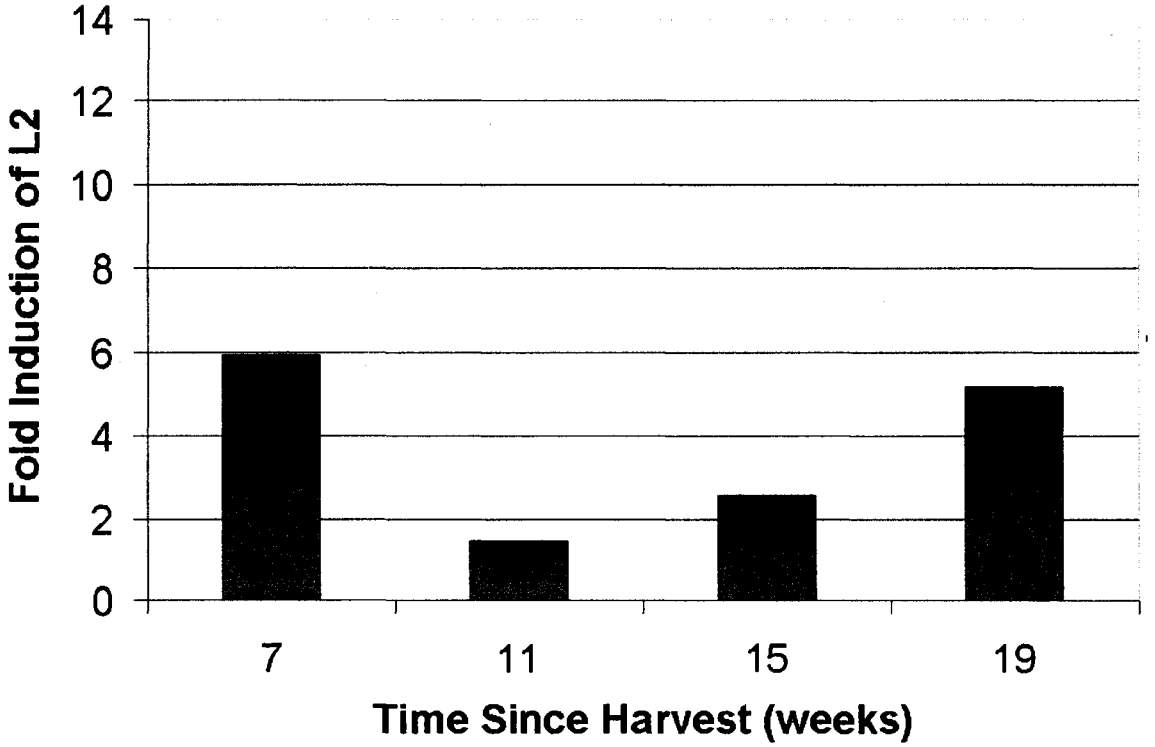

Figure 17: Housekeeping validation of *L2* is given as fold induction ($2^{-\Delta\Delta Ct}$) of *L2* when compared to the expression of *L2* at 2 weeks after harvest. (Mean, n = 2).

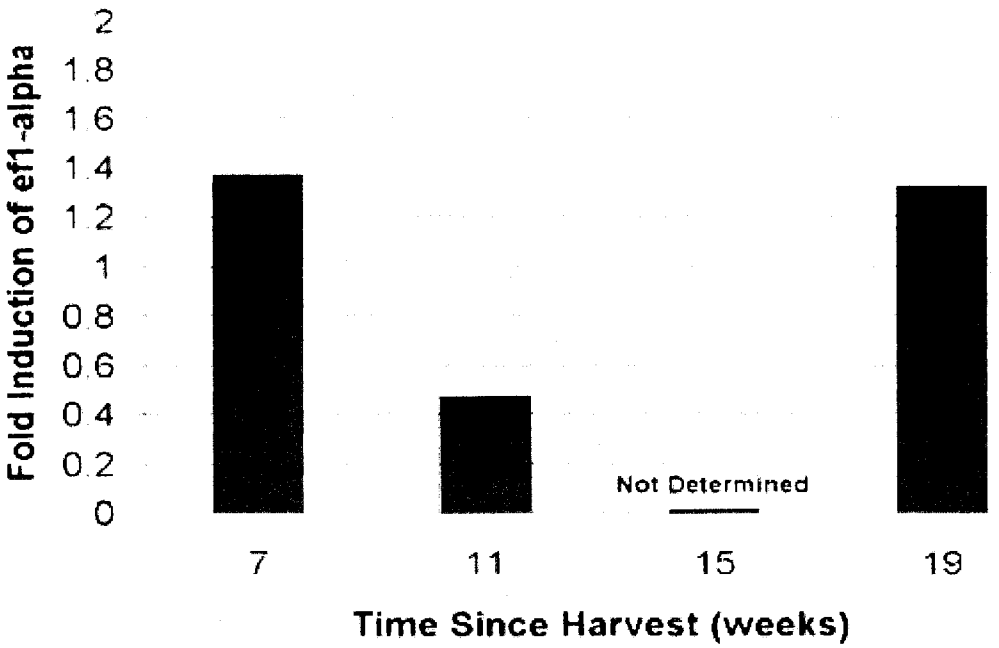

Figure 18: Housekeeping validation of *ef1-α* is given as fold induction ($2^{-\Delta\Delta Ct}$) of *ef1-α* when compared to the expression of *ef1-α* at 2 weeks after harvest. (Mean, n = 2).

