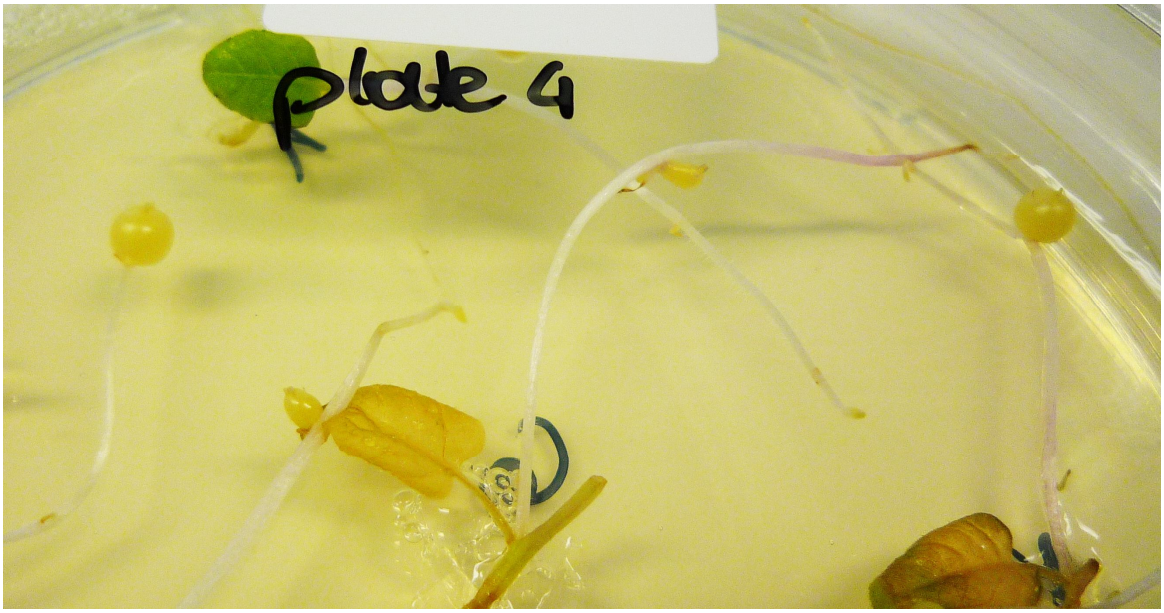
The Role of Auxin Related Genes in the Initiation of Potato Tuber Formation



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The Role of Auxin Related Genes in the Initiation of Potato Tuber Formation

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I would like to dedicate this work to my parents who have given me the opportunity to study in the Netherlands and support throughout my life.

Summary

Potato is the third most important food crop in the world. It is important to identify and characterize the genes that are involved in tuber initiation in order to regulate the quality and quantity of its production. Based on the previous studies that revealed the differential expression of auxin-related genes during the initiation of tuber formation, this thesis aims to understand the overall effect of auxin on tuber initiation through identification and exploitation of auxin-related genes. Genes such as YUCCA, PINs, TIR1 mediate the biosynthesis, distribution and perception of indole-3-acetic acid (IAA) in plants. Down regulation of StYUCCA19061 gene expression during potato tuber developmental stages indicates its potential function in the initiation of tuber formation. Cloning StYUCCA19061 and over-expression in transgenic potato plants allows future analysis of the correlation between IAA biosynthesis and potato tuber development. Application of IAA on potato var. Bintje in an *in vitro* system results in a suppression effect on tuber formation. An increase of nearly 30% of tuber formation occurred when TIBA is added, while 20% increase when PEO-IAA is added. We demonstrated that auxin supplement from the stolon tip inhibits tuber formation, and strigolactone application also revealed a strong inhibition effect. We also observed that D2 as a strigolactone biosynthesis inhibitor lifts the inhibition effect of strigolacton on tuber formation. Finally, TIR1 RNAi and over-expression PIN1/PIN2 transgenic lines were tested with IAA in the *in vitro* system, but no significant differences were found between both treatments and transgenic lines.

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Chapter 1 Introduction

1.1 The initiation of potato tuber formation and auxin

Potato (*Solanum tuberosum* L.) is one of the main food crops in the world, its high value of protein quality and rich starch content makes it a good source for human consumption and cattle feed usage. Improving the potato tuber yield and quality requires a thorough investigation of the mechanisms underlying tuber induction and development. The formation of potato tubers were classified into four steps: 1) stolon induction and initiation 2) stolon growth 3) sessation of longitudinal growth 4) swelling of the stolon tip (Vreugdenhil and Struik, 1989). All of these processes are under hormonal control. Moreover, a tuber-inducing stimulus is required to induce the radial growth of the stolon tips. The initiation of tuber formation is associated with the production of signal molecules and environmental signals.

Auxins are known to play an important role in plant growth and tissue development. However, the role of auxin in potato tuber development is still not fully understood. Directional auxin flow through out Arabidopsis tissues was mapped based on the studies of PIN proteins distribution and the analysis of changes in localized auxini levels. Auxins are synthesized in meristem cells at shoot apex and transported to the root tip, where auxins which are redistributed basipetally through root cortical and epidermal cells. In the root elongation, auxins are transported back to the root tip again and the process is repeated, creating a 'reflux loop' (Blakeslee et al., 2005).

Sites of auxin biosynthesis are mainly in meristems, young primordial vascular tissues and reproductive organs as indicated by the expression of the YUCCA auxin biosynthesis genes (Cheng et al., 2006). In various shoot-derived organs, auxin is supplied through the outer layer and accumulates at the primordium tip, from where it is subsequently transported downward in a basipetal direction by trans-membrane auxin efflux transporters (PIN-FORMED: PIN) to specific tissues and trigger signaling cascade (Johnson and Morris, 1989). The vascular tissue differentiates with this auxin flow to form the new organ (Benková et al., 2003). Auxin is perceived by TIR1 to regulate auxin responsive gene expression (Benjamins and Scheres, 2008).

The potato tuber is a storage organ, which develops from an etiolated underground-stem like structures, called stolons. In a cDNA-microarray study of the potato tuber life cycle, a gene with homology to *adr11-2* (auxin down-regulated) shows a significant down-regulated expression profile at potato tuber organogenesis (Kloosterman et al., 2005). Previous studies implied the regulatory function of auxins in the initiation of potato tuber formation. Three crucial aspects of auxin, biosynthesis, transportation and perception are included in this thesis to elucidate the function of auxin in the initiation of potato tuber formation. The working hypothesis was based on the fact that stolon possesses the same properties of lateral shoot and is expected to be influenced by the auxin production and translocation. Auxin biosynthesis is assumed to take place in the tip of stolon, transported to the basal part of the stolon through the facility of PINs, and perceived by TIR1 to regulate the initiation of tuber formation.

1.2 Auxin biosynthesis

Although auxin is believed to regulate many aspects in plant developmental stages, the details of Auxin biosynthesis remain unclear. Multiple auxin biosynthesis pathways have been proposed, including tryptophan-independent and tryptophan-dependent. The YUCCA gene, which encodes a flavin monooxygenase-like enzyme, is crucial for a rate-limiting step in tryptophan-dependent auxin biosynthesis pathway (Zhao et al., 2001). The YUCCA gene family has been shown to have a regulatory function in auxin biosynthesis in many plants. Four of the 11 predicted YUCCA flavin monooxygenases (YUC1, YUC2, YUC4, and YUC6) have been identified in *Arabidopsis thaliana* and have a proven function in auxin biosynthesis. It was also observed that overexpression of AtYUC genes under the control of the Cauliflower Mosaic Virus 35S promoter leads to auxin overproduction (Cheng et al., 2006). Expression analysis of YUCCA-like genes also indicated that *OsYUCCA1* functionally predominates in rice and may be important in IAA biosynthesis. Plants overexpressing *OsYUCCA1* exhibited increased IAA levels and typical phenotypes (Yamamoto et al., 2007). Therefore, it is likely that the YUCCA-like candidate genes in potato also play an essential role in auxin biosynthesis because of the sequence homology. Recent findings demonstrate that the location of auxin biosynthesis is an important aspect for regulation of auxin functions. The special

expression of YUCCA genes is highly localized and is important for forming local auxin gradients (Cheng et al., 2006). Obtaining knowledge on the auxin biosynthesis in potato can provide a tool to regulate auxin levels in plants and control the tuber formation. Potato YUCCA knockdown mutants would also provide tools to elucidate other key factors that also involved in auxin mediated tuber formation. Therefore, selection of potato YUCCA homologs and analysis of the expression patterns of all these candidate genes will be the first step to uncover the roles for YUCCA gene in potato tuber formation. Furthermore, cloning the gene and producing overexpression StYUCCA transgenic lines provides a tool to investigate the molecular mechanisms of auxin synthesis in potato.

1.3 Auxin transport

Auxin distribution plays a key role in plant pattern formation. A classic experiment demonstrates that exogenous auxin inhibits the lateral bud outgrowth in decapitated *Vicia Faba* shoots where auxin which is synthesized in the leaf primordium, passed basipetally through the stem then inhibits the development of lateral bud growth (Thimann and Skoog, 1933). This directional auxin flow and distribution is regulated by specific auxin efflux carriers, PIN proteins, which are localized in certain polar positions in the cells. Functional analysis of six *Arabidopsis* PIN genes (PIN1, PIN2, PIN3, PIN4, PIN5, and PIN7) have been proven that encode important catalysts of auxin efflux (Křeček et al., 2009). PIN1 is localized primarily in the xylem parenchyma, and is essential for basipetal IAA transport in shoot tissues and for acropetal transport in root tissues (Blilou et al., 2005). Moreover, auxin permease AUX1 proteins are found localized asymmetrically in root protophloem cells at the opposite cell side from PIN1. These auxin influx carriers promote the movement of auxin to the root apex and facilitate basipetal auxin transport to distal elongation zone tissue(Swarup et al., 2001). At the root apex, auxin is redistributed in a PIN2-dependent transport stream to the root elongation zone where auxin which re-directed back into the PIN1-dependent transport stream. Once re-directed, auxin is transported to the root tip again and a ‘relux loop’ is created (Blakeslee et al., 2005).

Since stolon is an etiolated stem, it is likely that auxin flow is similar to the shoot. Auxin is expected to be synthesized in the stolon tip, followed by the downward transport towards the main

stem thereby inhibiting outgrowth of side tubers. Two potato PIN proteins were cloned and transferred in andigena potato (Solanum tuberosum L. subsp. andigena Hawkes) to obtain transgenic lines. Over-expression PIN1 and PIN2 transgenic potatoes were used in *in vitro* experiments to evaluate tuber formation of transgenic lines.

1.4 Auxin perception

Recently it was shown that Aux/IAA proteins interact with ARF proteins which bind to auxin-response promoter elements (Hagen and Guilfoyle, 2002). It was also demonstrated that auxins stimulate the interaction between SCR^{TIR1} and AUX/IAA proteins, promote the degradation of transcriptional regulators and lead to many downstream effects (Gray et al., 2001). Auxin promotes the AUX/IAA- SCR^{TIR1} interaction by binding directly to SCR^{TIR1} (Dharmasiri et al., 2005). By binding to a hydrophobic cavity at the protein interface of TIR1-ASK1, it was proven that auxins stabilize the TIR1-substrate interaction by acting as "molecular glue" (Tan et al., 2007). Once the Aux/IAA proteins are ubiquitinated and degraded by interacting with the SCR^{TIR1} complex through TIR1 protein, auxin related genes are subsequently transcribed.

