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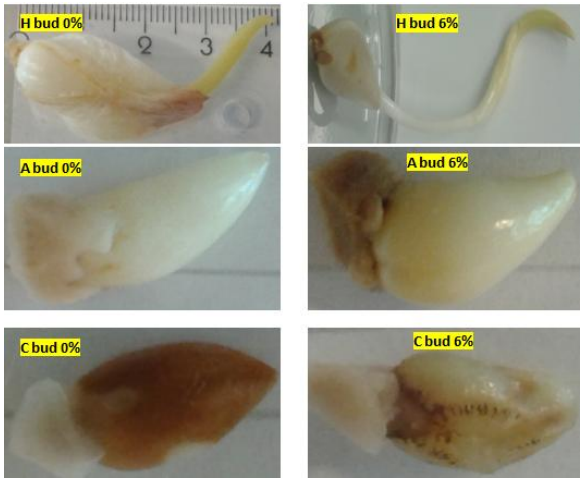

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Abstract

Background: Tulip *(Tulipa gesneriana* L.*)* is one of the top seven flower bulbs which are grown in the world and Netherlands is the leading producer of this plant. Tulip is mainly grown for cut flower production, commercial bulb production, outdoor and potted flowers, garden and landscape design. Tulip bulb is composed of fleshy scales (swollen and modified leaves) attached to a short modified stem ''basal plate'' that produces roots at the basal side. From inner to outer part, bulb is composed of apical flower bud in the case of adult bulb (or vegetative shoot apical meristem for young juvenile bulb) which contains differentiated leaves and all parts of the flower and several axillary buds. Usually, tulip propagates each year by natural vegetative way with the daughter bulbs developed from axillary daughter buds. However, natural tulip propagation rate is still too low because by the end of the growth cycle only 2 to 3 buds develop into daughter bulbs. In a preliminary study on the development of tulip axillary buds, which was done in Wageningen University, a two directional gradient in growth of tulip axillary buds was observed. The axillary buds closer to floral bud or closer to the outer H bud (the only buds that sprout and produce leaves) appear to grow the most. **Objective**: The aim of this present study is to understand the mechanism which controls this two directional gradient in growth of tulip axillary buds. **Hypothesis I**: Two directional gradient in growth could be due to the difference in photo-assimilate translocation in axillary buds. **Hypothesis II:** The difference in growth capacity could be due to the difference in endodormancy levels between buds. **Materials and Methods:** To test those hypotheses, *in vitro* culture of detached buds (A, D and H buds) was achieved at 0 and 6% sucrose and dry weight gain was measured. In addition, the expression of *BRANCHED1* (*BRC1)*, a dormancy related gene was analysed in A, H and D buds collected at different time points from bulbs grown under field conditions and the *in vitro* culture samples through qPCR. **Results:** *In vitro* culture, the absence of sucrose in the medium resulted in bud weight loss and death of D buds; while at 6% the buds absorbed the sucrose and gained dry weight. A and H buds gained dry weight at the same rate, while D buds grew hardly. In both samples from the field and *in vitro* culture, *BRC1* was up-regulated in D buds at initial time points. Moreover, down regulation in *BRC1* expression did not lead to the proper growth of D buds. **Conclusion:** Two gradient growth in tulip axillary buds depends on difference in endodormancy level in the buds. It could be a combination between both source to sink process and insufficient down-regulation of *BRC1* gene for proper growth of D buds. More evidence is needed to test if there is a preferential translocation of photo-assimilates from the leaves to the closest axillary buds.

Key words: Tulip morphology, tulip growth cycle, dormancy types, dormancy related genes in axillary buds

Table of contents

1. Introduction

1.1. Origin, description and morphology of tulip

Tulip (*Tulipa gesneriana* L.) is a monocotyledonous flower bulb which belongs to Liliaceae family (Kamenetsky and Okubo, 2013; Ptak and Bach, 2007; Jaap and Marjan, 2006). This ornamental geophyte originates from Irano-Turanian region (Kamenetsky and Okubo, 2013; Maarten, 1997). Tulip was introduced in Europe in the 16th century and nowadays, is one of the top seven geophytes genera which are grown in the world beside *Lilium*, *Gladiolus*, *Narcissus*, *Hyacinthus*, *Iris* and *Crocus* (Kamenetsky and Okubo, 2013; Department of floriculture Kashmir, 2012; Aurel, 1997) with the Netherlands as the leading producer (Jaap and Marjan, 2006). Tulip is mainly grown for cut flower production, commercial bulb production outdoor and potted flowers, garden and landscape design (Leeggangers *et al.*, 2013; Jaap and Marjan, 2006; De Hertogh and Le Nard, 1993).

Tulip is a geophyte plant with a bulb as an underground storage organ (Kamenetsky and Okubo, 2013). Tulip bulb is composed of five to seven fleshy scales (swollen and modified leaves) (Department of floriculture, Kashimir, 2012) attached to a short modified stem ''basal plate'' that produces roots at the basal side (Saniewski and Okubo, 2005). From inner to outer side, a bulb is composed of one apical flower bud in the case of adult bulb (or vegetative shoot apical meristem for young juvenile bulb) which contains differentiated leaves and all parts of the flower (Kamenetsky and Okubo, 2013) and several axillary buds (Figure 1A). The bulb is covered by a tunic or dry outer bulb scale (Department of floriculture, 2012). Axillary buds, also called daughter buds, are formed in the inner base of the scales with one bud per scale in general (De Hertogh and Le Nard, 1993). Tulip bulb contains the same parts as those of a standard plant (roots, stem which is basal plate, leaves which are scales, apical meristem, axillary meristems) with the only difference that for tulip, the organs are modified and clustered on the underground bulb (Figure 1).

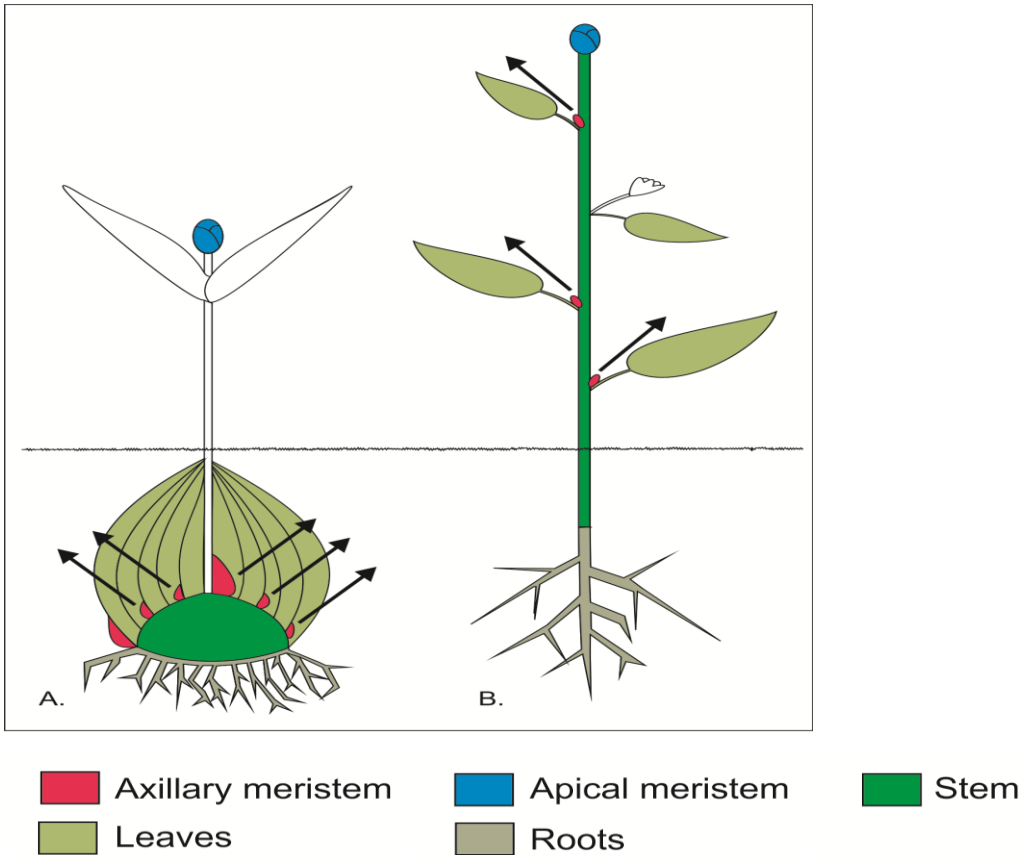

Fig. 1. Architecture of a tulip bulb (A) and standard plant (B). *Initiation of axillary meristems takes place in the axils of bulb-scales* ***(A)*** *or leaves* ***(B).*** *They form axillary buds which undergo a period of dormancy. Once bud dormancy release occurs, these buds grow out and develop into daughter bulbs in tulip plants, or lateral branches in a typical dicot plant (Leeggangers et al, 2013).*

1.2. Natural growth habit of tulip

According to life cycle, flowering and dormancy pattern *Tulipa* is classified in annual geophytes (Kamenetsky and Okubo, 2013), because it has an annual replacement cycle with three generations inside the bulb (Jaap and Marjan, 2006). The first generation is the mother bulb. The second generation are the axillary buds which will develop into daughter bulbs and will replace the mother bulb in the next season; and finally, the third generation are the grand-daughter buds inside the daughter bulbs, which will develop during winter of the following year (Department of floriculture, Kashimir, 2012; Jaap and Marjan, 2006). Normally, a mother bulb is planted in autumn and will develop the roots by November-December. During winter, shoot starts to elongate gradually and the daughter-bulbs start to grow slowly but there is no above-ground shoot. During spring, temperature increase triggers tulip floral bud elongation and blooming (Kamenetsky and Okubo, 2013; Lambrechts *et al.,* 1994).

