Physiology of tuber development and stolon architecture

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This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences

Physiology of tuber development and stolon architecture

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Thesis

submitted in fulfillment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus

Prof.dr. M.J. Kropff,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Friday 5 October 2012

at 11 a.m. in the Aula.

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Physiology of tuber development and stolon architecture

125 pages.

Thesis Wageningen University, Wageningen, NL, (2012)

With references, with summaries in English and Dutch

ISBN: 978-94-6173-383-2

The researcher of this thesis acknowledges financial support from the Bakalas Foundation and the Stichting Veenhuizen-Tulp fonds.

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

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General introduction

An adapted version of chapters 1 and 6 is accepted for publication in Plant Signalling & Behaviour as:

Efstathios Roumeliotis, Richard G. F.. Visser and Christian W. B. Bachem, 2012. A cross talk of auxin and GA during tuber development.

The potato crop

The potato crop production reached more than 324 million metric tons in 2010 ranking sixth among all agricultural commodities with a value of over 44 billion, establishing potato as the seventh most important food crop worldwide (www.fao.org). The potato crop (*Solanum tuberosum*) is cultivated for its tubers that are used for human consumption, animal consumption as well as for industrial use. Disease free homogenous potato tubers are also used as seed potatoes for agricultural use. The potato tuber is formed on the tips of the stolons that grow in the soil. During the stage of tuber growth, the developing tuber acts as a strong sink organ for assimilates making the potato tuber an excellent source of nutrition for man and animal. 60 to 80 percent of the dry matter of the potato tuber is starch, and on a dry weight basis the protein content of potato tubers is similar to cereals. Potatoes are a good source of vitamins B1, B3 and B6, and are rich in vitamin C especially in the skin, as well as a good source of potassium, magnesium, phosphorus and iron, while the fat content is low (US Department of Agriculture, National Nutrient Database). Potato starch is also used in paper, building, textiles and adhesive industries.

Potato originates from central and southern America and many wild *Solanum* species can be found throughout the continent from Uruguay to the United States (Hijmans and Spooner, 2001). Studies on the origin of the domesticated potato reveal that the domesticated potatoes probably originate from one center in Peru (Spooner et al., 2005). Since the discovery of the Americas by the Europeans the areas where the potato crop is cultivated have successfully expanded to Europe, Asia, Oceania and Africa. In the Netherlands, potato is the most important crop and the fourth most important commodity in terms of value after cow milk, pig meat and chicken meat. Netherlands is also one of the most important countries for the production of seed potatoes worldwide, controlling 70% of the world exports in 80 countries (http://www.lol.org.ua/eng/showart.php?id=50923). Furthermore, the Netherlands is an important potato breeding center, with many breeding companies located in the Netherlands where soil and climate conditions are optimal for potato breeding.

The increasing economic and nutritional value of the potato tuber has raised interest for the mechanisms that regulate the initiation and growth of the potato tuber. Research on potato tuberisation will help to enhance agriculture practice and to produce seed potatoes with less disease problems and greater agriculture value. In addition, the potato stolons serve as a model system to study underground storage organ formation. Therefore, potato tuber-initiation research is important because it combines research on a crop with great economic, agricultural and nutritional value with fundamental research.

Initiation of potato tuber formation

Grafting experiments, revealed that under favorable environmental conditions a signal is produced in the leaves and transported to the stolons to initiate the development of tubers (Gregory, 1956; Jackson, 1999). The nature of this signal, named tuberigen, in analogy to florigen that controls flowering in response to photoperiod remained elusive. Botanically, the tubers are shortened and thickened stems (Figure 1). Tubers are formed mainly on the tips of underground shoots called stolons that grow diagravitropically in the soil. Stolons, in contrast to roots, arise from de-repressed axillary buds at nodes in the lower part of the stem below soil level. Stolons have scale leaves structures and associated axillary side buds. Actively growing stolons have elongated internodes and the stolon apex has a characteristic bend, called the stolon apical hook (Figure 2).

Figure 1. Picture of potato plants producing stolons and tubers *in vivo* in an aeroponic system.

Potato tuber organogenesis takes place in the perimedullary region below the stolon apical meristem (Xu et al., 1998). Potato tuberisation can be divided in three stages: induction, initiation and tuber maturation (Ewing and Struik, 1992). The first visible signs of tuber

induction are the inhibition of the longitudinal growth of the stolon and the straightening of the apical hook due to swelling in the apical tip (Stage 3, Figure 2). A stolon tip is defined as having become a tuber when the swollen part of the stolon tip is at least twice the diameter of the stolon (Stage 4, Figure 2). In the initiation stage, the stolon tip is swelling by longitudinal cell divisions. (Xu et al., 1998). Finally, in the maturation stage, random cell division and cell enlargement results in the final size of the potato tuber. The major part of the mature potato tuber consists of storage parenchyma cells that are used to store a range of metabolites, proteins and starch in the tuber (Camire et al., 2009). The quality of the potato tuber is determined primarily by the size, shape and nutritional content of the potato tuber. These quality characteristics are dependent on the environmental conditions and the genotype.

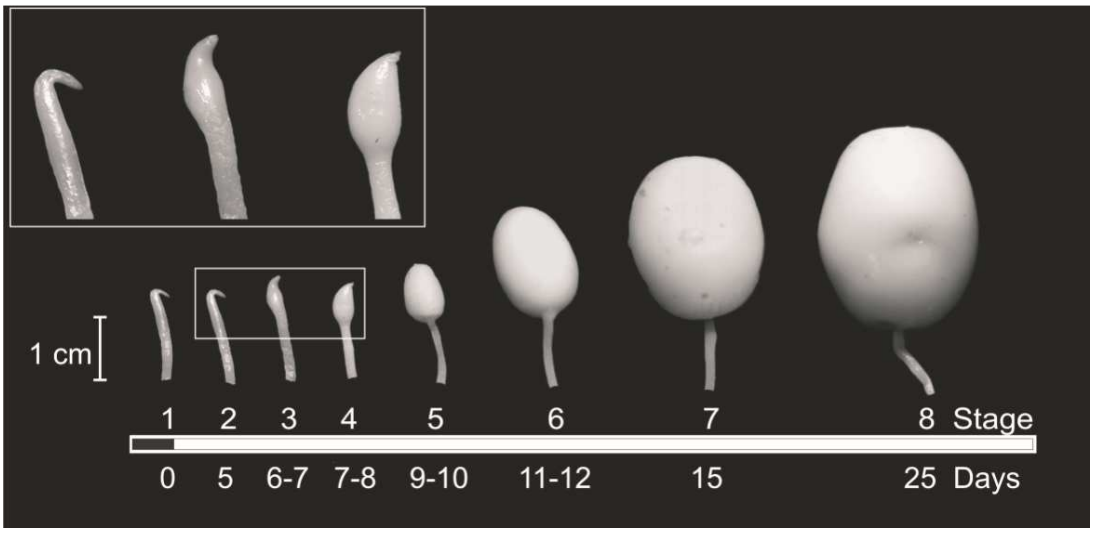

Figure 2. Potato tuber development stages (1-8) in a time period of 25 days after switch to inductive short days (8 hours of light) as described in Kloosterman et al. (2005).

Photoperiod

The origin of the Solanum species is located close to the equator, in central and south America, where day and night last 12 hours without large seasonal variation. Nevertheless, most of the wild potato species are photoperiod dependent to initiate tuberisation with short days (long nights) inducing tuberisation. Interruption of the long nights with a treatment longer than 30 minutes with red light at the middle of the dark period inhibits tuberisation, an effect that could be reversed by a far red light treatment (Batutis and Ewing, 1982). Photoperiod is sensed by photoreceptors such as phytochrome A (PHYA) and B (PHYB) in the aerial parts of the plant. In potato, PHYB has been shown to be involved in tuber initiation. PHYB antisense plants tuberise under long day and short day plus night break

(Jackson and Prat, 1996). In addition, constitutive expression of the *Arabidopsis CONSTANS* (*AtCO*) gene in potato impaired the inductive effect of short days on tuber initiation (Martínez-García et al., 2002). Grafting experiments verified that the *AtCO* overexpression lines exert their effect by acting in the leaves. In Arabidopsis, *AtCO* has been shown to promote flowering in response to long photoperiods (Onouchi et al., 2000). In addition, *AtCO* gene expression is regulated by photoreceptors and the circadian clock (Suarez-Lopez et al., 2001) strengthening a role for the *CO* gene as an important player in the pathway that regulates the initiation of tuber morphogenesis. *AtCO* interacts with *FT* genes to regulate flowering in *Arabidopsis* (Kobayashi et al., 1999). Recently, investigation of the role of *FT* genes in potato provided new insight on the molecular and genetic mechanism that regulated tuber initiation. Expression of a rice *FT* (*Hd3a*) ortholog in potato induced tuberisation under non-inductive long days. In addition, Hd3a-GFP protein was detected in stolons, even though no transcription of the Hd3a gene could be detected in the stolon tissue, providing a good candidate for the tuberigen signaling and suggesting that flowering and tuberising are two developmental events that are mediated by very similar mechanisms. Indeed, study of the potato *FT* homolog genes revealed that two different paralogs, (*StSP3D* and *StSP6A*) control flowering and tuberisation respectively (Navarro et al., 2011).

Phytohormones

Several plant hormones have been studied for their effect on tuber initiation. The class of plant hormones that has been studied most extensively is the group of Gibberellic Acids (GAs). More than 120 GAs have been identified in plants, but only GA_1 and GA_4 are biologically active. GAs have long been thought to have an inhibitory role on tuber induction (Ewing, 1987; Vreugdenhil and Struik, 1989; Xu et al., 1998). Application of active GAs inhibits initiation of tuberisation (Vreugdenhil and Struik, 1989; Jackson and Prat, 1996). In potato, several GA biosynthesis genes have been identified and their role in tuber initiation studied. Analysis of GA biosynthesis and catabolism genes in potato, revealed that *StGA2ox1* gene that are involved in GA degradation are up-regulated five days after tuber induction is induced by short days (Kloosterman et al., 2007). Two *StGA20ox* genes that participate in the pathways that synthesize active GAs are also up regulated, in contrast to *StGA3ox2* gene which catalyses the final step in producing GA_1 and GA_4, two active forms of GA (Kloosterman et al., 2007). The change in the expression levels of the GA biosynthesis and degradation genes is likely to result in the decrease of *in vivo* levels of the active GAs in the stolon tip after tuber induction, as demonstrated *in vitro* (Xu et al., 1998).

In agreement with these results, *StGA20ox1* overexpression or antisense potato plants result in delayed or earlier tuberisation, respectively (Carrera et al., 2000). In addition, antisense transgenic plants for the *StGA2ox1* gene had increased levels of GA_{20} that is an inactive form of GA, reduced stolon growth and earlier *in vitro* tuberisation. In contrast, over expression of *StGA2ox1* delays *in vitro* tuberisation and alters tuber morphology (Kloosterman et al., 2007). *StGA3ox2* has been used in constructs with leaf, tuber specific or constitutive over expression with the CaMV 35S promoter. The leaf specific expression of *StGA3ox2* and the 35S over expression resulted in earlier tuberisation, in contrast to the tuber specific expression that had slightly delayed tuberisation (Bou-Torrent et al., 2011), suggesting that as shown in pea (Proebsting et al., 1992), different forms of GA are transported in a different manner. However it is clear that active GAs have an inhibitory effect on tuber induction. In addition, in wild type plants after tuber induction, an up-regulation of expression of the GA degradation genes results in a decrease of the active GA content in the swelling young tuber.

Apart from GAs, several other plant hormones have been studied for their effect on tuber initiation. Exogenous application of cytokinin (zeatin riboside) to an in vitro tuberisation system, resulted in increased tuber formation (Mauk and Langille, 1978). In addition, plants overexpressing a cytokinin biosynthesis gene (*ipt*) yielded more tubers with reduced tuber weight and nitrogen content (Tao et al., 2010), suggesting that cytokinins may act more on promoting stolon branching rather than on tuber induction.

Auxin is also a plant hormone that has been studied for its effect on tuber development. Application of auxin on *in vitro* tuberisation resulted in earlier tuberisation with sessile smaller tubers (Xu et al., 1998). A study in auxin responsive genes, revealed that auxin responsive factor-6 gene (*ARF6*) has a peak in expression prior to visible swelling followed by a down-regulation during subsequent tuber growth (Faivre-Rampant et al., 2004). A different approach to identify candidate genes using a gene expression study on early stages of tuber development revealed that several auxin related genes exhibited differential expression profiles (Kloosterman et al., 2008). Among those genes were *ARF* genes, *Aux/IAA* genes as well as members of the *PIN* gene family. Auxin plays a very important role in almost all developmental events in a plant that require changes in meristem identity and asymmetrical cell divisions, such as lateral root formation (Marchant et al., 2002), embryogenesis (Luo et al., 2011), and flower development (Krizek, 2011). During the stage of tuber initiation, changes in the plane of cell division occur in the region that give rise to the young tuber through swelling (Xu et al., 1998).

Other hormones such as jasmonic acid (JA) have also been implicated in the initiation of tuberisation. Application of JA in *in vitro* explants enhanced tuberisation (Pelacho and

Mingo-Castel, 1991). In addition, an important increase in JA content was noticed at tuber set, but in tubers no changes in the content of JA was noticed (Abdala et al., 2002). Nevertheless, JA application experiments showed promotion or inhibition of tuberisation, depending on the concentrations applied (Vreugdenhil and Struik, 1989).

Abscisic acid applications on *in vitro* tuberisation systems have produced contradicting results. Koda and Okazawa (1983) reported that application of abscisic acid (ABA) in an *in vitro* tuberisation system with 2% sucrose resulted in slight swellings in the sub apical region that did not develop into proper tubers. In contrast, Xu et al. (1998) reported that ABA application resulted in higher frequencies of tubers only in 1% sucrose, but estimation of the ABA-like substances in tuberising explants were not different compared to non tuberising explants.

Other factors

One of the most important factors used to induce tuberisation is sucrose. The exact mechanism by which sucrose induces tuberisation is not yet known. Nevertheless, research on the role of sucrose transporters in potato (Weise et al., 2000) revealed that RNA interference of a sucrose transporter in potato (SUT4) resulted in higher tuber production and release of the photoperiodic inhibition of tuber induction on the strict photoperiodic *Solanum tuberosum* group *Andigena* (Chincinska et al., 2008), revealing that sucrose can be an important factor in the tuber inductive pathway. Sucrose has been used extensively in *in vitro* tuberisation systems (Koda and Okazawa, 1983; Hendriks et al., 1991; Ewing and Struik, 1992; Xu et al., 1998; Xu et al., 1998; Kloosterman et al., 2007). *In vitro* tuberisation with the use of sucrose has a series of advantages. It is a system that has the capability to produce synchronously a great number of tubers in a time and cost efficient manner. In addition, the aseptic conditions and the control on the nutrition of the *in vitro* propagation help to avoid infections and other abiotic stresses that may interfere with the tuber initiation procedure. It is not clear yet how sucrose induces tuberisation. It is likely that sucrose merely provides the energy source to produce the tuber to a system that induces tuberisation through other stimuli, such as short photoperiod, or that sucrose is the stimulus that induces tuberisation. It is also a possibility that sucrose induces tuber induction through osmotic pressure (Ewing and Struik, 1992).

Another environmental factor that is known to affect tuber induction is nitrogen (Krauss, 1985). Higher levels of nitrogen supplied to the roots inhibit tuberisation, while lower levels can promote tuberisation. It is important to note that low nitrogen levels under non inductive

long days do not promote tuberisation, indicating that nitrogen levels are not a main factor that control tuber formation (Krauss, 1985) but exert an inhibitory effect. High shoot temperatures are also known to inhibit tuber induction under short photoperiods but especially under long days (Jackson, 1999). High air temperature can cause secondary growth of a tuber, known as heat sprouting. Cool temperatures reinitiate tuber growth, forming consecutive tubers. The variety and complexity of the factors influencing or mediating tuberisation indicates that tuber formation is a delicate and complicated developmental event.

The role of auxin in plant development

Auxin is the most extensively studied plant hormone and is an important player in various developmental events. Auxin trafficking is important for cell identity in the embryo (Hakman et al., 2009; Luo et al., 2011), flower development (Cheng and Zhao, 2007; Krizek, 2011), phyllotaxis (Reinhardt et al., 2003) and root development (Marchant et al., 2002; Okushima et al., 2007). In addition, auxin has a profound role in shoot branching (reviewed in Domagalska and Leyser, 2011). Early shoot ablation experiments showed that auxin has a dominant role in shoot branching. Decapitation of the shoot apex enhanced branching, while application of auxin on the ablated shoot restored the branching phenotype (Snow, 1937). Main sites of auxin biosynthesis are shoot apical meristems, along with the expanding leaves, cotyledons and roots (Chen et al., 1998; Gälweiler et al., 1998). Auxin is transported from the sites of biosynthesis to the lower parts of the plant through a Polar Auxin Transport (PAT) stream that is mediated by influx and efflux carriers. Influx has been assigned to the AUX1 protein (Bennett et al., 1996; Marchant et al., 1999). Studies revealed that AUX1-mediated auxin influx in the root cells is responsible for differential expansion of the epidermal cells (Swarup et al., 2005) establishing a role for AUX1 in auxin transport and in gravitropic response in the roots. Auxin efflux is mediated mainly by members of the PIN (Gälweiler et al., 1998; Muller et al., 1998) and to a lesser extent by the MULTIDRUG RESISTANCE (MDR) − p-glucoprotein(PGP) family of proteins (Gil et al., 2001; Noh et al., 2001). Asymmetrical distribution of the influx and efflux carriers mediates the maintenance of the growth axis of the plants by regulating the PAT stream (Gälweiler et al., 1998), as well as responses to gravitropic (Okada et al., 1991; Muller et al., 1998) and phototropic (Okada et al., 1991) stimuli, root development (Friml et al., 2002) and apical shoot formation (Okada et al., 1991). In *Arabidopsis*, many members of the PIN family of proteins have been studied extensively. The members of the PIN family of proteins are divided to two groups according to the protein length; the long “canonical” PINs and the short PINs (reviewed in Krecek et al., 2009).

Cloning, functional analysis and localization studies of one member of the short PINs, AtPIN5 protein, revealed that AtPIN5 protein is localized on the endoplasmic reticulum and is involved in the compartmental distribution and homeostasis of auxin providing evidence that short *PIN* genes play a role in regulating auxin levels in the plant cell (Mravec et al., 2009).

There is at least one tryptophan dependent (Zhao et al., 2001) and one tryptophan independent (Seo et al., 1998) pathway that result in auxin biosynthesis in the plants. These pathways have not been fully elucidated yet, but many genes that participate in these pathways have been studied (reviewed in Lehmann et al., 2010). One of the best studied families of genes that participate in auxin biosynthesis is the *YUCCA* gene family. The *YUC* genes encode flavin monooxygenases that have been proven to be directly involved in auxin biosynthesis (Kendrew, 2001; Zhao et al., 2001; Mashiguchi et al., 2011; Stepanova et al., 2011).

There are two different hypotheses explaining how auxin mediates shoot branching. The canalization hypothesis supports that the PAT stream in the main stem mediates branching with the flow of auxin from the shoot apex to the lower parts of the plant. As long as this main auxin flow remains strong, the side buds are unable to channel their locally produced auxin in the main stem, thus remaining unable to shoot (Leyser, 2009; Prusinkiewicz et al., 2009; Domagalska and Leyser, 2011). In this hypothesis, other factors that are known to participate in the branching mechanism, such as cytokinins (Sachs and Thimann, 1967; Tanaka et al., 2006) or the recently discovered group of phytohormones, strigolactones (Hayward et al., 2009), act after the connection between the vascular system of the bud and the main vascular system of the stem is established or by dampening Polar Auxin Transport, respectively. The second messenger hypothesis supports that auxin produces a second messenger, and this second messenger is responsible for inhibiting shoot branching. Strigolactones are strong candidates for the second messenger (Gomez-Roldan et al., 2008). Both hypotheses have a series of arguments to support their validity (reviewed in Kohlen et al., 2011).

Outline of this thesis

The scope of this thesis is to elucidate the role of important phytohormones such as auxin, GAs and strigolactones in potato tuberisation. There are several lines of evidence leading to a possible role for auxin in tuber development. Application of auxin on *in vitro* tuberisation systems has given contradicting results (Koda and Okazawa, 1983; Xu et al., 1998). Still, a

potato *ARF6* gene was found to have a peak in expression in the stolon apex prior to visible swelling (Faivre-Rampant et al., 2004). In addition, use of new technologies allowed a broader scale investigation of the genes involved in tuber initiation. A microarray chip experiment revealed that several auxin related genes exhibit a differential expression profile during early stages in tuber development (Kloosterman et al., 2005). Auxin is known to have a profound role in various developmental events that require changes in cell identity and reorientation of the plane of cell division (Cheng and Zhao, 2007; Okushima et al., 2007; Hakman et al., 2009; Krizek, 2011; Luo et al., 2011) as shown to occur in early stages in tuber development (Xu et al., 1998; Vreugdenhil et al., 1999). Therefore, the possibility that auxin may somehow be involved in the initiation of tuberisation was gaining ground. We used several tools and different approaches to clarify the role of auxin, including reverse genetics, hormonal treatments and scoring of hormone concentrations. We also tried to integrate tools and emerging knowledge from the field of developmental plant physiology such as strigolactones, as well as the information provided by the sequencing of the potato genome.

In *chapter 2*, we present data about the role of auxin in an *in vitro* tuberisation system. In addition, we monitored the changes in auxin content in the stolon and in the shoot of the potato plant during early stages of tuber development. The direction of auxin transport in the stolons is also investigated and a role for strigolactones in stolon development is assessed.

In chapter 3, the *PIN* family of genes is investigated in potato. All family members are identified based on sequence similarity to known *PIN* genes, as well as by identifying characteristic features of the *PIN* genes. Expression studies based on the RNA-seq data provided by the potato sequencing project and with RT-PCR provides information about the role of the PIN proteins in tuber development. Finally, cloning of the promoter of two *PIN* genes into a *promPIN*::GUS construct revealed the sites of expression of those two genes.

In *chapter 4*, a member of the YUCCA family of proteins in potato, *StYUC-like1*, which is involved in auxin biosynthesis, is cloned into an overexpression construct. Changes in auxin content, shoot branching, root structure and tuber yield are investigated.

In *chapter 5*, the role of gibberellins in tuberisation is investigated. A GA biosynthesis gene, *StGA3ox2*, that is known to be expressed in the potato tuber, is cloned into an RNAi construct to reduce the expression of the gene. Changes in GA content, the above ground phenotype and the effect of the down regulation of *StGA3ox2* are described. In addition, a *promGA3ox2*::GUS construct is used to visualize the sites of *StGA3ox2* gene expression.

Finally, in *chapter 6*, a summary and discussion of the most important results and future perspectives of the research on tuberisation are presented.

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The effects of auxin and strigolactones on tuber initiation and stolon architecture in potato

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Published as:

Roumeliotis et. al., (2012) Journal of Experimental Botany, Vol. 63, No. 12, pp. 4539–4548

Abstract

Various transcriptional networks and plant hormones have been implicated in controlling different aspects of potato tuber formation. Due to its broad impact on many plant developmental processes a role for auxin in tuber initiation has been suggested but never fully resolved. Here, we measured auxin concentrations throughout the plant prior to and during the process of tuber formation. Auxin levels increase dramatically in the stolon prior to tuberization and remain relatively high during subsequent tuber growth, suggesting a promoting role of auxin in tuber formation. Furthermore, *in vitro* tuberization experiments showed higher levels of tuber formation from axillary buds of explants where the auxin source (stolon tip) had been removed. This phenotype could be rescued by application of auxin on the ablated stolon tips. In addition, a synthetic strigolactone analogue applied on the basal part of the stolon, resulted in fewer tubers. Our experiments indicate that a system for the production and directional transport of auxin exists in stolons and acts synergistically with strigolactones to control the outgrowth of the axillary stolon buds, similar to the control of above ground shoot branching.

Introduction

Potato tuber formation is the result of interplay between environmental cues and endogenous signals. In many potato species, short photoperiods promote the initiation of potato tuber organogenesis whereas high night temperatures and high nitrogen levels have an inhibiting effect (Gregory, 1965; Demagante and Vander Zaag, 1988; Ewing and Struik, 1992; Benjamins and Scheres, 2008). Under favourable environmental conditions a graft transmissible signal is produced in the leaves and transported to the stolon where it induces tuber formation (Gregory, 1956). This graft transmissible signal has recently been identified to be an FT-like protein, encoded by an *FT* ortholog in potato called *StSP6A* (Navarro et al., 2011). Several plant hormones have been implicated in tuber initiation, particularly gibberellic acid (GA) was shown to have a strong inhibiting effect and degradation of active GA's in the stolon tip at tuber formation is important for tuberization to proceed normally (Ewing and Struik, 1992; Carrera et al., 2000; Kloosterman et al., 2007). Abscisic acid (ABA) has been shown to have a promoting effect on tuberization when applied exogenously and may act antagonistically to GA (Xu et al., 1998). Application of a cytokinin (zeatin riboside) to an *in vitro* tuberization experiment, resulted in an increase of tuber formation (Mauk and Langille, 1978). Moreover, potato plants expressing the cytokinin biosynthesis gene *ipt* yielded more tubers, but with reduced tuber weight and nitrogen content (Tao et al., 2010). Xu *et al*. (1998) reported that when auxin was applied to single nodal *in vitro* potato explants, an earlier tuberization phenotype was observed with sessile and slightly smaller tubers. These findings suggest a role for auxin in tuber formation. However, the precise mode of action for auxin in conjunction with other plant hormones in stolons has not been established partly due to lack of knowledge on auxin concentrations in the stolon. A microarray based expression study provided additional data to support an important role for auxin in tuber development. Many auxin related genes are differentially expressed during early tuber developmental stages. Genes involved in auxin transport (*PIN* gene family), auxin response factors (*ARF*) and *Aux/IAA* genes exhibited differential expression profiles (Kloosterman et al., 2008). The *ARF*6 gene was found to have high expression prior to visible swelling followed by down regulation during subsequent tuber growth (Faivre-Rampant et al., 2004). Auxin has been shown to play a key role in many different aspects in plant architecture, such as lateral root formation (Marchant et al., 2002), embryogenesis (Luo et al., 2011) and flower development (Krizek, 2011). Tuber organogenesis is divided in three stages, tuber induction, tuber initiation and tuber growth (Ewing and Struik, 1992). During the stage of tuber initiation, changes in the plane of cell division occur in the region of the stolon that will give rise to the young tuber through swelling (Xu et al., 1998). Auxin is produced in plants by at

least two different metabolic routes, a tryptophan dependent and tryptophan independent pathway (reviewed in Lehmann et al., 2010). Although these pathways are not yet fully understood, several biosynthetic genes have been identified, such as the *YUCCA* gene family (Steven G, 2001; Zhao et al., 2001; Cheng et al., 2006). Recently, YUC proteins were identified to be catalysing a rate limiting step in the main IAA biosynthesis pathway in Arabidopsis (Mashiguchi et al., 2011). Shoot apical meristems (SAMs) are the main sites of auxin biosynthesis, along with cotyledons, expanding leaves and root tissues (Ljung et al., 2001). Specific subcellular localization of auxin influx and efflux carriers, modulate the transport directionality (Chen et al., 1998; Gälweiler et al., 1998). Auxin transport inhibitors, such as 2,3,5- triiodobenzoic acid (TIBA) which interrupt the constitutive cycling of the PIN proteins between the plasma membrane and the endosomes, interfere with directional auxin transport, and have been essential tools in formulating the concept of asymmetrical auxin distribution (Dhonukshe et al., 2008). In addition to polar auxin transport inhibitors, auxin antagonists such as PEO-IAA competing for the same binding site as auxin, has been used to unravel the role of auxin in various developmental procedures, such as root node development (Abel and Theologis, 2010) and gravitropic response (Nishimura et al., 2009).

Studies on plant stem architecture have led to a model that describes the principles of shoot branching (reviewed in Domagalska and Leyser, 2011). Two key players in the model are auxin and strigolactones (SLs) (Cook et al., 1972; Bouwmeester et al., 2003; Gomez-Roldan et al., 2008; Rameau, 2010). Auxin was the first plant hormone shown to have an inhibitory effect on shoot branching (Snow, 1937), through the establishment of polar auxin transport (Gälweiler et al., 1998). SLs have recently been identified as the secondary signal that, in concert with auxin, appears to regulate shoot branching (Gomez-Roldan et al., 2008). Moreover, SLs have been detected in root exudates and extracts of several plant species including *Arabidopsis* and tomato (López-Ráez et al., 2008; Kohlen et al., 2011). It has been proposed that SLs act either directly on axillary bud outgrowth or indirectly via dampening of auxin transport and canalization of auxin from the buds (Brewer et al., 2009; Prusinkiewicz et al., 2009). In this study, we determined auxin concentrations during early tuberization events in potato plants *in vivo*, in parts of the stolon and in parts of the stem. Furthermore, we examined the impact of auxin, auxin transport inhibitors and a synthetic strigolactone analogue on tuber formation using a modified *in vitro* tuberization approach. Based on these results we propose a similar system of apical dominance in underground stolons as described for shoots.