TIR1 is an F-Box protein which characterized as substrate receptor of SCF-type ubiquitin protein ligase (E3) complex. Studies have shown that loss of TIR1 results in auxin resistance, therefore SCR^{TIR1} was thought to regulate the degradation of negative regulators of auxin signaling (Calderon-Villalobos et al., 2010). The mechanism of auxin perception in potato tuber formation was examined by evaluating loss of TIR1 transgenic lines. Transgenic TIR1 RNAi potato karnico lines were obtained from previous studies. In this thesis, an *in vitro* experiment was conducted on those transgenic plants to elucidate the impact of loss-function of TIR1 on tuber development.

1.5 Strigolactones

After axillary bud formation, the dormant buds require internal and environmental cues to induce the outgrowth. Many of these signals have been identified such as cytokinin which promotes branching, auxin that indirectly inhibits branching. Other cues such as nutrient supply, photoperiod, and developmental status are all factors affecting shoot branching. Novel carotenoid-derived

terpenoid lactones, strigolactones, were first identified as rhizosphere signals and have recently been found shown to be secondary messengers that inhibit shoot branching in pea (*Pisum sativum*) (Gomez-Roldan et al., 2008; Umehara et al., 2008). Auxins may inhibit axillary bud growth by relieving repression of strigolacontes biosynthetic genes, which may prevent auxin transport from the bud. Interaction between auxin and strigolactones maintain hormonal homoeostasis in plants, auxin is basipetally transported through the stem whereas strigolactone moves upwards in the xylem (reviewed in(Stirnberg et al., 2010)). Screening of highly branched mutants also identified a class of branching inhibiting genes that show varying degrees of regulation by auxin and auxin-dependent feedback loop, and has highlighted the role of graft-transmissible signals that are produced in roots and stems (Beveridge, 2006). The complexity of interaction between hormones and other environmental factors still remains unknown. Tuber induction is assumed to be influenced by multiple regulatory controls including auxin and strigolactone interactions. In this thesis, we focused on the function of strigolactones in tuber initiation to elucidate its importance in this specific developmental stage. Fluridone, an inhibitor of an enzyme early in the biosynthetic pathway of carotenoids and second more specific inhibitor D2 (obtained from Physiology Department, Wageningen UR), were also used. D2 is a carotenoid cleavage dioxygenase (CCD) inhibitor which only reduced strigolactone but not ABA content in roots (López-Ráez et al., 2010).

1.6 Scope of this thesis

The objective of this thesis is to get an overall understanding of the role of auxin in the initiation of tuber formation. Previous studies have shown that the complexities of shoot branching include a range of factors. Microarray analysis gave a differential expression of auxin-related genes in the initiation of potato tuber development, indicating that auxin may play an key role in specific time point of tuber formation (Kloosterman et al., 2005). In order to understand how auxin regulate the formation of potato tubers, we investigated the role of auxin in the initiation of tuber development by using reverse genetic approach with genes involved in auxin biosynthesis, transportation, and perception. Since YUCCA proteins are known as a rate-limiting role in auxin biosynthesis, we need to identify StYUCCA in potato and characterize its function in regulating tuber formation. For auxin

distribution and transportation, transgenic andigena plants overexpressing PIN1 and PIN2 were used to study the performance of tuber formation. And TIR1 RNAi transgenic karnico lines were studied to understand if auxin perception is important for tuberisation. In addition, strigolactones that function with auxin in the regulation of shoot branching were also used in the *in vitro* experiment to uncover its role in initiation of tuber development. This overall view of auxin related genes in the initiation of potato tuber formation contributes to enrich the understanding of this hormone regulatory function in specific tuber developmental stage and provide the preliminary knowledge to exploit IAA supplement for modulating potato tuber quality and production.

Chapter 2 Auxin inhibits tuber formation in a *in vitro* system

2.1 Scientific context and objective

To understand the role of auxin in the initiation of tuber formation, a pilot experiment was first conducted on untransformed potato cv Bintje explants in *in vitro* system. In a study on decapitated pea with exogenous auxin supply has shown that the shoot apex is an important source of auxin where play a role in the inhibition of axillary bud (Thimann and Skoog, 1934). Therefore, apical stolon is expected to be the main source of regulatory control for tuber development. To investigate this hypothesis in potato, stolon tips of *in vitro* explants were removed. The control explants were used to exam the importance of the apical stolon tissue to control growth of tubers. In previous studies, rapid IAA breakdown was found during tissue culture. In order to distinguish the effect of IAA degradation, two different auxin treatments were designed. The Bintje plantlets were first grown on the tuber-inducting medium in both IAA 1A and 1B treated with 1μM IAA. After 18 days, the first treatment, the plantlets in group IAA 1B were taken out and put on a new medium with same concentration of IAA. The IAA was only applied on the ablated tip of stolons in both cases. Also ablated tips were used in the control, stolon tips were put in the tuber-inducing medium. The error bar was calculated by the formula: $\mathrm{Var} = \frac{p(1-p)}{n}$, p stands for the percentage of tuber formation and n is the total number of plantlets per treatment. The hypothesis that IAA inhibits potato tuber formation was further investigated by monitoring the amount of tuber formed in treatment with two auxin inhibitors, 2,3,5-triiodobenzoic acid (TIBA) and PEO-IAA. PEO-IAA is an auxin antagonist that binds to TIR1/AFBs to suppress the expression of auxin-responsive genes (Hayashi et al., 2008; Nishimura et al., 2009). TIBA reduces polar auxin transport in stem segments by blocking cellular auxin efflux, specifically interfere with the efflux carriers (Estelle, 2001).

2.2 Results

2.2.1 Auxin inhibition effect on tuber formation

Amount of tuber formed in control with ablated tips (64%) was significantly higher than control

with non-ablated tips (51%) Explants with ablated tips formed more tubers than control under identical growing condition. The percentage of tuber formation of controls is consistently higher than IAA 1A and 1B in the first 4 weeks after treatment. However, a significant increase of tuber formation was found in IAA 1A after 3 weeks (Figure 2.1) From this time point, IAA 1A and 1B show a distinct differences indicating that 1A had a significant increase in the amount of formed tubers, while 1B had similar tuber numbers compared to the control. After 6 weeks incubation in the dark, plantlets in IAA 1A had reached a higher percentage of tuber formation (82%) in comparison to the control with ablated tips (64%) Only 16% of the stolons in IAA 1B had formed tubers. This result indicated the importance for additional supplementation of IAA in potato *in vitro* system which helps maintaining the inhibition effect of IAA on tuber formation.

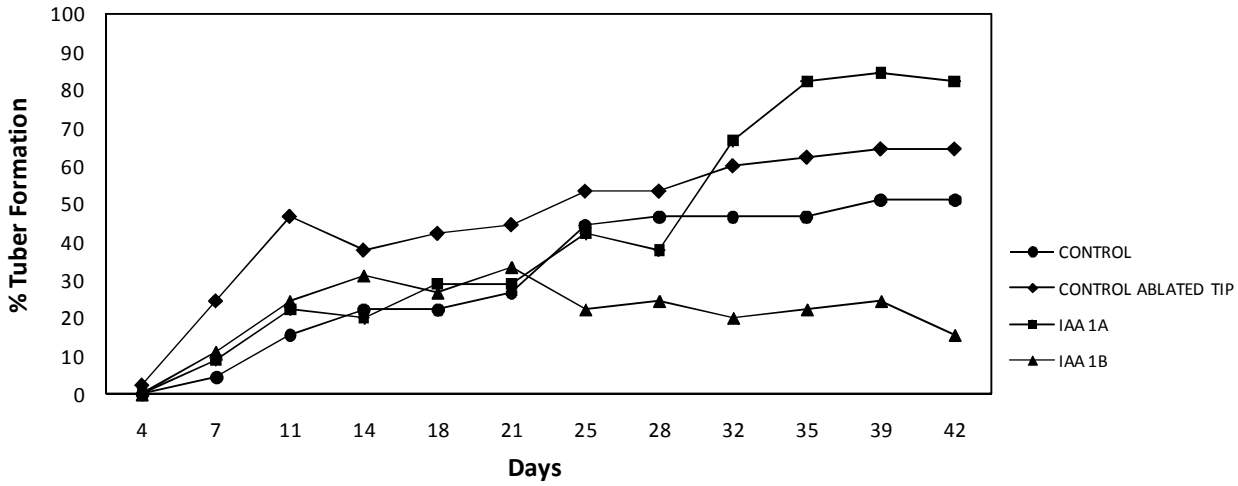

Figure 2.1 *In vitro* tuberisation of explants with ablated tips grown in tuber inducing medium (C;♦) and with 1µM IAA from the basal (IAA 1A; ■) and additional 1µM IAA supplement on day 18 (IAA 1B; ▲)

2.2.2 Auxin inhibitors lift auxin suppression on tuber induction

It is clear that both auxin inhibitors TIBA and PEO-IAA increased the percentage of tuber formation (Figure 2.2) After 6 weeks incubation in the dark, plantlets treated with TIBA (82%) had higher amount of tuber formation than PEO-IAA (71%) As these two inhibitors were applied from the basal of the explants without tip ablation, intact explants were used as controls. Control explants showed a steady increase of tuberisation over 42 days of culture and only reach to 51% by the end of

the experiment period.

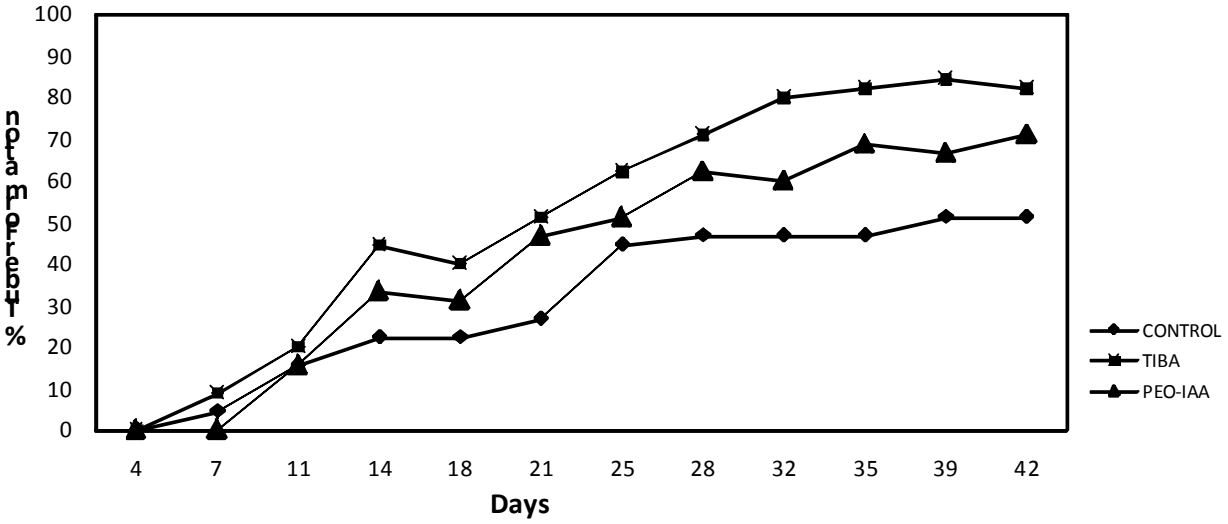

Figure 2.2 *In vitro* tuberisation of untransformed potato Bintje plant with auxin transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) and auxin antagonist PEO-IAA supplementation. (Control; ♦, TIBA; ■ and PEO-IAA; ▲)

2.3 Discussion

It is generally accepted that dormancy gradually develops in potato tubers from the time cell division in the stolon tip has stopped and the tuber starts to develop. Tuber induction is a complex process which involves a wide variety of physiological and environmental inputs (Claassens and Vreugdenhil, 2000). Endogenous level of auxin was higher in the initiation of tuber formation and decrease in the later stage of tuber growth (Obata-Sasamoto and Suzuki, 1979). Growth of lateral buds of stolons are inhibited when the growing apical bud is replaced by IAA application, indicated the inhibitory influence of the auxin (Thimann, 1939). This thesis used *in vitro* system to elucidate the effect of auxin in potato tuber formation.