After blooming of the mother bulb in spring, the daughter buds are transformed into daughter bulbs (Department of floriculture, 2012; De Hertogh and Le Nard, 1993). Normally, two or three daughter buds grow into daughter bulbs with a reasonable size, while the others show hardly any grow and might remain dormant (Leeggangers *et al.*, 2013; Department of floriculture, Kashmir, 2012). It has been reported that the innermost bud (A bud) produces the largest bulb, while the other buds grow into smaller bulbs with fewer scales (Department of floriculture, Kashmir, 2012). Daughter bulbs' growth reaches the maximum in early spring. At the end of the spring, growth of the daughter bulbs is complete and all the aerial organs of the mother bulb senesce. Thereby, the mother bulb is replaced by a cluster of new bulbs (daughter bulbs which contain an apical bud in vegetative state) (Saniewski and Okubo, 2005).

In the summer at high temperature (17-20°C or higher), daughter bulbs undergo initiation and differentiation of the central vegetative bud into a floral bud and initiate the axillary vegetative buds (grand-daughter buds) and root primordial. Once flower initiation and differentiation are complete, daughter bulb enters in dormancy state i.e from early autumn and they cannot develop into shoots until the early spring of the following year (Kamenetsky and Okubo, 2013; Podwyszynska, 2012). In the case that low temperature occurs in the summer, floral initiation inside the bulb will be prevented and vegetative meristem formation will benefit (Jaap and Marjan, 2006). In the case temperature remains high in autumn, this leads to low shoot growth and flower abortion in the next season (Kamenetsky and Okubo, 2013).This explains that tulip bulb requires cold conditions (4.5°C between 14-16 weeks) for dormancy release and sprouting (Kamenetsky and Okubo, 2013; Department of floriculture, Kashmir, 2012).

1.3. Tulip propagation

There are two methods that can be used for tulip propagation: sexual propagation by seed production and vegetative propagation. Like for other plants, three developmental phases namely juvenile, adult, and reproductive phases are also encountered in tulip propagated from seeds. However, in this species, juvenile phase takes several years (Kamenetsky and Okubo, 2012; Podwyszynska and Marasek, 2003). Thus, sexual propagation in tulip is usually only used by breeders in order to create and introduce new cultivars to the market and this can take more than 20 years (Podwyszyńska and Sochacki 2010; Podwyszynska and Marasek, 2003). Vegetative reproduction is divided into natural vegetative propagation (outgrowth of daughter buds) and artificial propagation by scaling or micropropagation *in vitro* of cells, tissues or organs (Kamenetsky and Okubo, 2013). The rate of natural vegetative propagation in tulip through daughter bulbs is also insufficient to meet the demand of the market (Leeggangers *et al.*, 2013; Maslanka and Bach, 2013). This insufficiency is due to the restricted number of lateral

buds which will rise into new adult bulbs for the next generation. Artificial propagation in tulip is not easy through traditional vegetative techniques like scaling, cuttings, etc compared to other flower bulbs, such as lily and gladiolus (Aurel, 1997; Podwyszynska and Marasek, 2003).

Although plant tissue culture can increase the reproduction rate compared to natural vegetative propagation, it seems to be difficult and growth rate still too low for tulip (Jaap and Marjan, 2006). For instance, shoot and bulb production from tulip axillary buds was also studied by Rice and colleagues (1983) and the multiplication rate was reported to be low. Moreover, Ptak and Bach (2007) and Podwyszynska and colleagues (2011) revealed that the only successful technique in tulip micropropagation is the culture of stalk explants as it gave 60-90% regeneration rate although some researchers observed that many cultivars are not able to regenerate shoos via this technique (Klerk *et al*, 2014, unpublished). Propagation of tulip by axillary bud is the most suitable to ensure genetic stability of the regenerated plants since meristems resist more to genetic changes than other tissues and it is also the most used as tool to study tulip development (Ngezahayo and Liu, 2014).

1.4. Dormancy in tulip bulb

Dormancy is a physiological mechanism in which plant adapts to survive during unfavorable environmental conditions, and by which there is no visible growth of plant organs (Kamenetsky and Okubo, 2013). Usually, they are three different kinds of bud dormancy in standard plants such as endodormancy, ecodormancy, and paradormancy. Endodormancy or innate dormancy refers a growth inhibition due to an endogenous signal, while ecodormancy is growth suppression due to an external stimulus like temperature, drought, etc. Paradormancy is considered as a growth reduction due to an internal signal in the plant and the signal is transported to a target tissue. Axillary bud dormancy is an example of paradormancy by which bud growth is suppressed by the dominance of shoot tip or apical bud referred as apical dominance (Kamenetsky and Okubo, 2013). In tulip, axillary buds undergo probably all types of dormancy. They have paradormancy because their dormancy level seems to be controlled by other tissues such as the floral bud. The outgrowth of the buds is also controlled by ecodormancy because it has been reported that they need cold winter for breaking dormancy. Finally, they probably have a certain level of endodormancy, meaning that the dormancy is regulated inside the bud tissue itself, for instance when the dormancy correlates with expression of dormancy related genes.

It is already known that harvested daughter bulbs undergo a period of dormancy and are not able to grow until the early spring of the following season. However, during this period, flower initiation and other physiological changes occur inside the bulb (Kamenetsky and Okubo, 2013). Hence, bulb dormancy can be defined as a period in which organ differentiation occurs inside the bulb, with minimal shoot elongation or any physiological changes outside the bulb (Kamenetsky and Okubo, 2013). Rees (1981) suggested that a true dormancy occurs when meristematic tissues become inactive. According to him, there is no true dormancy in tulip bulbs as flower and bud differentiation continues inside the bulb even there are no apparent morphological changes. Maybe, it is the reason why Kamenetsky (1994) referred this period of bulb dormancy as ''Intrabulb development''.

1.4.1. Dormancy release in tulip bulb

1.4.1.1. Dormancy release by cold treatment (pre-cooling)

It has been observed that during the dormancy period there is no visible external growth from tulip bulb. Temperature is considered as the main factor that affects dormancy and dormancy release and subsequent developmental processes in tulip plant. For instance, high temperature (17-25°C) is required for flower initiation and development inside the adult daughter bulbs (Khodorova and Boitel-Conti, 2013). In addition, temperatures ranging between 14-16°C are required for tulip aboveground growth (Leeggangers *et al.*, 2013; Kamenetsky and Okubo, 2013). However, cold temperature triggers physiological and molecular changes inside the bulb and is required for dormancy release in apical bud (Kamenetsky and Okubo, 2013; Khodorova and Boitel-Conti, 2013; Lambrechts *et al.*, 1994). For instance, full cooling of tulip mother bulbs (5°C for seven months) of tulip was noticed to be necessary for the growth of daughter bulbs and this affects flower bud differentiation, shoot growth, flowering and bulbing of axillary buds in the next season (Saniewski and Okubo, 2005). This explains the requirement of pre-cooling treatment of tulip bulbs (4°C) (Department of floriculture, Kashmir, 2012) at least for eight weeks before planting or low winter temperature for dormancy release and activation of internal changes necessary for stem elongation and successful blooming in the next season (Leeggangers *et al*., 2013; Saniewski and Okubo, 2005; Rietveld *et al*., 2000).

Some authors reported a correlation between temperature and hormonal balances, sugar mobilization and other physiological changes which are required for bulb sprouting (Jaap and Marjan, 2006). Thereby, a correlation between cold treatment (8-12 weeks) and decrease of abscisic acid (ABA) and starch level in tulip bulbs was observed while there was an increase in soluble sugars, cytokinin and gibberellin contents (Ptak and Bach, 2007; Podwyszynska, 2012).

It was observed that external dormant state in tulip does not affect internal processes and internal active metabolisms are achieved through hormonal balance, water flux and carbohydrate mobilization inside bulb. All these processes in geophytes plants are affected by temperature (Khodorova and Boitel-Conti, 2013) (Figure 2). It was observed that ABA and cytokinins phyotohormones in bulbs are not dependent on temperature conditions, while production of auxin and gibberellin is. Auxin is the main hormone involved in initiation of tulip stalk elongation and plant response to auxin sensitivity was reported to correlate to cold treatment (Rietveld *et al*., 2000). Thus, in the absence of cold treatment shoot growth in tulip is too slow (Khodorova and Boitel-Conti, 2013).

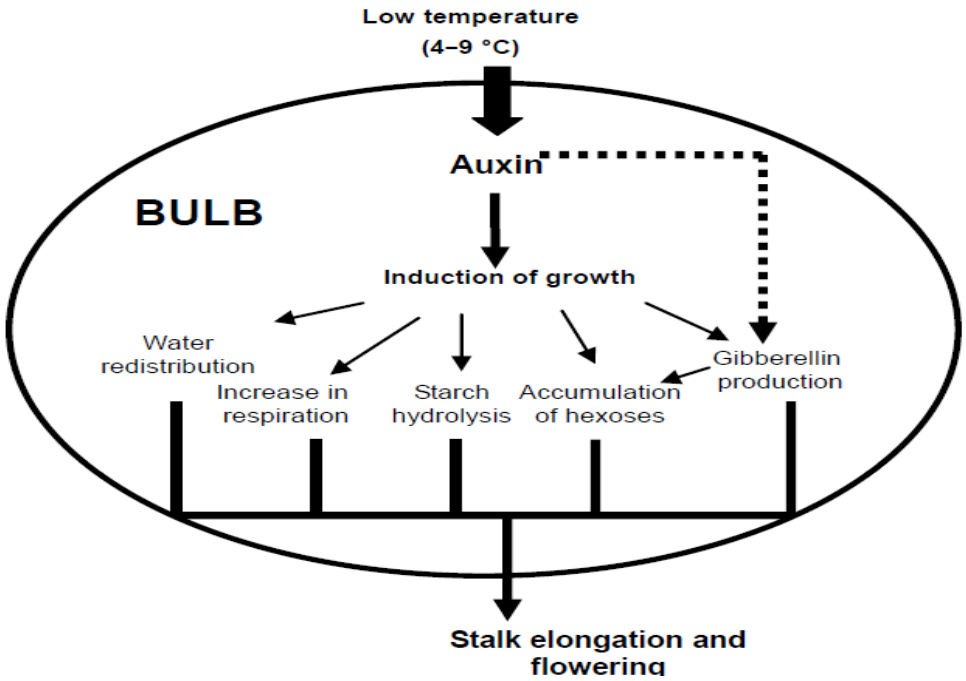

Figure 2. A proposed model of low temperature effect on growth in geophytes. Low temperature increases plant sensitivity to auxin. Auxin induces shoot growth and this triggers storage reserves remobilization in bulb, an increase in water flux, respiration and gibberellin biosynthesis. The latter process enhances expression of invertase genes, which are required for hexoses production. The sugars are then used for shoot elongation and flowering. Auxin also triggers onset of gibberellin biosynthesis (Khodorova and Boitel-Conti, 2013).