Materials and methods

Plant materials and in vitro tuberization

Single-node cuttings from short-day-grown potato plants (*Solanum tuberosum* L. var Bintje) were propagated *in vitro*, on standard Murashige and Skoog medium (Murashige and Skoog, 1962) 2% (w/v) sucrose. Potato plantlets were grown for 4 weeks prior to harvesting single node explants. After 10 to 12 days of growth in the dark on 2% sucrose MS medium with 1 mg/l BAP, the explants formed etiolated shoots/stolons which were transferred to petri dishes containing different tuberization media and kept in the dark at 18°C. Explants with stolons were placed into petri dishes containing dual media based on KI medium (Hendriks et al., 1991). Media supplemented with compounds under investigation were applied to the base of the explants or to the stolon tip (Figure 4, inset). Final concentrations of studied compounds were: 80 µM TIBA, 1 µM PEO-IAA, 1 µM IAA, and 5µM GR24. Benzylaminopurine (BAP), indole-3-acetic acid (IAA) and 2,3,5- triodobenzoic acid (TIBA) were purchased from Sigma. PEO-IAA (Hayashi et al., 2008) was a kind gift from Dr. Kenichiro Hayashi. The explants for each treatment were divided into four groups of two Petri dishes each, with 6 explants in each Petri dish (total 48 explants per treatment). The number of explants producing tubers was monitored for a period of 39 days.

Detection of strigolactones in potato roots

Solanum tuberosum L. var. Karnico, Bintje and *S. tuberosum* group *andigena* were grown *in vitro* for 20-30 days and then transferred to an aeroponic system (Nutricolture co. UK) on Hoagland's solution, as previously described (López-Ráez et al., 2008). After 24 days of growth, the plants were exposed to phosphate starvation as described for tomato (López-Ráez et al., 2008). 7 days after the phosphate starvation, roots were harvested for extraction of SLs. The extraction of strigolactones (SL) and the LC-MS/MS analysis were performed as described previously (López-Ráez et al., 2008).

Assessment of the auxin concentrations

Potato plants (*S. tuberosum* group *andigena*) were propagated *in vitro* and grown for 2 1/2 weeks in MS20 before being transferred to soil filled pots in the greenhouse. After 9 weeks, the shoot apex, middle and basal internode stem segments, the stolon region 1 cm below the apex (sub swelling region: SSR) and the stolon apical meristem (STAM), were harvested and immediately frozen in liquid nitrogen (day 0). The remaining plants were then transferred to short day (SD) conditions (8 hours light). The same set of plant tissues was harvested 5, 16 and 26 days after the switch to SD conditions. Fully matured tubers were harvested 8 weeks after switch to SD and tuber apex, heel, pith and the perimedullary region samples were collected. For all samples, two biological repeats were collected and tissues from 5 different plants were pooled for each repeat, except for the stolon tip on day 5 where only one biological repeat was collected due to low sample weight. All samples were ground to a fine powder and stored at -80°C. The auxin extraction was performed starting with 200-250mg of ground material. 1 ml of MeOH with labeled auxin (IAA-IS) (0.1nmol/ml-> in sample 0.5nmol/ml) was added and samples were briefly vortexed. The samples were then sonicated for 10 minutes. After sonification, the samples were placed in a shaker (~150 rpm) in a cold room (4^{o} Celsius) for 1 hour. The samples were then centrifuged at 2000 rpm for 10 minutes and the supernatant was transferred to a 4ml glass vial. Extraction was repeated with 1ml MeOH without IAA-IS, shaken for 1 hour in a cold room, centrifuged and the supernatants of the same sample were pooled. The columns (Solid Phase Extraction cartridges, Grace Davison Discovery Sciences) were placed on a Solid Phase Extraction Apparatus and pre-equilibrated by applying in order: 5ml Hexane, 5ml acetonitrile, 5ml deionizer water and 5ml imidazole buffer. The sample was applied on the column and washed by applying the following in this order: 5ml hexane, 5ml ethylacetate, 5 ml acetonitrile and 5ml methanol. The samples were eluted with 4ml 98% and methanol 2% acetic acid. Solvents were evaporated in a speedvac. The samples were finally eluted in 200µl acetonitrile:H_2O:formic acid=25:75:0.1 and filtered in vials using a RC4 Minisart 0.2 µm filter. The LC-MS/MS analysis was performed as described in (Ruyter-spira et al., 2010).

DR5::GUS transgenic plants and GUS staining

Transgenic plants harbouring the DR5::GUS (Ulmasov et al., 1997) and the promoter from a potato PIN gene fused to the GUS reporter (p*StPINV*::GUS) were obtained by *Agrobacterium* mediated transformation of *S. Andigena*. *StPINV* is a potato PIN gene located on chromosome five (genome sequence coordinates PGSC0003DMB000000051: 1580317-1584014 V.3.0), previously found to be expressed during early stages of tuber initiation (Kloosterman et al.,

2008). A region of 1310bp upstream of the translational start site of *StPINV,* was used to drive the GUS gene expression in the p*StPINV*::GUS construct. The GUS staining assays were done as described previously (Stomp, 1992). The incubation of the tissues in GUS substrate X-Gluc was performed overnight at 37^{o}C. The tissues were washed with 70% ethanol prior to imaging.

Quantitative RT-PCR.

S. tuberosum L. group *andigena* plants were grown in the greenhouse transferred to short day conditions to induce tuberization. Stolon tips were harvested at day 0 (switch to short days), 2, 4, 6 and 8. RNA was extracted using the Qiagen RNaesy Plant mini kit and DNAse I treated. cDNA synthesis was performed using Bio-Rad iScript cDNA synthesis kit and qRT-PCR was performed using the Bio-Rad cycler. As a reference gen eIF3e was used (forward primer seq GGAGCACAGGAGGAAGATGAAGGAG, reverse primer seq CGTTGGTGAATGCGGCAGGAAGGAG). StYUC-like1 (GeneBank accession number JN935396) gene expression study was performed with the following primers: forward primer seq TGTTTTGGACATTGGTGCAT, reverse primer seq AACGGTGCCACATGAAAACT.

Auxin transport assays

Polar auxin transport was measured as previously described (Okada et al., 1991), with some modifications. Stolon tips were cut to 25 mm pieces, put into 1 mL brown glass vials and incubated at either the proximal or distal end with 200 µL ¼ strength Hoagland medium containing ^{14}C-labeled IAA (American Radiolabeled Chemicals Inc., St. Louis, USA) with or without 2.5 µM NPA, for 18 hours at room temperature. The final concentration of IAA was adjusted to 1 µM (0.2 nCi/mL). For comparable access the tip at the distal end (0.5 mm) was removed before introduction to the radioactive medium. The 5 mm end not in direct contact with the radioactive medium was incubated at 50˚C for 2 hours using 0.5 mL Lumasolve (Lumac Systems AG, Basel, Switzerland) and radioactivity was counted in 4 mL Ultima GoldTM (PerkinElmer Life Sciences, Inc., Boston, USA) using a tri-carb 2100TR liquid scintillation counter (PerkinElmer Life Sciences, Inc., Boston, USA).

Results

Auxin measurement in potato plants under inductive and non-inductive conditions

We measured the concentration of the free auxin (IAA) in *in vivo* plants at different time points after a switch from long day (LD, non-inductive) to short day conditions (SD, inductive). Under non-inductive long day conditions, the IAA concentration increased from the apex to the lower parts of the plant (Figure 1A). Average IAA concentrations of 560, 2510 and 3250 pmol of IAA*gram^{-1} fresh weight (FW) were measured for the shoot apex, middle and basal part of the stem, respectively. After the switch to short day conditions, the IAA concentration gradient decreased and at day 26, the difference in IAA concentrations between shoot apex and basal stem was lost (Figure 1A). The concentration of oxidized auxin (oxindole-3-acetic acid; referred as OxIAA hereafter) was complementary to that of IAA (Figure 1C). At day 0, the concentration of OxIAA in the shoot apex, middle stem and basal stem was 2630, 220 and 400 pmol, respectively. As with free IAA, the concentration of OxIAA reduced over time such that by day 26, the concentrations were 590, 280 and 0 pmol in the shoot apex, middle stem and basal stem, respectively.

IAA concentrations were also measured in *in vivo* grown stolons at the same developmental stages as the above ground tissues. IAA was measured in the stolon apical meristem (STAM) and the stolon sub-swelling region (SSR) (Figure 1B). Under long day conditions, free auxin concentration in the STAM was 270 pmol*g^{-1} FW. After a small initial decrease after the switch to SD conditions (day 5; 70 pmol*g^{-1} FW), IAA levels increased dramatically to a maximum of 1050 pmol*g^{-1} FW on day 16, at which time the first tubers were observed. On day 26 when tubers are around 1 cm in diameter, IAA concentrations were reduced to 510 pmol*g^{-1} FW (day 26 Figure 1B). Interestingly, the increase in auxin concentration is not restricted to the STAM but also extends to the section behind the stolon tip (SSR) that follows a similar profile and reaches a peak in auxin concentration at day 26 (2430 pmol*g^{-1} FW; Figure 1B)

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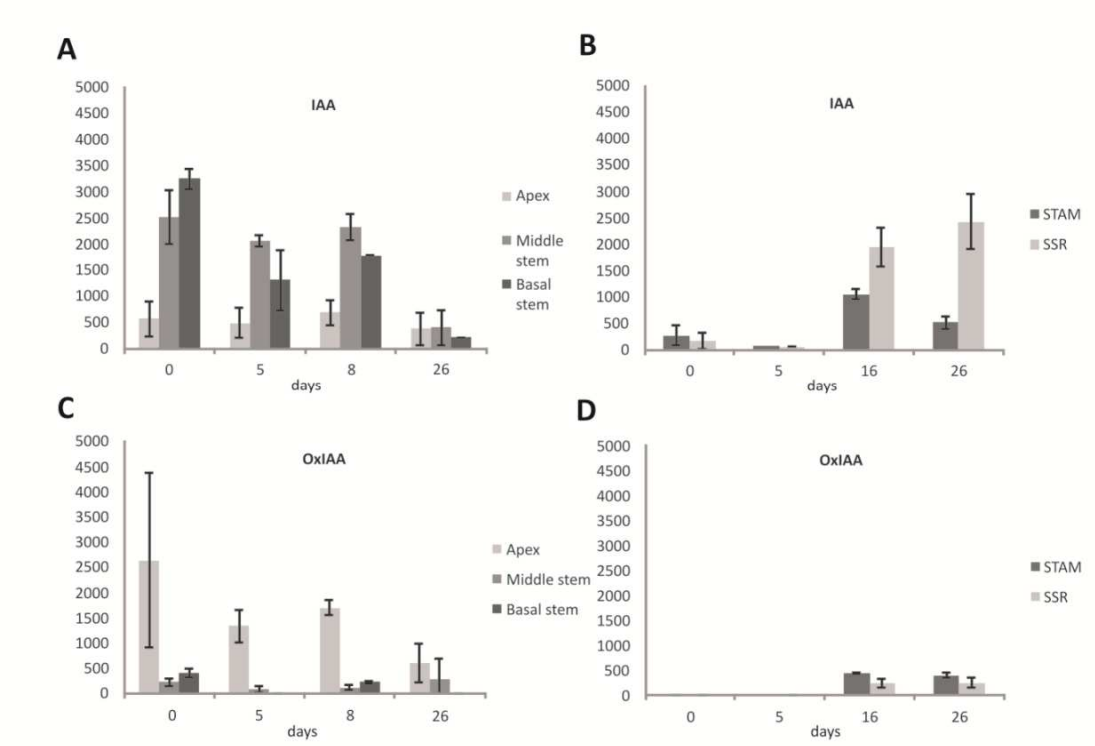

Figure 1. Concentration of free IAA and OxIAA (A, C) in three different parts of the stem (apex, middle and base) and two parts of the stolon (Stolon Apical Meristem and Sub-Swelling Region) (B, D). Plants were grown for 9 weeks under non-inductive conditions, before switch to inductive short day conditions. IAA and OxIAA concentrations are in pmol per gram of fresh weight. Samples were harvested under long day conditions (LD day 0) just before switching to inductive short days and after 5, 8 and 26 days in short day conditions (SD day 5, 8 and 26 respectively). Error bars represent standard error of the mean of two replicated measurements.

Potato plants were transformed with the DR5::GUS construct to visualize local auxin concentration. In the stolon a strong GUS staining was observed in the SSR and around the vascular tissues clearly visible in the swelling tip (Figure 2A). In young tubers, blue staining is clearly visible around the vascular bundles and a strong staining is observed in the stolon region below the growing tuber (Figure 2B) consistent with the auxin measurements.

Potato plants were also transformed with a construct comprising 1.3 Kbp of the regulatory sequences of a PIN gene from chromosome 5 of potato (p*StPINV*) driving the GUS reporter gene (p*PINV*::GUS construct) in order to visualize the locations of expression of *StPINV* gene that is involved in auxin transport. In the stolon apical hook, GUS staining was observed around the vascular bundles, depicting that the *StPINV* expression is strongly correlated with the DR5::GUS staining and indicating auxin transport from the stolon apical meristem (Figure 2C).

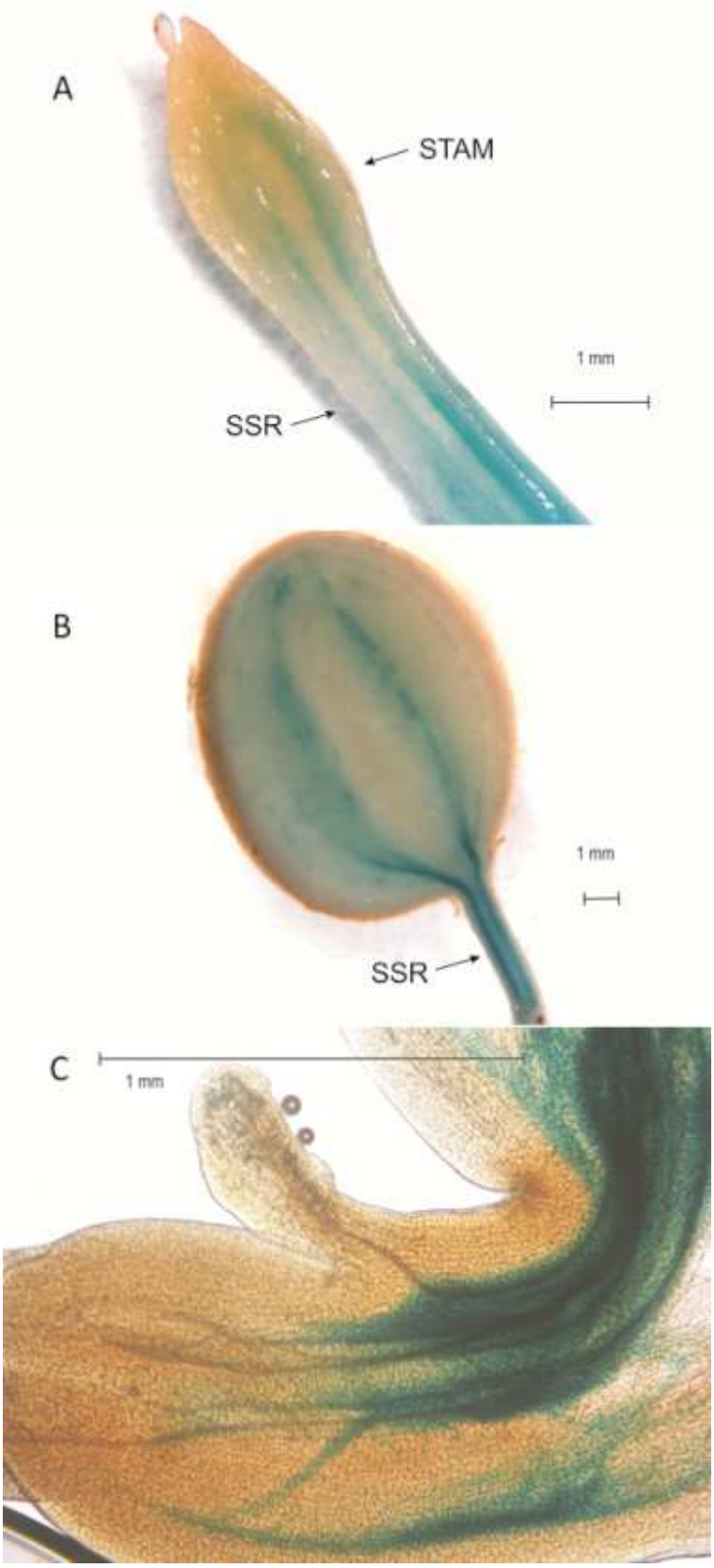

Figure 2. Staining of DR5::GUS in transgenic stolon after 9 days in short days (A) and in a young tuber after 20 days in short days (B). The arrows indicate the Sub Swelling Region (SSR) and the Stolon Apical Meristem (STAM). In (C), staining of promPINV::GUS transgenic plants in the stolon apical hook under long day conditions. The GUS staining is located around the vascular tissue in the STAM, indication of auxin transport from the site of biosynthesis in the STAM to the basal parts of the stolon.

IAA levels were measured in different parts of mature tubers to evaluate auxin distribution within the tuber. The tuber apex had the lowest concentrations of free IAA, (110 pmol*g^{-1} FW) but in similar concentration ranges as the perimedullary region (120 pmol*g^{-1} FW) and the pith (170 pmol*g^{-1} FW). The highest concentration of IAA observed in the tuber heel (240 pmol) is consistent with the GUS staining of younger tubers (figure 2B). IAA levels of whole tuber samples were around 160 pmol*g^{-1} FW significantly less than at tuber swelling (1050 pmol*g^{-1} FW).

Interestingly, there were no detectable levels of OxIAA in STAM or in the SSR at days 0 and 5. Only at day16, OxIAA was detected (430 and 220 pmol*g^{-1} FW for STAM and SSR respectively) but concentrations were lower than those found for free IAA and remained relatively stable during further tuber growth (day 26; 390 and 240 pmol*g^{-1} FW for STAM and SSR, respectively).

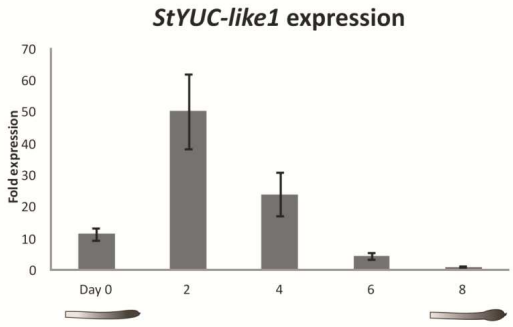

Figure 3. Relative fold expression of the StYUC8-like 1 gene, under LD conditions (day 0) and 2, 4, 6 and 8 days after switch to short days in a climate chamber. Error bars show the standard error of the mean of three technical repeats.

Expression of an auxin biosynthesis gene (*StYUC-like1*) in the potato stolon

In order to identify genes responsible for the increase in IAA content in the stolon prior to tuber onset, we identified the potato orthologs of an auxin biosynthesis gene family (YUCCA) using the potato genome sequence. Based on expression analysis of five *YUCCA-like* gene orthologs over the tuber development stages described by Kloosterman et al. (2000) one *YUC*-like gene (here referred to as *StYUC-like1*) gene showed a 200 fold increase at stage 2, 5 days after induction to tuberise compared to stage 8, 25 days after tuber induction (Supplementary Figure 1). The expression profile of this *StYUC-like1* gene in stolon tips during tuber development was verified in *Solanum tuberosum* L. var Andigena plants we used for auxin measurements, by qRT-PCR (Figure 3). A peak in the expression of *StYUC-like1* was observed already 2 days after the switch to SD conditions. After day 2 the expression levels decreased rapidly (Figure 3). At the tuber swelling stage (day 8) expression levels were almost 50-fold lower in comparison to the peak at day 2 and 10-fold less compared to non-inductive LD conditions on day 0. *StYUC-like1* expression peaks prior to visible stolon swelling while the strong increase in IAA levels was not observed before swelling. However,

it is important to note that auxin measurements were performed on plants grown in the greenhouse and required a longer period in short day conditions before stolon swelling could be observed in comparison to plants grown in the climate cell used for the tuber developmental series (day 0 to 8). Therefore StYUC-like1 remains a strong candidate for the involvement in the increase of local auxin concentrations in the stolon tip after the switch to SD conditions. Expression data for alternative auxin biosynthesis pathways using the publically available RNA-seq data (PGSC, 2011), revealed that either these orthologs are poorly expressed in the case of potato *TAA and TAR* orthologs, or their expression in stolons and tubers does not change over time as in the case of potato *AMI1* gene orthologs (data not shown). Therefore we conclude that alternative pathways are unlikely to play a significant role in the initiation of tuber formation.

The effects of IAA, SL and their inhibitors on stolon axillary bud outgrowth and tuberization in vitro

In standard *in vitro* tuberization, apical tubers and subsequently lateral tubers are formed on etiolated shoots/stolons in the dark due to the promoting effect of high sucrose levels. Based on the *StYUC-like1* gene expression profile and the IAA measurements in the stolon, the stolon apical meristem (STAM) is a likely site of auxin biosynthesis. To study the role of the stolon apical meristem and auxin biosynthesis in the process of lateral tuber initiation under tuber inductive conditions, the stolon tip was removed from the in vitro stolon explants. As a result, the axillary buds of the stolons grew out and formed more tubers in comparison to the control (Figure 4A).

When IAA was applied on the ablated stolon tips, axillary bud outgrowth and thus tuberization was suppressed (Figure 4B). Interestingly, when explants were not transferred to fresh IAA containing media after 19 days final tuber numbers showed a substantial increase (Figure 4B, IAA A). These results indicate that stolon tips are a site of auxin biosynthesis and that auxin regulates the process of axillary tuberization in a similar way as axillary shoot growth in the aerial stem.

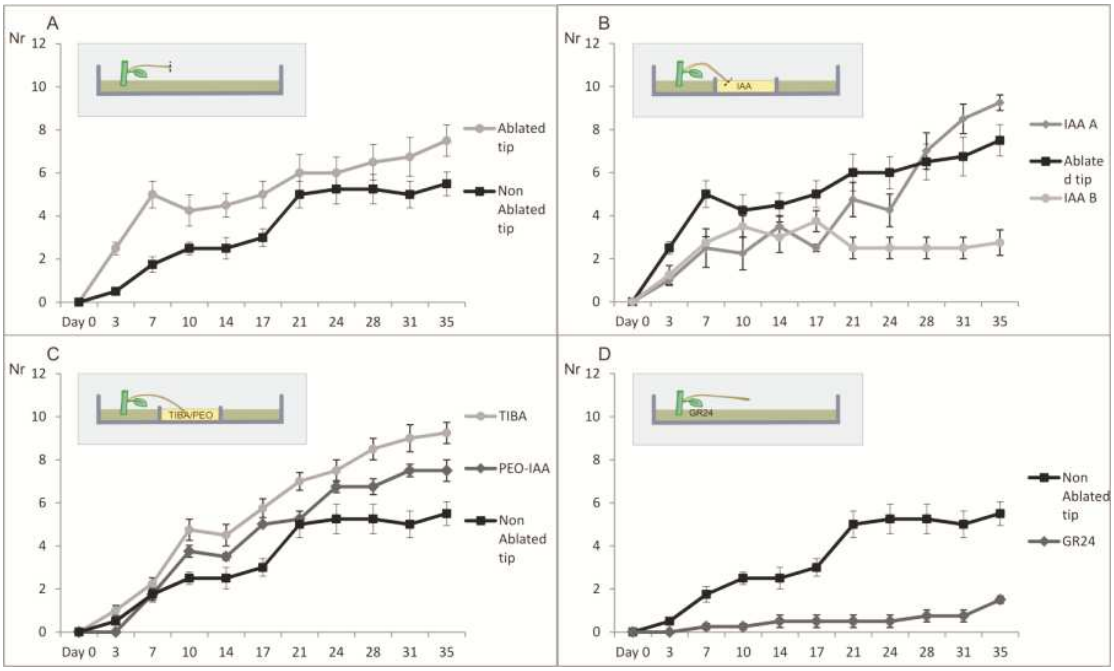

Figure 4. Comparison of in vitro tuberization frequency between the ablated and not ablated stolon tips exposed to various treatments (A-D). In all panels the Y-axis, (labeled; "Nr") represents the number of explants and the X-axis, (labeled "Days") represents the days under tuber inducing conditions. (A) The effect of stolon tip ablation on the production of in vitro tubers. (B) Effect of the application of 1µM auxin on ablated stolon tips where explants were either transferred to fresh medium after 19 days (IAA B) or remained on the same medium over the experimental period (IAA A) The controls (black graph) were explants with ablated tips without hormone treatment. (C) Numbers of explants that produced tubers after applying TIBA and the IAA antagonist PEO-IAA. The controls are the same as in (B). (D)The effect of GR24 on in vitro tuberization efficiency. In all cases, error bars represent standard error of the mean of four replicated measurements. The insets are diagrammatic representations of the location of treatment application, and the ablation of the stolon (dotted line).

TIBA is a widely used auxin transport inhibitor that interrupts polar transport of auxin. When TIBA was applied on the basal part of the explants, a higher number of explants formed tubers in comparison to the control (Figure 4C). Furthermore, the tubers were sessile and formed on the distal axillary buds of the stolons. Similarly, when the auxin antagonist PEO-IAA was applied on the basal part of the explants, a promoting effect on the tuberization was observed, but not as strong as with TIBA treated explants (Figure 4C). TIBA and PEO-IAA are likely to decrease the inhibitory effect of auxin, as auxin transport (TIBA) or perception (PE0-IAA) is reduced. As a result, the inhibition on stolon bud outgrowth is reduced and axillary buds are able to grow out and form tubers under inductive conditions.

To investigate a possible role of strigolactones (SLs) in the stolon axillary bud outgrowth and initiation of lateral tuber formation, we applied GR24, a synthetic SL analogue in the *in vitro* tuber induction system. Application of GR24 on the basal part of the stolon explants resulted in significantly less tubers (Figure 4D). At day 39, less than 2 explants per group of 12 treated

with GR24 formed tubers, in contrast to more than 5 in the control plants. This result reveals a strong inhibitory effect of GR24 on stolon axillary bud outgrowth

Root extracts of potato plants were investigated for the presence of SLs known to be present in tomato roots (López-Ráez et al., 2008). In all three potato cultivars analysed, two different SLs namely orobanchol and orobanchyl acetate were detected (Figure 5). Phosphate starvation using an aeroponics system increased the level of both strigolactones. Interestingly, the potato cultivars exhibited different levels of the two SLs in both normal and phosphate depleted growing conditions.

IAA transport assays

In order to investigate the direction of movement of auxin in the stolons, we scored the transport of ^{14}C-labeled IAA in the presence or absence of the polar auxin transport inhibitor NPA (2.5 µM) in stolon tips placed vertically in Hoagland medium, with their basal or the apical part placed in the medium containing the ^{14}C-labeled IAA. Scoring the transport of ^{14}C-labeled IAA revealed that the efflux of IAA from the stolon tip is 3.5 times more compared to the influx into the stolon tip (Figure 6). This indicated that the main direction of IAA movement in the stolon tissue is from the stolon tip to the basal part of the stolon. NPA is a polar auxin transport inhibitor that acts by disturbing the localization of the PIN proteins. Application of NPA did not alter the transport of IAA into the stolon tip, but decreased the outflux of IAA to the same levels as the influx indicating that the polar movement of IAA out of the stolon tip is mediated by the PINs, and influx in probably due to diffusion. The results of the ^{14}C-labeled IAA assays verified IAA is moving in a polar direction out of the stolon tip

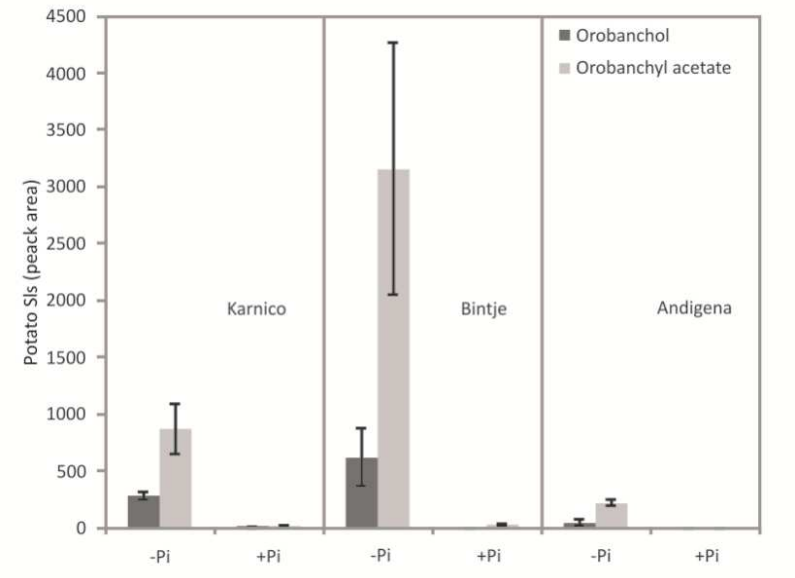

Figure 5. Identification of Strigolactones orobanchol and orobanchyl acetate in potato root extracts of three genotypes (Karnico, Bintje and Andigena) under normal growth conditions (+P) and under phosphate starvation (-P). The relative concentration is calculated by the peak areas measured by LC-MS/MS analysis. Error bars represent standard error of the mean of two replicated measurements.

a movement that is mediated by the PIN proteins.