Comparison between ablated and non-ablated plants proved that stolon tip is the source of auxin and is important for regulating the formation of tubers. When stolon tip is removed, the amount of tuber formation increased around 10% in comparison to non-ablated stolon. Removing auxin source may shut down the polar auxin flow in stolon subsequently induce the formation of tubers. The percentage of tuber formation did not change significantly in both IAA 1A and IAA 1B before IAA re-supplement. A significant increase of tuber formation in IAA 1A without IAA re-application

indicates that auxin is degraded in potato *in vitro* system and raised the percentage of tuber formation. Moreover, it is clear that explants in IAA 1B maintained lower percentage of tuber formation in comparison to the controls and also indicating that IAA suppresses the induction of tuber.

TIBA (2,3,5-triiodobenzoic acid) can inhibit auxin polar transportation. In the *in vitro* system, it has been found that TIBA improved adventitious shoot regeneration in sugar beet (Zhang et al., 2004). Application of TIBA from basal part of plantlet cuttings increased the number of tuber formed. This promotion effect may indicate that the distribution of auxin is interrupted by TIBA; a subsequent shut down the auxin stream in the main stolon then may enable tubers to develop from axillary buds. PEO-IAA is an auxin antagonist, which blocks the perception of auxin. It has been shown that the transient reduction of auxin signaling by PEO-IAA rapidly down-regulates the expression of many core cell cycle genes and partially suppresses an early initiation of cell differentiation in the *Arabidopsis* root meristem (Ishida et al., 2010). By binding to TIR1/AFBs protein complex, PEO-IAA perception is blocked thus auxin inhibition effect on tuber formation is interfered. Consistent with these results, we show a promoting effect on tuber formation in TIBA and PEO-IAA application. Auxin inhibitors increase the number of tubers formed, further verifying the inhibitory effect of auxin shown in previous chapter.

Chapter 3 Strigolactone inhibits potato tuber formation

3.1 Scientific content and objective

Similar to shoot branching, the initiation of tuber formation may also involve a complex regulation loop. Axillary branching is known to be controlled by auxin and strigolactone. Furthermore, auxin and strigolactone are mutually controlled in a complex dynamic feedback loop (Hayward et al., 2009). Several studies on mutants with increased shoot branching were found in several species and have revealed the deficiency of these mutants in the synthesis of branch-inhibiting signal, strigolactone. These are *decreased apical dominance1* (*dad1*) in petunia (*Petunia hybrid*a) *ramosus1* (*rms1*) to *rms5* in pea (*P. sativum*) *more axillary growth 1-4* (*max1-4*) in Arabidopsis (*Arabidopsis thaliana*) and *dwarf* and *high tillering dwarf* (*d/htd*) in rice (*O. sativa*) (review in(Xie et al., 2010)). Based on this research, we suggest that strigolactone may act as a branch-inhibiting signal together with auxin to inhibit potato tuber induction. To investigate this research question, *in vitro*-grown Bintje plantlet cuttings were treated with basally supplied GR24, a synthetic strigolactone. There were two carotenoid cleavage dioxygenases (CCDs), CCD7 and CCD8, were proposed to be involved in the biosynthesis of shoot branching inhibiting signal which are later known as strigolactone (Booker et al., 2004; Gomez-Roldan et al., 2008; Sorefan et al., 2003; Umehara et al., 2008). Two carotenoid-biosynthesis inhibitors were used in this experiment, fluridone is known for herbicide that interrupts carotenoid biosynthesis (Schwartz et al., 1997) and D2 (provided by Laboritory of Plant Physiology, Wageningen University) is a carotenoid cleavage dioxygenase (CCD) inhibitor which only reduced strigolactone but not ABA content (López-Ráez et al., 2010).

3.2 Results

Plantlets grown in *in vitro* system with synthetic strigolactone GR24 supplementation had shown a significant inhibition on tuber formation that only yield 13% tuberisation (Figure 3.1) In comparison with tuberisation of control plants, D2 supplementation had no inhibitory affect, with similar tuberisation levels as control (58% and 51% respectively) Another strigolactone inhibitor,

fluridone, also inhibits the formation of tuber (27%) However, the inhibition effect on tuber formation of fluridone is not as strong as GR24.

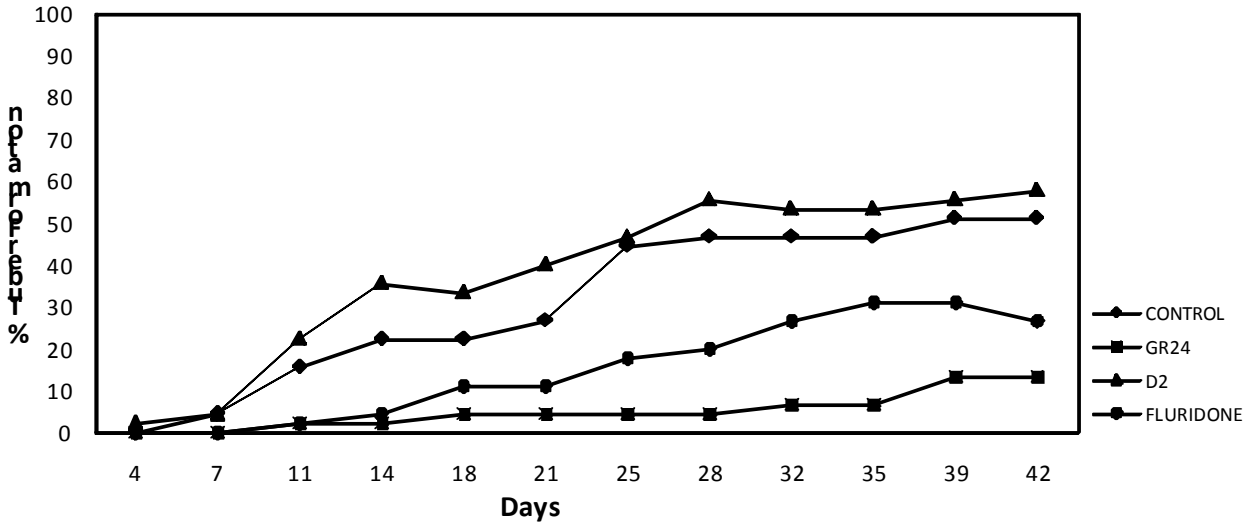

Figure 3.1 *In vitro* tuberisation of Bintje control (C;♦) and with strigolactone analog GR24 supplementation (GR24; ■) and inhibitors supplementation (D2; ▲ and Fluridone; ●)

3.3 Discussion

In chrysanthemum, it has been demonstrated that the activation of bud can be inhibited by GR24 (Liang et al., 2010). The inhibition effect of GR24 on tuber induction supports the idea that strigolactones inhibit shoot branching. The final percentage of tuberisation after the 42 days of treatment showed that GR24 has a significant suppression effect on tuber formation during the first five weeks and reached only 13% by the end of the experiment. This result proved a clear and strong inhibition effect of strigolactones on tuber formation. Interestingly, the carotenoid cleavage dioxygenase (CCD) inhibitor D2 lifted the inhibition effect of GR24. However, the effect of D2 even resulted in a higher percentage of tuberisation than in control explants. It is important to note that explants used in these experiments do not include roots, the source of strigolactone production *in vivo*. Statistically, tuberisation of plantlets in D2 supplied medium has no significantly difference to control.

Fluridone not only inhibits the biosynthesis of strigolactones but also another carotenoid derivative, abscisic acid (ABA) (Matusova et al., 2005). Studies in tomato ABA-deficient mutants have shown that reduction in strigolactone production correlated with down-regulation of LeCCD7

and LeCCD8 genes, and suggest that ABA play a role in the regulation of strigolactone biosynthesis in roots (López-Ráez et al., 2010). Furthermore, ABA was suggested to play a role in regulating the dormancy of potato tubers, however, the direct role of ABA remains unclear (Fernie and Willmitzer, 2001). The antagonistic effects of ABA and GA, may indicate a tuberisation promoting role for ABA (Xu et al., 1998). This may be an indirect explanation for the differential affect of the higher tuberisation found in D2 treatment compared to the Fluridone treatment.

Chapter 4 Evaluation of TIR1 RNAi transgenic potato lines

4.1 Scientific context and objective

The F-box protein TIR1 is an auxin receptor that plays an essential role in auxin perception by mediating Aux/IAA degradation and auxin-regulated transcription (Dharmasiri et al., 2005). When auxin binds directly to TIR1, AFB1-3 (Auxin Signaling F-box 1-3) promotes the recruitment of Aux/IAA repressor and ubiquitin-dependent proteolysis(Gray et al., 2001; Kepinski and Leyser, 2005). Consequently, the activation of ARFs leads to auxin-reponsive gene expression or down regulation(Hagen and Guilfoyle, 2002). The mechanism of auxin signalling in *A. thaliana* has been investigated. However, only two *Aux/IAA* members (*StIAA* and *StIAA2*) have been studied in *Solanum tuberosum* (Kloosterman et al., 2006; Zanettia et al., 2003). Since auxin inhibition effect on tuber initiation has been indicated in chapter2, decreasing the expression of *TIR1* by RNAi mechanism may interrupt auxin perception subsequently display a distinct phenotype on tuber induction of *TIR1* RNAi transgenic potato lines. Considering *TIR1* involved in auxin perception, two levels of IAA concentration were used to evaluate the capacity of auxin perception in three transgenic lines. Auxin antagonist PEO-IAA was used in this experiment to help to confirm the interruption of auxin perception in *TIR1* RNAi transgenic lines. In this thesis, we designed this *in vitro* experiment to evaluate the tuber initiation of three *TIR1* RNAi transgenic potatoes lines in order to have an overview of the influence of *TIR1* on tuber formation.