In addition, cold treatment was reported to influence sugar mobilization and transport (Figure 2) from bulb scales to the buds. This is due to the temperature effect on expression and activity of α-amylase activity which are required for starch hydrolysis into hexoses (Lambrechts *et al.*, 1994). This was observed in tulip bulb where a rapid starch degradation was found in precooled (5°C for 12 weeks) scales than in non-cooled ones (17°C for 12 weeks). In the same experiment sucrose and fructants were the main soluble sugars identified in precooled scales. The simple sugars, from which especially glucose, are involved in cell division and shoot elongation. It has been suggested that in plant, slow shoot elongation in absence of cold

treatment is a result of inadequate sugar supply (Khodorova and Boitel-Conti, 2013) and this has been confirmed by a study on tulip growth which was conducted by (Lambrechts *et al.*, 1994) in which floral stalk of pre-cooled bulbs elongated and produced flowering faster than non-cooled bulbs.

Beside hormonal sensitivity and carbohydrate status, low temperature also affects water flux in tulip bulb and axillary buds. It was revealed that low temperature (5°C) during storage, induces water flux from the basal plate and scales to the developing buds. One of the reasons of these results could be due to the fact that low temperature leads to expression of aquaporin γTIP gene in stalks, a gene involved in water transport (Khodorova and Boitel-Conti, 2013). Moreover, low temperature enhances the number of mitochondria and subsequently higher respiration and energy production from pre-cooled buds compared to non-precooled ones (Lambrechts *et al.*, 1994). Furthermore, low water content was analysed in tulip buds which were stored at 17°C. It is known that a proper water flux between bulb and buds affects subsequent growth and developmental stages. Therefore, low water state in the bulbs at high storage temperature, results to flower bud abortion. Although the mechanism is not clear, high temperature in contrast inhibits water transport to the buds (Khodorova and Boitel-Conti, 2013).

1.4.1.2. Dormancy release by internal signals

Beside temperature conditions, hormone balance and carbohydrate status were also reported as factors affecting dormancy release in higher plants. The most hormones which are related to dormancy release in geophytes plants are auxin, cytokinin, and gibberellin. Auxin is the main hormone involved in initiation of tulip stalk elongation. The main source of auxin is the flower or apical bud. Therefore, the removal of floral bud even in pre-cooled bulbs led to poor stalk growth whereas auxin application to that removal site resulted to normal stem growth (Khodorova and Boitel-Conti, 2013). Gibberellin is a hormone which accelerates cell elongation and when it was applied to non-cooled tulip bulbs this resulted to shoot elongation and flowering. The same results were also obtained in lily bulblets propagated *in vitro* (Kamenetsky and Okubo, 2013). On the other hand, cytokinin was revealed to play role in dormancy breaking especially in tubers and corms. This was confirmed by a complete dormancy release in potato tubers when the level of cytokinin increased in apical buds. This hormone is essential for cell division (Kamenetsky and Okubo, 2013).

A. Theory of auxin and apical dominance

It is already mentioned above that tulip bud outgrowth can be controlled by paradoramancy, a dormancy controlled by apical dominance. The term apical dominance refers as the growth of shoot tip or apical bud over axillary buds (Mason *et al.*, 2014; Cline, 1994). Usually, apical dominance induces dormancy in axillary buds and the removal of apex releases this dormancy and induces bud outgrowth (Rietveld *et al*., 2000; Romano *et al.,* 1993). The main phytohormones involved in apical dominance and the control of lateral bud outgrowth are auxin and cytokinin (Cline, 1994). Auxin maintains apical dominance and inhibits lateral bud growth, while cytokinin induces it (Beveridge, 2006). Therefore, the level of lateral bud release or shoot branching correlates to apical dominance (Cline, 1994). It is known that auxin is mainly produced in apical bud (apex) (Rietveld *et al*., 2000; Romano *et al*., 1993). Some authors reported that auxin basipetal transport from shoot tip to roots (Mason *et al.,* 2014) leads to its export to the axillary buds where it suppresses the bud growth (Cline, 1994). This plant hormone also has a negative effect on regulation of hormones involved in lateral bud outgrowth (Cline, 1994) (Figure 3).

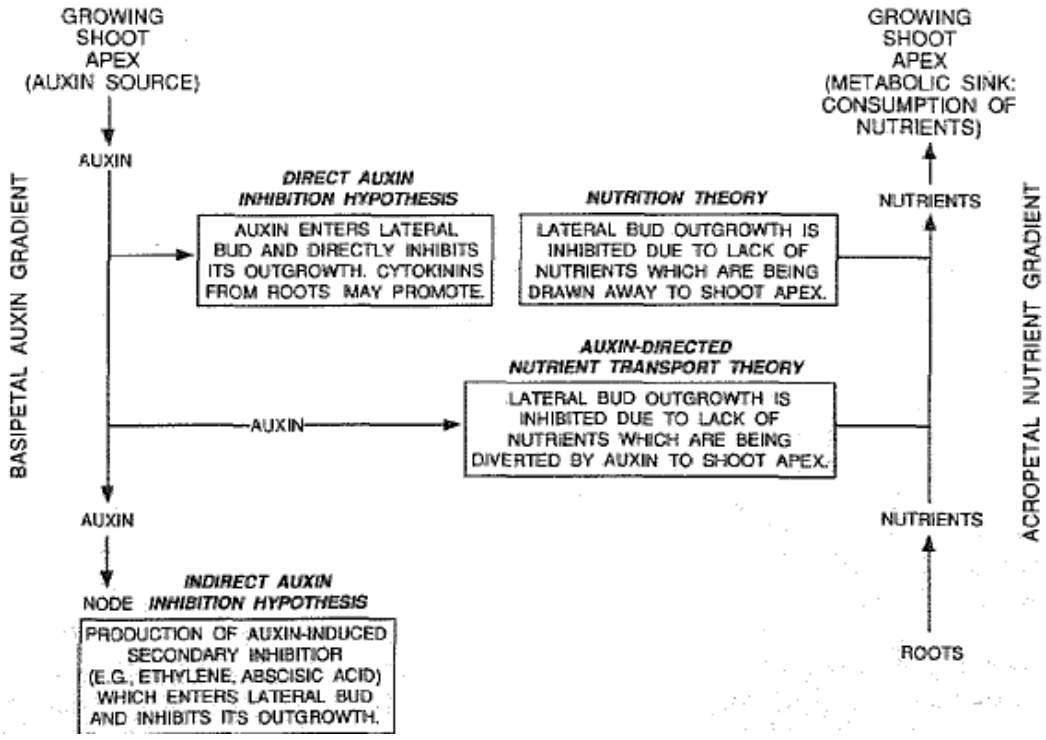

Figure 3. Hypotheses to explain the mechanism of apical dominance (reviewed by Cline, 1994)

Some researchers thought that auxin stimulates the biosynthesis of secondary inhibitors of lateral buds such as ethylene and ABA and that these hormones move toward axillary buds where they prevent bud outgrowth (Chatfield *et al.*, 2003; Cline, 1994). However, it was revealed that ethylene is not a second messenger for auxin as no lateral bud release was correlated to ethylene level in plant (Li and Bangerth, 1999). However, ABA was found to be associated with bud dormancy but no relevant study showed its role as second messenger of auxin (Chatfield *et al.*, 2003). Other authors reported that auxin negatively affects the regulation of cytokinin transport from roots to the buds (Cline, 1994).

Auxin is basipetally transported from apex to roots whereas cytokinin is acropetally transported from the roots and this transport gradient plays a major role in outgrowth of lateral buds. It has been reported that cytokinin export from the root to the buds is dependent on apical auxin. Other researchers suggest that auxin can prevent cytokinin synthesis. Therefore, reduction of auxin level after decapitation of the apical bud triggers either cytokinin export from the roots to the buds or increase cytokinin biosynthesis in lateral buds and subsequent budbreak (Beveridge *et al*., 1997a). Furthermore, a study conducted on peas showed that beside the auxin and cytokinin contents in plant, another cue could be associated with lateral bud release. The model proposed that auxin level may directly influence nutrient transport along stem (Beveridge *et al*., 1997a) while cytokinin was supposed to enhance nutrient sink in axillary buds and then induces nutrient mobilization toward the buds (Cline, 1994). In summary, auxin plays central role in the growth of shoot tip and apical dominance over lateral bud outgrowth by its basipetal transport from apex towards the roots. Depletion of auxin in stem promotes the synthesis of cytokinin (hormone involved in branching) (Mason *et al.,* 2014; Beveridge, 2006) as well as it inhibits strigolactone biosynthesis (hormone which inhibits bud growth) (Mason *et al*., 2014).