3.2.2 Fold Induction of STM during meristem development

The induction of *STM* was calculated using both *L2* and *ef1-α* as reference genes and the results are shown in Figure 19. The fold induction of *STM* expression is shown for both reference genes in figure 29. Similar to figure 16, there was a large increase in *STM* induction between 7 and 11 weeks after harvest. When corrected to *ef1-α* and *L2*, *STM* transcript levels increased 42.5-fold and 17.4-fold, respectively. *STM* mRNA levels continued to increase thereafter until they reached 89-fold and 28-fold, respectively, 19 weeks after harvest. Given that *ef1-α* showed less variation in fold induction compared with *L2* (Figures 17 & 18), it is likely to be a better choice as a housekeeping gene in potato meristems emerging from dormancy. However, the same trend was seen with both housekeeping genes (Figure 19).

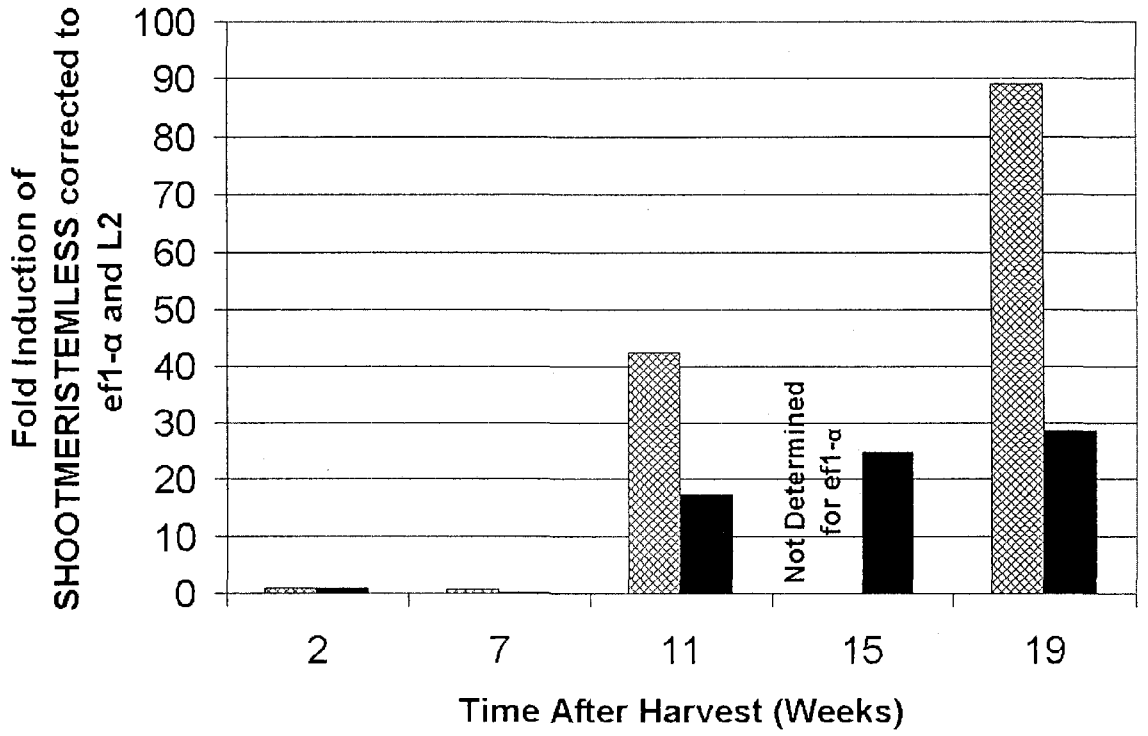

Figure 19: The fold induction of *STM* when *L2* (black bars) and *ef1-α* (cross-hatched bars) are used as reference genes. (mean, n=2).

Chapter 4: Discussion

4.0.0 Results of Gene Expression Analysis

4.0.1 Housekeeping Gene Analysis

The assay of the variation in expression of at least 2 reference genes is recommended for a reliable qPCR assay (Farrell, 2005). Figures 17 and 18 show the expression of *L2* and *ef1-α* during dormancy emergence compared to the expression of these genes 2 weeks after harvest. The fold induction of both genes remains consistently moderate although that of *ef1-α* is less variable. In addition, the expression of these genes was found to vary little in whole potato tubers during biotic and abiotic stress by Nicot *et al.* (2005). It is concluded that these two genes are suitable reference genes for the preliminary study of dormancy emergence in the microtuber model system.