4.2 Results

Three *TIR1* RNAi transgenic karnico potato lines were obtained from previous work (Line17, 39, 42) Line 42 grown with tuber-inducing medium performed a higher percentage of tuber formation as untransformed control plants, line17 and line 39 showed less tuber formation (Figure 4.1) All these transgenic lines showed a relatively high percentage of tuberisation in comparison with the results from Bintje experiment in Chapter 2 (Line42; 100%, Untransformed karnico; 87.5%, Line17; 83.3%, and Line39; 75%)

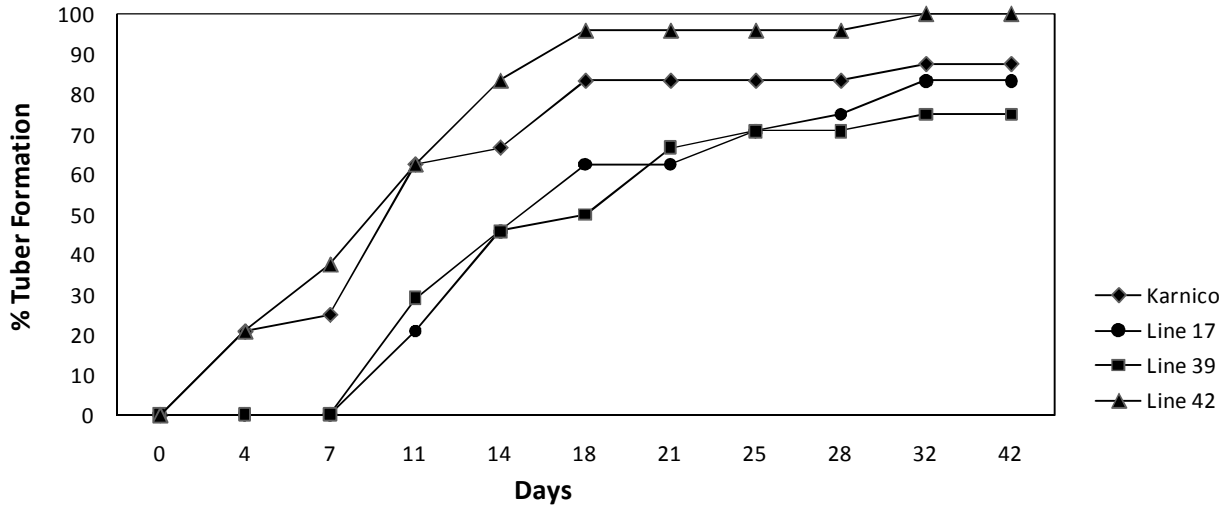

Figure 4.1 *In vitro* tuberisation without hormone supplement of potato karnico control (Karnico;♦) and three transgenic lines (Line17; ●, Line39; ■ and Line42; ▲)

Tuber formation of potato lines with supplement of 1µM IAA was shown in Figure 4.2. Percentages of tuber formation of three transgenic lines and untransformed control plants were all close to 100% on the 42th days after treatment. Similar result was also seen in plants grown in the medium with ten times IAA concentration supplement but the rate of tuber formation was more different in each line (Figure 4.3) No transgenic line showed significant difference in comparison to control plants in treatment with PEO-IAA supplement (Figure 4.4)

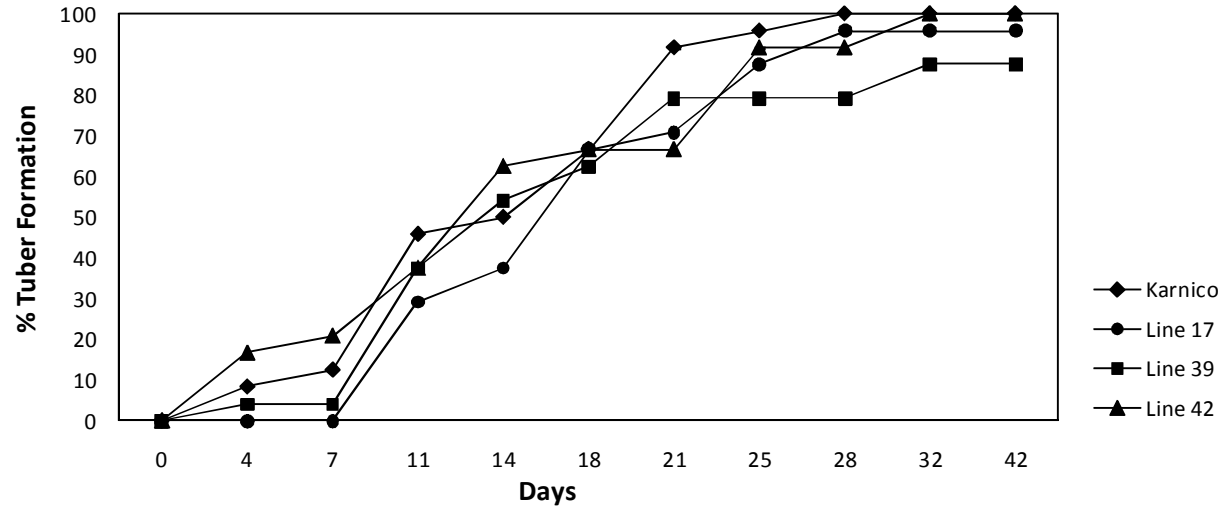

Figure 4.2 Tuber formation of untransformed potato karnico (Karnico;♦) and *TIR1* RNAi transgenic lines (Line17; ●, Line39; ■ and Line42; ▲) grown in *in vitro* system with 1µM IAA supplement.

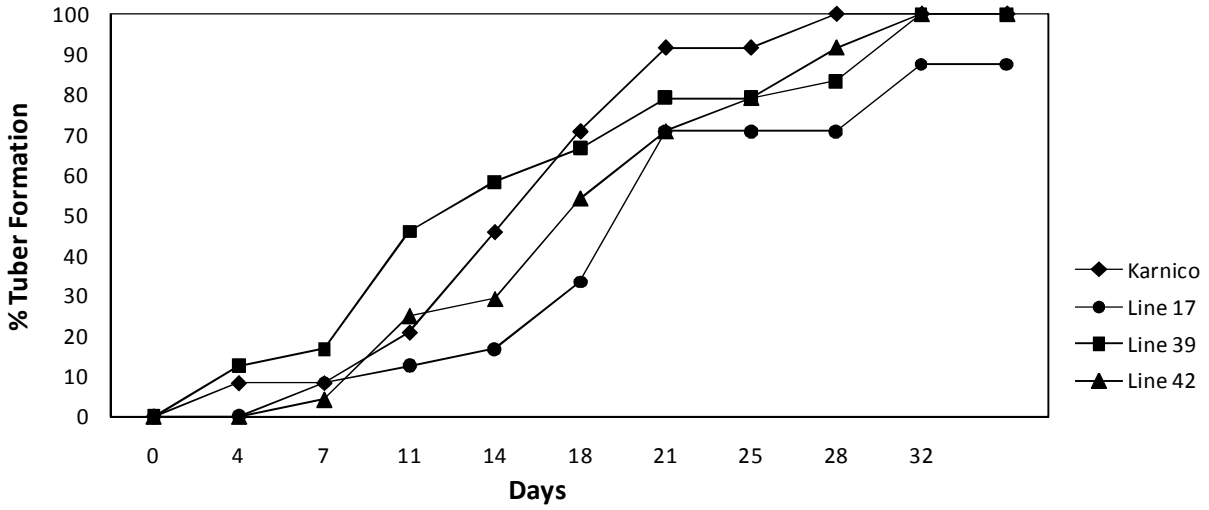

Figure 4.3Tuber formation of plantlets grown in medium with 10µM IAA supplement (Karnico;♦, Line17; ●, Line39; ■ and Line42; ▲)

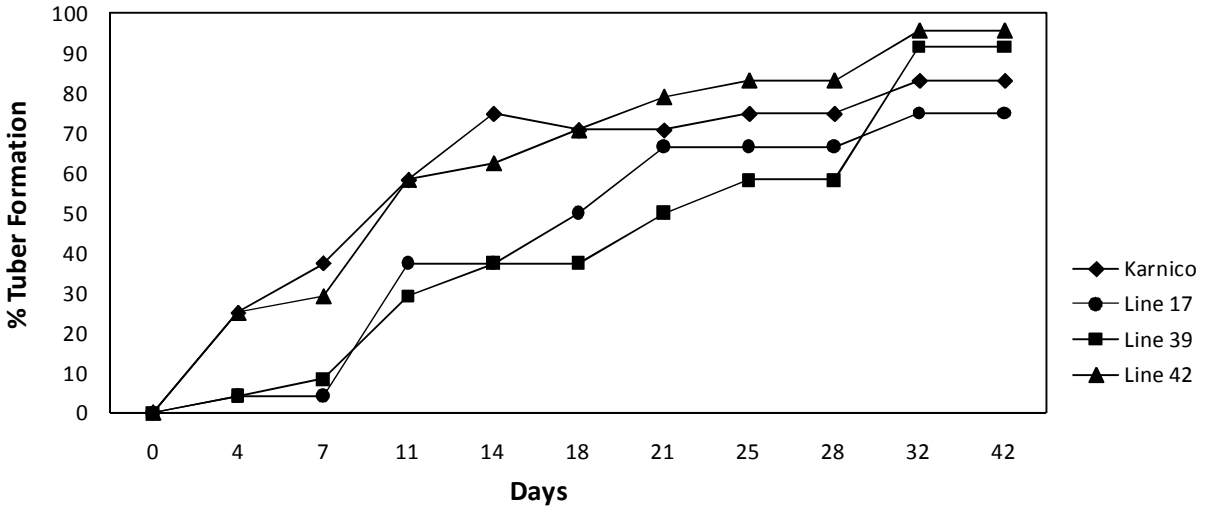

Figure 4.4 Tuber formation of plantlets grown in medium with PEO-IAA supplement (Karnico;♦, Line17; ●, Line39; ■ and Line42; ▲)

4.3 Discussion

Exploiting RNA interference pathway, to knock out expression of the *TIR1* gene in transgenic potato was hoped to generate plants with inactive auxin perception. RNA interference pathway may inactivate gene expression completely. Therefore, using RNAi mechanism to target *TIR1* is a feasible way to show a phenotype after knockdown of the *TIR1* gene expression. Three *TIR1* RNAi transgenic lines were selected by evaluating the qRT-PCR result (Figure 4.5) It was shown that all the transgenic plants express low levels of *TIR1*, three lines which express the lowest level of *TIR1*

were chosen for evaluating its performance of tuberisation in the *in vitro* system.

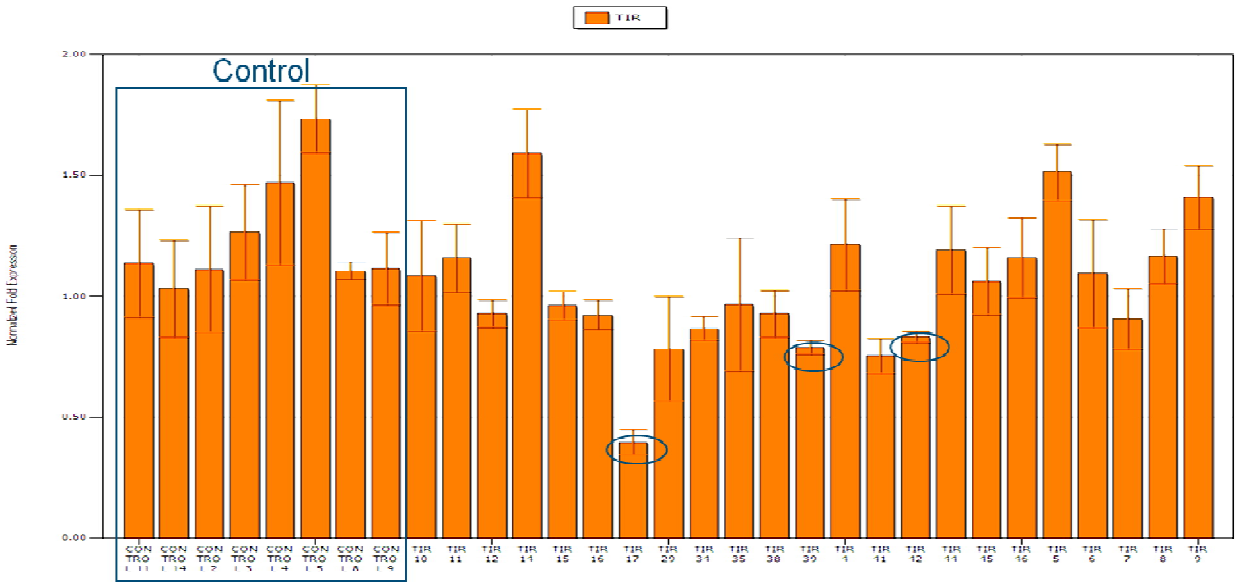

Figure 4.5 *TIR1* gene expression of all *TIR1* RNAi transgenic potato lines.