B. Theory of sugars and axillary bud outgrowth

In contrast to auxin, sugars were noticed to be necessary nutrients not only required for energy supply but also as signals for many physiological processes such as flowering, dormancy release, stress tolerance, etc (Moghaddam and Van den Ende, 2013). It was reported that carbohydrates are important for bud growth by acting with phytohormones and by controlling apical dominance (Lockhart 2014; Rolland *et al.*, 2006; Cline, 1994). For instance, in a study conducted on budbreak in *Gentebiana* species, a correlation between bud release and disaccharide sugar ‘’gentabiose’ was observed. This sugar was reported to act as signal to release dormancy through ascorbate (AsA) and GSH sulphur-containing amino acids. The two

latter amino acids are also antioxidant and reduce ROS via AsA-GSH cycle. The synthesis of AsA-GSH was observed in active buds which were fed with gentabiose. However, it is not known if other sugars have the same effect on dormancy release in *Gentabiana* or other plant species (Lockhart, 2014).

In study conducted by Mason and colleagues (2014) on auxin and sugar supply in dormancy release, after decapitation of pea plant, they have observed that bud release from dormancy was independent on apical auxin. This was confirmed by observation of bud release earlier before the change of auxin level in stem was detected. However, after decapitation, change in sugar distribution and accumulation in lateral buds was observed within a timeframe of bud outgrowth. In addition, sucrose increase in plant suppressed *BRANCHED1* (*BRC1*) expression, a key gene for bud dormancy maintenance which is synthesized in buds through strigolactone and cytokinin. The same study showed that the transport of sugars to axillary buds was inhibited by apical dominance which is controlled in turn by auxin. On the other hand, high sink in assimilates in apex over buds limits the amount of sugars distributed into lateral buds for growth (Mason *et al*., 2014; Kelly *et al*., 2012).

Thereafter, suppression of apical dominance leads to sugar redistribution in the buds and subsequently to meristematic activity to release bud growth. This explains that increasing of sugar supply in the plant enhances bud release from the dormancy caused by apical dominance. Thereby, application of exogenous sucrose induces bud release from dormancy. Increase of sugars in plant was reported to be important and sufficient regulators for lateral bud growth from dormancy by regulating cell cycle and meristematic activity. The findings from the same experiment supported the hypothesis that assimilates produced in leaves during photosynthesis are transported to shoot tip (apical dominance); and suppression of the shoot tip enables long-distance redistribution of sugars into lateral buds for bud release (Mason *et al.*, 2014). A minimal amount of sugars must reach axillary buds to remove apical dominance and for initial bud growth or apex for flowering induction (Mason *et al.,* 2014; Beveridge, 2006) (Figure 4).

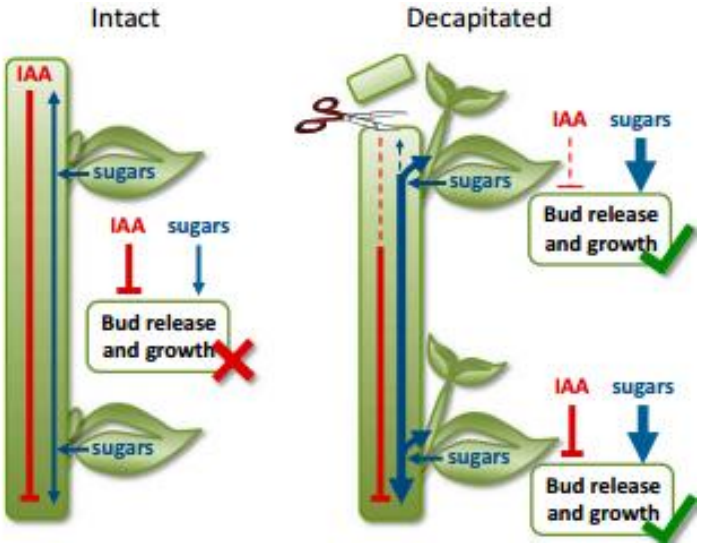

Figure 4. Apical dominance is controlled by sugar responses. In intact plant, apical dominance is maintained over axillary bud growth by limiting sugar transport to buds. Decapitation of apex led to rapid sugar accumulation in axillary bud and subsequently to bud outgrowth (Mason et al., 2014)

Preliminary research on tulip axillary buds done in Wageningen University

The outgrowth of tulip axillary buds has been studied in three cultivars during two growth cycles in a study conducted by Moreno-Pachon (2013; unpublished). The main finding from her study is a two gradient direction of growth in axillary buds according to their position in the bulb. The inner axillary bud (A bud) which is close to flower bud produced the biggest daughter bulb at the end of the season. The second and third place in growth was taken by B and H bud (assimilate source itself) while as long as the axillary buds locate far from either flower bud or H bud, they produced smaller bulbs (Figure 5). It was also clear that A, B and H buds grew during the cold period while C, D and E had limited or not apparent growth (Figure 5). In addition, she found that only apical (floral bud) and H bud sprout and produce leaves during the growth cycle, with the leaves of the floral bud being bigger than the ones of the H bud. Another observation was that axillary buds increase dramatically in size once the flower opens and is removed from the plant.

Since the active leaves of a plant are the source of assimilates produced through photosynthesis, it can be said that tulip bulbs have two opposite located sources of photo-assimilates and that after blooming the axillary buds closer to those sources become strong organ sinks (Figure 5).

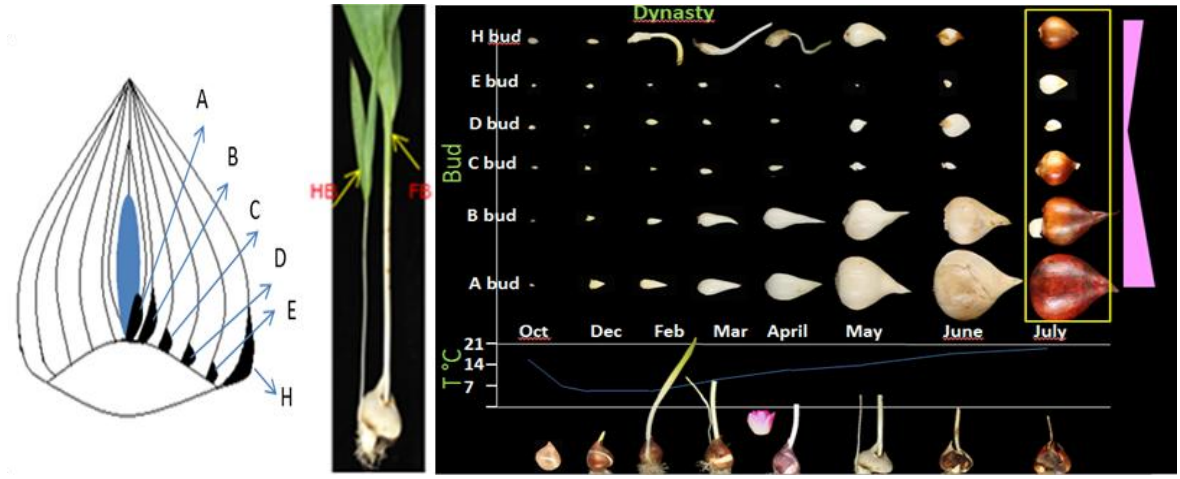

Figure 5. Gradial growth between axillary buds due to their location in tulip bulb. Flower bud (FB) or H bud (HB) are the only buds that produce leaves (source of photo-assimilates). The buds closer to the sources grew more than the others.

From the just explained findings, some hypotheses were formulated and were tested during this internship project:

1. The two directional gradients in growth of the axillary buds is caused by a source to sink process: there is a higher translocation of photosynthesis assimilates from the leaves of the floral bud to A bud and B bud; and from the leaves of the H bud to itself and to E bud.

2. The two directional gradients in growth of the axillary buds are caused by different levels of bud endodormancy controlled by dormancy related genes.

1.5. Aim of the present study

The aim of this present study is to understand the control of the two directional gradients in growth of tulip axillary buds. To achieve this objective, the two before mentioned hypothesis were tested.

2. Materials and methods

2.1. Bud tissue culture

The present study was conducted in molecular laboratory of Wageningen University. The experiment was performed through tissue culture of three axillary buds: the mid located bud, D bud, which does not grow out during the bulb growth cycle; the most inner located bud, A bud, which reach the highest size and weight at the end of the growth cycle; and the most outer located bud, H bud, which produces leaves during the bulb growth cycle and reaches a mid-size after the bulb growth cycle. All buds were collected from pre-cooled bulbs (using Strong Gold cultivar). Pre-cooled bulbs consisted of adult bulbs stored at 1°C for 3 months after lifting. This precooling treatment simulates the winter period required for the bulbs to break ecodormancy.

Before bud culture in growth medium, a surface sterilization was achieved by dipping the buds in 70% ethanol, transferring them in 2% sodium hypochloride for 20 minutes followed by washing with sterile water for 1, 5 and 10 minutes, respectively. The sterile buds were then air dried and individually cultured in MS medium supplement with agar and with, or without sugar (control), according to treatment. A medium was composed of half strength MS (0.22% MS w/v), 0.4 % phytagel (w/v) and 0.1 % DMSO (v/v) and with or without sucrose accordingly to treatment. Then, the pH was adjusted at 5.8 before it was autoclaved. After culturing the buds, the pots were incubated at 24°C in dark in a tissue culture room.

2.2. Measurement and calculations of dry weight (DW) gain

In this study, bud growth (dry weight gain) was measured for 2 months with three weeks between sampling points. The detached buds had a big variation in initial size; therefore, to calculate DW gain, we must first normalize the final DW (fDW) of each bud against its initial DW (iDW). However, initial dry weight is not possible to be measured otherwise you kill the bud, but it can be estimated. Therefore, we used initial fresh weight (iFW) of each detached bud (measured before culture) and index (water content index: Calculations below) to estimate the initial DW.