4.0.2 STM Gene Expression During Development

In potato tubers, synthesis of DNA, RNA and proteins occurs continuously and these activities increase as the tubers progress through dormancy break (McDonald & Osbourne, 1988). Promoter regions in the genome are hypothesized to require histone multi-acetylation in order to allow transcriptional activators to bind correctly (Struhl, 1998). Histone acetylation has been shown to occur during natural and chemically induced dormancy break in the meristems of tubers, indicating a change in the chromatin architecture state that could underlie concomitant changes in gene expression (Law & Suttle, 2004). In Arabidopsis, another Solanaceous species, the genes *WUS* and *STM* are expressed the earliest during seed germination and are required for establishment or

maintenance of stem cell fate, whereas genes expressed later are involved in regulating the size of the central zone (Bowman & Eshed, 2000). Figure 19 shows the fold induction of *STM* in tuber meristems when normalized to both *L2* and *ef1-α*. As is seen, the fold induction of *STM* remains near 1 until the 11th week after harvest, when it then increases (Figure 19). It continues to increase for measurements taken with both reference genes after the 11th week until the 19th week. This result suggests that there is a significant and sustained increase in the expression of *STM* between the 7th and 11th weeks after harvest. Measurements of meristem size in Figure 16 correlate with this suggestion, as a statistically significant increase in growth is seen between weeks 7 and 11. However, it must be noted that the sample size for both target and reference genes is small. In addition, the fold induction for week 15 could not be determined due to a lack of replicable data for *ef1-α*. It is possible, as *STM* is one of the first transcripts to be observed during *Arabidopsis* shoot apical meristem development, that *STM* could be expressed earlier than was observed in this experiment (Bowman & Eshed, 2000). The divergence between the meristems and tubers developmental stages implicate that sets of genes are being turned 'on' and 'off' during transition. More genes are expressed during tuber formation and sprouting than during dormancy (Trindad *et al.* 2004; Bachem *et al.* 2000). Sprouting is expected to be controlled through interacting genes throughout the plant including the genes which are directly involved (Trindade *et al.* 2004). In addition, meristems are regulatory structures in plants, and therefore have the capacity to control changes in development (Sussex,

1989). The increase in *STM* transcript levels in the meristem during development is therefore an interesting result as it is known to encode a homeodomain protein in *Arabidopsis* (Francis, 2008). This may indicate that its expression causes a cascade of downstream effects. Therefore, an increase in *STM* expression during endodormancy break could reflect the increase in metabolism observed in other studies (Kloosterman *et al.* 2005). Since *STM* is expressed in the shoot apical meristem of *Arabidopsis* during embryogenesis, it is possible that dormancy break in the potato meristem occurs in a similar fashion. It is of interest to continue to examine dormant meristems and understand how they develop in comparison to dormant *Arabidopsis* seeds.

Coleman & Coleman (2000) have shown that dormancy emergence occurs between 6 and 12 weeks after harvest, depending on cultivar and sucrose concentration. Work by Suttle (1995) showed sprouting in two storage regimes of 20°C and 3°C beginning at approximately 7 weeks postharvest. Given that the dormancy emergence shown in other published data agrees with these findings across cultivars and treatments, the period between 7 and 11 weeks post harvest will be examined more thoroughly to begin elucidating the molecular mechanisms which govern dormancy emergence in this system. The experimental data presented here agrees with these findings as the breaking of endodormancy in our microtubers appears to have occurred between the 7^{th} and 11^{th} week after harvest.

4.0.3 WUS and aprt

As mentioned in chapter 3, *WUS* and *aprt* were not included in the gene expression analysis because of problematic qPCR analysis. Figure 10, when compared with Figures 6 through 9, showed a fuzzy band underneath the putative *WUS* target band. The other gene's PCR products did not possess such bands underneath the putative target bands (Figures 6, 7, 8, 9). This may indicate that an undesirable product was being formed during endpoint PCR. Given that qPCR requires sensitive fluorescent detection and SYBR green fluoresces when bound to double stranded DNA, any double stranded products in the reaction tube will fluoresce whether they are specific or non-specific (Farrell, 2005). In addition, the low abundance of the *WUSCHEL* gene transcript may have contributed to competitive reactions such as primer dimerization or hairpins initiating transcription during PCR (Bowman & Eshed, 2000). Also, poor quality mRNA, a consistent problem when isolating plant RNA, may have contributed to the failure of aquiring data on both *WUS* and *aprt*. Plant tissues may have secondary metabolites such as phenolics and high molecular weight polysaccharides which can co-precipitate during nucleic acid isolation (Camacho-Villasana, 2002). The meristem samples used in this study included some tuber tissue. This tissue, which is high in starch, co-precipitates with RNA. This can interfere with the isolation of biologically active nucleic acids. This problem is currently being addressed in our lab by the use of a plant specific RNA isolation method (Lashbrook *et al.* 2002; Wilkins & Smart, 1996). Finally, we have been

using a T7 RNA amplification kit (Ambion) to increase transcript levels and thus the sensitivity of the *WUS* qPCR reaction (results not shown).

4.1.0 Objectives

The first objective of this research was to develop a microtuber model system that provides tubers for research that are biochemically similar to field grown tubers. The second objective was to develop primers for *WUS* and *STM* homologues in *Solanum tuberosum*, in order to successfully amplify these genes using PCR. The present results suggest this has been successful for *STM* but not for *WUS*. Cloning and sequencing data for both amplified fragments would increase confidence. The third objective was the use of quantitative measurement data and a qPCR assay to determine when dormancy break occurs in our microtuber culture system such that we can further investigate the molecular events which accompany this developmental shift. This objective has been only partially met.