A drastic decrease in the expression of TIR1 should leads to severe defect in auxin perception subsequently showed a different phenotype of tuber formation in this experiment. However, from the results of *in vitro* experiment, tuber formation showed no significant difference between transgenic lines and control plants. Possible explanation is that full knockout of *TIR1* by RNAi was not achieved, since RNAi may not fully abolish expression of TIR1, the expression may only be knockdown for a small portion and unable to distinguish the difference of tuberisation. Alternatively, it is possible that due the large number of TIR-genes in potato individual homologues may ameliorate the detrimental effects of TIR1 knock-down.

Chapter 5 Evaluation of over-expressing *PIN1* / *PIN2* transgenic andigena potatoes

5.1 Scientific context and object

The distribution of auxin establishes an essential gradient of this hormone in plants and regulates organ development. From the results of quantitative RT-PCR analysis (Figure 5.1) the differential gene expression of *PIN1* and *PIN2* within potato tuber development indicates potential function of these PIN proteins in tuber formation. To identify the function of PIN proteins in tuber formation, we designed an *in vitro* experiment to evaluate the performance of tuber formation of over-expressing *PIN1*/*PIN2* transgenic andigena lines.

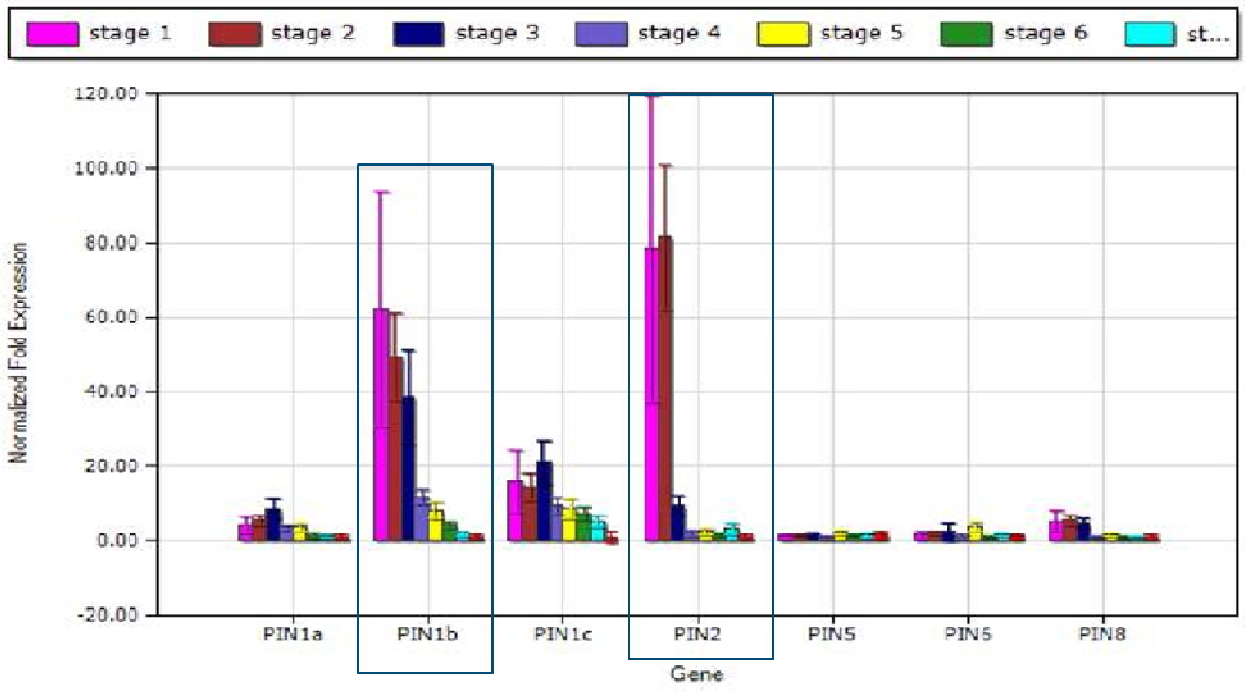

Figure 5.1 *PIN* gene expression of transgenic lines.

5.2 Results

5.2.1 *In vitro* tuberisation of 35S:*PIN1*:RFP transgenic lines

Two lines of *PIN1* overexpressed transgenic plants, Line12 and Line34, were obtained from previous experiments. Overexpressing *PIN1* transgenic lines and untransformed andigena plants in control 1 were grown on the tuber inducing medium to evaluate the phenotype and tuber formation. It is assumed that the up-regulated level of *PIN1* expression in 35S::*PIN1*::RFP transgenic lines would regulate tuber initiation by influencing auxin transportation. To exam this hypothesis, IAA was supplied from the ablated tips of the stolons to evaluate the effect of enhanced PIN1 protein distribution on tuber formation. A double treatment experimental design was used for IAA supplement, basal part of plantlet cuttings were grown in a larger petri dish filled with tuber-inducing medium and ablated tips of plantlet cuttings were put in a smaller petri dish which filled with tuber-inducing medium and 1μM IAA supplemen (Figure 5.3)

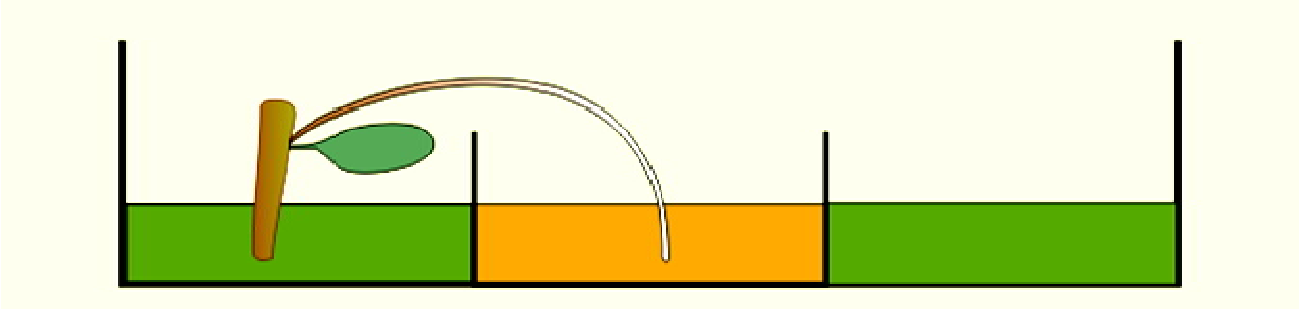

Figure 5.2 Double treatment for *in vitro* experiment with IAA supplement.

Therefore, plants with ablated tips were put in the tuber inducing medium were used as control 2 for comparing tuber formation of plants grown in IAA supplement. However, tuber formation is hardly seen in both control 1 and control 2 in this *in vitro* system (Figure 5.3 andFigure 5.4) Untransformed andigena showed a large amount of formed tubers under the IAA supplied condition and reach to 66% of tuber formation (Figure 5.5) Both transgenic line 12 and line 34 showed very low percentage of tuber formation (20% and 0%, respectively)

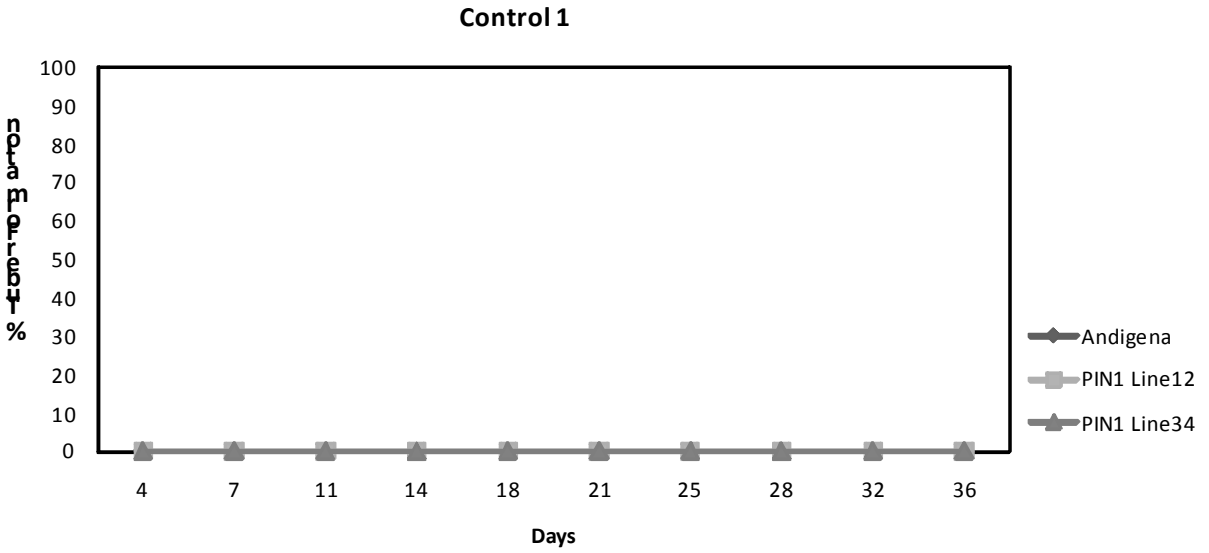

Figure 5.3 *In vitro* tuber formation of untransformed andigena and transgenic lines over-expressing *PIN1*gene. Tips of stolons were put in the medium without decapitation.

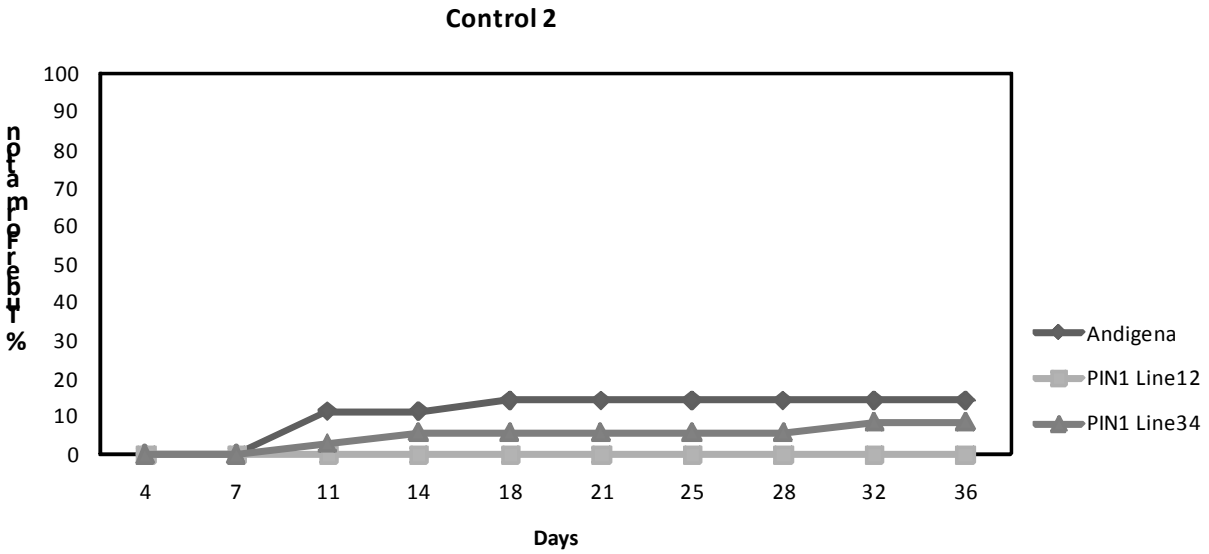

Figure 5.4 Tuberisation of untransformed andigena and transgenic lines. Stolon tips were ablated and put into tuber inducing medium.