Equations used to estimate initial DW:

iDW= iFW/index

Where iDW: initial dry weight, iFW: initial fresh weight (measured before culture)

Index= FW/DW

Where FW: initial fresh weight, DW: dry weight

To get the index of each bud type, we measured initial FW of A, H and D buds (15 replicates for each) then we put them in oven overnight. Next, we measured the dry weight and from this we calculated the water content index for each bud type.

Table 1. Index used to estimate initial DW of axillary buds

Index	A bud	D bud	H bud
FW/DW	2.71	2.4	3.15

From here, estimated initial dry weight (iDW) was used to normalize the DW gain.

DW gain (%) = ((fDW-iDW)/iDW)) *100

Where fDW: final dry weight of each bud (see how it was measured below); iDW: initial dry weight (estimated: see above explanation).

The following process was used to measure the final dry weight (fDW) of the buds. For every time point we measured the weight of an empty tube, after adding a fresh bud, we put it immediately on liquid nitrogen and dried in freeze drier. After drying, we measured the tube with dry mass and the difference gave us the final dry weight.

2.2. RNA extraction

Plant materials (H, D and A buds) sampled from different time points during experiment were collected in liquid nitrogen and immediately freeze dried for two days. After grinding them, around 60 mg of tissue was used for RNA extraction. RNA extraction was performed with Hot-Borate method. The hot borate buffer was supplemented with 792 mg PVP and 26.4 mg DTT to 13.2 ml of Hot-borate stock for every 15 samples. The buffer was incubated at -80°C to enable complete homogeneity of the solution. Tissues were then mixed with 800 µl XT-buffer. After adding 4 µL Proteinase K, samples were incubated at 42°C for 15 minutes. Subsequently, 64 µl of 2M KCl was added and samples were incubated on ice for 30 minutes followed by a centrifugation at 12000g at 4°C for 20 minutes. The supernatant was transferred to a new tube and 259 µL of 8M LiCl was added and incubated 1 hour or overnight at 4°C on ice. Subsequently, samples were centrifuged at 12000g at 4°C for 30 minutes and the remaining pellet was washed with 750 µL of 2M LiCl and after a new centrifugation for 10 minutes, air dried pellet was resuspended in 80 µL DEPC-treated water. In order to remove DNA, after measuring the RNA

concentration with the Nanodrop, 10 µg of RNA was treated with DNAse enzyme by adding 10 µL of DNAse buffer and 10 µL of DNAse enzyme to a final volume of X µL of RNA.

In the next step, samples were incubated at 37°C for 20 minutes. Then, 100 µL phenol chloroform was mixed to RNA and 200 µL of this RNA/phenol cholorophorm mixture was transferred to phaselock eppendorf tube and after spinning for 5 minutes, around 85 µL of the upper phase was transferred to an eppendorf tube in which 1/10 volume of 3M NaAc and 2.5x volume 100% ice-cold ethanol (8.5 µL and 212.5 µL respectively) was added. This was followed by a precipitation for at least 1 hour at -20°C. RNA extraction was continued with centrifugation at 12000 g for 30 minutes at 4°C followed by washing the pellet with 250 µL cold 70% ethanol and a new centrifugation for 10 minutes. Evaporation of ethanol was performed by air drying for 10 minutes in a fume hood. Finally, the pellet was dissolved in 20 µL DEPC-treated water. RNA concentration and quality were assessed by using Nanodrop spectrophotometer and RNA integrity was checked by agarose (1%) gel electrophoresis after diluting 1 µL of RNA sample with 1.6 µL of loading buffer and 7.4 µL DEPC-MQ water.

2.3. cDNA synthesis

From 500 ng of isolated RNAs, cDNA synthesis was performed by using a cDNA synthesis kit (Biorad) for first-strand cDNA synthesis. It contained the RNase H^+ iScript reverse transcriptase for sensitivity and iScript reaction mix (a buffer) to prevent indiscriminate degradation of RNA template. cDNA synthesis was achieved as following. First, a reaction solution was prepared by mixing 4 µL 5x iScript reaction mix (buffer), 1 µL iScript Reverse Transcriptase enzyme, RNA template volume calculated from the RNA concentrations obtained on Nanodrop in order to use 500 ng RNA and DEPC-MQ water to get a total volume of 20 µL. Then, the reaction solution was incubated for 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C and finally hold it at 6°C. After performing the reaction, cDNA was diluted (1:20) by adding 95 µL of nuclease free water to 5 µL of cDNAs.

2.4. Gene expression analysis

Gene expression in both tulip samples from field and *in vitro* was performed via qPCR (quantitative polymerase chain reaction) with Bio-Rad program. By this method, 2.5 µL of cDNA was mixed with 0.5 µL of primer (10µM), 2 µL of DEPC-water and 5 µL of SYBRGREEN to make a total volume of 10 µL reaction mixtures for gene amplification. SYBRGREEN was a mixture of enzyme, buffer and chemical dye to that fluoresce when bound to double-stranded DNA. A series of heating and cooling cycles (95°C for 3' and for 30'' respectively, 60°C, 95°C, 55°C, 95°C for 30'', 1', 10'' and 5'' respectively) allows the amplification of cDNA and production of double-

stranded DNA. Three housekeeping genes were used to normalize the expression of *BRANCHED1* (*BRC1*). Those housekeeping genes were *Protein phosphatase 2A* (*PP2A)*, *Actin* (*ACT*) and *Elongation factor 1 alpha* (*EF1α*) (Table 2).

Table 2. Sequence of housekeeping genes and gene of interest

Gene	Primer name	Sequence
Elongation factor 1 alpha	qEF1a_fw	TGA GAA GGA GGC TGC TGA AATCA
	qEF1a_rev	TCA CGA TGA CCA GGA GCA TC
Actin	qAct1_fw	AGC AAC TGG GAT GAC ATG GA
	qAct1_rev	GGA CAG CCT GAA TTG CAA CA
Protein phosphatase 2A	qPP2A_fw	TGG CGA GTG GTT TAC TGC TA
	qPP2A_rev	CCG TCT TCA AAT GGT TTG GT
BRANCHED1 (BRC1)	tBRC1_fw	ATGAGGCTCTCCCTGGATGT
	tBRC1_rv	ACATGGTGAGAAGCCACTGG

2.5. Statistical analysis

Data from growth parameters were arranged in Excel program. Then, results were statistically analysed via analysis of variance and standard deviation with GenStat software. Significant differences were calculated at 5% (*P=0.05*) and Tukey's test was used to make different groups from the means. Bio-Rad program was used to normalize and to calculated gene expression in the samples.

3. Results

3.1. Tulip bud growth *in vitro* culture

3.1.1. Choice of sugar concentration to test hypothesis 1

Before we tested the two hypotheses, a pilot experiment was placed to have a general understanding on how tulip buds respond to tissue culture conditions and to different sucrose concentrations. This could mainly help in making a decision on which sugar concentration would be better for the main experiment for bud growth. Although there was no significant difference in fresh weight gain due to sugar concentrations (*P=0.201*), the curve of growth increased with around 2 to 3 units from 0-6% sucrose concentrations (27.2, 29.2 and 32.9% respectively) and only 1 unit of fresh weight gain when sugar concentrations was increasing from 6% to 9% (32.94 to 33.57%) (Figure 6). Moreover, a significant difference in fresh weight was observed between axillary buds. Thus, higher FW gain was obtained in H buds (51.63%) followed by A, B and C (35.4, 35.1 and 29.20% respectively) while the lowest gain was found in D buds (2.37%).

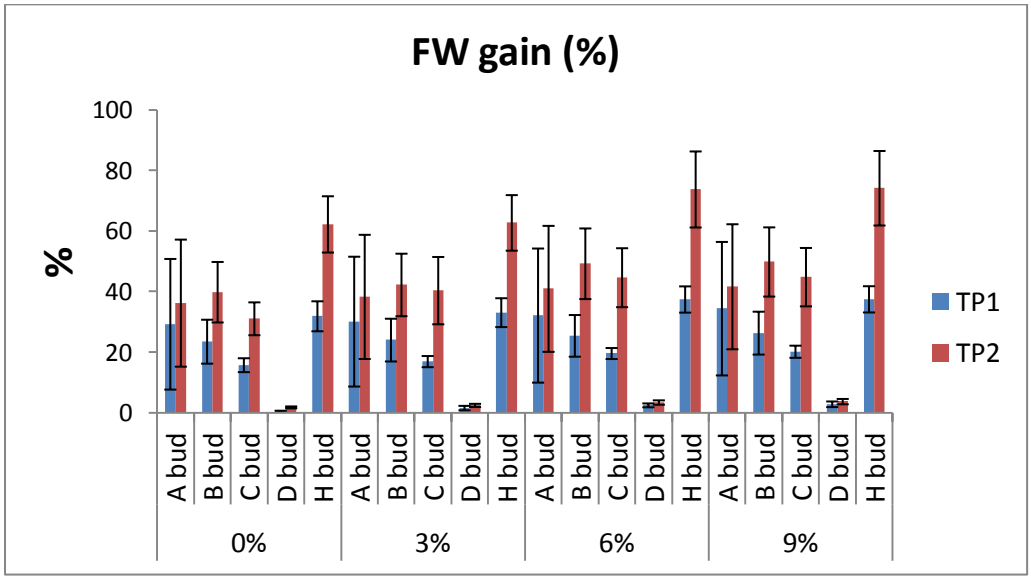

Figure 6. Fresh weight (FW) gain of detached tulip axillary buds under different sucrose concentrations (0, 3, 6 and 9%) during pilot experiment. Error bars were added via standard deviation between treatments.