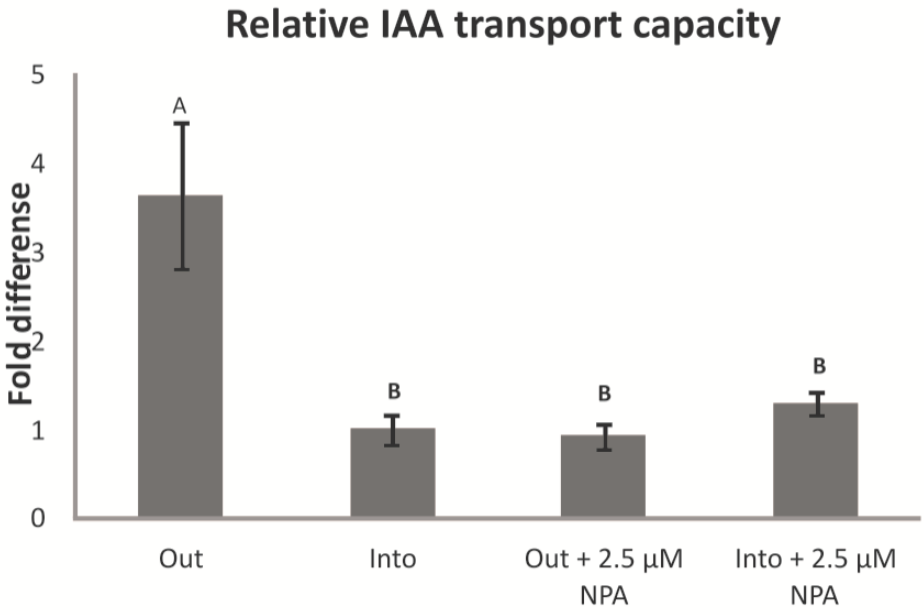

Figure 6. Fold difference in the transport of auxin out and in the stolon tip in the absence and presence of the auxin transport inhibitor NPA. Influx of IAA into the stolon tip is set as 1. Error bars are standard error of the mean for ten replicates per treatment. Statistically significant differences are noticed between treatments that are noted with different letter, A or B (t test, a=0.05)

Discussion

We studied the free IAA and oxidised IAA (OxIAA) content in three different parts of the stem of potato. The concentrations of free IAA in these three stem parts were 10 to 50 fold higher than the concentrations found in Arabidopsis (Mashiguchi et al., 2011). Furthermore, we show a negative correlation between the concentrations of free IAA and OxIAA in the same tissue. It is particularly noteworthy that in potato, we found a gradient of IAA levels in the stem with the highest concentration at the base (Figure 1A). Since the basipetal transport of auxin is well established, in potato the high IAA concentration in the basal stem at the time of harvest may indicate an auxin accumulation due to a bottleneck of transport in the lower stem. This gradient is diminished after the switch to short day conditions. Whether this is due to a reduced biosynthesis of auxin, degradation or increased transport into other tissues such as the roots remains to be resolved.

Indeed, assessment of the concentration of free IAA in stolon tips before and in three different stages after the switch to short days shows a peak of IAA concentration in the stolon apical meristems and the sub swelling region 16 days after the induction to tuberise. Auxin is known to participate in many developmental processes, such as flower development and lateral root formation involving cell division and changes in the orientation of plane of cell division (Marchant et al., 2002; Krizek, 2011; Luo et al., 2011). During stolon swelling the plane of cell division also changes from transversal to longitudinal (Xu et al., 1998). The change in cell division plane is likely to be at least partly controlled by auxin and the increase in auxin content that we noticed in these cells.

The question whether *in planta* auxin is transported from the shoot apex into the stolons or whether auxin is synthesised autonomously by the stolon tip was answered by the finding that the auxin biosynthesis gene *StYUC-like1* increases transiently after the switch to short days and by the IAA transport assays. The *StYUC-like1* gene expression appears to be highly specific for stolons (PGSC, 2011) and thus may be a causative factor in the increase in IAA concentration in the stolon tip that was noticed a few days later. The phase-shift between *StYUC-like1* gene expression and auxin accumulation is consistent with the delay between expression and enzyme activity. The difference in *StYUC-like1* gene expression at day 0 and day 26 may in part be due to the dilution of cells with active transcription of this gene in tubers in comparison to the stolon tissue. However, a gradual reduction of *StYUC-like1* gene expression in the young tubers is in line with a stabilisation of IAA concentration and slow decrease during further tuber growth. The finding that the expression of genes from alternative IAA biosynthesis pathways are not tissue specific or differentially expressed during tuber development, makes it unlikely that these pathways play a key role in tuberisation. In addition, ^{14}C-labeled IAA assays verified that the main direction of IAA movement is from the stolon tip to the basal part of the stolon. NPA application that is known to disrupt PIN protein polarity also disrupts IAA efflux from the stolon tip (Figure 6). As a result, the direction of IAA movement and the mechanism that mediates polar auxin transport in stolons and in stems appear to be similar.

The high auxin levels in the swelling stolon are confirmed by the induction of DR5::GUS (Figure 2). It is interesting to note that during further tuber development, high levels of GUS staining are maintained in the tuber heel and proximal stolon section. The p*PINV* driven GUS staining in the apical hook indicates that auxin is transported from the STAM, being the probable site of auxin biosynthesis, towards the basal part of the stolon (Figure 2C). The high auxin levels in the stolon sections, not destined to form a part of the tuber both prior and after tuberization implies the need for additional factors for development and differentiation of this

organ. Clearly, other hormones, sucrose and a recently identified FT-ortholog all play essential roles in tuber induction and formation of the potato tuber (Navarro et al., 2011).

In vitro tuberization has been extensively used for studies concerning tuberization (Koda and Okazawa, 1983; Hendriks et al., 1991; Ewing and Struik, 1992; Xu et al., 1998; Xu et al., 1998) and has been proved to be a good representation of what is happening *in vivo*. It combines the advantages of producing many tubers, synchronously, in a disease free, cost and time efficient manner. Explants in our experimental design were grown in the dark to produce stolons that would produce tubers upon induction by high sucrose concentration. It is possible that sucrose merely provides the energy source needed to produce the tuber and the dark condition is the stimulus that induces tuber induction, or alternatively that sucrose might be the stimulus itself (Ewing and Struik, 1992). Here we describe the use of both mechanical and chemical ablation of the stolon tip in *in vitro* potato stolons to investigate the role of auxin in the architecture of the stolon under tuber inducing conditions. In addition, the ^{14}C-labeled IAA assays verified that IAA is polarly transported from the stolon tip to the basal parts of the stolon (Figure 6). When auxin transport is inhibited either through ablation or chemical inhibition, there is a release of dormancy of axillary stolon buds and an increase in the number of tubers (Figure 4). The application of IAA on the mechanically decapitated stolon tips reduces the numbers of explants producing tubers compared to explants with decapitated stolons. This suggests that the re-establishment of polar auxin transport restores the apical dominance on the distal axillary buds.

Another major player in the control of shoot branching are the strigolactones (SL) (Hayward et al., 2009). SLs, working together with auxin, have been shown to have an inhibitory role on shoot branching (Gomez-Roldan et al., 2008). In our *in vitro* tuberization experiments a synthetic SL analogue (GR24) was applied, leading to a marked inhibition of axillary stolon bud outgrowth and subsequent potato tuber formation. Whether SLs are present in stolons of *in vivo* growing plants and the role these may play in tuberization remains unclear. However, we measured SL for the first time in potato roots and were able to demonstrate their presence in root extracts. Taken together, these results point to a novel role of auxin in tuber formation. Furthermore, in vitro tuberization experiments indicate an additional role for auxin together with strigolactone in the regulation of stolon axillary bud growth.

Acknowledgements

We thank Tatsiana Charnikhova, Francel Verstappen and Wendy Jo-Chen Liao for their technical support. The synthetic strigolactone analogue GR24 was kindly provided by Binne Zwanenburg (Department of Organic Chemistry, Radboud University, Nijmegen, The Netherlands). The strigolactone standards used in this study were kindly provided by Koichi Yoneyama (Weed Science Center, Utsunomiya University, Japan). PEO-IAA was kindly provided to us by Dr. Kenichiro Hayashi, Department of Biological Sciences, Tokyo Metropolitan University, Tokyo, Japan. We thank Dr. Hendrik Rietman for providing the plant material for the the ^{14}C-labeled IAA assays. We thank Prof. Salome Pratt for providing the *Solanum tuberosum* L. var. Andigena RNA of the developmental range, days 0 to 8. Part of this work was done in the framework of the EU-SOL project (PL 016214-2 EU-SOL) and co-financed by The Netherlands Organization for Scientific Research (NWO; VICI grant, 865.06.002 and Equipment grant, 834.08.001 to H.B.). H.B. was co-financed by the Centre for Bios stems Genomics (CBSG), which is part of The Netherlands Genomics Initiative/Netherlands Organization for Scientific Research. E.R. gratefully acknowledges financial support from the Bakalas Foundation, and the Stichting Veenhuizen-Tulp fonds.

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The PIN family of proteins in potato and their putative role in tuberisation.

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Submitted for publication in Plant Signalling & Behaviour

Abstract

The PIN family of transmembrane proteins mediates auxin efflux throughout the plant and during various aspect of plant development. In *Arabidopsis*, the PIN family is comprised of 8 members, divided into 'short' and 'long' PINs according to the length of the hydrophilic domain of the protein. Based on sequence homology using the recently published potato genome sequence (*Solanum tuberosum* group Phureja) we identified ten putative potato *StPIN* proteins. Mining the publicly available gene expression data, we constructed a catalogue of StPIN tissue specificity and analysed their expression level during the process of tuberization. A total of four *StPIN* genes exhibited increased expression four days after induction to tuberise which is prior to the first signs of stolon swelling. For two PIN genes, StPINV and *StPINVII*, promoter sequences were cloned and placed in front of a GUS reporter protein to study tissue specificity in more detail. prom*StPINV* driven GUS staining was detected in the flower stigma, in the flower style, below the ovary and petals, in the root tips, in the vascular tissue of the stolons and in the tuber parenchyma cells. *StPINVII* promoter driven GUS staining was detected in flower buds, in the vascular tissue of the swelling stolons and in the storage parenchyma of the growing tubers. Based on the obtained data in this study and extending on current knowledge of auxin transport we postulate a role for the *StPINs* in redistributing auxin to the proper sites in the swelling stolon during early events in tuber development.

Introduction

Auxin is involved in various developmental processes, such as flower development (Krizek, 2011), root development (Rahman et al., 2007) and embryo patterning (Mashiguchi et al., 2011). The presence of auxin in the plant cell and the differential effect of auxin on the cell in terms of cell division, growth and differentiation, is controlled by two determinants: the rate of metabolism and transport. The main sites of auxin biosynthesis are the shoot apical meristems along with cotyledons, expanding leaves and roots (Ljung et al., 2001). From the sites of biosynthesis, auxin is transported to other parts of the plant by diffusion or through active transport. The directional Polar Auxin Transport (PAT) system distributes auxin from the sites of biosynthesis to lower parts of the plant and is mediated by influx and efflux carriers. The influx of auxin in the plant cells is mediated by the influx carriers auxin resistant 1/like aux1 (AUX/LAX)(Marchant et al., 1999; Marchant et al., 2002), while efflux carriers have been identified as the PIN family of proteins (Gälweiler et al., 1998). The PIN family has been investigated extensively in Arabidopsis (reviewed in Krecek et al., 2009) rice (Yamamoto et al., 2007), and tomato (Pattison and Catalá, 2011). The asymmetric distribution of the influx and efflux proteins on the plasma membrane results in the directional movement of IAA from the upper to the lower parts of the plant. In *Arabidopsis*, the family is comprised of 8 members (AtPIN1-8), and is divided in to two groups. *AtPIN1* to *AtPIN4*, *AtPIN6*and *AtPIN7* represent the canonical (long) PINs. The long PIN proteins have a relatively long central hydrophilic loop and share high sequence similarity, especially in the hydrophobic domains of both N- and C- termini. *AtPIN5* and *AtPIN8* form the second group of the PIN family ('short' PINs). Short PINs have a virtually absent central hydrophilic loop (PIN structure and evolution reviewed in Krecek et al., 2009). The first PIN mutant, *AtPIN1*, exhibited a naked, pin-forming inflorescence with no or just a few defective flowers indicating the importance of proper auxin localization and the role of PINs in plant development (Gälweiler et al., 1998). *AtPIN2* and *AtPIN4* are involved in the gravitropic response and root development respectively (Muller et al., 1998; Friml et al., 2002), while *AtPIN3* has a role in gravitropic and phototropic responses as well as apical shoot formation (Okada et al., 1991). In contrast to the long PINs, which are located on the plasma membrane, the short *AtPIN5* is localized on the endoplasmic reticulum and participates in the compartmental localisation and homeostasis of auxin (Mravec et al., 2009). The intron/exon structure bears similarities within the PIN family members across plant species (Krecek et al., 2009). In *Arabidopsis* the typical example is comprised of six exons, and exceptions are found only in the short PINs or in PINs similar to *AtPIN2*

In the *Solanaceae* family, the PIN family of proteins has only been studied in tomato (Pattison and Catalá, 2011) where the role of the PIN proteins in auxin distribution during fruit development was evaluated. The SlPIN group is comprised of 10 members. Six of those are long PINs and SlPIN5, 6, 8 and 10 are short PINs. In potato, the *PIN* genes have not been functionally studied or systematically compared on a genome-wide scale. Two potato genes, highly homologues to Arabidopsis PIN proteins, were shown to be up-regulated in the stolon tips just prior to tuber swelling and down regulated soon after (Kloosterman et al., 2008). In addition, it was recently shown that auxin content increases locally in the stolon tip just prior to stolon swelling (Roumeliotis et al., 2012). These results indicate a prominent role for auxin and auxin transport in the regulation of potato tuberization although a coherent sequence of events still has to be established.

In this paper, we describe the identification of additional PIN family members using the recently published potato genome. Furthermore, we used available RNA-seq data to study variation of StPINs expression in a number of potato tissues (PGSC, 2011).We studied the expression of all the StPINs during the early stages of tuberisation using quantitative RT-PCR. In order to study the expression pattern further we cloned two *PIN* promoters and fused these promoters in front of a reporter GUS protein.

Materials and methods

Identification of *StPIN* gene sequences

The potato *PIN* sequences were retrieved by blasting all identified Arabidopsis *PIN* gene sequences against the potato genome sequence (Supplementary table 1). Identified Potato PIN sequences were aligned (Crystal W2 alignment, http://www.ebi.ac.uk/Tools/msa/clustalw2/) with the *Arabidopsis thaliana* PIN genes and were screened for the presence of the conserved PIN hydrophobic/hydrophilic structure. Prediction of the location of the transmembrane domains within the protein sequence was performed with TMHMM program (v2) (http://www.cbs.dtu.dk/services/TMHMM/. The predicted intron-exon structure of the StPINs genes data was retrieved from the potato genomics browser (http://solanaceae.plantbiology.msu.edu) The StPIN genes were named according to their chromosomal location to avoid erroneous associations linked to the *Arabidopsis* numbering. Where two PIN genes are located on the same chromosome these are identified with *a* and *b*.

Cloning and analysis of *STPINV* and *StPINVII* promoter sequences

The promoter regions of *STPINV* (2741bp) and *StPINVII* (2983 bp) were cloned from *S. tuberosum group andigena* using Gateway technology (Invitrogen Europe BV, Blijswijk, NL). All primers used are provided in Supplementary Table 3. Vector pKGFS7 (Karimi et al., 2002), harbouring the GUS reporter protein was used for transformation and promoter expression studies. Transgenic plants harbouring the prom*StPINVII*::GUS and prom*StPINV*::GUS construct were obtained by Agrobacterium mediated transformation (*AGL0*) of *S. tuberosum group andigena in vitro* plantlets as described previously (Visser et al., 1989). The GUS staining assays was done as described previously (Stomp, 1992). The incubation of the tissues in GUS substrate X-Gluc was performed overnight at 37ºC. The tissues were washed once with 70% ethanol prior to imaging.

Expression analysis of *StPIN* genes

Solanum tuberosum group andigena plants were grown in the greenhouse and transferred to short day conditions when the plants were at the 6th fully expanded leave stage. Stolon tips were harvested under long days conditions (day 0; LD 16h light) after which plants were transferred to short days conditions (SD 8h light), and harvested at day 2, 4, 6 and 8 after the switch to SD. Total RNA was extracted using the QiagenRNaesy Plant mini kit (QIAGEN Benelux B.V.Venlo, NL) and DNAseI treated (Invitrogen). 1µg of RNA was used for cDNA synthesis and the final product was diluted 20 times in a total volume of 400 µl (BioradiScriptcDNA synthesis kit, Bio-Rad Laboratories B.V., Veenendaal, NL). qRT-PCR was performed using the BioradiQ™ SYBR® Green Supermix on a Bio-Rad cycler. The reactions were performed in triplicate in a final volume of 10 µl, containing 5 µl of SYBR® Green Supermix, 100 nM of each primer, PCR-grade water and 2 µl of cDNA sample. Reactions were incubated at 95°C for 5 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. *eIF3e* was used as a reference gene (all qRT-PCR primer sequences are provided in Supplementary Table 2). RNA-seq data of various tissues of the RH genotype was retrieved from the potato genomic browser and includes flower, leaves, shoot apex, stem stolon, young tuber and root tissue. For each tissue, RNA-seq reads are mapped against the predicted gene structures indicating relative expression levels (nr of fragments per kb per million reads or FPKM).

Results and discussion

Identification of the *PIN* gene family members in potato

Based on sequence similarity with Arabidopsis PIN proteins and the presence of trans membrane domains, a total of ten potato PIN genes could be identified using the potato reference genome (Material and Methods). As in Arabidopsis, the potato PINs are divided in two groups based on the length of the protein, the long PINs and the short PINs. The group of

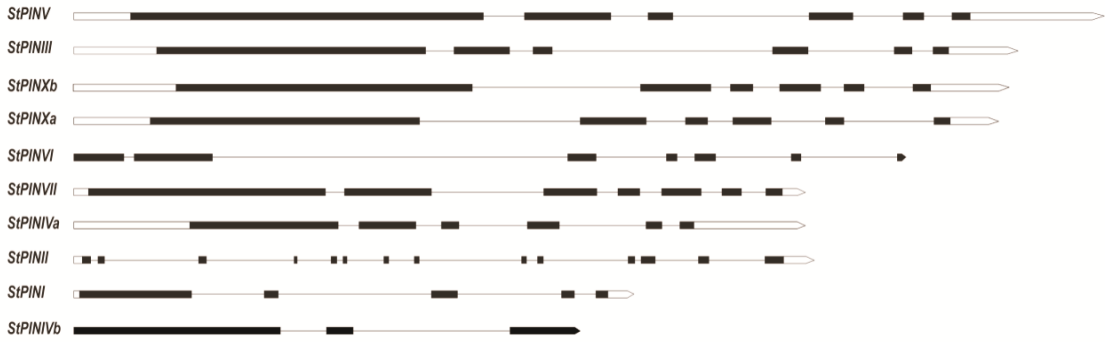

Figure 1.The predicted gene structure for the StPIN family of genes (BGI gene model). The direction of transcription is from left to right. Black boxes represent the exons, white boxes represent the 5´ and 3´ UTR and the lines represent the introns.

the short StPINs is comprised of three members (StPINIVb (321aa), StPINI (355aa) and StPINII (358aa)), same number of members as the Arabidopsis group and one less than the tomato group (Pattison and Catalá, 2011). The remaining 7 StPINs that form the group of long PIN's have a size range of 412 to 631 amino acids. The difference in length between the proteins is mainly due to the difference in length of the hydrophilic region located between the transmembrane domains present at both ends of the protein. The N-terminus and C-terminus regions that contain the transmembrane domains of the proteins are highly conserved in the Arabidopsis and Potato PIN proteins (Supplementary Figure 1). For 8 out of the 10 PIN genes, the protein prediction model predicts 5 to 7 exons. Exceptions are *StPINII* with 14 exons and *StPINVb* with 3 exons, two of the three short StPINs (Figure 1). In the N-terminus of the proteins, all StPINs have 4 to 5 trans membrane domains followed by the hydrophilic loop that varies in length. The C-terminus end has 3 to 4 trans membrane domains, with the exception of *StPINII* that has two trans membrane domains (Figure 3).

The phylogenetic analysis reveals the evolutionary relationship between the AtPIN and StPIN predicted PIN proteins (Figure 2). In three cases, an *AtPIN* gene was found to group with a single *StPIN* gene (*AtPIN2* with *StPINVII, AtPIN6* with *StPINVI* and AtPIN8 with *StPINII*). In contrast, *AtPIN1* clusters together with potato *StPINIII, StPINXa*, StPINIIIXb, while *AtPIN3, 4, 7* form a group with *StPINIVa* and StPIN*V*. The short *AtPIN5* is located in a branch with *StPINI* and *Vb*. These results can indicate the evolutionary paths that resulted in the *PIN* family in *Arabidopsis* and in potato. It seems likely that *AtPIN1* share a common ancestor with *StPINIII*, *StPINXa* and *StPINXb*. The fact that *StPINXa* and *Xb* are very similar in sequence level and are located on the same chromosome indicates a recent duplication event. In addition, the fact that *AtPIN3, 7* and *4* are located on the same branch indicates a common ancestor with *StPINV* and *StPINIVa* that are also located on the one branch. Clustering of proteins based on sequence similarity between *AtPIN* and *StPIN* genes could indicate similar functional roles or sub-functionalization in species dependent developmental processes.

Tissue specificity expression of potato *PIN* family members

Together with the potato genome sequence, additional data including RNAseq generated expression data became available, targeting a number of different potato tissues or developmental stages (material and methods). Based on the RH RNAseq tissue libraries, we built a heatmap that shows sites of *StPINs* expression throughout the plant (Figure 4a). Only *StPINV* and *StPINIII* are expressed in all tissues, with *StPINV* expressed at a high level. All remaining potato PINs seem to have some degree of tissue specificity. *StPINVII* is expressed predominantly in the stolon tissue, and *StPINIVa* is present at a low level in all tissues with the exception of roots. *StPINI*, which is highly similar in protein sequence to *AtPIN5*, is predominantly expressed in the roots.

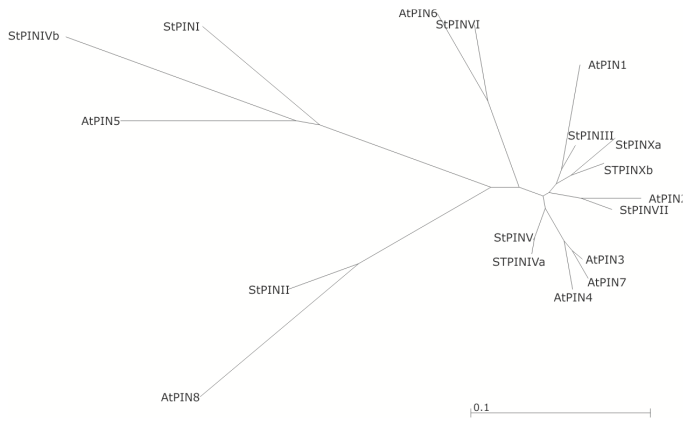

Figure 2. Phylogenetic tree of the PIN proteins of Arabidopsis thaliana and Solanum tuberosum.

In each tissue, a different set of *StPINs* appears to be expressed (figure 4a). In the roots, StPINI, Xb, V and III gene expression was detected,

while in the stolon *StPINIVa* and *StPINV* are predominantly expressed with *StPINIII*, *Xb* and *VII* present at lower levels. In the stolon 5 out of 10 StPINs were detected and with *StPINIII* and *StPINV* exhibiting the highest expression levels. In flowers, expression of 6 StPINs was observed but only *StPINV* and *StPINIVa* show relative strong expression. In the shoot apex, stem and roots 7 StPINs exhibit expression (*StPINI, III, VI, V, IVa, Xa* and *Xb).* In the apex, *StPINV* is expressed at the highest level. The fact that several *StPIN* genes can be expressed in each tissue indicates there is a level of redundancy between the *PIN* genes. Redundancy between the *PIN* genes is a mechanism that can ensure that the flux of auxin can remain undisrupted. The polar auxin transport mediates translocation of auxin from the sites of biosynthesis to the lower parts of the plant and the roots. Little knowledge exists on the fate of auxin after it reaches the root tips and enters the outer epidermis cells. StPINI is predominantly expressed in the root which is interesting as the Arabidopsis ortholog (*AtPIN5*) has been shown to participate in the subcellular localization and homeostasis of auxin (Mravec et al., 2009). Based on sequence similarity between *AtPIN5* and *StPINI* it is likely that *StPINI* is involved in auxin homeostasis in potato. Still, the role for *StPINI* in auxin homeostasis and subcellular localization in the roots needs to be confirmed.

Stolon is an underground etiolated stem that grows diageotropically. The Arabidopsis PIN2 is known to have a role in the gravitropic response in roots (Luschnig et al., 1998; Muller et al., 1998) by directing auxin flow to one side of the root and thus mediating differential growth across both sides of the root. In a similar manner, light effects distribution of AtPIN3 protein that regulates auxin distribution and differential growth in response to light (Okada et al., 1991). As a result, the comparison between the StPINs expressed in stem and stolon becomes very interesting as stolons are generally deprived of light. StPINVII seems to be highly homolog to AtPIN2 (72% similarity at the amino acid level). In potato PINVII is expressed in the stolon but not in high levels the stem, providing a possible explanation for the different gravitropic response of the stolon in comparison to the aerial shoot. These results provide useful leads to start understanding the diageotropic growth response of the stolon in comparison to the aerial shoot growth and the PINS potentially involved.

Figure 3. Hydropathy test for the 10 StPINs found in the potato genome. Red peaks and blocks represent the transmembrane domains, pink lines the areas of the proteins predicted to be outside the cell, and the blue lines represent the areas of the proteins predicted to be inside the cell.

In potato, previous research on tuber development revealed two *StPINs* (later named *StPINV* and *StPINVII*) that have a peak in expression 4 days after induction to tuberise, indicating a role for auxin in the development of the potato tuber (Kloosterman et al., 2008). In order to investigate the expression profile of the PIN family of genes in the early stages of tuber induction, we performed qRT-PCR on stolon tip samples collected 0, 2, 4, 6 and 8 days (stages T0 to 8 respectively) after plants were transferred to short days to induce tuberisation (Figure 4b). The results revealed that several *StPINs* are highly expressed in the swelling stolon (stage T8). In addition, a peak in expression levels is noticed for six *PIN* genes at stage T4 (*StPINIII*, *IVa*, *Xb*, *VI*, *VII* and *II*) 4 days after induction to tuberise, ranging from 2 to 10-fold increase in comparison to T0. By stage T8 (day 8), the expression levels of *StPINIII StPINVI* and *StPINII* are the same as at stage T0, but a 2 to 5-fold decrease was observed for *StPINVI*, *StPINIVa* and *Xb*. The expression levels of two more *StPINs* (*StPINV*, *Vb*) remain relatively stable in all stages while *StPINXa and StPINI* show a gradual increase of expression. Auxin has been shown to be a positive regulator of PIN gene expression (Vieten et al., 2005). The up regulated profile of six *StPIN* genes at stage T4 is in accordance with our previous findings where auxin was shown to accumulate in the potato stolon tip after induction to tuberise and prior to first visible swelling (day 8) (Roumeliotis et al., 2012). Auxin is known to participate in many developmental events, such as embryogenesis (Luo et al., 2011), flower development (Krizek, 2011), lateral root formation(Marchant et al., 2002) and tuber initiation (Roumeliotis et al. 2012) where auxin has been associated with the establishment of meristem identity. This peak in expression is probably important to distribute auxin to the correct sites

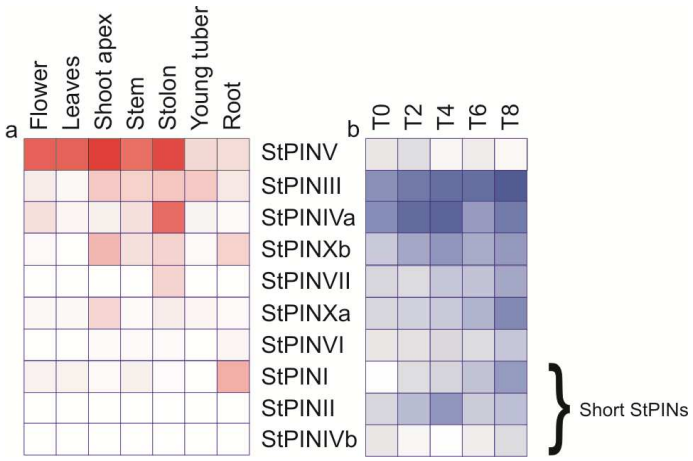

Figure 4. Heat map of expression of the StPINs in the corresponding tissues according to the RNA-seq data of the RH and in the tuber developmental series (time points T0 to T8). In Figure 4a, expression levels in the various tissues are indicated by shades of red, where white indicates no expression. In Figure 4B, for stages T0 to T8 of the developmental series, 0 to 8 days after induction to tuberise, shades of blue represent fold increase in the expression of the corresponding gene and white indicates the lowest expression detected. Lowest expression is detected for StPINVb at stage T4 (C(t)=36.29), and highest expression is detected for StPINIII at stage T8 (C(t)=25.14).

where auxin may be required in the formation of a new organ, the tuber.