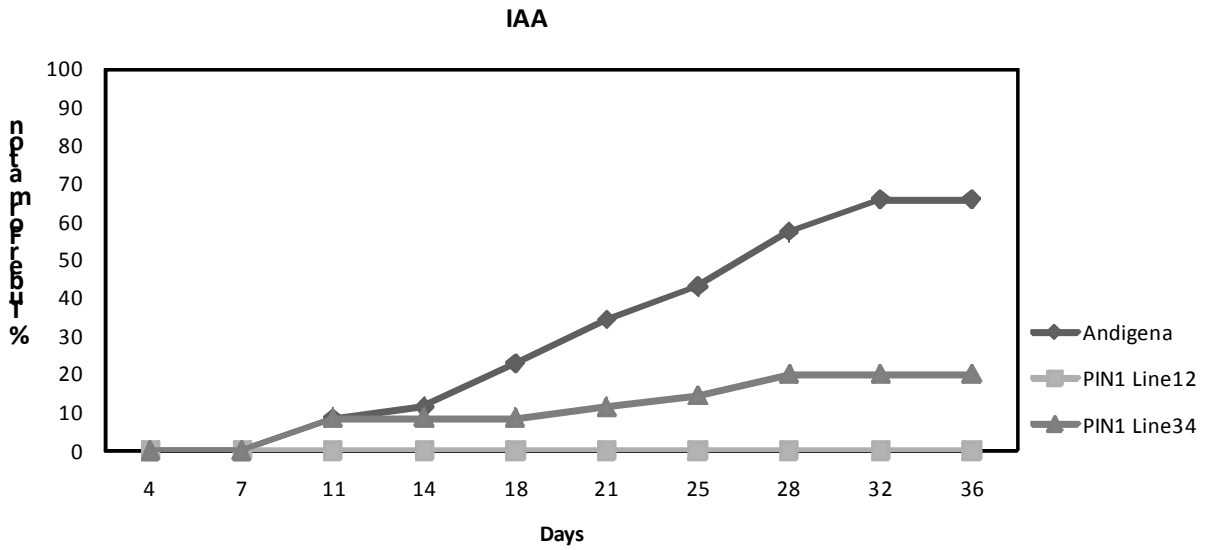

Figure 5.5 Tuber formation of untransformed andigena and transgenic lines. Stolon tips were all ablated and put in tuber-inducing medium with 1µM IAA supplement.

5.2.2 Selection of 35S:*PIN2*:RFP transgenic candidate lines

Forty-four transgenic andigena lines over-expressing *PIN2* gene were produced. Total RNA were extracted from all the lines and cDNA were produced for *PIN2* expression analysis using qRT-PCR. Ten lines with high levels of PIN2 expression were collected and propagated under controlled conditions in 10 replicates. Infected plants were eliminated during propagation. Line 11, line 19, and line 37 were chosen for tuber formation evaluation by the high level of *PIN2* expression (Figure 5.6) The RFP expression analysis was also conducted with qRT-PCR which showed a similar pattern as *PIN2* expressions, reconfirming the candidate lines have high gene expression.

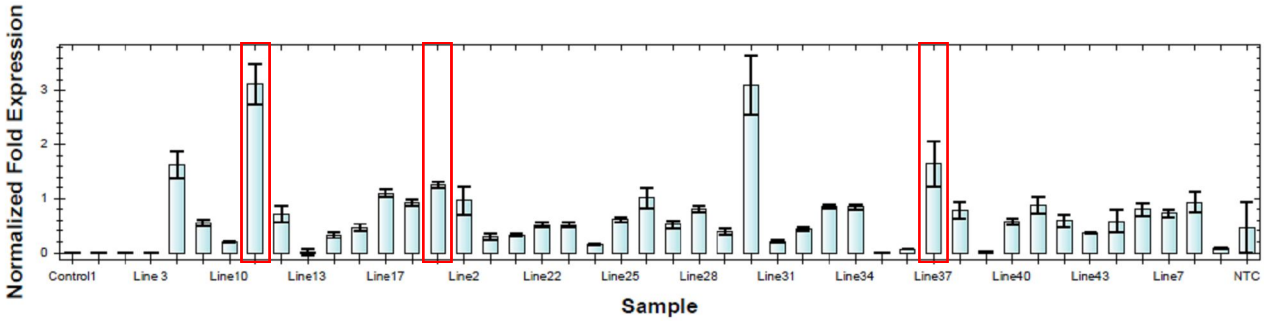

Figure 5.6 *PIN2* expression in 44 lines of 35S:*PIN2*:RFP transgenic andigena potatoes.

5.2.3*In vitro* tuber formation of 35S:*PIN2*:RFP transgenic lines

Three over-expressing PIN2 transgenic lines (Line 11/19/37) were used in the *in vitro* experiment for evaluating the performance of tuber formation. Plantlet cuttings in Control 1 had non-ablated stolon tips and were not treated. In control 2, stolon tips were non-ablated and were put into the tuber-inducing medium for comparing the IAA effect on tuber formation. The tuber formation in control 1 was very low (Figure 5.7) Transgenic line19 and untransformed andigena showed a slightly higher tuberisation in control 2 (Figure 5.8) Under IAA supplied condition, tuber formation of untransformed andigena reached to 65% (Figure 5.9) All the transgenic potatoes showed a higher percentage of tuberisation under IAA supplied condition in comparison to control 2.

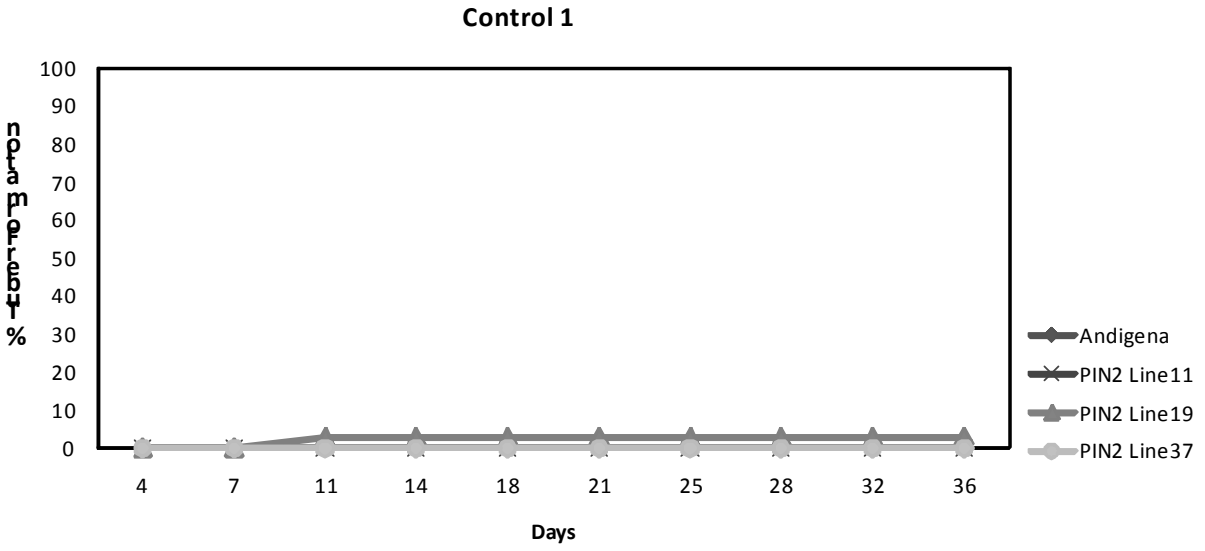

Figure 5.7 Tuberisation of andigena and transgenic lines with 35S::*PIN2*::RFP in the *in vitro* system.

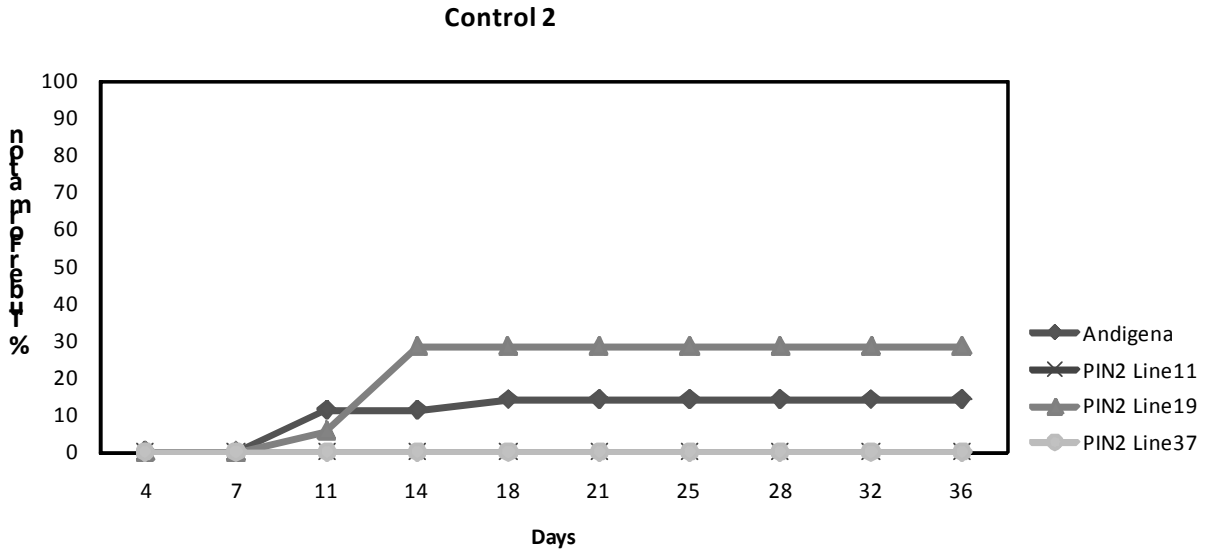

Figure 5.8 Experimental setting for Control 2 was designed for comparing IAA treatment on the ablated tips. Stolon tips of plantlet cuttings were ablated and put in tuber inducing medium.

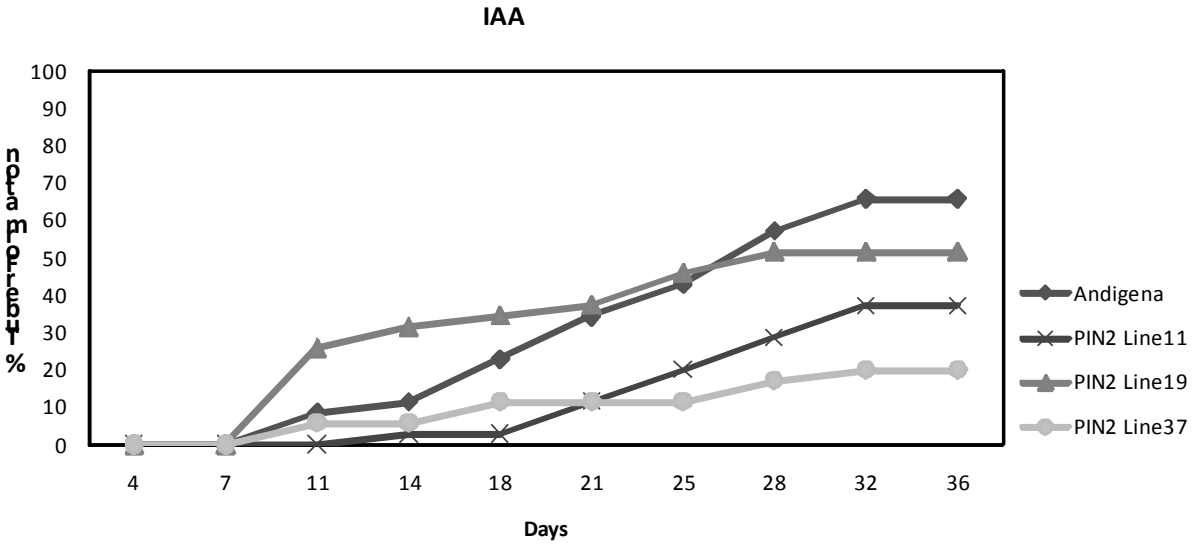

Figure 5.9 Tuber formation of overexpressing *PIN2* transgenic lines and untransformed andigena which were grown on tuber inducing medium with IAA supplement from the ablated tips.