4.2.0 Conclusions

First, the microtuber culture and harvest system developed has the potential to model postharvest physiology in potato tubers at a cellular level. Second, gene expression analysis can be conducted using the genes *L2* and *ef1-α* as references. Third, meristem measurement data suggests dormancy emergence occurs between the 7^{th} and 11^{th} week after harvest in this system. Finally, gene expression analysis of the putative *STM* homolog conditionally indicates that meristem dormancy emergence occurs between the 7^{th} and 11^{th} week after harvest in microtubers. Validation of these findings could be

accomplished by the successful measurement of *CLV* and *WUS* expression levels. The expression pattern of novel genes such as *HANABA TARANU* (*HAN*) known to be required for the precise expression pattern of *WUS* in *Arabidopsis,* could be investigated in tuber meristems. The cloning and sequencing of PCR products could provide further confidence in the results of these experiments.

4.3.0 Future work

A number of directions have been made possible by the current research. First, the sample size of the current assay must be larger to increase confidence in the results. Second, T7 RNA amplification should be conducted in order to produce a consistently reliable *WUS* qPCR assay. RNA amplification could increase the sensitivity of the assay for *WUS* and decrease the possible non-specific primer binding side reactions occurring. Third, the fragments amplified by PCR should be sequenced to ensure the assay is measuring the *STM* homolog. Fourth, since we have preliminary information from the meristem length measurements, a subtractive hybridization library of cDNAs comparing gene expression of the timeline of microtuber dormancy emergence before and after this period should be investigated. Finally, specific proteins can potentially be investigated in order to uncover new information about the process of vegetative dormancy emergence.

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Appendix 1: Calculation of Fold Induction

The equation $X_n = X_o \times (1 + E_x)^n$ describes PCR amplification, Where X_n is the number of target molecules at cycle n, X_o is the initial number of target molecules, E_X is the efficiency of the reaction, and n is the number of cycles (Applied Biosystems, 1997). The threshold cycle (ct) in qPCR is the number at which the amplified target reaches the fixed threshold and replaces n in the equation (Applied Biosystems, 1997). Dividing this equation by one describing the reference amplification, $R_T = R_o \times (1 + E_R)^{Ct, R}$, gives $(X_o/R_o) \times (1+ E)^{Ct, X - Ct, R} = K$, assuming the efficiencies of both reactions are equal to 1, where K is a constant (Applied Biosystems, 1997). (X_o/R_o) can be described as X_N, or the normalized amount of target, and Ct, X – Ct, R can be described as ΔCt, or the difference between the cycle threshold of the target and the reference transcript (Applied Biosystems, 1997). Rearranging the equation gives $X_N = K \times (1 + E)^{-\Delta Ct}$, which, when divided by the same equation for the control point, gives $(1 + E)^{-\Delta\Delta Ct}$ (Applied Biosystems, 1997). For amplicons close to 150bp, the efficiency is close to 1, which gives the original equation (Applied Biosystems, 1997).

Appendix 2: Multiple Sequence Alignments of Primers With Known Gene Sequences of Homologs