By the same experiment, shoot production was also observed but only in H buds when they were grown under the medium supplemented with sucrose (3, 6 and 9%) (Figure 7). The results obtained showed that sugar is required for bud growth and 6% sucrose was taken as the best amount to be supply to axillary buds during the main experiment.

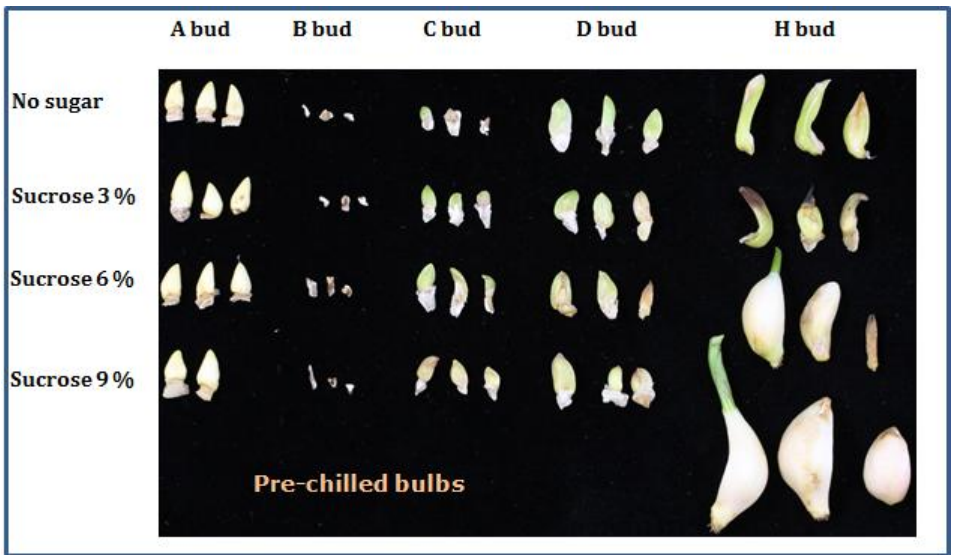

Figure 7. Shoot production of detached tulip buds under different sucrose concentrations (0, 3, 6 and 9%) during pilot experiment.

3.1.2. Tulip bud dry weight gain during *in vitro* culture

As mentioned before, to test if the two way gradient in growth of axillary buds is caused by higher translocation of sucrose to the buds closer to the sinks, we detached A, H and D buds and cultured them *in vitro* on 0 and 6% sucrose. We predicted that at equal source of sugar, the DW gain of all three buds was going to be similar. DW gain was only observed at 6% sucrose compared to the control. In control conditions all buds lost mass (DW gain < 0). This suggests that without sugar supply, axillary buds lost mass instead of growing, while when sugar is supplied they absorb it for growth. Besides DW gain, other physiological changes were observed in these tulip buds. For instance, it was observed that only H buds produced the shoots during this experiment, while A and D buds did not. The H buds produced shoots under both growth medium without sugar or with sufficient sucrose but with long shoots in the last one. In addition, the D buds died on the medium without sugar whereas when sucrose was supplied, the buds survived (Figure 8). This showed that sugar is required for proper growth and survival of tulip buds.

However, at 6% sucrose , H and A grew at the same speed but they gained higher DW (54.4 and 41.5% respectively) than D buds which only gained on average 6.3 % of their initial DW (Figure 8). These results suggested that D bud growth may be controlled by additional mechanisms than simply sucrose availability.

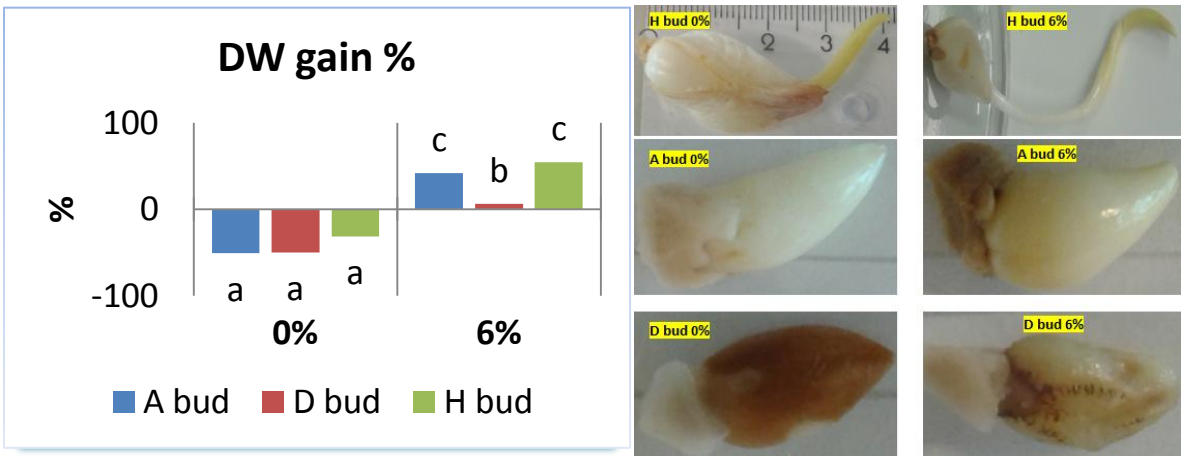

Figure 9. Dry weight gain (right) of detached A, H and D tulip buds in vitro culture on medium without sugar (0%: control) and sucrose 6%. The difference was calculated by using averages of DW gain of each type of bud for 2 months and groups with different letters are significantly different. Tulip buds responses under growth medium without sugar (control) and the one supplemented with sucrose (6%).The samples used on the picture are the ones collected 2 months after culture (left).

3.2. *BRC1* expression in tulip axillary buds

3.2.1. *BRC1* expression in samples collected from the field

Since we did not get enough evidence to accept or reject hypothesis 1, we suspected that D bud growth may be blocked by dormancy related genes, which was stated before as our hypothesis 2. Therefore, we profiled the gene expression of *BRC1* in A, B and D buds but from samples collected in the field under natural growth conditions. By using qPCR, we found out that at the moment of planting (TP0) *BRC1* gene expression was up-regulated in D buds and down-regulated in H and A buds (Figure 10). *BRC1* expression level diminished dramatically after blooming (TP2) and disappeared by TP3 (Figure 10). This showed that D buds are endodormant until blooming time whereas H and A buds seem to be active from the early stages of development. This higher expression of *BRC1* in D buds can explain the cause of their limited growth from planting time until blooming. However, D buds growth was still limited even when *BRC1* expression is gone (TP2 and 3). This suggests that maybe; *BRC1* was not sufficiently down-regulated to allow metabolic activities needed for growth and development in those buds.

Alternatively, no sugars are left over in this stage for the D-bud or the other buds became too strong sinks for the available sources.

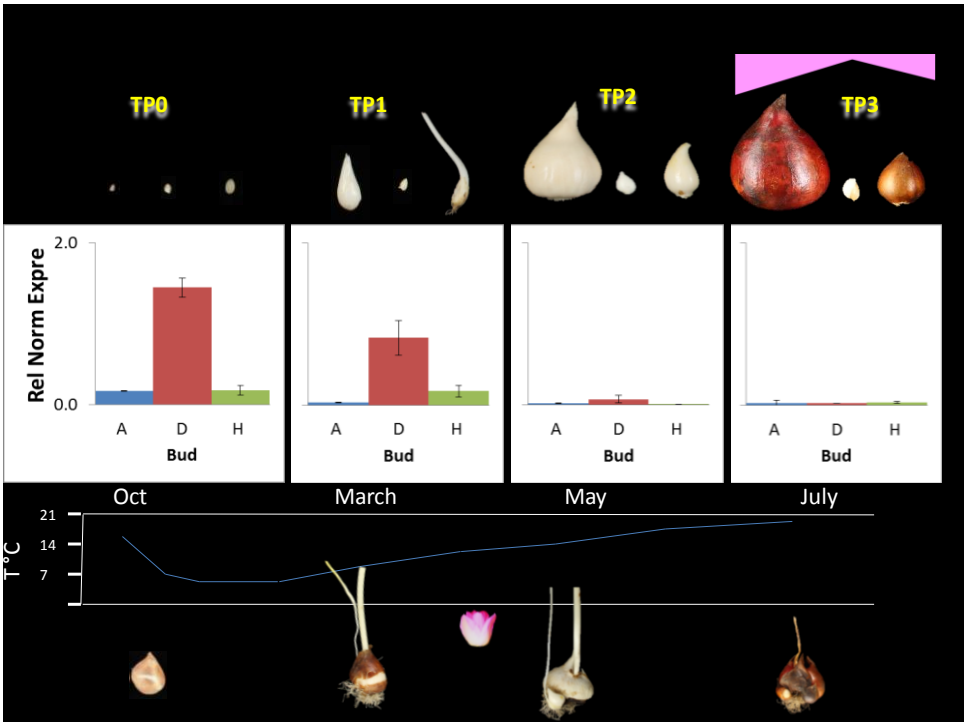

Figure 10. BRANCHED1 (BRC1) gene expression in tulip axillary buds. The samples were collected in the field (Dynasty cultivar) at different time points: TP0: October (before planting), TP1: March; TP2: May; TP3: July. Error bars were added on the graph via standard deviation between treatments.

3.2.1. *BRC1* expression in samples collected from *in vitro* culture

BRC1 expression was also analysed in the samples (A, H and D buds) collected from *in vitro* culture. In these samples, *BRC1* gene expression was higher in D and H buds before culture (T0). The expression of this gene in A buds was low since the beginning and remained low during the whole period of this experiment regardless the sucrose concentrations. In H buds, *BRC1* expression level diminished through the time and it was almost completely down-regulated at the end of the experiment (9WAC) under both 0 and 6% sucrose. The expression of *BRC1* in D buds during culture was only analysed under 6% sucrose because at 0% the buds died. Then, the level of *BRC1* expression in D buds also decreased after 3 weeks of culture but was higher than in A and H buds (Figure 11). This indicates that as in field conditions, *BRC1* gene is generally highly expressed in D buds at the beginning of the growth cycle, which correspond to before planting and during cold period. Under *in vitro* conditions, *BRC1* expression did not reach a complete down-regulation in D buds whereas under field conditions, the gene was

almost gone after blooming (TP2 and 3) and this may be the reason why we did not see the same growth capacity of D bud compared to A and H.