5.3 Discussion

Previous studies found that auxin transport in the inflorescence shoot was greatly reduced in *pin1* mutants, the result suggested that the normal level of polar transport activity is required for floral bud formation in early developmental stages (Okada et al., 1991). In the initiation of potato tuber formation, auxin transport proteins would be one of the determining factors that regulate the distribution of IAA. However, due to low tuber formation on andigena potato, it is difficult to conclude the influence of overexpressing PIN proteins from transgenic lines evaluation. Before tuber formation, production of a transmissible signal is required to induce tuber initiation (Gregory, 1956). The transmissible signal vary by cultivar, it has been shown that phytochrome B is involved in the photoperiodic control of tuberisation in *S. tuberosum* subsp. *Andigena* (Jackson et al., 1996). This light sensitive characteristic of andigena potato tuberisation may be a crucial condition in this *in vitro* system, whereas the experimental procedure did not include this factor and might be the reason for non tuber formation. A double treatment approach was used for supply IAA from the ablated tip. However, plantlets were damaged in the middle part of stolons which decrease the authenticity of the

results. The slight increase of tuberisation in control 2 might caused by the damage of stolon tips. Beside the possibility of human error, no significant difference of tuberisation was observed between overexpressing *PIN1*/*PIN2* transgenic plants and untransformed plants.

Chapter 6 Selection and analysis of potato YUCCA homologs

6.1 Scientific context and objective

The biosynthesis of auxin is a complex mechanism that includes multiple pathways and has not been clearly elucidated. Two major IAA biosynthesis pathway have been proposed: Trp-dependent and Trp-independent pathways (Zazimalova and Napier, 2003). The *YUCCA* gene was suggested to play a key role in Trp-dependent auxin biosynthesis because it catalyzes the N-oxygenation of tryptamine and convert it into N-hydroxytryptamine (NHT) a rate-limiting step in auxin biosynthesis (Zhao et al., 2001). Studies on defects of *yuc1yuc4* and *yuc1yuc2yuc6* also indicating the importance of *YUCCA* genes in auxin biosynthesis and demonstrating the expressions of *YUCCA* genes are very tissue specific (Cheng et al., 2006). In this thesis, we mainly focused on *YUCCA* gene because of its importance in Trp-dependent auxin biosynthesis pathway and its potential function in the initiation of tuber formation.

Many approaches were carried out to select a *YUCCA*-like gene in potato and have this gene cloned to provide the material for further studies. First, BLAST searches and phylogenetic tree were used to identify a *YUCCA*-like gene in potato, *StYUCCA19061*. To test whether the YUCCA homolog has functions in the initiation of tuber formation, we amplified this gene from andigena potato genomic DNA and made the construct with 35S over-expression promoter. Thus, we overexpressed *StYUCCA19061* under the control of CaMV 35S promoter in transgenic andigena potato plants for further studies.

6.2 Results

6.2.1 Phylogenetic tree construction

To investigate the molecular mechanism of IAA synthesis in potato, first step is identifying a *YUCCA*-like gene in potato genome. In order to select a group of candidate genes for potato YUCCA, we performed a tBLASTn search against *Solanum phureja* scaffold v3 Databases using the known Arabidopsis functional *YUCCA* mRNA as a query sequence. The results contain 11 potato homologs. A phylogenetic tree was constructed by aligning full-length amino acid sequences of these candidates

and Arabidopsis YUCCA proteins (Figure 6.1) By comparing the phylogenic relationship, all potato YUCCA proteins cluster into two groups. First group includes functional AtYUCCA4 and seven StYUCCA proteins; second group includes functional AtYUCCA1 and four StYUCCA proteins. It is showed in the phylogenetic tree that the YUCCAs in *Arabidopsis* and potato are located on the same branch, suggested evolutionary similarity in genetic characteristics. The degree of similarity between AtYUCCAs and StYUCCAs amino acid confirm a conserved sequence motif is among lineages and may possess similar protein structure and function.

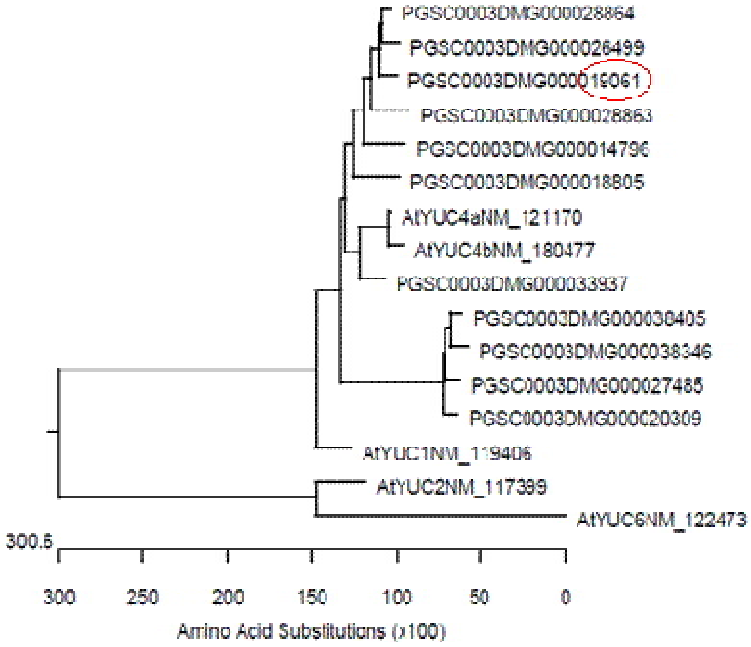

Figure 6.1 Phylogenetic analysis of potato and *Arabidopsis* YUCCA proteins. GenBank accession numbers are shown.

6.2.2 Gene expression profiles by using quantitative RT-PCR

To confirm the expression of these *StYUCCA* genes in potato tissues, a quantitative RT-PCR was performed using gene specific primers with potato Bintje cDNA template. The synchronous potato tuber induction system was established and cDNA were produced from the total RNA isolated from eight tuber developmental time points. These time points are ranging from non-induced stolons, induced stolons, swelling stolon tips, tuber initiation stage and four different tuber growth stages(Kloosterman et al., 2005). In this thesis, cDNA from eight developmental stages were used to determine *StYUCCA* genes expressions in specific time point during tuber formation. According to the output of phylogenetic relationship analysis of all YUCCA homologs in potato, seven homologs

were analyzed by quantitative RT-PCR to obtain the expression profiles. Significant differential expression was only shown in *StYUCCA19061* during tuber development (Figure 6.2) The highest gene expression was found in the induced stolons stage then decrease by the following growth stages. For other *StYUCCA* homologs, gene expressions were hardly detected in whole of tuber development process.

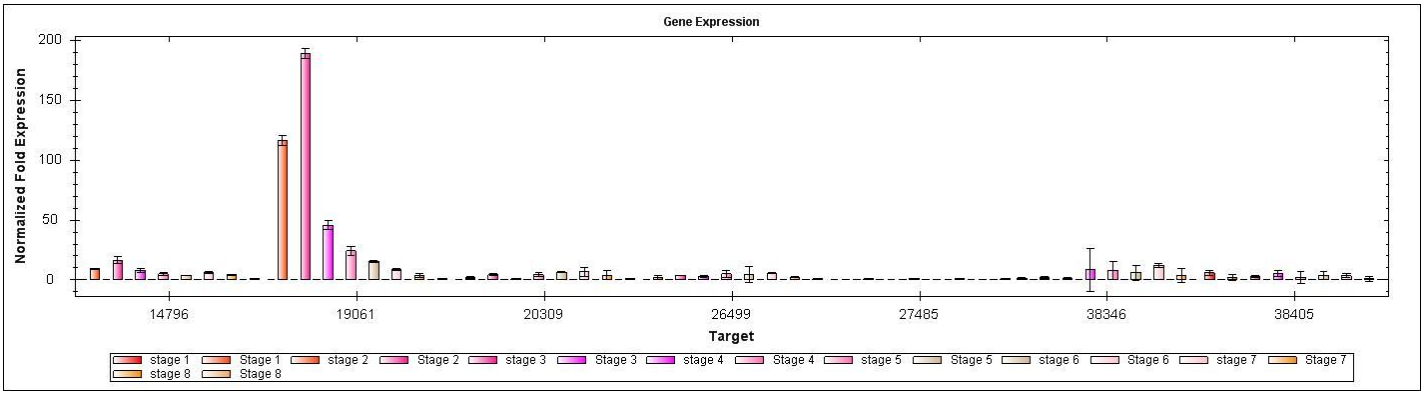

Figure 6.2 Expression profile of *StYUCCA* genes in eight tuber developmental stages.

6.3 Discussion

Phylogenetic analysis and qRT-PCR were conducted to elucidate the most promising *YUCCA* homologs in potato. By comparing the result from phylogenetic anaylsis and gene expression profiles of all *StYUCCA* homologs, we suggested that *StYUCCA19061* may play a key role in auxin biosynthesis and involved in the initiation of potato tuber formation. Conserved amino acid sequence of StYUCCA19061 suggested a similar function of AtYUCCAs, which catalyze a rate-limiting step in auxin biosynthesis. By changing the level of endogenous auxin, overexpressing StYUCCA19061 transgenic plants may show the impact of YUCCA in tuber initiation. Result of qRT-PCR confirmed that the expression of *StYUCCA19061* is regulated during tuber development. Transgenic potatoes that overexpress *StYUCCA19061* were produced for revealing its function in tuber initiation. After transformation, calli were kept in the growth chamber for growing *in vitro* plantlets. According to the results in chapter 2 that auxin has an inhibition effect on tuber initiation, overexpressing *StYUCCA19061* potatoes are assumed to give higher percentage of tuberisation. *In vitro* experimental design can also be used on these transgenic lines to exam the hypothesis. Similar evaluation on overexpression YUCCA transgenic plants was conducted in rice. Overexpressing *OsYUCCA1*

transgenic rice demonstrated an increased level of IAA and showed auxin overproduction phenotypes. The expression of *OsYUCCA1* is restricted to tips of leaves, roots, and vascular tissues (Yamamoto et al., 2007).