The RNA-seq data obtained from tubers and the qRT-PCR expression of PIN genes during tuber development are not directly comparable, as sampling was done on different genotypes, time points and tissue types. Nevertheless, *StPIN* genes that are predominantly expressed in the stolon tissue from the tissue panel were also t found to be expressed in the stolon developmental stages, with the exception of *StPINV*. In the tissue panel, *StPINV* seems to be the predominant *PIN* gene expressed in the stolon as well as in almost all other tissues. Surprisingly, in the stolon developmental series the *StPINV* expression is lower compared to the other *StPIN* genes. It is possible that *StPINV* is down regulated once the potato plant is induced to tuberise, as shown by the lower *StPINV* expression in the tuber. All other PINs found to be expressed in the stolon tissue in the RNA-seq data, such as *StPINIII*, *IVa*, *VII*, *Xb* are detected in the RT-PCR data. The compartmental distribution of auxin close to the vascular system in the stolon and swelling tuber (Roumeliotis et al., 2012) may be a result of the combined expression of all the PIN genes during early stages of tuberisation.

StPINV/VII promoter GUS staining

In order to identify the regions of expression of the St*PIN* genes in stolons and in young tubers in more detail, the promoters of two *StPIN* genes were cloned in front of a GUS reporter gene (Material and Methods). In transgenic plants harbouring the *StPINV* promoter construct, GUS staining was detected in flowers, stolon tips, root tips and swelling tubers (Figure 5a-d). More specifically, GUS staining was visible in the stigma and the style of flowers and in the stem just below the ovary and petals (Figure 5a). In stolon tissue, GUS staining was detected in the vascular tissue (Figure 5b) in the sub apical region where the first visible swelling if the stolon takes place (Xu et al., 1998), while in roots GUS staining was restricted to the root tip (Figure 5c). In tubers, GUS staining was visible in the vascular system, and in the perimedullary region (Figure 5d). GUS staining was also detected in the basal part of the pith close to the heel of the tuber where it attaches the stolon. *StPINVII* promoter driven GUS staining was also detected in flower buds, roots, swelling stolons and young tubers (Figures 5e-h). In mature flowers, no GUS staining was detected. In addition, stolon tips did not have any GUS staining, until the stolon tip started to swell (first visible sign of tuberisation), with the staining to be restricted to the vascular tissues (Figure 5f).

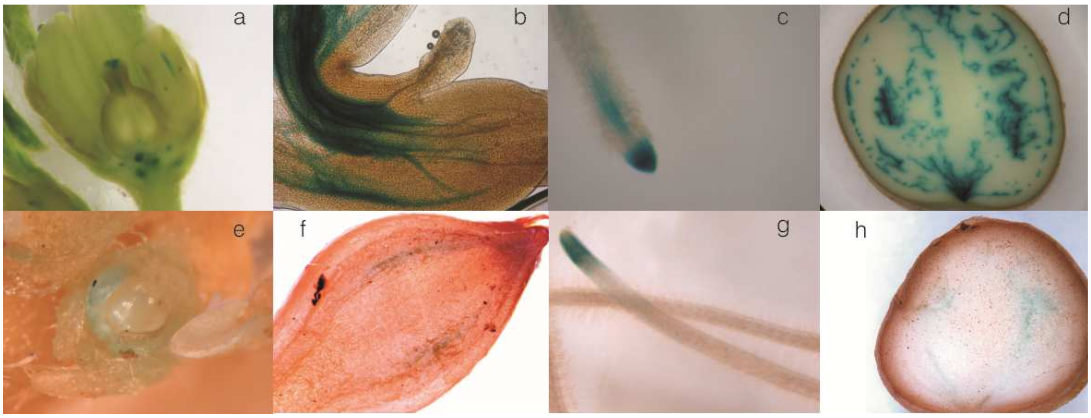

Figure 5. PIN promoter expression study. Gus expression driven by the StPINV promoter is detected in flowers (a), stolon tip (b), root tip (c) swelling tuber (d) and StPINVII expression was found the in flower bud (e), swelling stolon (f), root tip (g) and in swelling tuber (h).

In the mature tuber (Figure 5h), GUS staining was observed in the pith and in the perimedullary region, partially overlapping with the *StPINV* promoter driven GUS staining. In some tissues such as the young tuber, *StPINV* promoter and *StPINVII* promoter driven GUS staining is overlapping in the same regions in the vascular tissue, in the pith and in the perimedullary region. A large portion of the tuber growth is attributed to the thickening of the perimedullary region (Xu et al., 1998) therefore distribution of auxin in this tissue by the PIN proteins can be of importance. In contrast, in root tips and in flowers, GUS staining driven by the *StPINV or StPINVII* promoter seem to have a different spatial expression pattern. In roots, *StPINV* promoter driven GUS staining is observed primarily in the root cap and in the stele, while *StPINVII* promoter GUS staining is observed in the apical meristem adjacent to the root cap, close to the elongation zone. It seems that the two *PIN* genes might have partially overlapping roles in distributing auxin in some tissues, and unique functionality in others. Redundancy between different members of the PIN family has been reported before in tomato (Pattison and Catalá, 2011).The overlap of expression of the *StPINs*, as suggested also by the expression heatmap (Figure 4a) in the various tissues implies that *StPINs* in potato might act synergistically to direct plant growth and development.

Conclusion

In this research we present the PIN family of proteins in potato and discuss possible roles based on the homology to the Arabidopsis *PINs*. Based on the potato genome sequence, we identified ten potato PIN family members and studied their relative expression levels in various tissues and during early potato tuberization. Promoter analysis of two of two potato

PIN homologues revealed the sites of location in aerial parts of the plant, as well as in the stolons during the first days after tuber induction. Based on these results and what is known about the changes in auxin content during early stages of tuber development, we discuss a possible role for *StPINs* in redistributing auxin in the swelling stolon and developing tuber.

Acknowledgements

We thank Prof. Salome Pratt and José A Abelenda for providing the S. tuberosum group andigena RNA of the developmental range, days 0 to 8. Part of this work was done in the framework of the EU-SOL project (PL 016214-2 EU-SOL) and E.R. gratefully acknowledges financial support from the Bakalas Foundation, and the StichtingVeenhuizen-Tulpfonds.

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Down regulation of *StGA3ox2* gene in potato results in altered GA metabolism and effect plant and tuber growth characteristics

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Submittion in preperation

Abstract

GA biosynthesis and catabolism has been shown to play an important role in regulating tuberisation in potato. Degradation of active GAs is occuring in the stolon tips short after induction to tuberisation. Overexpression of a GA degradation gene, results in earlier tuberisation phenotype, while reducing expression of the same gene results in delayed tuberisation. In addition, overexpression of genes involved in GA biosynthesis results in delayed tuberisation, while decreased expression of those genes results in earlied tuberisation. The final step in GA biosynthesis is catalysed by *StGA3ox1* and *StGA3ox2* activity, that convert incative forms of GA into active GA_1 and GA_4. In this study we cloned *StGA3ox2* gene in an RNAi construct and used this construct to transform potato plants. The *StGA3ox2* silenced plants were smaller and had shorter internodes. In addition, we assayed the concentrations of various GAs in the transgenic plants and showed an altered GA content. No difference was noticed on the time point of tuber initiation. However, the transgenic clones had increased number of tubers with the same yield, resulting in smaller average tuber weight. In addition, we cloned the promoter of *StGA3ox2* to direct expression of the GUS reporter gene to visualize the sites of GA biosynthesis in the potato plant. Finally, we discuss how changes of several GA levels can have an impact on shoot, stolon and tuber development, as well as the possible mechanisms that mediate feed-forward and feed-backward regulation loops in the GA biosynthetic pathway in potato.

Introduction

The genes that participate in GA biosynthesis and their effect on plant development have been extensively studied in *Arabidopsis* (Huang et al., 1998; Fleet et al., 2003; Schomburg et al., 2003). The biosynthesis of GAs is a complex network of interacting pathways comprising more than 120 precursors and bioactive GAs that have been identified *in planta* so far (revewed in Hedden and Phillips, 2000). GA 20-oxidase and GA 3-oxidase, catalyse the last two steps of active GA biosynthesis, converting inactive forms of GA such as GA_{20} into active forms such as GA_1, while GA2ox catalyzes the breakdown of bioactive GA's. Overproduction of active GAs results in a series of phenotypes, such as longer hypocotyls, increased stem elongation, earlier flowering and decreased seed dormancy (Huang et al., 1998), while plants over-expressing of *GA2ox* genes show a GA dose dependent stem length (Schomburg et al., 2003).

Potato tubers are formed at the etiolated tips of diageotropically growing shoots, called stolons. The formation of tubers is a result of a signaling cascade involving environmental inputs, hormonal signaling and transcript regulation. GA has been associated with a role in potato tuber formation, in which high concentrations inhibit or delay tuberisation while, low levels promote tuber formation (Kumar and Wareing, 1974; Ewing, 1987; Xu et al., 1998). In an *in vitro* tuberisation experiment, application of GA results in delayed, reduced and less synchronous tuberisation. An estimation of the endogenous GA levels revealed that GA content is lower under tuber inductive conditions (Xu et al., 1998). Moreover, degradation of the active GA content in the potato stolon tip by induction of the *StGA2ox1* gene occurs soon after induction to tuberisation and prior to tuber swelling both *in vitro* and *in vivo* (Kloosterman et al., 2007). Cloning and over-expressing the *StGA2ox1* gene in potato, resulted in a dwarf phenotype reduced stolon growth and earlier *in vitro* tuberisation phenotype, while plants with reduced expression of *StGA2ox1* showed delayed *in vitro* tuberisation and altered tuber morphology, concomitant with altered GA content during the process of tuberization (Kloosterman et al., 2007). Over expression of the *StGA20ox1* gene that is involved in GA biosynthesis, resulted in taller plants with elongated internodes that had reduced dormancy period while antisense plants were shorter with shorter internodes and an earlier tuberization phenotype (Carrera et al., 2000). In contrast, expression of *StGA3ox2* under a constitutive or leaf specific promoter results in taller plants that tuberise earlier under short day conditions. Similarly, *phyB* RNAi knock-down transgenic plants are able to produce tubers even under unfavorable long-day conditions and exhibit increased GA content in the stem (Jackson and Prat, 1996), again in contrast with the common notion that higher levels of

GAs delay tuberisation. However, the site of accumulation of GA is crucial in this respect and a higher level of GA in the stem is often associated with a lower level of bioactive GA's in the stolon most likely due to differential mobility of the various GA's and feedback regulation (Bou-Torrent et al., 2011). Over-expression of StGA3ox2 using a tuber specific promoter had a small effect on plant height and a slightly delayed tuberisation phenotype. New evidence on auxin content and auxin related genes during early stages of tuber development support a role for auxin and a crosstalk with GAs after tuber induction to regulate the plane of cell division in the stolons tips to seize longitudinal growth and initiate tuber growth (Roumeliotis et al., 2012).

In this study we cloned the *StGA3ox2* gene and transformed potato plants with a *StGA3ox2* RNAi construct, to modify GA metabolism and study the impact on potato tuber formation and plant growth. In addition, we cloned the promoter of *StGA3ox2* to direct expression of the GUS reporter gene to visualize the sites of GA biosynthesis in the potato plant. We discuss how changes of several GA levels can have an impact on shoot, stolon and tuber development, as well as the possible mechanisms that mediate feed-forward and feed-backward regulation loops in the GA biosynthetic pathway in potato.

Materials and methods

Construction of the *StGA3ox2* RNAi and *promStGA3ox2*::GUS plasmids

Gateway technology (Invitrogen Europe BV, Blijswijk, NL) was used for the construction of the *StGA3ox2* RNAi and the promStGA3ox2::GUS constructs. The *StGA3ox2* gene sequence was retrieved from the NCBI (http://www.ncbi.nlm.nih.gov/), (gene annotation number gb|FJ792643). For the construction of the StGA3ox2 RNAi silencing construct, a 623 bp long fragment was amplified from stolon cDNA library (S. tuberosum L. var. *Andigena)* using StGA3ox2 specific primers (5'- caccGTGACGTCATCGAAGAGTAC and 3'-TTAACCTACTTGGACGCCAC) and cloned into destination vector pK7GWIWG2 (Karimi et al., 2002). The promoter region of *StGA3ox2* (3528bp) was cloned from *S. tuberosum group andigena* using specific primers (5'-GGG-GCA-ACT-TTG-TAC-AAA-AAA-GTT-Gaccaaaaagggaaattctagag and 3'- GGG-GCA-ACT-TTG-TAC-AAC-AAA-GTT-Gtactcttcgatgacgtcactga). Vector pKGFS7 (Karimi et al., 2002), harboring the GUS reporter protein was used for transformation and promoter expression studies. Transgenic plants harboring the *StGA3ox2* RNAi construct and the prom*StGA3ox2*::GUS construct were

obtained by Agrobacterium mediated transformation (*AGL0*) of *S. tuberosum group andigena in vitro* plantlets as described previously (Visser et al., 1989). Several transgenic clones were obtained for the *StGA3ox2* RNAi, and the three best clones based on StGA3ox2 expression levels and phenotype were selected for further study. Similarly, several clones harboring the prom*StGA3ox2*::GUS construct were obtained and selection for the three best clones was based on the performance of the GUS assays. The GUS staining assays was performed as described previously (Stomp, 1992). The incubation of the tissues in GUS substrate X-Gluc was performed overnight at 37ºC. The tissues were washed once with 70% ethanol prior to imaging.

Plant material

Single-node cuttings from short-day-grown potato plants *(Solanum tuberosum* L. var *Andigena*) transformed with the *StGA3ox2* RNAi construct and the prom *StGA*3*ox2*::GUS as well as the untransformed control were propagated *in vitro*, on standard MS medium (Murashige and Skoog, 1962) 2% (w/v) sucrose. Potato plantlets were grown for 2 weeks before being transferred to 5L soil filled pots. Plant height and the number of branches for each plant were monitored weekly. After 10 weeks, plants were transferred to a climate chamber with 8 hours light 21^{o}C and 16 hours dark 18 ^{o}C in order to induce tuberization. 17 days after plants were placed in the climate chambers, the underground parts of the *StGA3ox2* RNAi transgenic clones plants were harvested, and tuber yield was estimated. For statistical analysis we used the SPSS Statistics v19 (© SPSS, Inc., 2001, Chicago, IL, www.spss.com) software package, (post hoc comparisons LSD, a=0.05)

Quantitative RT-PCR

Estimation of the level of expression of the *StGA2ox*, *StGA3ox2* and *StGA20ox* genes was performed with quantitative RT-PCR (qRT-PCR). Gene specific primers used were (primer sequences in Supplementary Table 1). RNA was extracted from the same samples GA content was estimated using the Qiagen RNaesy Plant mini kit (QIAGEN Benelux B.V. Venlo, NL). The Invitrogen DNAse I Amplification Grade was used to avoid DNA contamination, and the Bio-Rad iScript cDNA synthesis kit was used for cDNA synthesis (Bio-Rad Laboratories B.V., Veenendaal, NL). 1µg of RNA was used per cDNA synthesis reaction, and the product was dissolved 20 times. The Bio-Rad iQ™ SYBR® Green Supermix was used for the qRT-

PCR with the Bio-Rad cycler. The reactions were performed in triplicate in a final volume of 10 μl, containing 5 μl of SYBR® Green Supermix, 100 nM of each primer, PCR-grade water and 2 μl of cDNA sample. Reactions were incubated at 95°C for 5 min to activate the enzyme, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. eIF3e was used as a reference gene (primer sequences in Supplementary Table 1).

GA measurements

For GA measurements, plants transformed with the *StGA3ox2* RNAi construct and the untransformed control were propagated *in vitro* and placed in the greenhouse as described above. Samples from apex and middle stem were collected from three plants with two biological repeats for two independent clones and the untransformed control. Samples were harvested on two time points. The first harvest took place before the switch to inductive short day conditions. The second harvest was performed a week after the plants were placed in inductive short days. Material was immediately frozen in liquid nitrogen and ground into a fine powder. GA measurements were performed as described elsewhere (Lange et al., 2005). Standards were used for the following gibberellins: GA_1, GA_4, GA_8, GA_9, GA_{19}, GA_{20}, GA_{29}, GA_{34}, GA_{44}, GA_{51} and GA_{53}.

Results

With the purpose of examining the role of altered GA content in the stolon tips on tuber development, we cloned a fragment of *StGA3ox2* gene, which is involved in active GAs degradation, in an RNAi construct and we transformed the construct into potato plants.

Expression levels of the *StGA2ox2* gene in the RNAi clones.

In order to investigate the transcript levels of the *StGA3ox2* in the transgenic clones, RNA was extracted from the apices of independent clones and qRT-PCR was performed with specific primers (primer sequences in Supplementary Table 1). The transcript levels of *StGA3ox2* revealed that the transgenic clones exhibited 4 (clone 14) to more than 20-fold (clone 24) decrease, verifying that the RNAi construct conferred decreased expression for the

targeted gene (Figure 1).

Figure 1. Expression levels of the StGA3ox2 gene in the apex in four transgenic clones and the untransformed control grown in the greenhouse. The StGA3ox2 gene expression for clone 24 is set to one. Error bars represent standard error of the mean using three technical repeats.

StGA3ox2 RNAi transformed clones have shorter plants with smaller leaves and smaller average tuber weight.

Potato plants transformed with the *StGA3ox2* RNAi construct and their untransformed control were placed in the greenhouse and monitored weekly for their height to identify possible differences between the clones and the untransformed controls. After week four, all four transgenic clones exhibited shortened stem length (Figure 2A) and smaller leaf size (Figure 2B) compared to the control. At week nine, the plants were transferred to climate chambers and grown under short day conditions in order to induce tuberisation. The differences between the stem length of the transgenic plants and the untransformed control continued to increase, resulting in significantly shorter plants for all transformed clones (Figure 2C). 17 days after the plants were placed in the short day climate chambers to induce tuberisation, tuber fresh weight and number of tubers was scored. Overall the differences in tuber formation were not very large, especially regarding total tuber weight. The control plants had an average of 43 g per plant, while the transgenic clones had 44 g for clones 27 and 24 and 50 g for clone 14. Statistically only clone 14 showed a significant increase in total fresh weight. On average however, all transgenic plants produced either more tubers or exhibited reduced average tuber

weight (Figures 3A, 3B and 3 C).

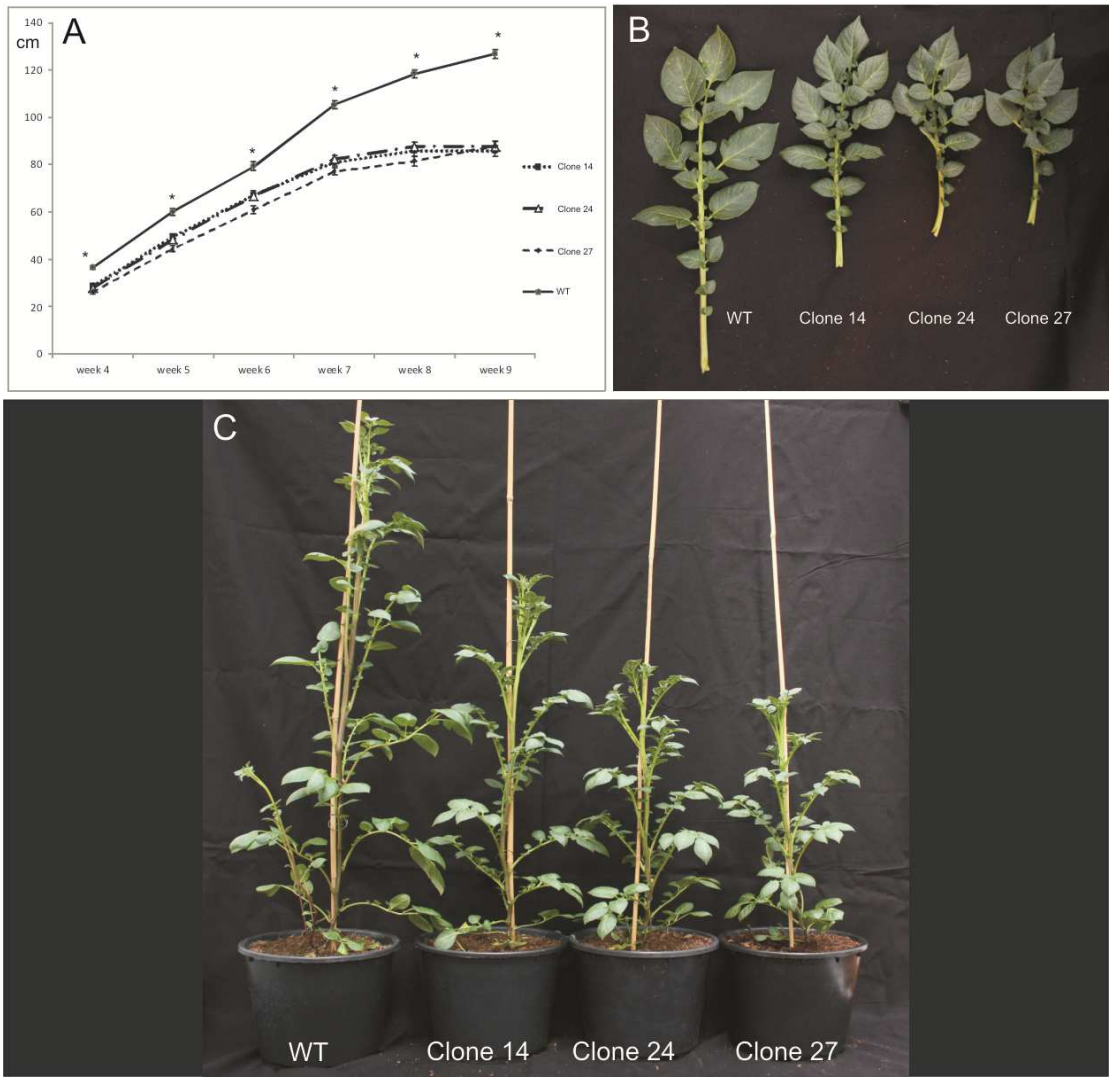

Figure 2. (A) Scoring of plant height (cm) of the Wild Type (WT) Solanum tuberosum L. var. Andigena and three transgenic clones 14, 24 and 27. Error bars represent standard error for nine repeats per clone and six repeats for the wild type control. Asteriks (*) represent statistical significant difference between control and transgenic clone (post-hoc comparisons LSD, a=0.05). (B) Comparison between leaves of the Wild Type Solanum tuberosum L. var. Andigena and transgenic clones 14, 24 and 27. (C) Comparison between control and transgenic clones 14, 24 and 27 .

Localisation of *StGA3ox2* expression.

In order to identify the regions of expression of the StGA3ox2 gene, a 3528 kb putative regulatory region of the StGA3ox2 gene was cloned and fused to the GUS reporter

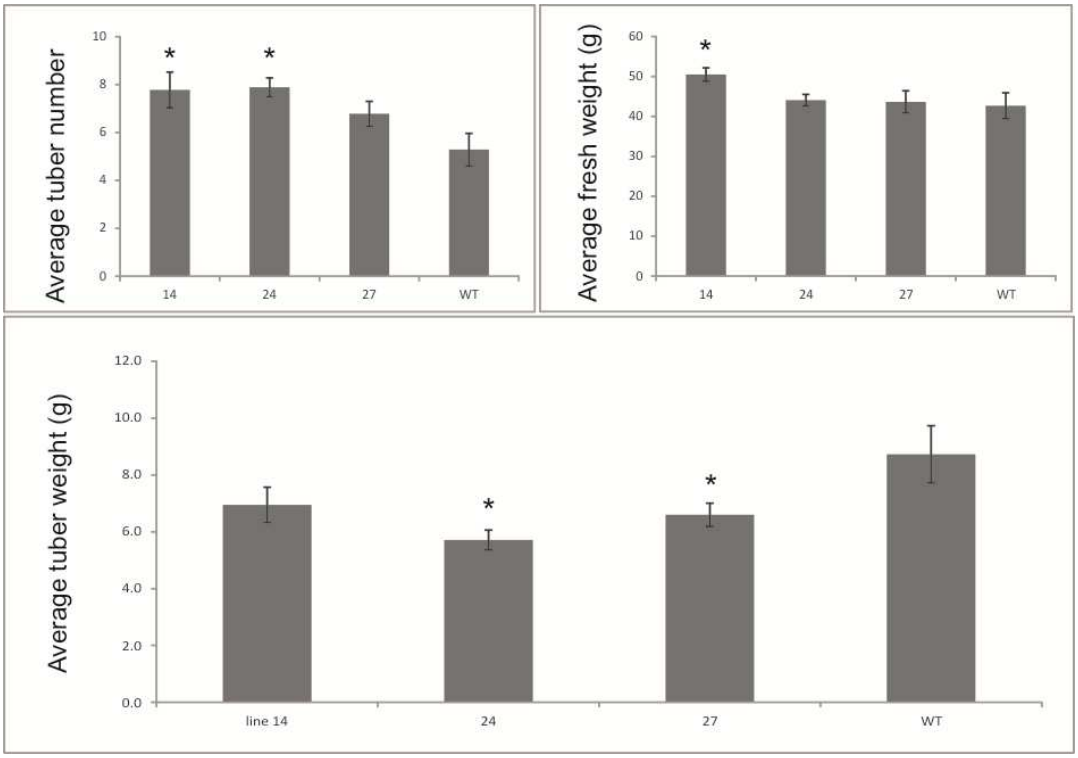

Figure 3. (A) Scoring of the number of tubers, (B) the fresh weight and (C) the average tuber weight of the Wild Type (WT) and the transgenic clones 14, 24 and 27. Error bars represent standard error for nine repeats per clone and six repeats for the wild type control. Stars represent statistical significant differences (post-hoc comparisons LSD, a=0.05).

gene. GUS activity was found in the tips of young leaves (Figure 4A), young flower buds (Figure 4B), around vascular tissues in a cross section of the stem taken from the region 1 centimetre directly below the shoot apex (Figure 4C) and in non-tuberising stolon tips (Figure 4D). In the stolon tip, GUS staining is highest in the stolon apex and small leaf scales, and appears to be surrounding the vascular bundles similar to what was found in the shoot stem. No GUS staining was found in the lower stem, roots or in tubers (data not shown). These results indicate that *StGA3ox2* expression appears to be restricted to actively growing tissues.

StGA3ox2 RNAi construct alters the levels of the various GAs in the apex

In order to investigate the effect of the *StGA3ox2* RNAi construct on the GA content in the potato plant, we measured the concentration of five major GA precursors, the two bioactive GA species as well as four degradation products. These GAs were measured in the apex, middle stem and tuber in two independent transgenic clones and an untransformed control under long day conditions to achieve a special and temporal overview or GA metabolism

(Figures 5 and Table 1).

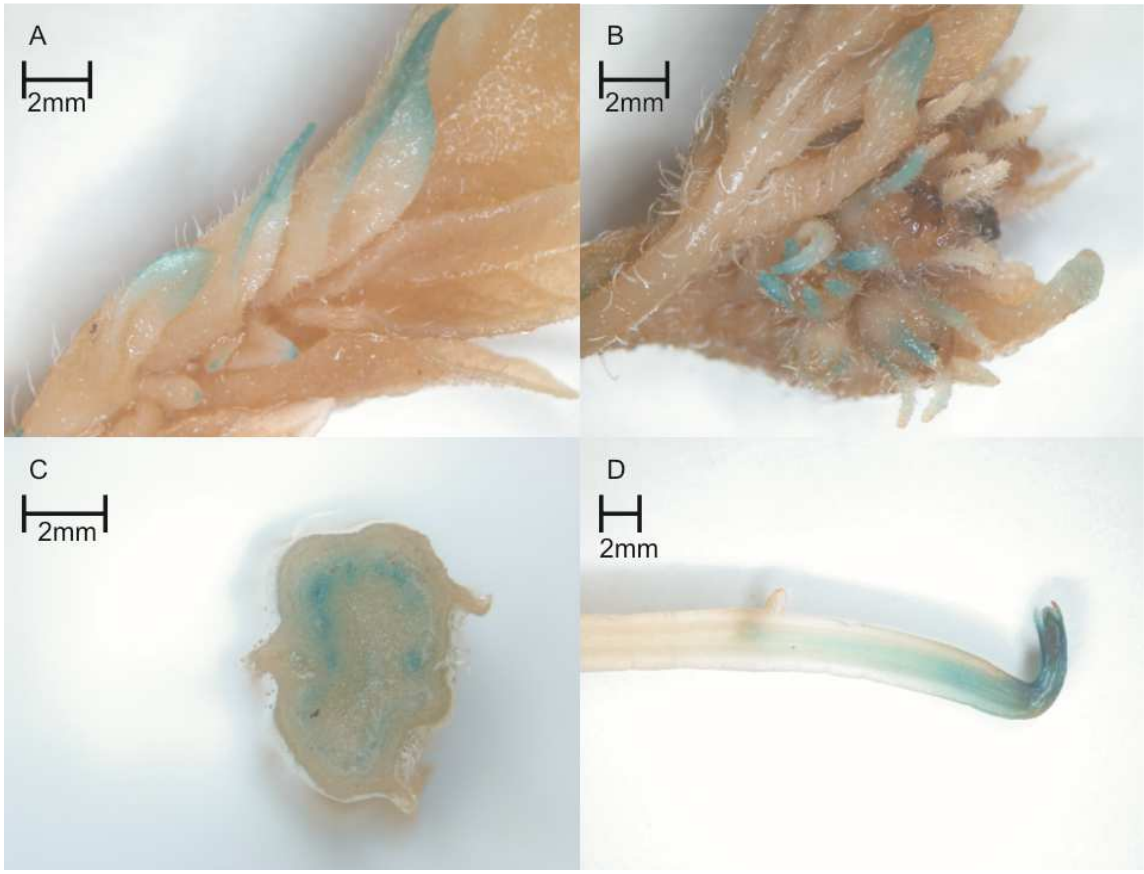

Figure 4. promStGA3ox2 directed GUS activity staining in the tips of leaf primordia in the apex (A), in the flower buds in the apex (B), in the vascular bundles in the shoot close to the apex (C) and in the stolon tips (D).