WUS primers aligned with the potato EST sequence gi|53700754|gb|CV47 and the known sequences AF481951 and AY162209.

```
Antirrhinum     ------------------------------------------------------------
Petunia         GCACGAGGCCATATTCTCTTCACTCTATCCCTTAGTTTGTTCTCTTCTTGTTTTAGTACA 60
Solanum         ------------------------------------------------------------


Antirrhinum     ------------------------------------------------------------
Petunia         GCTCTATACTTTATCATGGAAACAGCACAACACCAACAAAACAACCAACAACACTATCTT 120
Solanum         ------------------------------------------------------------


Antirrhinum     ------------CAAGATTCTCAAGGGA------TTGGCAAAATCAATA---ATGGGAGT 39
Petunia         CACCAACATTTATCAATTGGTCAAGGGACAAACATTGAAGATGGTAGCA---ACAAAAAC 177
Solanum         ------------CTAGTGGATCCCCGGC------CTGCAGGTATCCATGTACATGGGATC 42
                            *     **   **         **                *    *


Antirrhinum     GGCGGTAGTAGTTTCTTGTGCAGACAAAGCAGTACGAGGTGGACACCAACAACTGACCAG 99
Petunia         AACAGCAGCAATTTTATGTGTAGACAAAATAGTACAAGGTGGACACCAACAACTGACCAG 237
Solanum         ATCATCAAGAAGCTTAAGCATG---AAGGTTGCATCAATT---CACACGTGGATTCTTAG 96
                  *   *  *   *   *        **    * *  *  *   ***        *    **


Antirrhinum     ATAAGAATCTTGAAGGATCTTTACTACAACAATGGTGTTAGGTCTCCAACTGCTGAGCAG 159
Petunia         ATAAGAATATTAAAGGATCTTTACTACAACAATGGAGTTAGGTCTCCAACTGCTGAACAA 297
Solanum         AGCATGAAGCTGCTTCTCCTTCACTTACACT-TGGTTGCAAACGTTTAA-----GACCAC 150
                *  *  *   *        *** ***   **  ***    *    *  **     ** **


Antirrhinum     ATTCAGAGGATCTCTGCTAAGCTGA-GACAATACGGGAAGATTGAAGGCAAAAACGTGTT 218
Petunia         ATTCAGAGGATCTCTGCTAAGTTAA-GACAGTACGGAAAGATTGAAGGCAAGAATGTGTT 356
Solanum         TTGCTCCTAAGCTCAACACAACCAATAATGATACCACAACCACCATTGTTACTCCTCCTT 210
                 * *     * ***  *  *    *  *   ***   **     *  *  *       **


Antirrhinum     TTACTGGTTTCAGAATCACAAAGCTCGTGAGAGGCAGAAAAAGCGCTTTACTGCTGAT-- 276
Petunia         TTATTGGTTTCAGAACCATAAAGCCCGTGAAAGACAAAAGAAAAGGCTTATTGCTGCTGC 416
Solanum         TTGATCTTAAGAGTTTCATTAGGCCTGAAAGTAGCAATA-----GCCCTCCTAAACTTGC 265
                **  *  *   **   **  * **  *  *    **  *     *   *  *     *


Antirrhinum     CATCATC-ATCACATGAATGTGCCAAC---------AATTCACAATCATCATTATAAGCC 326
Petunia         TACCACTGATAACACTAACCTCCCCATGCAAATGCAATTTCAGAGAGGTGTTTGGAGATC 476
Solanum         TTACAATGAAGACAAGAAAGATTCCTCTCAGGTGGAATCTCACCCAGG-----AGGAACA 320
                   **   *  ***  **     *           *  ***


Antirrhinum     CCCTCCTGTTTATAACAAGTTCTCTAACATGAATAGTGGGAGTTTTCCATCTTCGTCTAA 386
Petunia         ATCTGCTGATGATCCCATTCACCACAAGTATACTAATCCAGGCGTTCACTGTCCATCAGC 536
Solanum         AGATGGAATCCAACACAAGAAC--AGATAGGGATACTGGAAATGTTGTATAGAGGTGGGA 378
                   *       *   **    *    *      ** *       **   *     *


Antirrhinum     TGGTTCACCAGGTTTTCTGACTACTCCAGGCTCTCATGTGGGAAACTATGGTTATGGATC 446
Petunia         TTCTTCTCATGGTGTCCTAGCAGTTGGA------CAGAATGGAAACCATGGTTATGGAGC 590
Solanum         TGCGCACACCCAACGCGCAACAAATAGA-------GCAAATCACAGCACAACTAGGGAAA 431
                *                  *   *  *              * *  *    ** ***


Antirrhinum     AGTGGCAATGGAGAAGAGTTTTAGGGAGTGTACCA-TATCATCAACAACAGATGCAAATG 505
Petunia         TTTAGCTATGGAGAAGAGCTTTAGGGACTGTTCAA-TATCACCAGGATCATCAATGAGTC 649
Solanum         TATGGGAAAATAGAAGGG---AAAAATGTGTTCTACTGGTTTCAAAACCATAAA--GCCC 486
                  * *  *   ***** *    *     *** * * *     **  * **


Antirrhinum     TTGGTGGATCAATG-AGCCAAAACATTGCCTGGATTGGAATCAACAATGAGTACCACAAC 564
Petunia         ATCATC-ATCATCA-AAACTTTGCATGGGCTGGAGTTGATCCCTACTCTTCTACTACAAC 707
Solanum         GTGAAAGACAAAAGCAGAAGAGGAATAGTCTTGGCCTTAGCCAAAGTCCAAGAACACCAC 546
                 *     *  *    *        ** * ** *     *  *          *  ** **
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Antirrhinum     CCCTACACTTTTATTGATACTAGAAAGTATATGAACGGTTATGATCAAACCCTAGAGATA 624
Petunia         T--TACCCTTTTCTTGA------AAAGACTAAACACTTTGAAAATGAAACCCTAGAAGCT 759
Solanum         C---ACCCAT-------------AGTCACTAGTTCCTTTGTCATTTGACACTAGGGG-A 588
                    ** * *              *          *  *    **   ** ****

Antirrhinum     GAGGAGGAGGCAGAAGAAAATTACACGGCTGAGATTGAAACTCTTCCCTTATTTCCTATG 684
Petunia         GATGAGGAACAGCAAGAAGAAGATCAAG--AAAACTACTACTATCAAAGGACCACTTCTG 817
Solanum         GAAGTAGTGAGGGATGAAGATAGTCCATACAAGAGAAAGTGTAGAGGATGGACATTTGAA 648
                ** *  *      * *** *           * *       *               *

Antirrhinum     CACGCAGATATAAAACAAGACACAGCAGATTATTTCAATGGGCGTTTGGAAAATGGGTGT 744
Petunia         CA-------ATAGAA----ACACTTCCACTTTTTCCAATGCATGAAGAGAACAT-----T 861
Solanum         TACCTGGAGGAAGAAC---AAACAACAACAAGGATGACGACGCAAATTATCAATTGGTAG 705
                 *         * **    * **  *          *               **

Antirrhinum     CCTAGGGCTTCACTGGAACTCACCCTCAATTCCTGGTTTGGAAATTCTAAGTATAATTAA 804
Petunia         TCCAG----TTTCTGTAAT-----CTGAAAC----ATCAGGAGTCTTCAGGTGGATTTTA 908
Solanum         AGAAAATGGTGATAGGACTT----CTTCAACTCTTTCCCATTGCATCCAGAAGGCATGAC 761
                   *     *    * *       **  *                 *  *       *

Antirrhinum     CTAAGTGTG-TTTCTAATTAAGGATTACCCCATATAGGTTGGAGTTTAATTATTAATTAC 863
Petunia         CACAGAATGGTATCGTGCCGATGACAACTTGGCTGCTGCTAGAGCTTCTCT-TGAACT-- 965
Solanum         CATGATGCA--TTTTTACACAGAATAATCTTTTTCTTTTTA---CTT------------- 803
                *           *       * *  *           *     **

Antirrhinum     TAGTGTATGCTTTGTACCTTGTAATTAGCTATTGACTATCTTATGTTAAGGGTGTTGGCA 923
Petunia         TAGTCTCAACTCTTTCATTGGAAATTCCTCTTAAAATGTTCTA-GTTATTAGTTCTCCTA 1024
Solanum         ------------------------------------------------------------

Antirrhinum     AGAGTAATCGGCCTTAATTTATTATATAATCATCATCTTGATTAAGCAGAAACTAAGATG 983
Petunia         GTATAATTTGCAGTTACTTTAATTTAGTCTAATTATGTTAGT---ACTAATTTGGCCATG 1081
Solanum         ------------------------------------------------------------

Antirrhinum     TAATCAAGCTGTTGAAGCTATACTATTGACTTGATTTAGTACTTAAAAAAAAAAAAAAAA 1043
Petunia         TACTACGTATCTTTGGACCATGGTAATG--TTAATTATCAATCTAATTAAAAAAAAAA-- 1137
Solanum         ------------------------------------------------------------

Antirrhinum     AAAA 1047
Petunia         ----
Solanum         ----
```
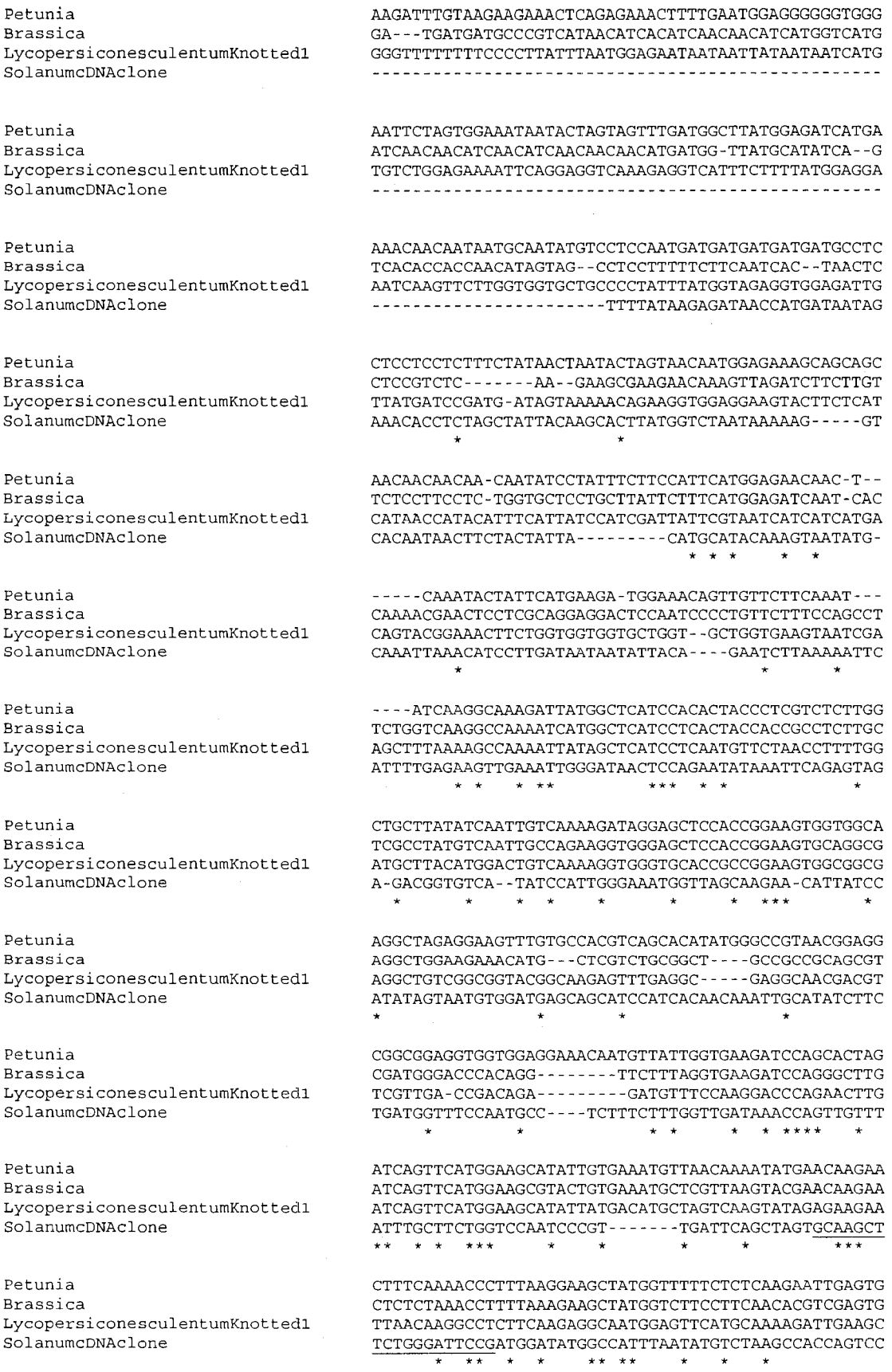