Chapter 7 Conclusion and future works

In this thesis, we present the analysis potato tuber formation under the control of phytohormone supplements in an *in vitro* system (Chapter2) Auxin, its transport inhibitor (TIBA) and antagonist (PEO-IAA) were used on untransformed potato plants and the results indicated an inhibition effect of auxin in tuber formation. Higher percentage of tuberisation was found when the stolon tip was removed. It can be explained that auxin biosynthesis site in the stolon was removed by ablating stolon tip, subsequently shut down the polar auxin stream in stolon thus allowed tuberisation to be initiated. It is known that proper auxin polar transport is required for many developmental stages in plants. By applying auxin transport inhibitor (TIBA) to the tuber-inducing medium, we find a higher level of tuber formation compared to the controls. Again, this result provides evidence supporting the importance of polar auxin stream in tuber initiation. Another crucial aspect of the effect of auxin is its perception mechanism. This was revealed by growing the explants with additional auxin antagonist PEO-IAA that inhibit auxin reception and signaling. We find that plants grown with PEO-IAA supplement yield higher amount of tuber. The result suggests that the inhibition of auxin on tuber initiation is not only caused by the directional transport but also by the perception of auxin. Moreover, we reconfirmed an auxin degradation phenomenon in potato *in vitro* system by observing the tuberisation in IAA re-supplied condition. The IAA degradation is discovered as a reason that can cause higher number of tuber formation. Re-supply IAA by transferring the plantlets into new medium on the 21^{th} day after first treatment can effectively maintain the auxin inhibition effect on tuber formation.

As discussed in Chapter3, low tuberisation of plants, which were grown with strigolactone supplement support, the view that strigolactone acts together with auxin to inhibit tuber formation. Tuberisation in strigolactone biosynthesis inhibitor (D2) treatment is as control, which confirmed the inhibition effect of strigolactone. Although the exact mechanism is unclear, the initiation of tuber formation may be controlled by the dynamic feedback loop of auxin and strigolactone. Fluridone is an inhibitor of carotenoid biosynthesis that suppresses both ABA and strigolactone biosynthesis and was used for comparing with D2 effect. However, the tuberisation in fluridone treatment is not as D2

treatment. The reason may explained from the results of previous research that ABA is not a dominant regulator in tuber formation but it stimulates tuber formation by counteracting gibberellin and sucrose(Xu et al., 1998). This stimulating effect may be inhibited by fluridone thus yield a lower tuberisation compared with D2 treatment.

It should be mentioned that, apart from investigating tuber formation, requirements of potato in this *in vitro* system may influence the results and needed further improvements. The difficulty is that the requirements for *in vitro* system may be potato subspecies specific. The etiolated stolons grown in appropriate light/temperature condition can ensure a continuous source of plant materials and be effectively used in further studies. Degradation of exogenous IAA in *in vitro* system was also confirmed by experiment on untransformed Bintje plants.

The mechanism of the initiation of tuber formation is a multiple signaling pathway which involving plant hormones and environmental conditions. In this thesis, we controlled the desirable environmental conditions for tuber inducing such as low concentration of nitrogen, high content of sucrose, short day time. Based on the suitable environmental conditions we are able to exam the effect of auxin and strigolactone on tuber initiatio (Figure 7.1)

The development of transgenic plants with regulated expression of auxin-related genes helps us to elucidate the role of auxin in tuber formation. Although transgenic plants with overexpressing PIN1/PIN2 and TIR RNAi did not provide significant differences in tuber development, the developed transgenic lines were grown in greenhouse after the experiment and the mature plants will be useful for further studies.

Studies of the mechanisms underlying the regulation of auxin and strigolactone in tuber formation may provide further information. It will also be interesting to measure the concentration of auxin/strigolactone in different part of the potato tubers. To understanding the PIN protein function, hypothetic PIN protein distribution differences between transgenic and untransformed plants may provide the positional information for auxin distribution. Determination of the relative contribution from auxin transport, perception and biosynthesis to tuber formation will be informative. Since these experiments were done in *in vitro* system, practical applications are dependent on the response to field tests. Although the number of tubers may regulated by auxin / strigolactone or its inhibitor,

acceptable quality and food safety along with proper cultural practices are all needed to be taken in to account for improving the quality and quantity of potato tuber production.

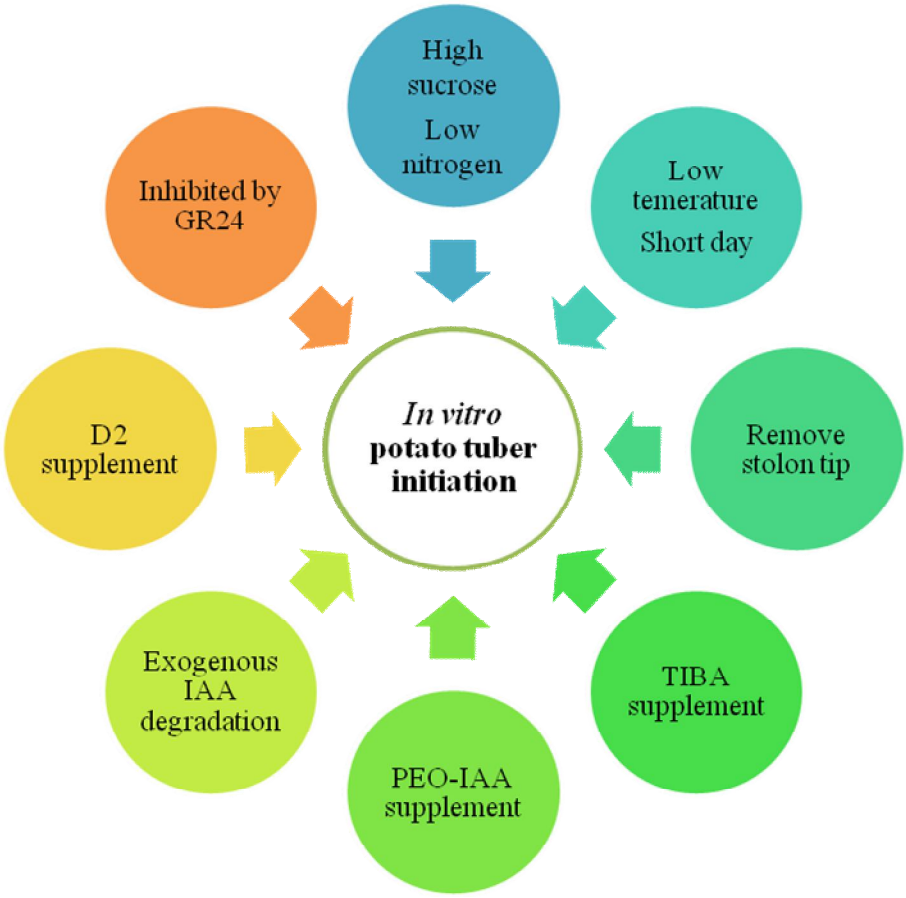

Figure 7.1 Environmental and endogenous factors in the initiation of tuber formation which were considered in this thesis.

Chapter 8 Materials and methods

8.1 Plant materials

For *in vitro* experiment, cv. Bintje was used for revealing the role of auxin and strigolactone during tuber formation. Cultivar Karnico was used as control for *TIR1* RNAi transgenic Karnico line17, line39, and line42 in the experiment for uncover the role of auxin perception in tuber initiation. Cultivar Andigena was used as control for 35S:*PIN2*:RFP transgenic Andigena line11, Line19, Line37 and 35S:*PIN1*:RFP transgenic Andigena line12 and line 34. Only 35S:*PIN2*:RFP transgenic Andigena lines were produced from this thesis, other transgenic lines were obtained by previous studies.

8.2 In vitro propagation condition

For keeping the population, potato plants were maintained in the MS medium (Murashige and Skoog, 1962) with 20g/l sucrose under 16h light period at 24℃ and propagated every 2-3 weeks. Before the *in vitro* experiment, explants were grown on BAP medium in 24h dark at 18℃ for 2 weeks to produce stolons. For evaluate the performance of tuber formation in *in vitro* experiment, tuber-inducing medium (MS mediuem with 80g/l sucrose) was used for growing tuber. After experimental treatment, all plant materials in *in vitro* system were covered by aluminium foil and kept at 18℃ for 7 weeks for observation.

8.3 In vitro experimental setting

Auxin supplement were applied from the ablated tips of plantlet cuttings because the stolon tips are assumed to be the site of auxin biosynthesis. A double treatment was used by growing the plant cuttings in a larger petri dish with tuber-inducing medium and ablated tips of plantlet cuttings were put in a smaller petri dish with tuber-inducing medium and IAA supplement. Except IAA supplement, additional supplements which used in the *in vitro* experiment was applied from the basal part of plant cuttings. The amounts we used are as following:

Supplement	Amount
TIBA	80uM
PEO-IAA	160uM
D2	5uM
Fluridone	5uM
GR24	5uM
IAA	1uM/10uM

8.4 Construction of *35S:StYUCCA19061*

The sequence of *StYUCCA19061* was obtained by blasting the *AtYUCCA* mRNA sequence into the potato genome browser. The complete coding region was amplified by Phusion PCR (Forward primer 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTatggttagctttaatggtcaagat-3' and Reverse primer 5'- GGGGACCACTTTGTACAAGAAAGCTGGGTtcacaaggttgagatttttgctt-3') The resulting PCR product starts with ATG start codon and ends with TGA stop codon. The 5' att site overhang was added to recombine the purified PCR product into pDONR221 entry vector (Invitrogen, Carlsbad, CA) This construct was further sub-cloned into Gateway^{TM} plant transformation destination vector pk7WG2 with 35S-CaMV overexpression promoter. The insert DNA was confirmed by amplifying with gene specific primers.

8.5 RNA isolation and quatitative RT-PCR

To estimate gene expression levels of *StYUCCAs* in differenct potato tissues, qRT-PCR analysis was performed. Genomic cDNA of Bintje including leaf, stolon, axillary buds, and roots were obtained from previous study.The primer sequences for quanticative RT-PCR are as follows:

primer	**sequence (** 5'----> 3')
19061 F	TGTTTTGGACATTGGTGCAT
19061 R	AACGGTGCCACATGAAAACT
14796 F	GGGGTTCCCTTTGTGATCTT
14796 R	AATTGTCGTGGGAGGTGAAG
26499 F	TCGCATCTCTATGGCAAAAA
26499 R	TGGGAATGGGAATTTTGGTA
27485 F	ATTGATGTTGGTGCCATTGA
27485 R	CGTTCTTCCCCATTCTCAAA
38405 F	GCCACATGCAACTTCATCAC
38405 R	GGCCGATTCAACACAAGTTT
20309 F	TGGAGAAATGGAATGGTATGC

20309 R	TTCCAACCACCTTCGGAATA
38346 F	CCTTGAAGATGGCACATTGA
38346 R	TCCATTGAAATACCCGCAAT

For selecting the transgenic lines with high *PIN2* expression, total RNA was extracted from 44 lines of 35S:PIN2:RFP transgenic plants. And the first-strand cDNA was synthesized from total RNA. According to the result of qRT-PCR, 10 transgenic lines with higher *PIN2* expression were selected out of 35S:*PIN2*:RFP population for regeneration. Three transgenic lines (Line11, Line19, and Line37) with higher *PIN* gene expression were used for *in vitro* experiment.

8.6 Tranformation on andigena potato

Construct 35S:*PIN2*:RFP and 35S:*St.YUCCA19061* were transformed into *Agrobacterium tumefaciens* strain Agl0. The *in vitro* stem cuts of *Solanum tuberosum* cv.Andigena was used for the transformation. Transformed plantlet cuttings were maintained in sterile culture for callus growing. The transgenic plants were first grown on basal MS medium(Murashige and Skoog, 1962) with 20g/l sucrose and will be transferred to greenhouse for producing mature plants.

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