The transgenic clone 14 had higher levels of GA_9 and GA_{20}, in comparison to the control plants, both substrates for *StGA3ox2* activity (Figure 5). In addition, GA_{44}, a precursor of GA_{20} two steps up in the biosynthesis pathway, had higher concentration in the transgenic clones compared to the untransformed control. GA_9 and GA_{20} are also substrates for the StGA2ox enzyme that degrades these two GAs into GA_{51} and GA_{29}, respectively. In clone 14, both degradation products GA_{51} and GA_{29} levels are increased. Contrary to expectations, active GA_4 and GA_1, products of the *StGA3ox2* step, were not lower compared to the untransformed control. Instead, GA_4 levels were even higher in clone 14 compared to the control. GA_4 and GA_1 are template for *StGA2ox* genes to produce GA_{34} and GA_8. Only GA_{34} levels were lower in clone 14.

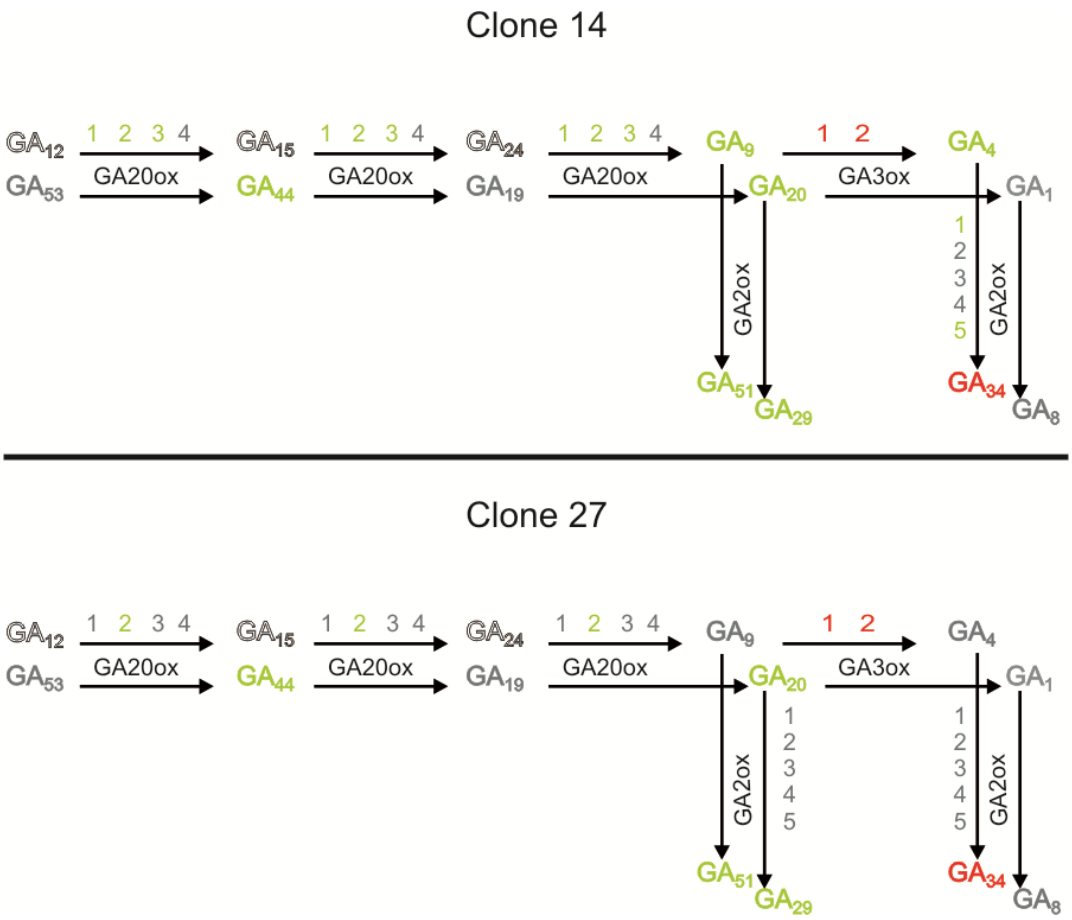

Figure 5. Changes in the gibberellins biosynthetic pathway for clone 14 (top) and clone 27 (bottom) in comparison to control plants under Long Day conditions. GA12, GA15 and GA24 were not scored (white letters with black outline). GAs indicated in green had higher concentration, GAs indicated with red had lower concentrations and GAs indicated with grey had no statistical changes compared to the untransformed control. The gene encoding the enzyme catalyzing each biosynthetic step is written next to corresponding step, and numbers represent the several members of the same gene family. The gene family members that were down-regulated are indicated with red colour, the family members that were up-regulated are indicated with green colour and the family members that did not show differences in expression are indicated with gray.

Gene expression analysis on the same samples as scored for GA content, revealed that in clone 14, three out of four identified *StGA20ox* genes had a two to three fold increase in expression levels compared to the untransformed control (Figure 5). In addition, out of five identified *StGA2ox* genes, only two had a 3-fold (*StGA2ox1*) or a mild 0.5 fold (*StGA2ox5*) increase (GA biosynthesis genes expression levels in Supplementary Figure 1).

In clone 27, down-regulation of StGA3ox2 was also observed. In addition, only GA20 but not GA9 content was higher. Moreover, as noticed for clone 14, concentrations of GA44, GA51 and GA29 were also higher. On the other hand, in contrast to clone 14, increase in GA9 and GA4 content was not observed. Furthermore, no increase in the expression levels of the StGA2ox genes was noticed, while only one out of four StGA20ox genes had higher expression levels compared to the untransformed control (Supplementary Figure 1). Our

	Apex - long days						Apex - short days					
	Clone 14		Clone 27		WT control		Clone 14		Clone 27		WT control	
ng/g FG	Average	SE	Average	SE	Average	SE	Average	SE	Average	SE	Average	SE
GA9	1.45	0.15	0.7	0.3	0.05	0.05	0.3	0.1	0.3	0	0.05	0.05
GA4	1.65	0.25	0.6	0.4	0.35	0.05	0	0	0.1	0.1	0.25	0.25
GA34	9.6	0.1	6.05	1.95	14.15	1.85	0.15	0.15	0.15	0.15	2.7	2.6
GA53	1.25	0.55	1.1	0.3	0.65	0.15	0.1	0	0	0	0	0
GA44	2.45	0.25	1.95	0.45	0.9	0.3	0.55	0.15	0.25	0.05	0.35	0.35
GA19	5.65	0.35	6.4	1.2	5.65	0.95	1.85	1.55	1.45	1.25	1.7	1.5
GA20	**>20**	**n/a**	**>20**	**n/a**	10.2	1.6	7.35	6.55	6.8	6.1	4.65	4.65
GA1	1.15	0.05	0.65	0.25	0.95	0.05	0.25	0.25	0.2	0.2	0.2	0.2
GA8	13.35	1.35	14.75	2.05	**>20**	**n/a**	2.45	2.25	2	1.9	8.7	8.4
GA51	4.15	0.45	3	0.8	0.6	0.2	1.5	1.3	1.45	1.25	0.55	0.55
GA29	**>20**	**n/a**	**>20**	**n/a**	9.05	0.65	15.35	14.85	14.55	14.25	9	8.9

Table 1. Concentrations of the GAs scored in the apices of two transgenic clones and the Wild Type untransformed control under long days and after one week in tuber inductive short day conditions and the Standard Error. The values are averages of two independent biological repeats.

measurements verified that in the apex during long days the GA_{20} content is higher in the partial silenced *StGA3ox2* transgenic clones, compared to the wild type control. In the middle stem the GA content was significantly lower for measured GAs but similar to the shoot apex. GA_{20} concentration tends to be higher in the transgenic clones. GA_8 and $GA_{34,}$ products of the GA_1 and GA_4 catabolism by StGA2ox, show no significant difference between the untransformed plants and the transgenic clones in the middle stem. In addition, the concentration of the active form GA_1 did not differ in the wild type and the transgenic plants (data not shown).

GA content was also measured after switch to tuber inductive short day conditions. The GA content in tuber inductive short days is much lower for all GAs measured in our research compared to the non-inductive long days. In the apex, the difference in the content of the precursor GA_{20} noticed in long days is decreased and the GA_1 content does not differ between the *StGA30x2* RNAi clones and the control (Supplementary Table 2). Significant differences are visible only in the content of GA_8 and GA_{34} that is lower in the knock-down clones compared to the untransformed control (Supplementary Table 2). GA levels in potato tuber samples were also measured under tuber inductive short day conditions in several tissues including the potato tubers, but virtually no GA was detected in any of the samples (data not

shown). Therefore, not concrete conclusions can be drawn from this set of results.

Discussion

Recently, the cloning and expression of a *StGA3ox2* under the control of 3 different promoters was described (Bou-Torrent et al., 2011). CaMV-35S regulated expression of this gene and the expression by the leaf specific promoter resulted in taller plants with altered GA content under tuber inductive short day conditions. A leaf specific expression resulted in shorter plants and the authors suggest that this was the result of PTGS co-suppression. In our study, the RNAi mediated reduction in expression of *StGA3ox2* resulted in shorter plants with shorter internodes, a similar phenotype to the *StGA20ox1* antisense clones (Carrera et al., 2000) and *StGA2ox1* overexpression clones (Kloosterman et al., 2007). The *StGA20ox1* and *StGA3ox2* RNAi clones seem to have reduced capacity to produce GA_1 and GA_4 while the *StGA2ox1* overexpression results in increased conversion rate of GA_1 and GA_4 to GA_8 and GA_{34}. These results imply that the shoot and internode length of the potato is strongly correlated with the ability of the potato plant to produce and catabolise the bioactive GA_1 and GA_4.

The concentration of several GAs was examined in two independent *StGA3ox2* RNAi clones. GA_{20}, the substrate of *StGA3ox2* was found to be higher in both clones and GA_9 also substrate for *StGA3ox2* was found to be higher in clone 14, likely due to the reduced *StGA3ox2* expression levels. GA_{20} and GA_9 are a substrate also for *StGA2ox* in a biosynthetic step that produces GA_{51} and GA_{29}. In both clones, GA_{51} and GA_{29} were found to have higher concentrations suggesting that this metabolic pathway is working in higher rates in the transgenic clones compared to the untransformed control.

Additional evidence that the metabolic flux of active GAs is altered is provided by levels of GA_8 and GA_{34} in short day conditions (Figure 6). Switch to tuber inductive short day conditions are known to induce *StGA2ox1* gene expression that regulates catabolism of the active GA_1 and GA_4 into GA_8 and GA_{34} (Kloosterman et al., 2007). Interestingly, levels of GA_1 were not altered in the transgenic clones however its breakdown product, GA_8, is reduced, indicating a reduction of GA_1 turnover as a result of lowered GA_1 biosynthesis through GA3ox2.

In Arabidopsis, the presence of a possible feed-forward regulation of GA2ox genes through GA_1 and GA_4 to stabilize GA concentrations has been described (Thomas et al., 1999). In clone 14, higher concentration of GA_4 is observed. Expression analysis of five *StGA2ox* genes

in clone 14 revealed that two *StGA2ox* genes had higher expression levels. In clone 27 no differences in GA_1 and GA_4 content was observed and *StGA2ox* gene expression was not altered. Thus, at least for clone 14 there seems to be a positive correlation between the GA_4 levels and the *StGA2ox* gene expression.

In pea, there is evidence of a negative feedback mechanism that regulates *StGA20ox* gene expression (Martin et al., 1996; Ait-Ali et al., 1999). Application of GA_1 negatively effects *StGA20ox* and *StGA3ox* gene expression. In transgenic clone 14, two out of three *StGA20ox* genes investigated are up-regulated and in transgenic clone 27, one *StGA20ox* gene is up-regulated. These findings could be a result of the reduced capacity of the transgenic clones to produce GA_1 and GA_4. Without high capacity to produce GA_1 and GA_4 the negative feedback regulation would promote *StGA20ox* gene expression, resulting in higher levels of GA_9 and GA_{20} due to the inability of GA3ox to catabolize these pre-cursors at a sufficient rate.

As shown in Figure 5, both *StGA3ox* genes exhibit a down-regulated profile in the transgenic clones. It is likely that the RNAi construct, despite being specifically designed to target *StGA3ox2*, also affects transcript levels of *StGA3ox1*, due to sequence similarity. However, the steady state levels of GA_1 are not significantly different. This could also be attributed to the higher levels of the substrates of *StGA3ox* genes, which may be able to sustain this metabolic step despite the lower *StGA3ox* gene expression levels.

Leaf specific expression of *StGA3ox2* resulted in earlier tuberisation, while a leaf specific co-suppression line for *StGA3ox2* gene resulted in delayed tuberisation under tuber inductive short day conditions (Bou-Torrent et al., 2011). These results can largely be explained by the different modes of transport of the various GAs. Different GAs have been found to be differentially transported in Pea (Proebsting et al., 1992). Leaf specific expression of a *StGA3ox* gene would result in increased GA_1 biosynthesis in the stem, using a large part of the available and mobile GA_{20} substrate pool, resulting in reduced availability of the GA_{20} in the stolon. A reduction of GA_{20} in the stolon can result in decreased capacity to produce the less mobile GA_1, promoting earlier tuberization (Bou-Torrent et al., 2011). Consistent with that, the sink effect of the above-ground parts is not occurring in the tuber specific promoter driven *StGA3ox* gene expression, which results in local increase of GA_1 in the stolon tips, delay tuberisation and alter the number of tubers produced by each plant.

In the shoot apex, bioactive GAs show little or no changes in observed concentrations. Based on the physiological similarity between the shoot apex and stolon apex, a similar steady-state level of bioactive GAs could be expected to occur in the stolon top. The fact that we could not observe changes in the time point of potato tuber initiation may be explained by this assumption.

Scoring the number of tubers and tuber yield produced by the transgenic clones revealed that two out of three of the *StGA3ox2* RNAi clones had smaller average tuber weight (Figure 3C), due to increased number of tubers (Figure 3A) rather than reduced yield (Figure 3B). Tuber specific promoter driven *StGA3ox2* expression also resulted in increased number of tubers but not altered yield (Bou-Torrent et al., 2011). In our transgenic clones, *StGA3ox2* gene expression is down regulated, while two *StGA2ox* genes are up regulated (Figure 5). In Pea, decreased auxin content through shoot decapitation results in increased *PsGA2ox1* and decrease *PsGA3ox1* gene expression resulting in reduced GA_1 content (Ross et al., 2000). Auxin is known to be one of the major factors controlling branching (Ongaro and Leyser, 2008; Hayward et al., 2009) and the mechanisms that control branching in stolons are similar to the mechanisms that control branching in shoots (Roumeliotis et al., 2012). Altered GA:Auxin ratio within the stolon due to reduction of GA3ox2 expression may therefore result in increased stolon branching and tuber formation. This might provide a possible explanation for the increased tuber numbers observed on the stolons for some of the transgenic clones (Figure 3A).

In this study we investigated the effects of the *StGA3ox2* RNAi transformation on the plant development, tuber formation and GA content in potato plants. In agreement with previous studies, potato clones transformed with the *StGA3ox2* RNAi construct were shorter with reduced internode length, and had altered content for various GAs, but did not alter the total yield produced or the time point of tuber initiation.

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Over-expression of a YUCCA-like gene results in altered shoot and stolon branching and reduced potato tuber size

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Submittion in preperation

Abstract

Auxin is known to be involved in various developmental events, including meristem identity, shoot branching and initiation of potato tubers. The previously identified *StYUC-like1* gene in potato, that exhibits a peak in expression after tuber induction and prior to tuber swelling was cloned and over-expressed in order to study the effects of altered auxin content on shoot and stolon architecture and tuber development. The potato plants transformed with the 35S::*StYUC-like1* construct exhibited increased shoot and stolon branching, reduced leaf size, lower average tuber weight and enhanced adventitious and lateral root formation. In addition, one transgenic clone, displayed a cracked tuber phenotype. Investigation of the IAA content on three independent transgenic clones revealed that the concentration of auxin was not altered in the shoot apex, but was significantly lower in the basal part of the stem. Our research helps to elucidate the importance of small changes of auxin content on several developmental events of the potato plant, such as shoot, stolon and root architecture.

Introduction

Auxin is the most extensively studied plant hormone, with effects on various aspects of plant development, including shoot branching, flowering, lateral root initiation, root growth and embryo patterning. Auxin biosynthesis and auxin transport are the two main determinants that control auxin content in the plant cell. Auxin distribution occurs by diffusion and/or by active transport involving influx and efflux carriers. The transport of auxin into plant cells is mediated by the influx carriers such as the AUX1-LAX gene family (AUXIN RESISTANT1, LIKE AUXIN RESISTANT; (Marchant et al., 1999; Marchant et al., 2002), while polarity determining efflux carriers have been identified as the PIN family of proteins (Chen et al., 1998; Gälweiler et al., 1998; Luschnig et al., 1998; Muller et al., 1998). Influx and efflux carriers sustain the Polar Auxin Transport (PAT) pathway that distributes auxin from the sites of biosynthesis to other tissues of the plant where auxin participates in developmental processes, such as lateral root formation (Marchant et al., 2002) or gravitropic response (Muller et al., 1998). In Arabidopsis it has been demonstrated that several tissues such as young leaves, cotyledons and roots have the capacity to produce auxin (Ljung et al., 2001). Two major auxin biosynthesis pathways have been proposed, a tryptophan (Trp)-dependent (Zhao et al., 2001) and a Trp-independent pathway (Seo et al., 1998). Indole-3-glycerol phosphate or indole is the likely precursor of IAA in the Trp-independent pathway but little is known about this pathway (Ouyang et al., 2000; Zhang et al., 2008). Several pathways have been proposed for Trp-dependent biosynthesis: the indole-3-acetamine (IAM) pathway, the indole-3-pyruvic acid (IPA) pathway, the tryptamine (TAM) pathway and the indole-3-acetaldoxime (IAOx) pathway (reviewed in Lehmann et al., 2010). These pathways have not yet been fully elucidated but several genes have been identified. For example, the members of the *YUCCA*-gene family (flavin mono-oxygenase) were shown to catalyse the last step of the IPA pathway, converting IPA into IAA (Mashiguchi et al., 2011) as well as catalyzing the hydroxylation of the amino group of tryptamine in the TAM pathway, a rate limiting step in auxin biosynthesis (Zhao et al., 2001) (auxin biosynthesis pathways and genes involved in these pathways reviewed in Mano and Nemoto, 2012).

The main sites of auxin biosynthesis are mainly apical meristems and the young leaves (Ljung et al., 2001). Auxin transport from the sites of biosynthesis to the lower parts of the plant mediates shoot branching. There are two main hypothesises on how auxin transport regulates shoot branching. In the canalization-based model, auxin is transported basipetaly from the site of biosynthesis to the lower parts of the plant in part mediated by PIN proteins (Vieten et al.,

2005). In this model, release of axillary bud dormancy requires transport of locally produced auxin into the main shoot in which strigolactones (SLs) play an inhibitory role (Prusinkiewicz et al., 2009). According to the second messenger model, auxin in the shoot mediates the production of a second messenger that has a direct effect on bud outgrowth (Snow, 1937; Sachs and Thimann, 1967). Cytokinins and strigolactones have been shown to have an effect on bud outgrowth when applied directly on the axillary buds (Sachs and Thimann, 1967; Gomez-Roldan et al., 2008). The biosynthesis of these hormones have both been found to be regulated by auxin (Nordström et al., 2004; Tanaka et al., 2006; Brewer et al., 2009; Hayward et al., 2009) and are good candidates for the role of secondary messengers.

In potato, stolons are underground diageotropically growing shoots that, under environmentally favourable conditions, develop tubers at their apical meristems (Ewing and Struik, 1992). Even though the relationship between stolon branching and the number of tubers produced is not extensively studied, it has been shown that the degree of stolon branching is one of the factors effecting number and size of the tuber (Celis-Gamboa et al., 2003). Recently, we showed auxin biosynthesis is involved in stolon branching and tuber development and that the branching mechanism in the stolon seems to be similar to the auxin mediated mechanism that mediates branching in shoots (Roumeliotis et al., 2012). *In vitro* application of auxin to single nodal potato explants resulted in an earlier tuberisation phenotype with sessile and slightly smaller tubers (Xu et al., 1998). *In vivo*, we have found that, in the stolon tips, there was a peak in auxin content a few days after induction to tuberise and prior to visible swelling. A role for auxin in the transition from stolon to tuber is accompanied by increased transcription levels of a *YUC* homologue in potato, *StYUC-like1*, and the *StPIN* family of genes during early stages of tuberisation (Roumeliotis et al., 2012).

Here we describe the analysis of transgenic potato plants over-expressing the *StYUC-like1* gene for altered IAA content in relation to potato developmental processes, including shoot and stolon branching and tuber formation.

Materials and methods

Identification of *StYUC-like* gene sequences

The potato *YUC* gene sequences were identified based on homology searches within the potato genome sequence for all known Arabidopsis YUC genes (NCBI). Potato *YUC* sequences were aligned with the Arabidopsis thaliana *YUC* genes using Crystal W2

alignment, (http://www.ebi.ac.uk/Tools/msa/clustalw2/). We have named the identified potato *StYUC-like* genes according to their sequence homology to *StYUC-like1* gene. Gene IDs for all *StYUC*-like genes and the Arabidopsis YUC genes used in this study are provided in Supplementary Table 1.

StYUC-like1 cloning and plant transformation

Gateway® technology was used for the cloning of *StYUC-like1* (GeneBank accession number JN935396) from *Solanum tuberosum* group *Andigena* genomic DNA (primer sequence in Supplementary Table 2). The plasmid pK7WG2 (Karimi et al., 2002) was used as the destination vector for the over-expression of the gene under the CaMV35S promoter (CaMV 35S:*YUC-like1)*. Agrobacterium (AGl0) mediated plant transformation was performed on stem cuttings as described in (Visser et al., 1989). A total of 12 transgenic clones were obtained. Three transgenic clones considered to be representative for all 12 different transgenic clones, based on the overground branching phenotype, shoot length and on the *StYUC-like1* transcript levels, were taken for more detailed studies.

Plant Material and measurement of auxin concentration

Single-node cuttings from short-day-grown potato plants *(Solanum tuberosum* group *Andigena*) transformed with the CaMV 35S::*YUC-like1* construct were propagated *in vitro*, on standard Murashige and Skoog medium (Murashige and Skoog, 1962) 2% (w/v) sucrose. Potato plants were propagated *in vitro* and grown for 4 weeks before being transferred to soil filled pots in the greenhouse. After 5 weeks, the shoot apex and basal internode stem segments were harvested and immediately frozen in liquid nitrogen. For all tissue samples, two biological repeats were collected and tissues from 3 different plants were pooled for each repeat. All samples were ground to a fine powder and stored at -80°C. The auxin extraction was performed as described in (Roumeliotis et al., 2012). The LC-MS/MS analysis, on the same tissue samples was performed as described in (Ruyter-spira et al., 2010).

Quantitative RT-PCR

The same material as used for auxin measurements was used for qRT-PCR. RNA was extracted using the Qiagen RNaesy Plant mini kit and DNAse I treated. cDNA synthesis was performed using Bio-Rad iScript cDNA synthesis kit and qRT-PCR was performed using the Bio-Rad cycler. The eIF3e gene was used as reference gene (primer sequence in Supplementary Table 2). Data analysis was performed using the Bio-Rad iQ5 software.

Assessment of the branching phenotype

Potato plantlets grown *in vitro* for 2 weeks were placed in 5 litre pots with soil. The height and the branching phenotype of the plants were monitored weekly. All side shoots longer than 5 centimetres were considered to be branches and their length was measured. After 8 weeks, tissue samples were harvested (shoot apex) to estimate expression of the *StYUC-like1* gene in the wild type plants and in the transgenic plants. After 10 weeks, plants were transferred to a climate chamber with 8 hours light (18ºC) and 16 hours dark (14ºC) to induce tuberisation. 17 days after plants were placed in the climate chambers, the underground parts of the plants were harvested. Tubers with a size larger than one 0.5 centimetre diameter were collected and weighed per plant to estimate fresh weight. For all the statistical analysis we used SPSS Statistics v19, (SPSS©, Inc., 2001, Chicago, IL), *post-hoc* comparisons DLS, a=0.05.

Growth conditions for estimating changes in root architecture

35S:StYUC-like1 over-expression transgenic and untransformed control plants were grown *in vitro* for four weeks before being transferred to an aeroponic system (Nutricolture co. UK) on Hoagland's solution. Root architecture was monitored daily for length and branching.

RNA-seq data for the *StYUC-like* genes

The potato genome browser (PGSC, 2011) was used to retrieve RNA-seq data of different tissues including shoot apex, flower, leaves, petiole, stem, stolon, tuber and root tissue of the RH genotype. For each tissue the number of reads of each RNA fragment corresponds to

expression values for each predicted gene (nr of fragments per kb per million reads or FPKM).

Results

Tissue specificity of the *StYUC-like1* gene and genes used in this study

Four annotated genes with high sequence homology to *Arabidopsis YUCCA* genes were identified within the potato genome sequence. Three of these (denoted *StYUC-like1-3)* fall into a single clade and show highest similarity to *AtYUC3* and *7* with a fourth gene (*StYUC-like4*) forming an out-group (Figure 1A). In a study of the transcript levels based on the RNA-Seq data of four potato *YUC-like* genes, it was revealed that *StYUC-like1* gene showed the highest expression in the stolon tip compared to the other *StYUC-like* genes (Figure 1B). Expression studies of transcript levels of *StYUC-like1* in the stolon tip during early stages of tuber development show a peak in expression after tuber induction and prior to visible swelling (Roumeliotis et al., 2012), establishing this gene to be the main candidate to study the role of auxin in stolon and tuber development. On an amino acid level, sequence of the potato *StYUC-like1* genes has 72% similarity to *Arabidopsis YUC*9 and 95% similarity to a tomato flavin monooxygenase-like protein (accession numbers in supplementary table 1).

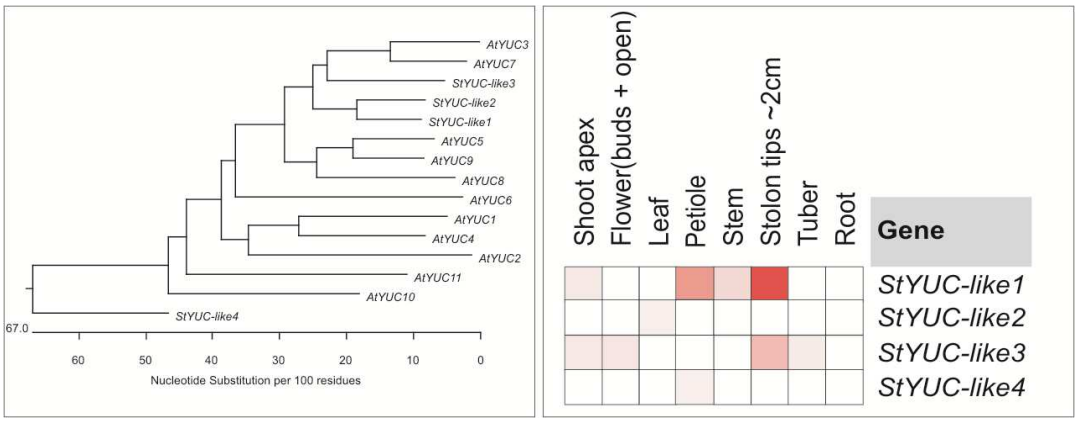

Figure 1. Heat map of expression of the StYUC-like genes in the corresponding tissues according to the RNA-seq data of the potato diploid genotype RH. Expression levels in the various tissues are indicated by shades of red, where white indicates no expression detected.