STM primers aligned with EST sequence DV624511 and Genbank sequences AF193813, AY112704 and LEU32247. Underlined portions are primer sequences.

```
Petunia                                 ----------------------------------------------GGC
Brassica                                ------------------------------------------------
LycopersiconesculentumKnotted1          TAAATATGCACAAAATTTGTTTATTTTTAGCCAATAAGAAAGAGATCTCG
SolanumcDNAclone                        ------------------------------------------------

Petunia                                 ACGAGGTGTGAGAGAGAGAGAGAGAGACAAAAAAGGGTTTATGGTACA
Brassica                                -AAAGCTTCCATGGAAAGTGGTTCCAACAGCACTTCTTGTCCGATGGCTT
LycopersiconesculentumKnotted1          TTGAGTAGTTGAAAAAAAAACTTTAATTAGTAGTTGAGGAATAATAAGTT
SolanumcDNAclone                        ------------------------------------------------

Petunia                                 TAGGCAAAAACAAAAGGAACAAAATTAAGTAACAC-------TTAAGAGT
Brassica                                TTGCCGGGGATAATAGTGATGGTCCGATGTGTCCT-------ATGATGAT
LycopersiconesculentumKnotted1          TCCTTTTTTTTATAGGGTTTTATTTAGTTATCATCATCATCTTGATTTA
SolanumcDNAclone                        ------------------------------------------------
```