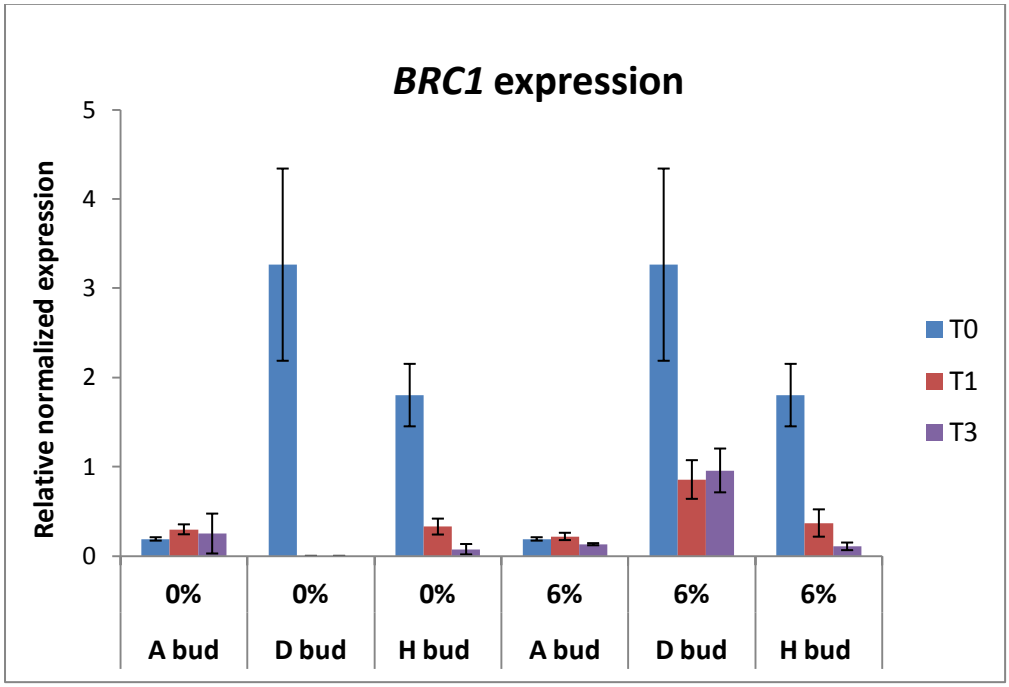

Figure 11. BRANCHED1 (BRC1) gene expression in tulip buds (A, H and D) in samples collected from in vitro culture (Strong Gold cultivar). The sampling was done at different time points: T0, T1 and T3 (before planting, 3 and 9 weeks after culture respectively). On 0%, in T1 and T3, no qPCR was performed in D buds because they have died already. Error bars were added via standard deviation between treatments.

4. Discussion

4.1. Tulip bud dry weight gain *in vitro* culture

It is already known that in living plant cells, sugars are required for energy production needed for growth and development. Usually, these assimilates are produced during photosynthesis process. *In vitro* culture, sugar is also required for plant tissue growth with the difference that in this case, tissue used sugars supplied in the growth medium as the photosynthesis is the limiting factor (Nowak *et al.*, 2004). Beside temperature, sucrose concentrations and the size of starting material are the most important factors for successful growth in vitro culture (Slabbert and Niederwieser, 1999).

This suggests that in the absence of sugar the tulip buds use their carbohydrate reserves for energy production to survive during tissue culture and here the size of starting material is also important. This explains why most of D buds (the ones with the lowest initial weight) died in the

absence of sugar. Beside the composition of growth medium, the size of starting material *in vitro* culture also contributes for tissue growth (Langens-Gerrits *et al*., 2002). In a study conducted by Langens-Gerrits and collegues (2002) on lily bulblet regeneration from scales *in vitro* culture, it has been reported that sugar uptake rate correlates with the sink activity of the tissues and that small tissues have higher sink than the larger ones. This means that D buds (the smallest buds) are able to uptake sugar at the higher rate than H and A buds. Thus, according to this theory D buds can get sufficient dry weight and grow as the other buds. Moreover, the results obtained for the A and H bud showed that sufficient sugar is required for survival and tulip bud growth and that tulip buds are able to uptake sugar from the medium through the cut surfaces for growth during in vitro culture.

4.2. *BRC1* expression in tulip axillary buds

BRANCHED1 (BRC1) is a transcription factor belonging to TCP family. TCP family is named according to *TEOSINTE BRANCHED1* (*TB1*), *CYCLOIDEA* and *PROLIFERATING CELL FACTOR1.* The genes which belong to this family have been reported to play role in architecture of plant inflorescence, leaf development, circadian rhythm, axillary bud outgrowth, etc (Danisman *et al.,* 2013; Braun *et al*., 2012). Those genes are grouped into two main classes, Class I and II. Class I mainly promote cell division whereas class II in which belong *BRC1* and *TB1* act as organ growth inhibitors by preventing cell proliferation (Braun *et al*., 2012).

The Arabidopsis *BRC1* gene has been reported to be highly expressed in axillary buds where it is up-regulated by strigolatone and down-regulated by cytokinin. It has been reported that its higher expression in axillary buds inhibits bud out growth (Braun *et al*., 2012). *BRC1* expression maintains bud dormancy and this was confirmed by a study conducted by Braun and colleagues (2012) on shoot branching in pea (*Pisum sativum*). In the same study, they have observed an increase of shoot branching in *Psbrc1* mutant. They revealed that *PsBRC1* acts with strigolactone to block cytokinin synthesis at transcriptional level and its transport from roots to axillary buds. In contrast to negative effect of both *BRC1* and strigolactone on bud growth, cytokinin promotes it.

The results on *BRC1* expression in tulip bud (samples collected from the field) showed that this gene was highly up-regulated in D buds and down-regulated in H and A buds. The high level of endodormancy in D buds was mainly observed in samples collected in October (before planting) until March and it decreased later from May. Ecodormancy is a common dormancy in stored and non-cooled tulip bulb, but which is removed by cold winter conditions. This explains that the limited growth in D buds after winter period (March to April) was only caused by a higher

expression of *BRC1* that made those buds inactive. Moreover, later during the growth cycle (July) *BRC1* expression in D buds was extremely low and it was almost gone in July. However, this did not result to high final growth in these buds. Braun and colleagues (2012) have reported that bud endodormancy release occurs only when *BRC1* reaches a certain minimum level in buds. Maybe, the low expression of *BRC1* in D buds is sufficient to block the bud growth.

Rees (1981) suggested that there is no true dormancy in tulip bulbs as flower and bud differentiation continues inside the bulb even there are no apparent morphological changes. This phenomenon has been referred as intrabulb development (Kamenetsky, 1994). This could be the reason of the growth which has been observed in A and H buds before the end of the winter (February). It is maybe because those buds were not truly ecodormant and they were already active from the beginning of the growth cycle. In addition, *BRC1* gene expression in those buds was also too low and seems to be removed earlier in Mach (in A buds) and in May (in H buds). Therefore, the main reason of difference in growth capacity between tulip buds which was observed under field conditions could be due to the difference in endodormancy levels between buds. However, the question which remains is to know another mechanism behind the limited growth in D buds later during growth cycle when *BRC1* expression is almost removed.

BRC1 expression was also analysed in detached tulip buds which were grown under *in vitro* culture conditions. The buds used have been pre-cooled 3 months ago to break the ecodormancy. In addition, the growth of detached buds was no longer controlled by the apical dominance. Thereby, endodormancy was the only dormancy that can control bud growth in this case. From the results, *BRC1* expression was also higher in D buds and H buds before culture but at different levels (too higher in D than in H buds). However, before *in vitro* culture, this gene was already down-regulated in A buds. Furthermore, the expression of *BRC1* in both A and H buds extremely diminished three weeks after culture and almost removed after 9 weeks regardless the sugar concentration supplied to the buds. In general, a correlation between *BRC1* expression in tulip buds and bud growth was observed *in vitro* culture. Thus, in A and H buds where the *BRC1* expression seemed to be low, the buds gained high dry weight and at the same growth rate. In D buds, although the expression of *BRC1* gene diminished three weeks after culture, its expression level remained constant until nine weeks after culture and this did not lead to proper growth of these buds. This explains that maybe the level at which *BRC1* was down-regulated was not sufficient to promote the growth of D buds.

In many literatures, sugars have been reported to not be necessary for only energy production but they also act as signals to trigger other physiological and molecular changes in plant tissues such as apical dominance control, bud dormancy release and flowering, etc (Lockhart, 2014; Takahashi *et al*., 2014; Moghaddam and Van den Ende, 2013). It was noticed that a minimal sugar amount must reach the axilllary buds for dormancy release and initiation of bud out growth in pea plant (Lockhart 2014; Mason *et al.,* 2014; Beveridge, 2006; Rolland *et al.*, 2006). In addition, sucrose increase in plant was reported to suppress *BRANCHED1* (*BRC1*) expression (Mason *et al*., 2014; Kelly *et al*., 2012). Although, there is not clear relationship observed between *BRC1* expression and sugar concentration, during this experiment most of D buds which were cultured in the medium without sugar died while at sufficient sugar they survived. This suggests that a combination between the positive effect of sugar in reducing *BRC1* expression (Mason *et al*., 2014; Kelly *et al*., 2012) and energy produced from sugar are required for tulip bud growth. In addition, because there is maybe a link between *BRC1* expression and preferential translocation of sugar into different buds, we could not completely test the hypothesis of sugars and we would need a situation in which D buds have no *BRC1* expression to be sure that difference in growth capacity resulted from insufficient carbohydrates in some tulip axillary buds.