Over-expression of the *StYUC-like1* gene results in altered above ground phenotypes

In order to analyse the function of the *StYUC-like1* gene in potato, we made a construct containing the entire coding region of the *StYUC-like1* gene and placed it under the control of a constitutive CaMV 35S promoter. Investigation of the transcript levels of the *StYUC-like1* gene in the apex of the transgenic clones revealed a several hundred fold increase in the transcript levels compared to the control (Figure 2). Three transgenic clones considered to be representative for all 12 different transgenic clones based on the over ground branching phenotype, shoot length and on the StYUC-like1 transcript levels, were grown under greenhouse conditions and monitored weekly for their height and overall above-ground phenotype to identify possible differences between the transgenic plants and the untransformed control plants. After 12 weeks, no differences in height were detected between the transgenic clones and the non-transgenic controls (Figure 3A). All three clones also exhibited extensive branching (Figure 3B and 4A) and smaller leave size (Figures 4B). While the control plants had an average of 3 branches per plant, all three transgenic clones had, on average, more than five branches (Figure 3B).

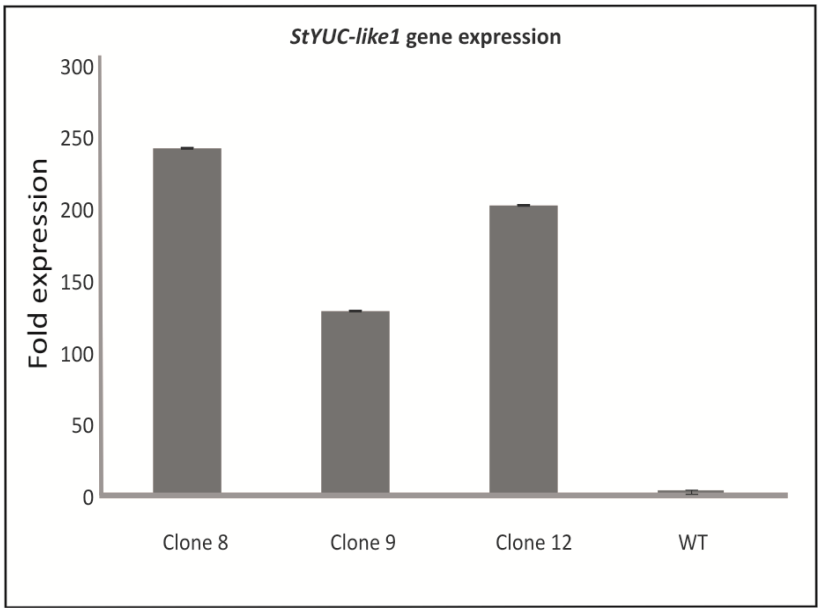

Figure 2. Fold increase of the expression of the StYUC-like1 gene in the apex in four transgenic clones and the untransformed control grown in the greenhouse. The StYUC-like1 gene expression in the untransformed control is set to one fold. Error bars represent standard error for two biological repeats with three plants pooled per biological repeat.

Examination of the length of the branches in each node revealed that in the first branch from the base, only a small difference of the branch-length was observed. In contrast, the transgenic clones exhibited significantly longer branch length for almost all higher branches (Figure 3C). The control plants had an average length of 43, 30 and 17 centimetres for branches one to three respectively, and no side branches above the fourth node. In contrast, for clone-8 the average length of branches one to six are 38, 21, 30, 23, 32 and 31 centimetres respectively and similar results were found for the other transgenic clones. These results show

that the transgenic clones exhibited increased branching and that for branches 3 to 6 the length of the respective branch is longer in comparison to the controls.

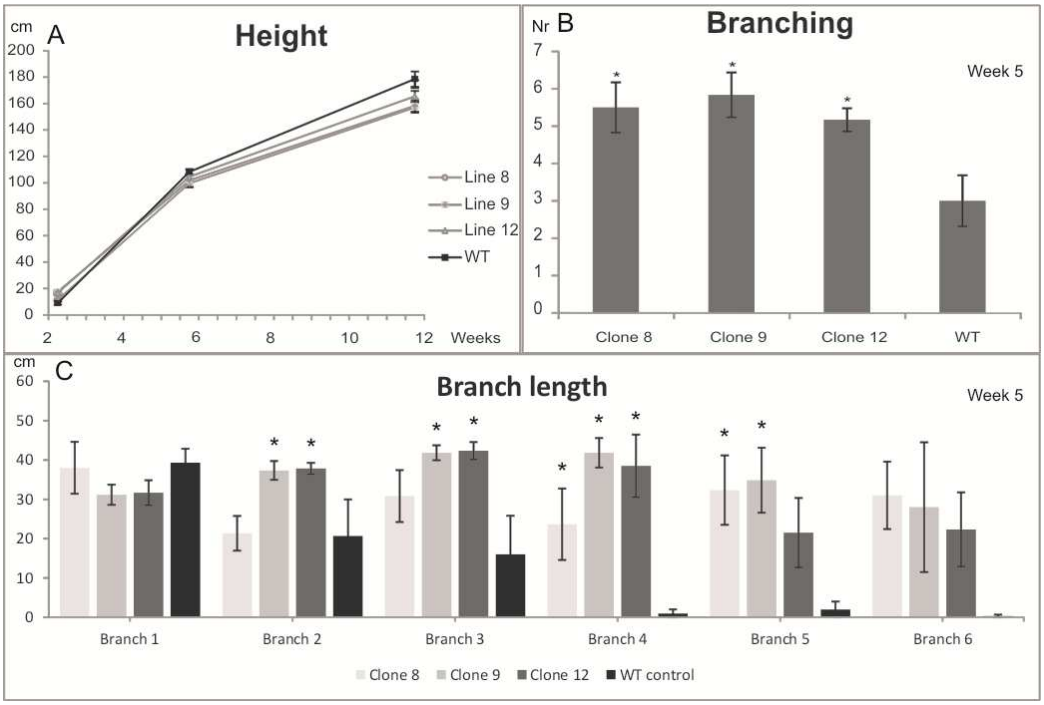

Figure 3. (A) Height measurements for four transgenic clones and the untransformed control over a period of 12 weeks of growth in the greenhouse. Error bars represent standard error for ten individual plants per clone. Dotted clone indicates the early stage when differences in height between three clones and the untransformed control are statistically significant. Significant differences (AVOVA test, a=0.05) 2.5 weeks after the plants were placed in the greenhouse are represented with a star on the left top corner. (B) Scoring of the branching frequency for three transgenic clones and the untransformed control. (C) Scoring of the length of the branches for three transgenic clones and the untransformed control. Error bars represent standard error for two biological repeats with three plants pooled per repeat and stars indicate statistical significant differences between the clone and the untransformed control (ANOVA test, a=0.05).

Over-expression of the *StYUC-like1* gene is altering the below-ground phenotypes

To investigate the effect of *StYUC-like1* over-expression on stolons and tuber formation, transgenic plants grown in the greenhouse for 10 weeks under non-inductive long-day conditions were transferred to short-day conditions to induce tuberisation (material and methods). After 2 weeks in the growth chamber, clone-8 showed numerous buds and sessile tuber-initials on almost all side buds indicating increased branching (Figure 5A). Moreover, transgenic clone-8 exhibited tubers with extensive cracks that were mainly parallel to the axis of the potato tuber from the heel to the tuber apex (Figure 5C). This cracked phenotype was noticed in all tubers in all plants of clone-8. For the estimation of final tuber numbers (Figure 6A) small tuber-initials were not included if smaller than 0.5 cm. For all three transgenic

clones, the average tuber weight was significantly lower compared to the control and the number of tubers was increased for clones 9 and 12 compared to the wild type plants (Figure 6C). Clones 9 and 12 had a significantly higher number of tubers, however, with the same total fresh weight as the untransformed control (Figures 6B).

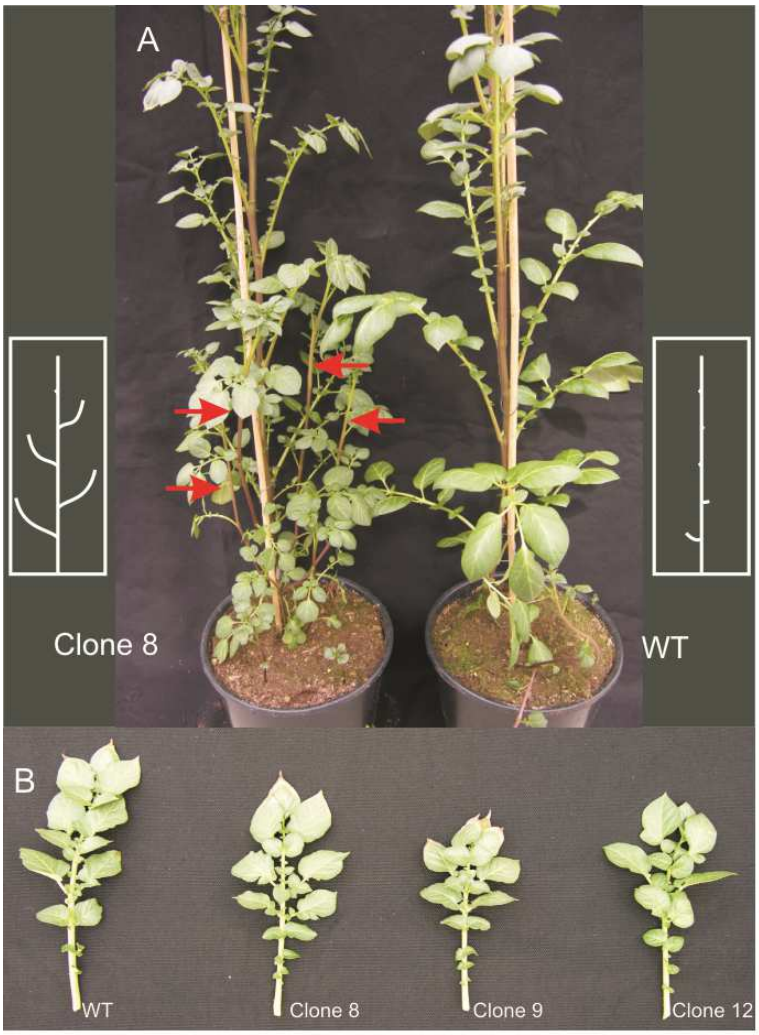

Figure 4. (A) Comparison of the branching phenotype between a transgenic clone and the untransformed control with a diagrammatic representation of the branching phenotype. Red arrows indicate the branches on the transgenic clone. (B) Comparison between the leaf size of the untransformed control and transformed clones 8, 9 and 12.

Preliminary observations on the root architecture of plants grown in soil indicated a bushy root structure for the 35S::*StYUC-like1* transgenic plants compared to the untransformed controls, however, the differences were difficult to quantify in a soil grown system. To be able to score for altered root morphology in a non-destructive way, plants were grown in aeroponic systems. Observation of the root network showed that the transgenic plants have a root system with increased adventitious rooting and lateral root formation compared to the untransformed control plants, exhibiting a bushier root phenotype (Figure 5B) in alignment with the initial observations of plants grown in pots with soil. The increased capacity of lateral root formation was evenly distributed over the entire root system established by the explants.

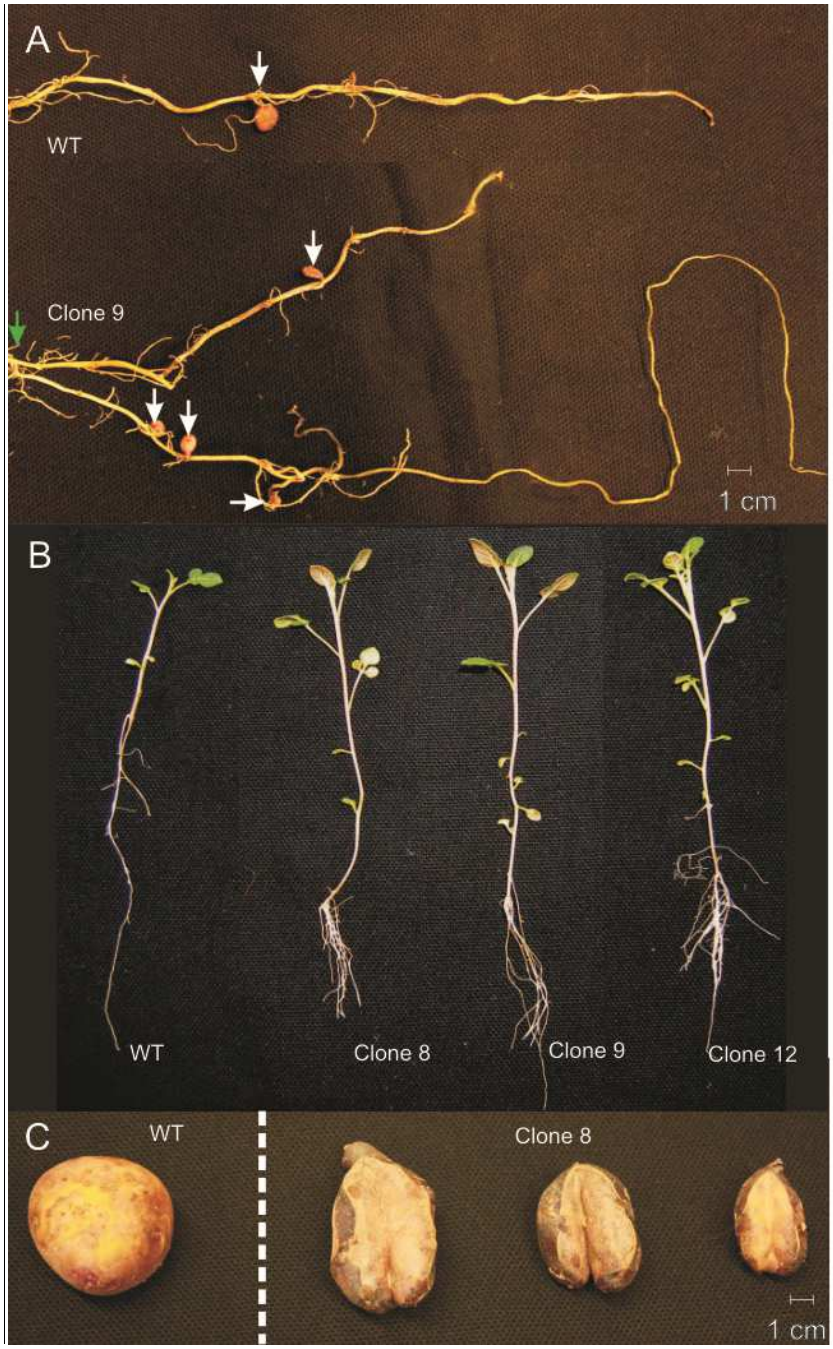

Figure 5. (A) Comparison between stolons of the untransformed control and transgenic clone-9. Arrows indicate stolon branching or tuber initials. (B) Comparison between the architecture of the root system of the untransformed control and clones 8, 9 and 12. (C) Potato tuber of the untransformed control next to clone-8 showing tubers with a cracked phenotype.

Transformed plants with the 35S::*YUC-like1* construct have altered auxin content

To investigate the effect of the 35::*StYUC-like1* construct in the transgenic potato clones on the auxin content we assayed auxin levels in the apex and in the basal part of the stem in mature plants grown in the greenhouse (Figure 7). Estimation of the IAA content in the apex where auxin biosynthesis is predominately taking place, showed no significant differences between the 35S::*StYUC-like1* plants compared to the untransformed plants (190 to 260 pmol/gr FW for the transgenic clones and 210 pmol/gr FW for the untransformed control;

Figure 7A). However, in the basal part of the stem, the 35S::*StYUC-like1* transgenic clones have less free auxin compared to the untransformed control plants (Figure 7). The untransformed control plants have an average IAA content of 130 pmol, while in the transformed clones IAA content ranges from 62 to 83 pmol. These results are contrary to expectations in plants where a higher level of IAA is anticipated due to the over expression of a gene participating in auxin biosynthesis.

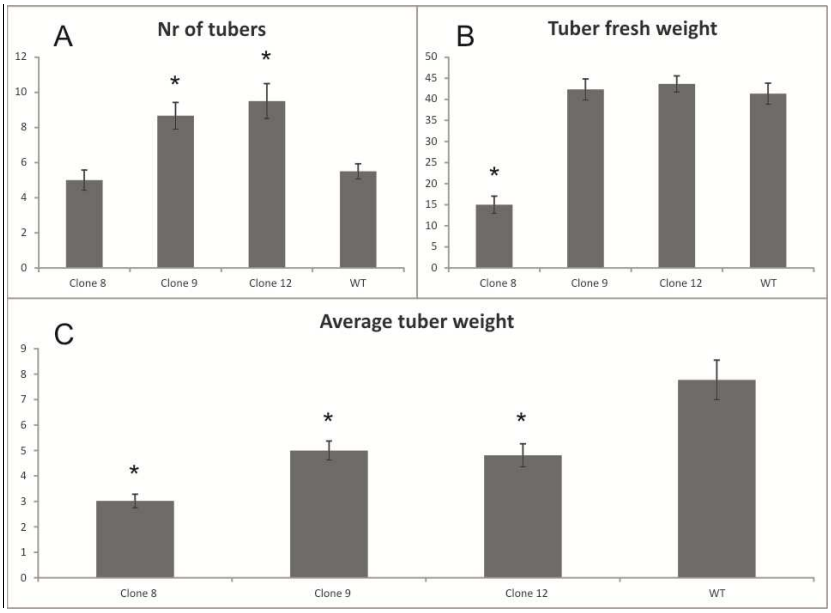

Figure 6. (A) Scoring of the average number of tubers per plant for each clone. (B) Scoring of the average tuber fresh weight per plant for each clone. (C) Scoring of the average tuber weight for each clone and the untransformed control. Error bars are standard error for 6 plants per clone, and stars indicate statistically significant differences between the clone and the untransformed control (ANOVA test, a=0.05).

Discussion

StYUC-like1 gene was previously shown to be expressed predominately in the stolon tip and exhibits a peak in expression just prior to tuber swelling, followed by a peak in auxin content (Roumeliotis et al., 2012). In this study, we investigated the effect of a 35S::*StYUC-like1* construct on shoot, stolon and tuber development in transgenic potato. Independent transformed clones with the 35S::*StYUC-like1* construct had decreased apical dominance in the shoot and stolon, smaller leaf size and enhanced adventitious and lateral root formation. These findings are not in agreement with the phenotypes observed for the rice and *Arabidopsis YUC* gene over-expressers (Cheng et al., 2006; Yamamoto et al., 2007). Moreover, decreased apical dominance is indicative of decreased IAA levels in the stem, which is in contrast to an anticipated increase of IAA content. Assessment of the IAA content in shoot apex and basal stem, revealed that while no differences were observed for auxin concentrations in the apex, IAA content in the basal stem was decreased in the transgenic clones compared to the untransformed control. In all three analysed transgenic clones, the number of shoot branches was increased from the third node and up and their length was

significantly longer compared to the untransformed control. Shoot branching and apical dominance in stems is mediated through polar auxin transport from the sites of biosynthesis to the lower parts of the plant. Lower levels of auxin in the basal stem is expected to result in decreased apical dominance and increased shoot branching (Prusinkiewicz et al., 2009), therefore low IAA levels provide a possible explanation for enhanced shoot branching in the transgenic clones. In addition to increased shoot branching, stolons also exhibited decreased apical dominance, resulting in increased stolon branching and higher number of tubers and tuber initials. Previously we presented evidence that the mechanism that mediates stolon branching is similar to the mechanism that regulates shoot branching (Roumeliotis et al., 2012). Therefore, lower auxin content in the basal shoot can also explain the decreased apical dominance in the stolons. A decreased apical dominance in the stolons, releases the stolon buds from dormancy allowing the development of secondary stolons that can develop into tuber-initials under inductive conditions, resulting in a larger number of small tubers.

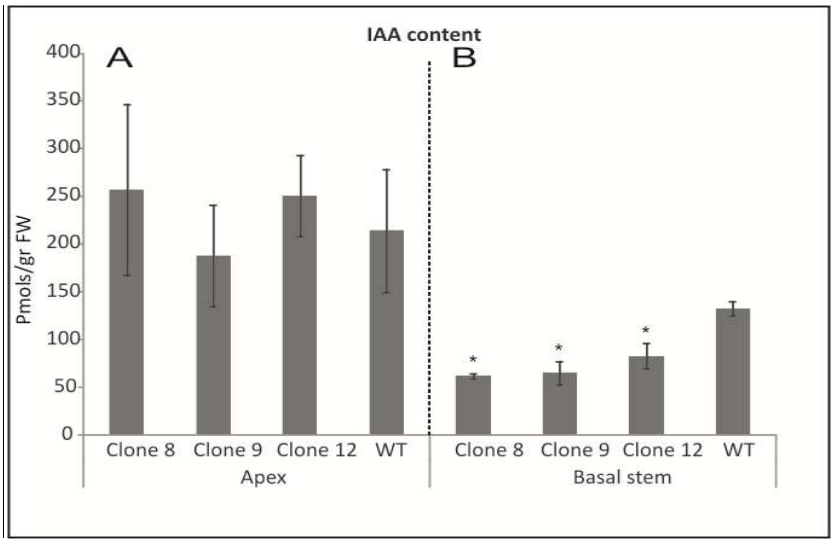

Figure 7. IAA content for 3 independent clones and the untransformed control in the apex (left) and basal stem (right). Plants were grown in the greenhouse for 5 weeks prior to harvesting the tissues. Error bars represent standard error for two biological repeats with three plants pooled per repeat and stars indicate statistical significant differences (ANOVA test, a=0.05).

In order to investigate whether the increased branching can be attributed to decreased auxin transport or changes in the levels of strigolactones, we examined the expression levels of the *StPINs, StCCD7* and *8* genes in the basal stem. The results did not revealed any significant differences in the expression levels of these genes in the basal stem compared to the untransformed control (data not shown). Therefore, additional experiments about the transport capacity of auxin and the levels of strigolactones are required in order to attribute the enhanced branching phenotype to changes in the auxin transport capacity or changes in strigolactone content.

Attenuating auxin biosynthesis by production of double, triple and quadruple *yuc* knockout lines in *Arabidopsis*, has been shown to result in decreased leaf size (Cheng et al., 2006). This links auxin biosynthesis with leaf size. Thus, the reduced leaf size observed in the 35S::*StYUC-like1* plants is consistent with lower auxin levels in the stem.

In all three transgenic clones, increased adventitious rooting was observed. It has been demonstrated that strigolactone content is negatively correlated with adventitious rooting (Rasmussen et al., 2012) and that *CCD7* and *8* genes that are involved in strigolactone biosynthesis are positively regulated by auxin (Hayward et al., 2009). Therefore it is possible that lower auxin content can result in lower strigolactone content and advanced adventitious rooting. Investigation of the expression levels of the *StCCD7* and *8* genes in the stem did not reveal significant differences in the expression levels of those two genes compared to the untransformed control (data not shown). Still, further investigation is required to examine auxin and strigolactone content as well as the *StCCD7* and *8* expression levels in the roots of the transgenic plants in order to be conclusive about the cause of this phenotype.

In addition to the production of larger numbers of smaller tubers, transgenic clone-8 showed uniformly aberrant tuber morphology. The observed cracked tubers are likely to be the result of disturbed coordination of cell division and expansion in different cell layers of the tuber. Since auxin is a primary factor in initiating cell division this phenotype is likely to be linked to locally altered auxin levels (Möller and Weijers, 2009).

Both the low auxin content in the transgenic plants and the phenotypes appears not to be consistent with *StYUC-like1* over expression, however, co-suppression (PTGS) is unlikely in the light of the very high *StYUC-like1* transcript levels in the transgenic clones. In other instances, for example in *Arabidopsis,* YUCCA gene over expression did not lead to significantly higher levels of auxin due to absence of substrate for the YUC protein (Stepanova et al., 2011). Therefore, it cannot be excluded that a feed-back regulation mechanism might be present and reduce the production of the substrate of *StYUC-like1*. Such feed-back and feed-forward mechanisms are known to exist in other plant hormone biosynthesis pathways, such as gibberellins. Such a feed-back regulation mechanisms could also reduce other compensating pathways for auxin production, resulting in the reduced IAA content in the stem.

In this research we present data concerning the effect of a 35S::*StYUC-like1* construct on various experimental events in potato. Overexpression of *StYUC-like1* gene resulted in small changes in IAA content in the basal stem, which enhanced shoot and stolon branching, increased lateral root formation and reduced leaf size. Our research sheds light on the effect that small changes in IAA content have in several developmental events of the potato plant and on our understanding of the mechanisms that mediate branching in shoots and stolons.

Acknowledgements

We thank Tatsiana Charnikhova, Francel Verstappen and Wendy Jo-Chen Liao for their technical support. Part of this work was done in the framework of the EU-SOL project (PL 016214-2 EU-SOL) and co-financed by The Netherlands Organization for Scientific Research (NWO; VICI grant, 865.06.002 and Equipment grant, 834.08.001 to H.B.). E.R. gratefully acknowledges financial support from the Bakalas Foundation, and the Stichting Veenhuizen-Tulp fonds.

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General discussion

The scope of the research presented in this thesis was to elucidate the role of auxin in tuber initiation, to describe a more accurate model of tuberisation physiology and to clarify the mechanisms that mediate stolon branching. In this chapter, the most important results are recapitalized with emphasis on tuber development and stolon architecture. Finally, future prospects of this research are discussed.

A role for auxin in tuber development

Auxin is the most well studied phytohormone and participates in a variety of developmental processes. Early on in plant development, auxin distribution mediates embryo patterning (reviewed in Möller and Weijers, 2009). Other developmental events that also require new meristem identity to be established, such as flower development (Krizek, 2011) and lateral root formation also require the action of auxin (Dubrovsky et al., 2008). Recently, in (Dhonukshe et al., 2012), it was shown that the re-orientation of the plane of cell division of *Arabidopsis* stem cells is auxin dependent, demonstrating a possible auxin mediated mechanism that regulates changes in the orientation of cell division in plants.

Cytological studies in the stolon tip revealed that upon tuber induction, longitudinal cell division is arrested, and the plane of cell division changes from a lateral to a longitudinal in order to terminate stolon elongation and achieve swelling of the tuber (Xu et al., 1998). Our hypothesis was that auxin is likely to participate in tuber development. Early experiments with applications of auxin in *in vitro* tuberisation systems did not provide a direct link between auxin and tuber development or had equivocal results (Koda and Okazawa, 1983; Xu et al., 1998). The advent of the genomic era in biology provided new tools to study tuber initiation and development though the study of gene expression and function. The differential expression of an auxin response factor gene showing a peak in expression just after tuber initiation provided a first indication that auxin plays a role in tuber initiation (Faivre-Rampant et al., 2004). A more comprehensive expression study using a microarray approach provided a much more detailed picture of the transcriptome wide regulation of genes during tuber initiation. This microarray experiment revealed that a large number of auxin related genes had a differential expression profile during early events in tuber development (Kloosterman et al., 2005). Examples of such genes are two *PIN*-like genes, an *adr11-2* (**a**uxin **d**own-**r**egulated) and an *acrA-like* (auxin regulated gene containing a GTP-binding site) genes. In *Arabidopsis*, transcript levels of *adr* genes were shown to be down-regulated in presence of auxin (Datta et al., 1993), while in tobacco, *acrA* expression levels are up-regulated after auxin application (Ishida et al., 1993). In potato, transcript levels of the *StPIN-like* and the *acrA-like* gene exhibited a peak in expression after tuber initiation, while the *adr-like* gene was down regulated. Therefore, this expression data indicates that auxin levels are likely to increase during early stages of tuber development. Scoring of auxin content in the stolon (Chapter 2, Figure 1) revealed that after tuber initiation, auxin content is indeed increased not only in the stolon tip, but also in the region just below the site of swelling. In the analysis of auxin

biosynthesis genes, as well as of *PIN* family of genes which is involved in auxin transport, we were able to verify that the at least one auxin biosynthesis gene, named *StYUC-like1,* is up-regulated during early stages of tuber development (Chapter 2, Figure 3). In addition, auxin transport assays revealed that IAA is polarly transported basipetally from the distal stolon apical meristem (STAM) to the proximal region of the stolon (Chapter 2, Figure 6), indicating that the stolon tip is a possible site of auxin biosynthesis. In chapter 2, we studied the expression pattern of the *PIN* gene family in potato, and we used prom*PIN*::GUS studies to visualize the locations of *PIN* gene expression for two different *PIN* genes in the swelling tubers (Chapter 3, Figure 5). Taken together, these results, combined with the known role for auxin in the development of other meristems, provides strong evidence for a role of auxin in tuberisation and more specifically in the events that take place after tuber initiation.

The group of phytohormones that has been extensively studied for their effect on tuber formation is the gibberellins. Application of GAs results in an inhibition of tuber initiation (Kumar and Wareing, 1974; Xu et al., 1998). In addition, *StGA20ox* genes have been shown to be up-regulated during early stages of tuber development (Kloosterman et al., 2005) and GA content is decreasing during stolon tip swelling (Xu et al., 1998). The first visible sign of tuber induction is the termination of longitudinal stolon growth (Xu et al., 1998). Active GAs are known to be involved in plant shoot elongation (Spray et al., 1996; Coles et al., 1999) including potato (Carrera et al., 2000; Kloosterman et al., 2007). Therefore, cessation of stolon elongation is likely to be a result of GA degradation. *In vitro* experiments showed that the termination of stolon elongation and initiation of stolon swelling occurs 5 days after induction to tuberise (Xu et al., 1998). Our observations on *in vivo* plant material on non-swelling stolon tips did not indicate an increase in auxin content at day 5. In contrast, in rapidly swelling stolon tips 16 days after tuber induction by short days, auxin content exhibits a four-fold increase compared to non-inductive conditions. Swelling of the stolon tip is attributed to random cell divisions and cell enlargement that are compatible with the role of auxin.