Petunia
Brassica
LycopersiconesculentumKnotted1
SolanumcDNAclone
AAGATTTGTAAGAAGAAACTCAGAGAAACTTTTGAATGGAGGGGGGTGGG
GA---TGATGATGCCCGTCATAACATCACATCAACAACATCATGGTCATG
GGGTTTTTTTTCCCCTTATTTAATGGAGAATAATAATTATAATAATCATG
AATTCTAGTGGAAATAATACTAGTAGTTTGATGGCTTATGGAGATCATGA
ATCAACAACATCAACATCAACAACAACATGATGG-TTATGCATATCA--G
TGTCTGGAGAAAATTCAGGAGGTCAAAGAGGTCATTTCTTTTATGGAGGA
AAACAACAATAATGCAATATGTCCTCCAATGATGATGATGATGATGCCTC
TCACACCACCAACATAGTAG--CCTCCTTTTTCTTCAATCAC--TAACTC
AATCAAGTTCTTGGTGGTGCTGCCCCTATTTATGGTAGAGGTGGAGATTG
----------TTTTATAAGAGATAACCATGATAATAG
CTCCTCCTCTTTCTATAACTAATACTAGTAACAATGGAGAAAGCAGCAGC
CTCCGTCTC-------AA--GAAGCGAAGAACAAAGTTAGATCTTCTTGT
TTATGATCCGATG-ATAGTAAAAACAGAAGGTGGAGGAAGTACTTCTCAT
AAACACCTCTAGCTATTACAAGCACTTATGGTCTAATAAAAAG-----GT
AACAACAACAA-CAATATCCTATTTCTTCCATTCATGGAGAACAAC-T--
TCTCCTTCCTC-TGGTGCTCCTGCTTATTCTTTCATGGAGATCAAT-CAC
CATAACCATACATTTCATTATCCATCGATTATTCGTAATCATCATCATGA
CACAATAACTTCTACTATTA---------CATGCATACAAAGTAATATG-
-----CAAATACTATTCATGAAGA-TGGAAACAGTTGTTCTTCAAAT---
CAAAACGAACTCCTCGCAGGAGGACTCCAATCCCCTGTTCTTTCCAGCCT
CAGTACGGAAACTTCTGGTGGTGGTGCTGGT--GCTGGTGAAGTAATCGA
CAAATTAAACATCCTTGATAATAATATTACA----GAATCTTAAAAATTC
----ATCAAGGCAAAGATTATGGCTCATCCACACTACCCTCGTCTCTTGG
TCTGGTCAAGGCCAAAATCATGGCTCATCCTCACTACCACCGCCTCTTGC
AGCTTTAAAAGCCAAAATTATAGCTCATCCTCAATGTTCTAACCTTTTGG
ATTTTGAGAAGTTGAAATTGGGATAACTCCAGAATATAAATTCAGAGTAG
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TCGCCTATGTCAATTGCCAGAAGGTGGGAGCTCCACCGGAAGTGCAGGCG
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CTTTCAAAACCCTTTAAGGAAGCTATGGTTTTTCTCTCAAGAATTGAGTG
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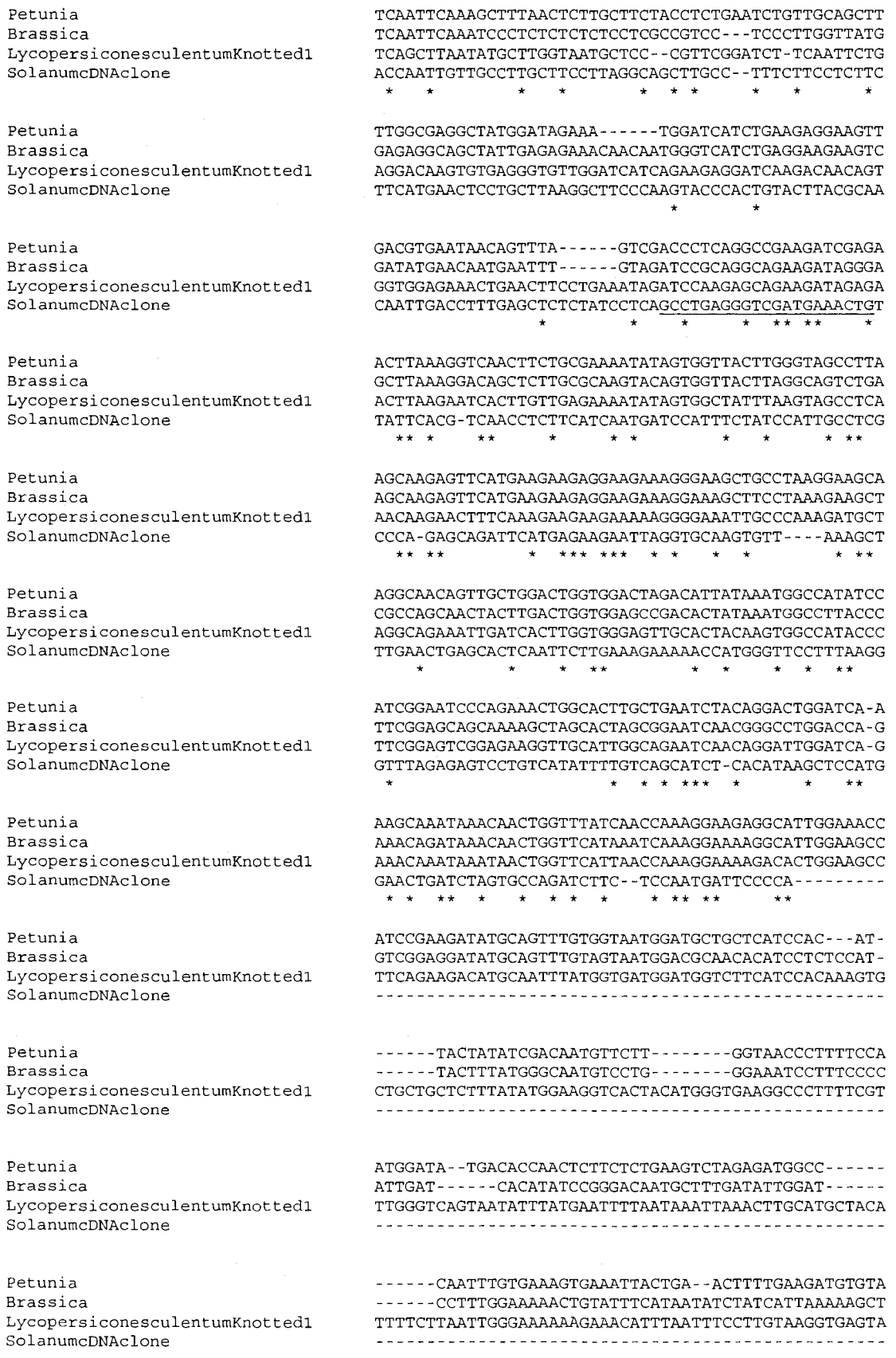
Petunia
Brassica
LycopersiconesculentumKnotted1
SolanumcDNAclone
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------CAATTTGTGAAAGTGAAATTACTGA--ACTTTTGAAGATGTGTA
------CCTTTGGAAAAACTGTATTTCATAATATCTATCATTAAAAAGCT
TTTTCTTAATTGGGAAAAAAGAAACATTTAATTTCCTTGTAAGGTGAGTA