5. Conclusions

The aim of this study was to understanding the mechanisms which are behind the two directional gradient in growth in tulip buds according to their location in mother bulb.

From the results, the following conclusions are made:

1. Two gradient growths in tulip axillary buds depend on difference in endodormancy level in the buds.
2. It could be a combination between both source to sink process and insufficient down-regulation of *BRC1* gene for proper growth of D buds.
3. More evidence is needed to test if there is a preferential translocation of photo assimilates from the leaves to the closer buds.

6. Challenges and recommendations

The first challenge encountered during this study was the high losses of H buds *in vitro* culture due to contamination by pathogens. By this, we recommend the application of antibiotics and fungus inhibiting chemicals in growth medium in the future. The second constraint was the high variation in size and weight of starting natural which can be overcomed by increasing the number of replicates. The final challenge was that even low expression of *BRC1* gene blocked the growth in D buds. So, it is not easy to conclude at which level *BRC1* inhibits bud growth or not and if there is a combination between the effect of source to sink process and *BRC1* down-regulation in bud growth. So, in the future, it is better to use the samples of almost null *BRC1* level or to apply *BRC1* repressor in the medium like cytokinin.

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References

Aurel, P. (1997). Biotechnology and molecular-based methods for genetic improvement of tulips. University of Pitesti, Faculty of Sciences, Department of Natural Sciences. 2-16.

Beveridge, C. A. (2006). Axillary bud outgrowth: Sending a message. *Current Opinion in Plant Biology, 9*(1), 35-40.

Beveridge, C.A., Murfet, I.C., Kerhoas, L., Sotta, B., Miginiac, E., and Rameau, C. (1997a). The shoot controls zeatin riboside export from pea roots: Evidence from the branching mutant *rms4*. *Plant J. 11*, 339–345.

Braun, N., Germain, A. S., Pillot, J. , Boutet-Mercey, S., Dalmais, M., Antoniadi, I., Rameau, C. (2012). The pea TCP transcription factor *PsBRC1* acts downstream of strigolactones to control shoot branching. *Plant Physiology, 158*(1), 225-238.

Chatfield, S., Booker, J., & Leyser, O. (2003). Auxin acts in xylem-associated or medullary cells to mediate apical dominance. *Plant Cell, 15*(2), 495-507.

Cline, M.G. (1994). The role of hormones in apical dominance: New approaches to an old problem in plant development. *Physiol. Plant. 90*, 230–237.

Danisman, S., Van Dijk, A. D. J., Bimbo, A., Van Der Wal, F., Hennig, L., De Folter, S., Immink, R. G. H. (2013). Analysis of functional redundancies within the arabidopsis TCP transcription factor family. *Journal of Experimental Botany, 64*(18), 5673-5685.

De Hertogh, A., & M. Le Nard. 1993. The Physiology of Flower Bulbs. Amsterdam: *Elsevier*

Department of floriculture Kashmir (2012). Tulip cultivation. www.thehindu.com/todays-paper/tp.../kashmir-tulip*. 21.October.2014.*

Jaap, M. V. & Marjan, G.M. (2006). TULIP. *Tulipa gesneriana* and *T. hybrids*. Biodiversity and Breeding, Plant Research International, Wageningen University and Research Centre, Wageningen, The Netherlands, 623-637.

Kamenetsky, R. (1994). Life cycle, flower initiation, and propagation of the desert geophyte *Allium rothii. Int. J. Plant Sci.* 155, 597-605.

Kamenetsky, R. & Okubo, H. (2013). Ornamental Geophytes: From Basic Science to Sustainable Production. CRC Press.6000 Broken Sound Parkway NW, Suite.

Khodorova, N.V., & Boitel-Conti, M. (2013). The Role of Temperature in the Growth and Flowering of Geophytes. *Plants review. 2*, 699-711 doi:10.3390/plants2040699

Geert-Jan M. De Klerk (2012). Micropropagation of Bulbous Crops: Technology and Present State. *Floriculture and Ornamental Biotechnology 6 (Special Issue 1), 1-8*

Lambrechts, H., Rook, F., & Kolloffel, C. (1994). Carbohydrate status of tulip bulbs during cold-induced flower stalk elongation and flowering. *Plant Physiology, 104*(2), 515-520.

Langens-Gerrits, M. and Geert-Jan M. De Klerk *(* 2002). Micropropagation of flower bublous. *Methodology in molecular biology, vol 111.*

Langens-Gerrits, M., Kuijpers, A., De Klerk, G., & Croes, A. (2003). Contribution of explant carbohydrate reserves and sucrose in the medium to bulb growth of lily regenerated on scale segments in vitro. *Physiologia Plantarum, 117*(2), 245-255.

Leeggangers, H. A. C. F., Moreno-Pachon, N., Gude, H., & Immink, R. G. H. (2013). Transfer of knowledge about flowering and vegetative propagation from model species to bulbous plants. *International Journal of Developmental Biology, 57*(6-8), 611-620.

Li, C.-J. & Bangerth, F. (1999). Autoinhibition of indoleacetic acid transport in the shoots of two-branched pea (*Pisum sativum*) plants and its relationship to correlative dominance. Physiol. Plant. 106, 415–420.

Lockhart, J. (2014). Bitter Taste of Winter: Gentiobiose Regulates Overwintering Bud Dormancy in Gentiana. *The Plant Cell*.
Preview,www.plantcell.org/cgi/doi/10.1105/tpc.114.132951.

Maarten, W. P. C. van Rossum (1997). Role of physiological factors in tulip bulb scale micropropagation. PhD research.

Maslanka, M. & Bach, A. (2013). Tulip propagation *in vitro* from vegetative bud explants. *Horticulture and Landscape Architecture*, 34, 21–26.

Mason, M. G., Ross, J. J., Babst, B. A., Wienclaw, B. N., & Beveridge, C. A. (2014). Sugar demand, not auxin, is the initial regulator of apical dominance. *Proceedings of the National Academy of Sciences of the United States of America, 111*(16), 6092-6097.

Moghaddam, M. R., & Van den Ende, W. (2013). Sugars, the clock and transition to flowering. *Frontiers in Plant Science, 4*(FEB).

Van den Ende, W. (2014). Sugars take a central position in plant growth, development and, stress responses. A focus on apical dominance. *Frontiers in Plant Science, 5*(JUN).

Ngezahayo, F. & Liu, B. (2014). Axillary Bud Proliferation Approach for Plant Biodiversity Conservation and Restoration. *International Journal of Biodiversity*, Vol 2014, Article ID 727025, 1-9 pages . http://dx.doi.org/10.1155/2014/727025.

Nowak, B., Miczyński, K., & Hudy, L. (2004). Sugar uptake and utilisation during adventitious bud differentiation on in vitro leaf explants of 'Węgierka zwykła' plum (*Prunus domestica*). *Plant Cell, Tissue and Organ Culture, 76*(3), 255-260.

Podwyszyńska, M. (2012). The mechanisms of in vitro storage organ formation in ornamental geophytes. *Floriculture and Ornamental Biotechnology, 6*(SPEC.ISS.1), 9-23.

Podwyszyńska, M., & Sochacki, D. (2010). Micropropagation of tulip: Production of virus-free stock plants. *Methods in Molecular Biology (Clifton, N.J.), 589*, 243-256.

Podwyszyńska, M. Wojtania, A., & Gabryszewska, E. (2011). The effect of growth regulators and sucrose concentration on in vitro propagation of camellia japonica L. *Propagation of Ornamental Plants, 11*(4), 177-183.

Podwyszyńska, M., & Marasek, A. (2003). Effects of thidiazuron and paclobutrazol on regeneration potential of tulip flower stalk explants in vitro and subsequent shoot multiplication. *Acta Societatis Botanicorum Poloniae, 72*(3), 181-190.

Ptak, A., & Bach, A. (2007). Somatic embryogenesis in tulip (*Tulipa gesneriana* L.) flower stem cultures. *In Vitro Cellular and Developmental Biology - Plant, 43*(1), 35-39.

Rees, A. R. (1981). Concepts of dormancy as illustrated by the tulip and other bulbs. *Ann. Appl. Biol.* 98: 544-548.

Rice, R. D., Alderson, P. G., & Wright, N. A. (1983). Induction of bulbing of tulip shoots in vitro. *Scientia Horticulturae, 20*(4), 377-390.

Rietveld, P. L., Wilkinson, C., Franssen, H. M., Balk, P. A., Van Der Plas, L. H. W., Weisbeek, P. J., & De Boer, A. D. (2000). Low temperature sensing in tulip (*Tulipa gesneriana* L.) is mediated through an increased response to auxin. *Journal of Experimental Botany, 51*(344), 587-594.

Rolland, F., Baena-Gonzalez, E., & Sheen, J. (2006). Sugar sensing and signaling in plants: Conserved and novel mechanisms. *Annu. Rev. Plant* Biol. 2006. 57:675–709.

Romano, C.P., Cooper, M.L., and Klee, H.J. (1993). Uncoupling auxin and ethylene effects in transgenic tobacco and Arabidopsis. *Plant Cell*, 5, 181–189.

Saniewski, M., & Okubo, H. (2005). Only full cooling of mother tulip bulbs is necessary for daughter bulb formation from daughter buds. *Acta Hort.* 673,501-508.

Takahashi, H., Imamura, T., Konno, N., Takeda, T., Fujita, K., Konishi, T., Uchimiya, H. (2014). The gentio-oligosaccharide gentiobiose functions in the modulation of bud dormancy in the herbaceous perennial Gentiana. *Plant Cell, 26*(10), 3949-3963.