Our findings on auxin content joint with the known role for GAs in tuber initiation and development, allows us to describe the physiological model that portrays the cross-talk between GAs and auxin during early stages of tuberisation. GAs and auxin seem to be involved in tuber development in two consecutive stages (Figure 1). At the stolon growth phase, GA content is relatively high and is mediating stolon elongation. The plain of cell division remains transversal during this phase. Auxin content is relatively low, and the role of auxin is to maintain stolon apical dominance. Short day conditions induce tuber initiation, and the mobile signal StSP6A is produced and transported from the aerial parts of the plant to the stolon tips (Navarro et al., 2011). When the StSP6A protein reaches the stolon tip, tuber formation is induced. GA levels are rapidly degraded and auxin content has a peak. This occurs simultaneously with termination of longitudinal stolon growth, change of the plane of cell division and swelling of the stolon to form the tuber. GA content is a very important switch in this developmental event. If GA degradation is hindered, a delay in the tuber initiation can occur, while greater GA degradation produces tubers earlier (Kloosterman et al., 2007).

At the tuber swelling stage, GA content has been degraded, while IAA content remains high and slowly decreases over time, correlated with a peak in the expression of a *StYUC* gene. It is during this stage that changes in the orientation of cell division in the tuber take place to conduct tuber growth. *In vitro* experiments with auxin application also point out the

importance of auxin in tuber initiation (Chapter 2). If auxin levels remain high by continuous application of auxin to the stolon tip, tuber formation is inhibited. On the other hand, a single auxin pulse *in vitro* stimulates tuberisation in comparison to the controls (Chapter 2, Figure 4B). Therefore, the auxin peak that we found *in vivo,* is an important factor for a stolon to start swelling. Recent evidence on the role for auxin in regulating the orientation of cell division in *Arabidopsis* (Dhonukshe et al., 2012), supports the notion that auxin is likely to be the key factor regulating changes in the orientation of cell division and tuber growth.

The direct connection between the signal transduction pathway resulting in the transport of StSP6A protein to the stolon tip and auxin accumulation remains unclear. However, un-published results showing protein-protein interaction between StSP6A and the potato StKANADI transcription factor (Dr. S. Prat; National Centre of Biotechnology, Madrid, personal communication) gives an intriguing link between day-length initiation of tuberisation and auxin. In *Arabidopsis*, KANADI has been shown to be involved in organ polarity (Kerstetter et al., 2001) and this polarity is likely to be mediated via relocation and differential recycling of PIN proteins (Ilegems et al., 2010). Hd3a the FT homologue in rice has also recently been shown to interact with the rice KANADI homologue OsKANADI1 (Taoka et al., 2011).

The StSP6A/KANADI interaction in stolon tips thus provides a possible mechanism for the reorganization of auxin flow and accumulation that we have demonstrated in our experiments. The elucidation of the light-regulated pathway for induction of tuberisation and the identification of the corresponding mobile signal together with the action of major plant hormones in this process now allows a novel model to be constructed for tuberisation and growth. Figure 2 represents a concise overview of the processes involved.

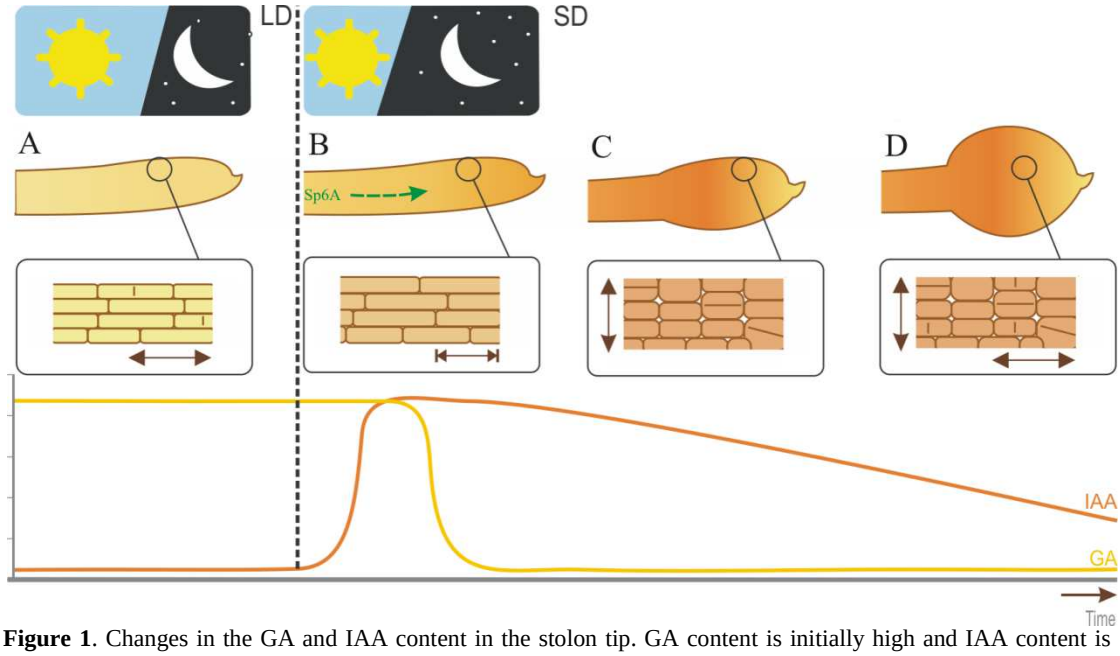

Figure 1. Changes in the GA and IAA content in the stolon tip. GA content is initially high and IAA content is low under non-inductive long day conditions. The stolon is elongating longitudinally (A). The black dotted line represents switch to tuber inductive long days. StSP6A transcript (green arrow) is the mobile signal that reaches the stolon tip. Elongation of the stolon is terminated (B), and GA content drops while IAA content is increased. Tuber swelling is achieved initially by cell transversal division (C) and finally by random cell divisions (D).

The mechanism that mediates stolon branching

Auxin produced in the apex is transported basipetally to the lower parts of the plant mainly through influx and efflux carriers (Blakeslee et al., 2005) that are localized in the plant cells that maintain the Polar Auxin Transport (PAT) stream (reviewed in Ongaro and Leyser, 2008). Release of dormancy of the axillary buds requires the export of the locally produced auxin from the bud to the main PAT stream. Ablation of a shoot apex, obliterates one of the main sites of auxin biosynthesis (Ljung et al., 2001) thus allowing auxin produced in the side buds, to be released in the main auxin transport stream (Snow, 1937; Ongaro et al., 2008). Auxin acts indirectly to mediate shoot branching, without entering the axillary bud (Booker et al., 2003), thus suggesting that a second messenger downstream of auxin may be required along with auxin to regulate side shoot outgrowth. Two candidates for the role of the secondary messenger have been described; cytokinins and strigolactones (Gomez-Roldan et al., 2008; Ongaro and Leyser, 2008). Cytokinins are transported acropetelly and can directly promote bud activation (Sachs and Thimann, 1967). In addition, auxin content is negatively correlated with cytokinin biosynthesis, suggesting that auxin might regulate shoot branching by controlling cytokinin content (Nordström et al., 2004; Tanaka et al., 2006).

Strigolactones are a recently discovered group of phytohormones with an effect on shoot branching (López-Ráez et al., 2008; Umehara et al., 2008; Brewer et al., 2009). The branching phenotype of several mutants with increased branching such as *decreased apical dominance* (*dad1*) in petunia and *more axillary branching* (*max*) in *Arabidopsis* can be rescued when these mutants are grafted on wild type roots, suggesting a mobile signal that moves from the roots to the above ground parts of the plant. In addition, SL levels in these mutants were lower, and SL application restored the phenotype to the wild type (Gomez-Roldan et al., 2008; Umehara et al., 2008) suggesting this mobile signal is SL, or is derived from SL. SLs have been shown to be transported through the xylem sap in *Arabidopsis*, and transcription of genes involved in SL biosynthesis are up-regulated by auxin (Foo et al., 2005; Hayward et al., 2009), making SLs a good candidate for the secondary messenger mediating shoot branching together with auxin. SLs are suggested to act directly in restraining the release of the side bud growth (Brewer et al., 2009) or indirectly, by modulating auxin transport (Prusinkiewicz et al., 2009).

Botanically, stolons are etiolated shoots that grow diageotropically in the soil. In chapter 2, studies on the directional movement of radio labeled auxin verified that auxin is transported from the stolon apical meristem to the basal parts of the stolon. Study of auxin biosynthesis genes revealed that at least one auxin biosynthesis gene is expressed in the stolon tip, and the expression study of the *StPIN* gene family revealed that the *PIN* genes are expressed in the stolon tip transporting auxin out of the apical meristem. The *in vitro* experiments with ablation of the stolon tip increased the number of side tubers grown on the explants (Chapter 2, Figure 1A), while application of auxin removed this promotion (Chapter 2, Figure 1B). Chemically ablating the stolon tip by applying auxin transport inhibitor or auxin antagonist had the same promoting effect (Chapter 2, Figure 1C). In addition, application of artificial SL in the *in vitro* system strongly inhibited tuber formation in the stolon apex as well as in the side buds, suggesting that the genetic machinery for SL perception and action is present in the stolons. SL detection assays in plants grown in aeroponic systems revealed that the potato is able to produce at least two different SLs (Chapter 2, Figure 5). These results suggest that a

similar system to the one described in shoots is present in the stolons and mediates the formation of tubers under tuber inductive conditions. In such a model, auxin is produced in the stolon apex and is moving basipetally through the PAT stream. Strigolactones act synergistically with auxin to prevent side bud outgrowth. Ablation of the stolon tip results in dampening of the PAT and the buds are released from dormancy by pumping their locally produced auxin in the stolon. Enhanced stolon branching will result in a higher number of secondary stolon apical meristems that are able to produce tuber initials, under inductive conditions. Therefore, the hormone regulated stolon branching is an important feature that controls the number of tubers produced by the potato plant (Figure 2).

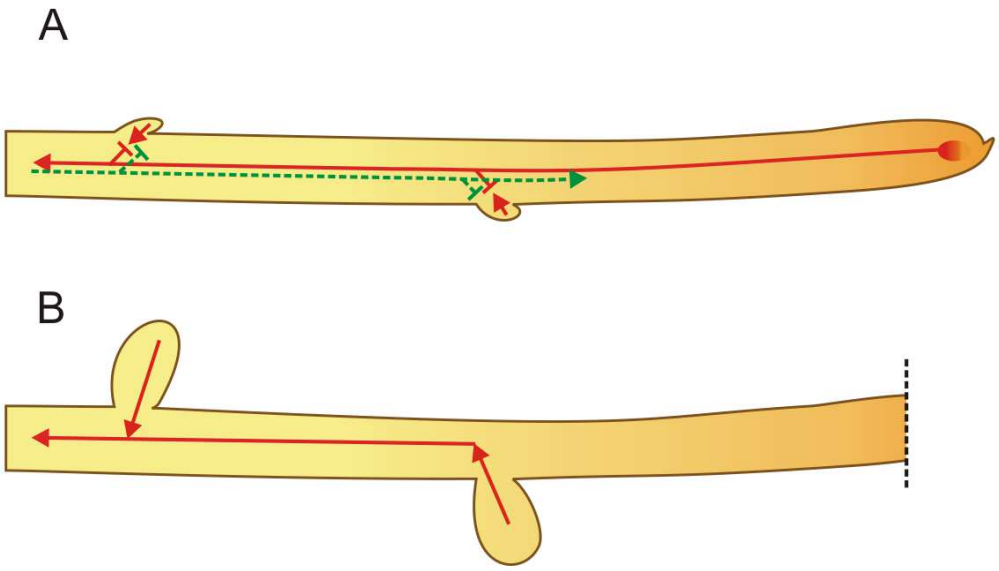

Figure2. Representation of the mechanism that mediates stolon branching. (A)Red arrow represents the Polar Auxin Transport that mediates basipetal movement of auxin from the sites of biosynthesis in the stolon tip to the basal part of the stolon not allowing the side stolon buds to shoot. Green arrow with dashed line represents Strigolactones that are moving in the opposite direction to auxin in the stolon and act synergistically with auxin to inhibit bud outgrowth. Small red arrows in the side buds represent the locally produced auxin that is not able to be transported into the main auxin stream. (B) Ablation of the stolon tips is enough to allow the locally produced auxin in the side buds of the stolon to be released from their dormancy and produce tubers under tuber inductive conditions.

Changes in GA content, as well as changes in auxin content can both result in an increase in the number of tubers, but not in total tuber yield

In chapters 4 and 5, we used reverse genetic approach to investigate the role of gibberellins and auxin in tuber development. Investigation of the average tuber weight and total tuber weight in the 35S::*YUC-like1* and in the *StGA3ox2* RNAi transformed plants revealed that in both cases, the number of tubers was altered but the total tuber weight was not significantly different.

Little is known about the role of auxin in potato tuber yield and the role of auxin has been investigated mainly on tuber initiation and development. In chapter 4, the 35S::*StYUC-like1*

transformed plants exhibited enhanced shoot branching, altered auxin content and altered root development (Chapter 4, Figure 3). In addition, in the stolons enhanced branching was noticed, and in several independent clones the number of tubers per plant was increased (Chapter 4, Figure 4A) or the yield per plant was reduced (Chapter 4, Figure 4B). Reduced yield in some clones was attributed to the fact that the stolons had numerous small tuber initials that were not scored as set tubers due to their small size. In all cases, the average tuber weight of the transgenic clones was significantly lower compared to the control plants (Chapter 4, Figure 4C). In addition, all tubers produced by clone 8 had cracks that extended from the tuber apex to the tuber heel, where the tuber is attached to the stolon. The phenotypes observed in stem and roots of the 35S::*YUC-like1* transgenic potato plants were not always in alignment with higher auxin content as noticed for *Arabidopsis* and rice (Cheng et al., 2006; Yamamoto et al., 2007) and fit better with lower auxin content as predicted in (Prusinkiewicz et al., 2009). Scoring of the auxin content revealed that the IAA content in the stem was lower than in the untransformed control (Chapter 4, Figure 7), possibly due to low substrate availability. In addition, lower auxin content can reduce the apical dominance of the stolon apical meristem, allowing a greater number of stolon side buds to shoot and form tubers under tuber inductive conditions, providing a possible explanation why an increase number of tubers is noticed in the transgenic clones.

In chapter 5, we investigated the effect of silencing *StGA3ox2* that is involved in GA biosynthesis on tuber initiation and tuber formation. The transgenic clones had shorter stem length, smaller leaf size (Chapter 5, Figure 2) and altered GA content (Chapter 5, Figure 4). The number of tubers in the transgenic clones was also investigated and in several transgenic clones was higher compared to the control Chapter 5, (Chapter 5, Figure 3A) but the average yield did not seem to be different in the majority of the transgenic clones (Chapter 5, Figure 3B), resulting in lower average tuber weight (Chapter 5, Figure 2C). Potato plants with antisense for *StGA20ox1* cDNA, a gene also participating in active GA biosynthesis, also exhibit shorter stem and internode length, but had an earlier tuberisation phenotype and increased yield (Carrera et al., 2000). In addition, potato clones down-regulated for *StGA2ox1*, a gene involved in active GA degradation, had no marked changes in the aerial part of the plant, but produced elongated tuber swellings or had a higher degree of stolon branching. Two of the transgenic *StGA2ox1* suppression clones had a lower yield. Over-expressing *StGA2ox1* also resulted in lower average tuber yield with plants having either fewer or smaller tubers (Kloosterman et al., 2007). Our finding that the tuber yield was not altered in the *StGA3ox2* RNAi transgenic clones is partially in contrast with the findings on changes on yield when other steps of the GA biosynthesis pathway are altered.

Based on these results, it seems likely that the physiological pathways described in this thesis exert their main affect on the time point of tuber initiation or alter the stolon development to deliver more or fewer tuber, but the yield capacity of the potato plant seems to be independent of these pathways. Yield capacity is more likely to be dependent on the nutritional and physiological status of the plant material, conditions that were similar in all the experiments described in this thesis.

Future perspectives

The research described in this thesis succeeds in assigning a role for auxin in tuber development, as well as in providing evidence on the mechanism that regulates stolon branching. In addition, studies with transgenic clones that have altered IAA and GA content revealed that tuber yield was not affected by the phytohormone levels, but there was a change in the number of tubers produced.

In this work, we discussed the phytohormone pathways that mediate tuber formation. However, several other pathways have been shown to exist that remain to be elucidated. For example, the *StBEL5* transcript is increased under tuber inductive conditions and *StBEL5* over-expression in potato plants overcomes the inhibitory effect of long days on tuber formation (Chen et al., 2003). StBEL5 transcript is transported from the aerial parts of the plant to the stolon tips in a photoperiod dependent manner. Furthermore, the *StBEL5* transcription is induced by light (Chatterjee et al., 2007). StBEL5 with its heterodimer partner, StPOTH1, have been shown to effect GA levels by regulating *StGA20ox1* gene expression (Chen et al., 2004) therefore constituting *StBEL5* a possible link between the photoperiodic and the phytohormone tuber inductive pathways.

The micro RNA miR172 has also been proposed to play an important role in tuber development. miR172 is involved in FT gene expression in *Arabidopsis* that regulates floral transition and miR172 transcript levels are mediated by the light receptor phyB (Martin et al., 2009). The *Arabidopsis FT* gene expression is regulated by *CONSTANS*. In addition, *CONSTANS* gene expression is repressed by *CDF1* (member of the *DOF* genes) gene and by the FKF/GI complex (Sawa et al., 2007). Is has been demonstrated in *Arabidopsis*, that one *DOF* gene, *DAG1*, acts as a negative regulator of *AtGA3ox1* by binding directly on the promoter and also that DAG1 acts functions as a negative component of phyB-dependent responses (Gabriele et al., 2010). If such a similar regulation system was present in potato, phyB dependent short day sensing would result in down regulation of the *StGA3ox1* gene and *StCO* gene would be repressed, resulting in a release of the potato *FT* gene expression, including *SP6A* that has been shown to regulate tuber initiation. Therefore, such a potato *DAG1* gene may have an additional role of the link between the photoperiod dependent pathway and levels of GA.

It remains challenging to try to explain how other factors that are involved in tuber initiation, such as sucrose content, nitrogen levels and temperature, fit in a more holistic model for tuber development. It cannot be excluded that environmental factors, like low N content and optimal temperatures are perceived by the plant and enhance the tuberising signal. On the other hand, it has been postulated that sucrose itself might even be an inductive signal for tuber development (Ewing and Struik, 1992). The effects of these parameters need to be examined in more detail in order to contribute to the full model that can describe tuber initiation and growth.

Another interesting point that grants further investigations is how auxin mediates tuber development. When the events are placed in a chronological order, IAA content peak in the stolon tip is followed by changes in the orientation of the plane of cell division. Auxin is

known to participate in a variety of events that require changes in the orientation of the place of cell division and changes in meristem identity. It is possible that the role of auxin in tuber development involves rearranging the direction and rate of cell division in the growing tuber. Therefore a more detailed study of the direction of auxin transport by the PIN proteins in specific cells in the swelling stolon as well as finely tuned auxin applications in the stolon apical meristem would be informative on the effect of auxin in the new growing organ.

From an agronomic point of view, elucidating the mechanism that mediates stolon branching is very important. For the first time, there is sound evidence that auxin is produced in the stolon tip and that stolon branching is mediated by a similar mechanism to shoot branching, but auxin or changes in the balance between the several GAs that are present in the potato plant do not affect tuber yield. Under tuber inductive conditions, the side shoots can result in tubers grown in the side of the stolon. It would be very interesting to study methods that can regulate stolon branching, depending on the desired results, small tubers that are suitable for seed potatoes or bigger tubers that are orientated for the food market. Research presented in this thesis is only the base for understanding and controlling auxin mediated stolon and tuber development.

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Summary

Potato (*Solanum tuberosum*) is one of the most important crop plants. It has great nutritional value for man and is used in industry in a wide variety of applications. Among agricultural commodities, potato is ranked 7th with a value of over 44 billion dollars at a worldwide scale. The edible part of the potato plant is the potato tuber that grows at the tips of etiolated shoots, called stolons. Study of the physiology of tuber initiation revealed a role for gibberellins (GAs) in tuber initiation. GA degradation genes are up-regulated prior to visible swelling and and GA levels decrease rapidly at the early stages of tuber induction. A more detailed investigation of the expression pattern of genes during early stages of tuber development with the use of a microarray chip, provided new insight on the phytohormone related genes involved in tuber initiation and development. Several auxin related genes exhibited a differential expression pattern, indicating that auxin might be involved in tuber organogenesis. Auxin is one of the most important and well studied phytohormones, with a role in many developmental events, such as embryo patterning, shoot branching and adventitious root formation. In several of these occasions, auxin mediated changes of the plane of cell division is an important feature to achieve organ development.

In this thesis we try to elucidate the role of auxin and its branching counterpart, Strigolactones (SLs) in tuber initiation and development. In addition, we study the physiological mechanisms that regulate stolon branching, and the effect of changes in the branching mechanism on tuber numbers and tuber yield. Furthermore, we study the effect of altered GA content on stolon branching and tuber development and yield.

In chapter one, we discuss the importance of the potato crop and introduce auxin, SLs and GAs, the biosynthesis pathways and their respective roles in plant development. In addition, we discuss the mechanisms that mediate shoot branching and the factors that regulate tuber initiation.

In chapter two, we used several different approaches to investigate the role of auxin in tuber initiation and development. Auxin applications inhibited *in vitro* tuber initiation when IAA was renewed in the medium. On the other hand, when a pulse of auxin was provided to the *in vitro* explants, the rate of tuberisation was increased. Measurements of the auxin content in *in vivo* stolon tips revealed that auxin content peaked just prior to tuber swelling. In addition, expression studies of auxin biosynthesis genes showed that at least one *YUC-like* gene that is likely to be involved in auxin biosynthesis, has a peak in expression prior to the increase in IAA content. Radiolabeled IAA transport assays indicated that auxin is polarly transported from the stolon tip to the basal part of the stolon. This finding indicates auxin is synthesized in the stolon tip. Furthermore, application of SLs in the *in vitro* tuberisation system strongly inhibited tuberisation, and measurements of strigolactone content in *in vivo* plants revealed that potato plants are able to produce SLs. Taken together, this data highlight a role for auxin in tuber development, and helped us to describe a mechanism for stolon branching analogous to the control of above ground shoot branching .

An earlier microarray chip experiment on potato tuber developmental stages had revealed that at least two *PIN-like* genes had a peak in expression during early stages of tuber development. In chapter three, we identify ten members of the *PIN* family of genes in potato based on

sequence similarity with known *PIN* genes. Using the RNA-seq data generated within the potato sequencing project, we investigate the expression pattern of the *PIN* family in various tissues in potato. Using the tuber developmental stages as described in chapter two, we investigated the expression pattern of the *PIN* gene family during the early stages of tuber development. We cloned the promoter regions of *StPINV* and *StPINVII*, and used them in promoter driven GUS constructs in order to visualise the locations of expression of those two *PIN* genes in various tissues of the potato plant such as flowers, roots and swelling stolon tips. Finally, we combine the results of chapters two and three to discuss the role of auxin distribution in tuber initiation and development.

GAs is a group of phytohormones that is known be involved in tuber initiation. In chapter four, we report the cloning of *StGA3ox2* and the use of this gene in an RNAi construct to silence its expression in potato. *StGA3ox2* is known to be involved in the degradation of active GAs into inactive forms. *StGA3ox2* RNAi plants exhibited shorted shoot length, smaller leaf size and smaller average tuber weight, due to increased number of tubers per plant. The content of several GAs was determined as well as the expression of GA biosynthesis and degradation genes in the transgenic clones and reveals changed metabolic fluxes of GA pathways due to the silencing of *StGA30x2* expression. Finally, we discuss our results with respect to the current literature on the role of GA in tuber initiation stolon architecture and tuber development.

In chapter five, we investigated the role of *StYUC-like1* gene in tuber initiation and development. *StYUC-like1* was identified to have a peak in expression during tuber induction and prior to tuber swelling in the tuber developmental stages from chapter two. *StYUC-like1* was cloned into a CaMV35S over-expression vector and transformed into potato plants. The transformed clones exhibited enhanced shoot and stolon branching, enhanced lateral root formation and decreased average tuber weight due to increased number of tubers per plant. In addition, one of the transgenic clones exhibited a cracked tuber phenotype. Measurements of the IAA content in the transgenic clones revealed that the transgenic clones had less auxin in the basal stem, providing explanation for the phenotype observed that are consistent with the lower auxin content phenotypes. Furthermore, we discuss the role of auxin biosynthesis in stolon branching and how it impacts on the number of tubers that are produced.

In chapter six, we summarise the most important results of this thesis. We discuss the role of auxin in tuber development based on the auxin content peak and the possible interaction with GA to regulate tuber initiation and growth. In addition, we portray an auxin mediated model, similar to the one that is present in shoots, that controls stolon branching. Furthermore, the effect of altered GA and IAA content on the number of tubers and tuber yield is discussed. Finally, we present the future prospects of the work on tuber initiation and development research as well as from an agronomic point of view.

Samenvatting

Aardappel (Solanum tuberosum) is een van de belangrijkste landbouwgewassen wereldwijd (rang 7) met een economische waarde van meer dan 44 miljard dollar. Het heeft een hoge voedingswaarde voor de mens alsook een grote verscheidenheid aan industriele toepassingen. Het eetbare deel van de aardappelplant is de knol die groeit op de toppen van geëtioleerde scheuten, de zogenaamde stolonen. In eerdere studies naar de gebeurtenissen voorafgaand en tijdens aardappelknolinitiatie werd al snel een centrale rol voor het plantenhormoon Gibberelline (GA) gevonden. GA degradatie genen worden al op-gereguleerd nog voordat de eerst stolon zwellingen zichtbaar zijn, met als gevolg dat GA niveaus snel afnemen tijdens de eerste stadia van knolvorming. Een meer gedetailleerd onderzoek naar de expressie van genen tijdens de eerste stadia van knolontwikkeling met behulp van een microarray, resulteerde in nieuwe inzichten met betrekking tot hormoon gerelateerde genexpressie. Naast GA genen werden ook verschillende auxine (IAA) gerelateerde genen geidentificeerd met differentiële expressie tijdens knolvorming. Dit resulteerde in de aanname dat er ook een regulerende rol voor auxine is weggelegd tijdens aardappelknolvorming. Auxine is een van de belangrijkste en best bestudeerde fytohormonen, met een rol in veel ontwikkelingsgebeurtenissen zoals embryo ontwikkeling, stengelvertakkingen en adventieve wortelvorming. In een aantal van deze processen is een verandering van de orientatie van celdeling een belangrijk kenmerk waarin auxine een belangrijke rol lijkt te spelen.

In dit proefschrift proberen we de rol voor auxine en zijn tegenhanger m.b.t stengelvertakkingen, strigolactonen (SLs) in de regulatie van knol initiatie te ontrafelen. Daarnaast bestuderen we de fysiologie van stolonvertakkingen, en het effect van veranderingen in stolonvertakking op knol aantallen en opbrengst. Ook doen we onderzoek naar de invloed van veranderde GA concentraties op stolonen vertakking, knolontwikkeling en opbrengst.

In hoofdstuk 1 bespreken we het belang van de aardappel als voedings gewas en beschrijven we de factoren van belang voor aardappelknol inductie. Daarnaast introduceren we de biosynthese routes van de planten hormonen auxine, SL en GA en hun functies in relatie tot plant ontwikkeling. Ook wordt het mechanisme van stengelvertakkingen besproken met de focus op bovengenoemde planten hormonen.

In hoofdstuk 2 gebruiken we verschillende onderzoeksmethoden om de rol van auxine te begrijpen tijdens het proces van knolinitiatie en ontwikkeling. Applicatie van auxine (IAA) aan het medium remt in vitro knol inductie als de auxine in het medium op tijd wordt ververst. Anderzijds, wanneer een enkele puls van auxine aan in vitro explantaten word gegeven, lijdt dit uiteindelijk tot een versterkte knolintiatie phenotype. Uit metingen van de auxine concentratie in de stolon tips bleek dat auxine piekt vlak voor stolonzwelling. Expressie studies van auxine biosynthese genen toonde aan dat ten minste een van de YUC-achtige genen, betrokken bij de biosynthese van auxine, een piek in expressie heeft nog voor de piek in auxine concentratie. Met radioactief gelabeld IAA wordt aangetoond dat auxine polair wordt getransporteerd van de stolon tip naar het basale deel van de stolonen. Op basis van dit resultaat kan worden geconcludeerd dat auxine in de stolon tip wordt geproduceerd. De applicatie van Strigolactones (SL) in het in vitro tuberisation systeem resulteerde in een

sterke inhibitie van knolzetting en via metingen van de strigolactone concentratie in in vivo planten wordt voor het eerst aangetoond dat aardappelplanten strigolactones kunnen produceren. Al deze gevens tesamen tonen een rol voor auxine in knol ontwikkeling, alsook een rol in stolonvertakkingen opeenzelfde manier zoals beschreven in bonvengrondse stengels.