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Petunia                          T---TATTGGGGATAT--AATTGAGGATGTTATTGCATTTACTTGTATGC
Brassica                         T---TGCAATATGTAT--AATGTATGGTTTCTTGGAATACTTAGGAACGT
LycopersiconesculentumKnotted1   TGAATAGTATGGATTTTTAATTAGTTGTAGTATGTATGTTTCTAGCAAGC
SolanumcDNAclone                 --------------------------------------------------

Petunia                          AGTAG----TAGTTGTTATTGGAACTTCAATGCAAGACCATAAGTACTTG
Brassica                         TGCACGTTCCGATTGATGAATAAATTTTAATGTATC-CTTTAATTATATA
LycopersiconesculentumKnotted1   CTTATAGGCCTAAGCACAAGGATATTTTATTGCTTG--TTCAATTGTATA
SolanumcDNAclone                 --------------------------------------------------

Petunia                          TAGTTAGAGCTATTT-ATATTATGGTTTACTTTATTAAAAAAAAAAAAAA
Brassica                         TGGGTTGTGTTGTGT-GACCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
LycopersiconesculentumKnotted1   TGGTGAGAATCCTTTTGTATTTAATATTGTAATGTTATTATTAGTGTACC
SolanumcDNAclone                 --------------------------------------------------

Petunia                          AAA---------------- 1375
Brassica                         AAAAAAAAAAA-------- 1367
LycopersiconesculentumKnotted1   AAAATCTAGTGTTAACTTA 1495
SolanumcDNAclone                 ------------------
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