Uit een eerdere microarray experiment met verschillenden aardappel ontwikkelingsstadia bleek dat ten minste twee PIN-achtige genen, betrokken bij auxin transport, een piek in expressie vertoonden tijdens de vroege stadia van knolontwikkeling. In hoofdstuk drie, beschrijven we de identificatie van tien leden van de PIN-familie van genen in aardappel op basis van sequentie-overeenkomst van beschreven PIN-genen uit andere species. Met behulp van de RNA-seq data, gegenereerd binnen het aardappel-sequencing project , onderzoeken we het expressiepatroon van de PIN-familie in de verschillende weefsels van de aardappelplant. Ook wordt de expressie van de verschillende PIN genen gevolgd tijden verschillende knolontwikkelingsstadia. De promoters van twee pin familie leden zijn gekloneerd en localisatie van expressie in de aardappel plant is uitgevoerd via GUS reporter constructen en gevisualiseerd in verschillende weefsels van de aardappelplant zoals bloemen, wortels en stolon tips. Aan het eind van hoofdstuk 3 worden de resultaten voor auxine distributie in de aardappel besproken in relatie tot de resultaten van hoofdstuk 2.

Gibbereline (GA) is een groep van fytohormonen waarvan bekend is dat ze betrokken zijn bij knolinitiatie. In hoofdstuk vier beschrijven we de klonering van StGA3ox2 en het gebruik van dit gen in een RNAi construct om StGA3ox2 expressie te verlagen. Voor StGA3ox2 is bekend dat het betrokken is bij de afbraak van actieve GAs naar inactieve vormen. StGA3ox2 RNAi planten vertoonde verkorte stengels, kleiner blad en een lager gemiddeld knolgewicht, dit laatste met name als gevolg van toename van het aantal knollen per plant. De veranderde concentraties van GAs en de expressie van GA biosynthese en afbraak genen in de transgene klonen, laat zien hoe de GA metabole routes veranderen als gevolg van de verlaagde expressie van StGA3ox2. Tot slot bespreken we onze resultaten over de rol van GA in knolinitiatie en ontwikkeling in relatie tot de huidige literatuur.

In hoofdstuk vijf onderzoeken we de rol van StYUC-like1 gen, een auxin biosynthese gen, in knol initiatie en ontwikkeling. StYUC-like1 werd geïdentificeerd met een piek in expressie tijdens knolinitiatie in de ontwikkelingsstadia zoals beschreven in hoofdstuk twee. StYUC-like1 werd gekloneerd in een CaMV35S over-expressie vector en getransformeerd in aardappelplanten. De getransformeerde klonen vertoonden versterkte stengel en stolonvertakkingen en meer laterale wortelvorming alsook en een lagere gemiddelde tuber gewicht door een verhoogd aantal knollen per plant. Een van de transgene klonen vertoonde ook een interessant gebarsten knol phenotype. Uit metingen van de IAA concentratie in de transgene klonen bleek dat de transgene planten minder auxine ophoopten in het basale stengel gedeelte, wat overeenkomt met de verhoogde aantal stengel vertrakkingen in dit gedeelte van de plant. Daarnaast beschrijven we de rol van auxine biosynthese in de regulatie van stolonen vertakking en hoe dit relateerd aan het aantal knollen die worden gevormd.

In hoofdstuk zes, vatten we de belangrijkste resultaten van dit proefschrift samen. We bespreken de rol van auxine bij knol ontwikkeling op basis van de auxine concentratie piek en de mogelijke interactie met GA om het proces van knol initiatie en groei te reguleren. We

beschrijven een model voor stolon vertakkingen overeenkomstig met het model zoals beschreven voor bovengrondse stengelvertakkingen met een centrale rol voor auxine. Bovendien wordt het effect van gewijzigde GA en IAA inhoud op het aantal knollen en knolopbrengst besproken. Tot slot presenteren we de onderzoeks mogelijkheden op het gebied van knol initiatie en ontwikkeling vanuit een landbouwkundig oogpunt.

Acknowledgements

I find it very difficult to make a selection of the people that I would like to thank for their help and support those four years. I will try to keep the acknowledgements short, hoping I will not forget someone.

The first person I would like to thank is my promoter, Prof. Dr. Richard Visser. I still remember the first attempts to communicate with him and see if a PhD for me would be possible sometime back in 2007. I would like to thank him for giving me the opportunity to get a PhD in Wageningen. I can now see that it has been a long way, and that this opportunity that was given in 2007 earned me experiences of a lifetime.

There is not enough to say about Christian Bachem, my co-promoter. I feel lucky that I had such a co-promoter during my PhD. It has been a long trial for me, and my learning curve took long to enter its exponential phase, but even during disagreements Christian has always been the voice of reasoning that would bring things back on track. I know many PhD students would like to be in my shoes and have Christian as their supervisor.

Bjorn Kloosterman was my daily supervisor for most of my PhD. The realization of this PhD was greatly part of his work as well. His guidance in the lab, in the greenhouse and in planning has been crucial in all stages of this PhD. Bjorn guided me carefully through many day to day steps in this PhD. Without Bjorn, I find it difficult to imagine how this PhD would have developed.

One more person that deserves a special paragraph is Marian Oortwijn. In so many cases you have been much more than a teacher in the lab with patience and helpful tips to make things work. I would like to thank you for all the things you taught me and for all your valuable help those four years.

Natalia, Giulia and Victoria, thanks not only for accepting me as a friend but also for accepting me in this madhouse you call house. Living in the living room or in the attic, doesn't matter, it is nice when you spend your time with friends. Antoine, wish I had the chance to be in that same house when you where there too!

There is a series of people I would like to thank for being there these four years. Arwa, Nasim, Freddy, Ping-Ping, my MSc student Wendy, Χρήστο, Μιχάλη, Νίκο, Matieu, Yusuf, Marcel, Alireza, Martin. Dirkjan I am done with my plants in the greenhouse. Wouter, I look forward to getting a chance to work with you again.

Special thanks to Harro J. Bouwmeester. Your input and expertise has been crucial for us. Francel and Tatsiana, thank you for your help with things we cannot do.

Θα ήθελα να ευχαριστήσω τους φίλους μου στην Ελλάδα που ήταν η βαλβίδα ασφαλείας μου όλα αυτά τα χρόνια. Στράτο, Τεό, Κώστα και Γωγώ, Κώστα, Αλέκο, Θανάση, Αντώνη, Αλίκη, Λίνα.

Ένα μεγάλο ιδιαίτερο ευχαριστώ στους γονείς μου, Πάνο και Χρηστίνα και τον αδερφό μου Κώστα. Σας ευχαριστώ για την υποστήριξή σας και την υπομονή σας, όχι μόνον αυτά τα τέσσερα χρόνια.

Πένη, δεν ξέρω πώς να σου πω ευχαριστώ για όλα. Έχεις υπάρξει κάτι περισσότερο από μια σύντροφος αυτά τα 9μιση χρόνια. Έχεις συμβάλει σε αυτό το διδακτορικό περισσότερο από όσο μπορείς να καταλάβεις.

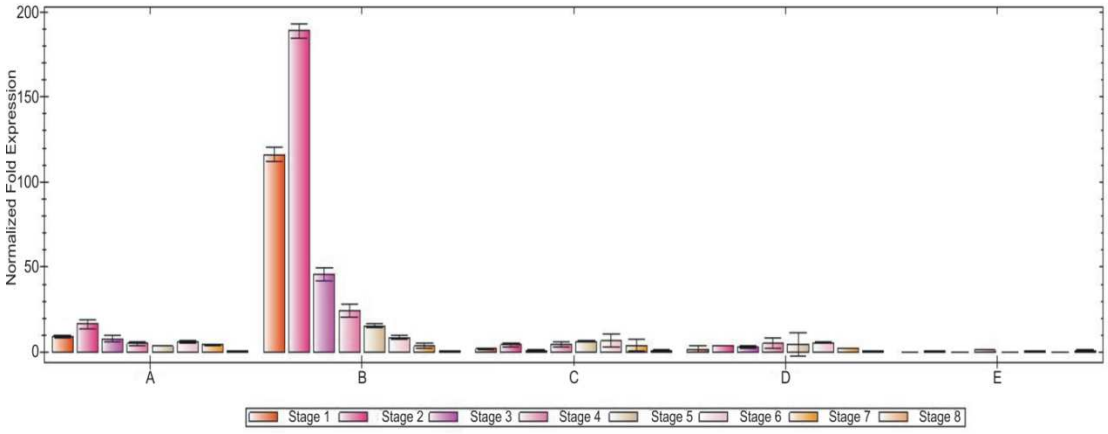
Curriculum vitae

Efstathios Roumeliotis was born on the 6th of August 1982 in Athens, Greece. In 2000 he graduated senior high school and participated in the national exams to enter the university. With a grade of 17.0 he succeeded to enter to the Agricultural University of Athens, in the department of Crop Production. He graduated the 5 year training in spring of 2006 with a grade of 6.97 with specialisation in Crop Protection and Environment. After fulfilling his military obligations and working for a short period of time in a business producing agricultural machinery, in summer 2007 he was granted a scholarship by the Bakalas bros. foundation for three years to continue his studied as a PhD student in Wageningen University. In 2011 he was granted a grant by Stichting Veenhuizen-Tulp fonds for one year to complete his research. This thesis is the result of four years of work in the field of potato tuber physiology and development.

Supplementary data

Chapter 2

Supplementary Figure 2.1. Expression analysis of five StYUC-like genes during early stages in tuber development. Stage 1; stolon tip grown under long day condition (16h) light. Stage 2; Stolon tip harvested 5 days after switch (5 DAS) to short day conditions (8 h light). Stage 3; First visible swelling harvested 6-7 DAS. Stage 4; tuber initiation (7-8 DAS). Stage 5; Tuber size 1 with diameter of about 0.8 cm (9-10DAS). Stage 6; Tuber size 2 (11-12 DAS). Stage 7; Tuber size 3 (15DAS) and stage 8; Tuber size 4 (25 DAS). On the X axis, A corresponds to transcript number PGSC0003DMT400030255 in the potato genome (PGSC, 2011), B to PGSC0003DMT400038139 (StYUC-like1), C to PGSC0003DMT400092778, D to PGSC0003DMT400051713 and E to PGSC0003DMT400097007.



Chapter 3

Supplementary Table 3.1.Accession numbers of the Arabidopsis PIN genes used in alignment.

Arabidopsis	Accession number
AtPIN1	AF089084
AtPIN2	AF086906
AtPIN3	AF087818
AtPIN4	NM_126203
AtPIN5	Q9LFP6
AtPIN6	Q9SQH6
AtPIN7	NM_102156
AtPIN8	NM_121514

Supplementary Table 3.2. Primers for qRT PCR used in this study.

Primer number	sequence (in 5' --->3' order)
StPINIII F	AGGCAGCTCTACCACAAGGA
StPINIII R	CAAGCCCAACAAACAAAACC
StPINVII F	TCATCTAAAGGGCCAACACC
StPINVII R	GTTGTATAGCTCCCCGCTCA
StPINIVa F	ACCAATGCTCAGGGATCAAC
StPINIVa R	GTCAGCTGCAATGAATCGAA
StPINV F	GGGACCCACTGGACTGACTA
StPINV R	ACTTGCTGGAGGCATCTGTT
StPINI F	CCCCAAGCAGTGACATCTTT
StPINI R	CGAAATTATGGTGCCAACAA
StPINVI F	GAATCCGCATTTTCATCCTC
StPINVI R	CCCGTTATGTAAAGGCGTGT
StPINXa F	CAGCCGAGCTGTTTCCTAAC
StPINXa R	TTTTCGCCACACCATAATCA
StPINII F	ATAGCATGCGGAACCAAAAA
StPINII R	TGTCCCCTTAGTCCAACAGC
StPINXb F	TTGGCCTAACTTGGTCCTTG
StPINXb R	AAACATTGCCATTCCGAGAC
StPINVb F	ATGTCAAAGGCAGGAAGTGG
StPINVb R	GTGCCACCAGCCATTACTTT
elF3e F	GGAGCACAGGAGGAAGATGAAGGAG
elF3e R	CGTTGGTGAATGCGGCAGGAAGGAG

Supplementary Table 3.3. Primers used for cloning.

StPINV prom	**F**	**CACCaaagttccaagttcaatc**
	R	tttcgcaaaaaaaattgtcaaatag
StPINV gene	F	CACCaaaatgatcacttggcacga
	R	caatggaggccttcaaaa
StPINVII prom	F	CACCgtcacatcctcagaccaag
	R	tcaaatttcgagaatccaacaa
StPINVII gene	F	CACCatgcacaactaaatgagtatga
	R	aaaagagagaaaagtatgaaag

Supplementary Table 3.4. Genomic location of the *StPIN* genes.

PIN gene	Genomic area
StPINV	PGSC0003DMB000000051:1583809..1580797
StPINIII	PGSC0003DMB000000026:1618465..1622012
StPINIVa	PGSC0003DMB000000322:55773..58731
StPINXb	PGSC0003DMB000000379:54866..546988
StPINVII	PGSC0003DMB000000613:194621..197391
StPINXa	PGSC0003DMB000000008:4038046..4041324
StPINVI	PGSC0003DMB000000227:699831..693452
StPINI	PGSC0003DMB000000068:6791..3618
StPINII	PGSC0003DMB000000004:2324342..2326593
StPINIVb	PGSC0003DMB000000123:720022..721629

Supplementary Figure 3.1. Alignment of the StPIN and AtPIN proteins was performed with ClustalW2-Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/) , alignment type: slow, and alignment options: default ("*": identical, ":": conserved substitutions, ".": semi-conserved substitution).

```
StPINIII        MITLSDFYHVMTAVVPLYVAMILAYGSVKWWKIFSPDQCSGINRFVALFAVPLLSFHFIA 60
AtPIN1          MITAADFYHVMTAMVPLYVAMILAYGSVKWWKIFTPDQCSGINRFVALFAVPLLSFHFIA 60
StPINXa         MITVSDLYHVLTAVVPLYVAMILAYGSVKWWKIFSPDQCSGINRFVALFAVPLLSFHFIA 60
StPINXb         MISLSDLYHVLTAVVPLYVAMILAYGSVKWWKIFSPDQCSGINRFVALFAVPLLSFHFIA 60
StPINIVa        MISWHDLYVVLTAVVPLYVAMILAYGSVRWWKIFSPDQCSGINRFVAIFAVPLLSFHFIS 60
StPINV          MITWHDLYVVLTAVVPLYVAMILAYGSVRWWKIFSPDQCSGINRFVAIFAVPLLSFHFIA 60
AtPIN3          MISWHDLYTVLTAVIPLYVAMILAYGSVRWWKIFSPDQCSGINRFVAIFAVPLLSFHFIS 60
AtPIN7          MITWHDLYTVLTAVIPLYVAMILAYGSVRWWKIFSPDQCSGINRFVAIFAVPLLSFHFIS 60
AtPIN4          MITWHDLYTVLTAVVPLYVAMILAYGSVQWWKIFSPDQCSGINRFVAIFAVPLLSFHFIS 60
StPINVII        MINGKDIYDVLAAIIPLYVAMILAYGSVRWWKIFTPDQCSGINRFVAVFAVPLLGFHFIS 60
AtPIN2          MITGKDMYDVLAAMVPLYVAMILAYGSVRWWGIFTPDQCSGINRFVAVFAVPLLSFHFIS 60
StPINII         MISLRDVYHVVAATIPLYVVMILAYISVRWGKLFSPEQCSGINKFVAKFSIPLLSFQVIS 60
AtPIN8          MISWLDIYHVVSATVPLYVSMTLGFLSARHLKLFSPEQCAGINKFVAKFSIPLLSFQIIS 60
StPINVI         ------------MVPLYFAMIVAYGSVKWWKIFSPEQCSGINRFVAVFAVPVLSFHFIS 47
AtPIN6          ------------------------------------------------------------
StPINI          MIGWDDIYKVVVGMMPLYVALILGYGSVKWWHMFKPEQCDTINRFNCFFILPFFNFQFIA 60
StPINVb         MIEWLDIYKVIEAMMPLYLALGLGYGSVKWWHKLSAEHCDAINRLNYFFVLPFFTFDFIS 60
AtPIN5          MINCGDVYKVIEAMVPLYVALILGYGSVKWWHIFTRDQCDAINRLVCYFTLPLFTIEFTA 60

StPINIII        ANNPYTMNIRFIAADTLQKLIVLGVLAVWANVSK-----RGSLEWSITLFSLSTLPNTLV 115
AtPIN1          ANNPYAMNLRFLAADSLQKVIVLSLLFLWCKLSR-----NGSLDWTITLFSLSTLPNTLV 115
StPINXa         SNNPYAMNYRFIAADTLQKVIVLVVLAIWSRISS-----RGSLEWSITLFSLSTLPNTLV 115
StPINXb         SNNPYAMNYRFIAADTLQKIIVLFVLAIWSRVSS-----RGSLEWSITLFSLSTLPNTLV 115
StPINIVa        MNNPYEMNFRFIAADTLQKVIMLIVLCIWSNVTK-----NGSLEWSITIFSLSTLPNTLV 115
StPINV          MNNPYEMNFRFIAADSLQKVIMLVVLSLWANLTK-----NGSLEWSITIFSLSTLPNTLV 115
AtPIN3          TNNPYAMNLRFIAADTLQKIIMLSLLVLWANFTR-----SGSLEWSITIFSLSTLPNTLV 115
AtPIN7          SNNPYAMNLRFIAADTLQKLIMLTLLIIWANFTR-----SGSLEWSITIFSLSTLPNTLV 115
AtPIN4          TNDPYAMNFRFVAADTLQKIIMLVLLALWANLTK-----NGSLEWMITIFSLSTLPNTLV 115
StPINVII        TNDPYSMNYHFIAADSLQKVVILFALFIWHVFSK-----RGSLEWVITLFSLSTLPNTLV 115
AtPIN2          SNDPYAMNYHFLAADSLQKVVILAALFLWQAFSR-----RGSLEWMITLFSLSTLPNTLV 115
StPINII         GSNLYKVNLKLLLADFIQKFLAVFLLAIFAKLKP-----KGNLTWIITGLSVSTLPNTLI 115
AtPIN8          ENNPFKMSPKLILSDILQKFLVVVVLAMVLRFWHPTGGRGGKLGWVITGLSISVLPNTLI 120
StPINVI         QNNPYQMDTKFILADTLSKILVLVLLSVWAICK-------GQLDWLITLFSVSTLPNTLV 100
AtPIN6          ----------FILADTLSKIFVFVLLSLWAVFFK-----AGGLDWLITLFSIATLPNTLV 45
StPINI          NINPYNLNYLFLTGDVIAKALVILILVLWANFYR-----KGSFSWGITTFSLSTLNNTLV 115
StPINVb         QVNPYKMNYPFICGDLIAKAIIGFFLTLWANFYS-----KGNFSWSITTFSFCSLTNALV 115
AtPIN5          HVDPFNMNYRFIAADVLSKVIIVTVLALWAKYSN-----KGSYCWSITSFSLCTLTNSLV 115
                          :: .* : * .    * :            *   * ** :*.. * *:*:

StPINIII        MGIPLLKGMYGEFSGSLMVQIVVLQCIIWYTLMLFMFEFRGARLLISEQFP-DTAGSIVS 174
AtPIN1          MGIPLLKGMYGNFSGDLMVQIVVLQCIIWYILMLFLFEYRGAKLLISEQFP-DTAGSIVS 174
StPINXa         MGIPLLKGMYGDASGSLMVQIVVLQCIIWYTLMLFLFEYRGARMLIAEQFP-DTGGSIVS 174
StPINXb         MGIPLLKGMYGDASGSLMVQIVVLQCIIWYTLMLFLFEYRGARMLIAEQFP-DTGGSIIS 174
StPINIVa        MGIPLLIAMYGEYSGSLMVQVVVLQCIIWYTLLLFLFEFRGAKMLIMEQFP-ETAGEIVS 174
StPINV          MGIPLLIAMYGEYSGSLMVQVVVLQCIIWYTLLLFLFEYRGAKMLIMEQFP-ETAASIVS 174
AtPIN3          MGIPLLIAMYGEYSGSLMVQIVVLQCIIWYTLLLFLFEFRGAKMLIMEQFP-ETAASIVS 174
AtPIN7          MGIPLLIAMYGEYSGSLMVQIVVLQCIIWYTLLLFLFEYRGAKILIMEQFP-ETGASIVS 174
AtPIN4          MGIPLLIAMYGTYAGSLMVQVVVLQCIIWYTLLLFLFEYRGAKLLIMEQFP-ETGASIVS 174
StPINVII        MGIPLLKAMYGDFSGNLMVQIVVMQSVIWYTLMLFMFEYRGAKLLIGEQFP-ETAASITS 174
AtPIN2          MGIPLLRAMYGDFSGNLMVQIVVLQSIIWYTLMLFLFEFRGAKLLISEQFP-ETAGSITS 174
StPINII         LGIPLIKAIFGDAAAELLAQLIALQSLVWYNLLLLLFELN-----ATKES------YMMS 164
AtPIN8          LGMPILSAIYGDEAASILEQIVVLQSLIWYTILLFLFELN-----AARALP-SSGASLEH 174
StPINVI         MGIPLLNAMYGDFTQSLMVQLVVLQCIIWYTLLLFLFEYRAATILIKNQFPGNVAASITK 160
AtPIN6          MGIPLLQAMYGDYTQTLMVQLVVLQCIIWYTLLLFLFELRAARLLIRAEFPGQAAGSIAK 105
StPINI          VGVPLMKAMYGDLGVDLVVQAAVIQALLWLTSLLFALEFWKTKMNNNINGI--------- 166
StPINVb         MGIPVMNAMSPQVGVDLVIQSLAIQFLIWSIIIQFMMELKNAKDEIMACEG--------- 166
AtPIN5          VGVPLAKAMYGQQAVDLVVQSSVFQAIVWLTLLLFVLEFRKAGFSSNNISD--------- 166
                :*:*:  .:       :: *  .:* ::*   : : :*
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StPINIII        IHVDSDVMSLDGRQVLETEAELKEDGKLHVTVRKSNASRSDIFSRRS-----GGFSSTTP 229
AtPIN1          IHVDSDIMSLDGRQPLETEAEIKEDGKLHVTVRRSNASRSDIYSRRS-----QGLS-ATP 228
StPINXa         FKIDSDVISLDGKEPLETQAEVGDDGKLHVVVRKSTSSRSEIFSRMSH-GHNTGGLSMTP 233
StPINXb         FKVDSDIISLDGKEPLETQAEVGDDGKLHVTVRKSTSSRSEIFSRRSH-GPNSG-LSLTP 232
StPINIVa        FKVESDVVSLDGQDFLETDAELGQDGKLHVTVRKSNVSRRSFAM--------------TP 220
StPINV          FKVESDVVSLDGHDFLETDAEIGQDGKLHVTVRKSNASRRSFAM--------------DH 220
AtPIN3          FKVESDVVSLDGHDFLETDAEIGDDGKLHVTVRKSNASRRSFCG-----------PNMTP 223
AtPIN7          FKVESDVVSLDGHDFLETDAQIGDDGKLHVTVRKSNASRRSFYGGGG--------TNMTP 226
AtPIN4          FKVESDVVSLDGHDFLETDAEIGNDGKLHVTVRKSNASRRSLMM--------------TP 220
StPINVII        FRVESDVISLNGREPLQADAEIGDDGKLHVIVRRS-SASSIISSYNK---GILQSNNMTP 230
AtPIN2          FRVDSDVISLNGREPLQTDAEIGDDGKLHVVVRRSSAASSMISSFNKSHGGGLNSSMITP 234
StPINII         PSEVAVELEVPGEPELEKD---------------------------------------- 183
AtPIN8          TGNDQEEANIEDEPKEEEDEEE------------------------------------- 196
StPINVI         FEIDNDVISLDGRNPLCTESEINGNGRIHVRIRRSTSSAPESAFSSS--------IGITP 212
AtPIN6          IQVDDDVISLDGMDPLRTETETDVNGRIRLRIRRSVSSVPDSVMSPS--------LCLTP 157
StPINI          ANNNSVELGNINTTTQM------------------------------------------ 183
StPINVb         ANQDLEGNDNNNAS--------------------------------------------- 180
AtPIN5          VQVDNINIESGKRE--------------------------------------------- 180

StPINIII        RPSNLTNAEIYSLQSSRNP-TPRGSSFNHTDFYSMVAGTAGRNSNFG-ANDVYGMSN--- 284
AtPIN1          RPSNLTNAEIYSLQSSRNP-TPRGSSFNHTDFYSMMASGGGRNSNFGPGEAVFG------ 281
StPINXa         RPSNLSNTEIYSLQSSRNM-TPRDSNFNHNDIYSMVNGKNN--ATMSPRTSNFG------ 284
StPINXb         RPSNLTNAEIYSLQSSRNP-TPRGSSFNHTDFYSMVNGKN---ANMSPRNSNFG------ 282
StPINIVa        RPSNLTGAEIYSLNSSRNP-TPRGSNFNHTDFYAMMGFPG-RLSNFGPADS--------- 269
StPINV          RPSNLTGAEIYSLSSSRNP-TPRGSNFNHNDFYSMMGFPGGRLSNFGPADMYSVQS---- 275
AtPIN3          RPSNLTGAEIYSLST-----TPRGSNFNHSDFYNMMGFPGGRLSNFGPADMYSVQS---- 274
AtPIN7          RPSNLTGAEIYSLNT-----TPRGSNFNHSDFYSMMGFPGGRLSNFGPADMYSVQS---- 277
AtPIN4          RPSNLTGAEIYSLSS-----TPRGSNFNHSDFYSVMGFPGGRLSNFGPADLYSVQS---- 271
StPINVII        RASNLTGVEIYSVQSSREP-TPRASSFNQSDFYAMF-ASKTASPKHGYTNSYG------- 281
AtPIN2          RASNLTGVEIYSVQSSREP-TPRASSFNQTDFYAMFNASKAPSPRHGYTNSYGGAGAGPG 293
StPINII         ------------------------------------------------------------
AtPIN8          ------------------------------------------------------------
StPINVI         RASNLSNAEIFSVHTP---------------LHNG--DIPFGHGDLG-VGFR-------- 246
AtPIN6          RASNLSNAEIFSVNTPNNRFFHGGGGSGTLQFYNGSNEIMFCNGDLGGFGFTRPGL---- 213
StPINI          ------------------------------------------------------------
StPINVb         ------------------------------------------------------------
AtPIN5          ------------------------------------------------------------

StPINIII        -------NSRGPTPRPSNYEEESGK---SRFNNYLGGAAAPAPTQSNSN-------TNYP 327
AtPIN1          --------SKGPTPRPSNYEEDGGP---AKP-TAAGTAAGAGRFHYQSGGSGGGGGAHYP 329
StPINXa         ---------------NLGFDEES------GFGKTN---------------------VGYP 302
StPINXb         ---------------NYGHDEESGV---AGFGRGNGVYGQGN--------------AGYP 310
StPINIVa        ------------TPRPSNFEENCTQGALT-SSPKFGFYPAQS---------------NYP 301
StPINV          --------SRGPTPRPSNFEENCAPGGLVQSSPRFGYFPAQQP-----------APGSYP 316
AtPIN3          --------SRGPTPRPSNFEENCA----MASSPRFGYYPGG-------------GAGSYP 309
AtPIN7          --------SRGPTPRPSNFEESCA----MASSPRFGYYPGG-------------APGSYP 312
AtPIN4          --------SRGPTPRPSNFEENNA----VKYGFYNNTNSSVP------------AAGSYP 307
StPINVII        GDVFSVQSSKGPTPRTSNFEEEMSKIGSNKKNRGGRSMSGELYNSGSNAST---NGLVYP 338
AtPIN2          GDVYSLQSSKGVTPRTSNFDEEVMKT-AKKAGRGGRSMSGELYNNNSVPS--------YP 344
StPINII         ------------------------------------------------------------
AtPIN8          ------------------------------------------------------------
StPINVI         ---------AASTRLSGGYASSDAYS-------------------------------LQP 266
AtPIN6          ---------GASPRRLSGYASSDAYS-------------------------------LQP 233
StPINI          ------------------------------------------------------------
StPINVb         ------------------------------------------------------------
AtPIN5          ------------------------------------------------------------

StPINIII        AP-NPGMFS----PSNNG------TKAHKNTA------KKGD--QEG-GKDLHMFVWSSS 367
AtPIN1          AP-NPGMFS----PNTGGG---GGTAAKGNAP------VVGGKRQDGNGRDLHMFVWSSS 375
StPINXa         APTNAGIFS----PVTGG----PGTKKKANG-------TESG-------KDLHMFVWSSS 340
StPINXb         APTNAGIFS----PATG-----PVTKKKANGG------TEGG-------KDLHMFVWSSS 348
StPINIVa        AP-NPEIASIVPKNTKNQQ-----QLQVHSQHQHQQQQQQNGKAS-HDAKELHMFVWSSS 354
StPINV          AP-NPEISSAVPKSTKPQQPNVQTQKQEVQQQQQQQQQQPNAKANNHDAKELHMFVWSSS 375
AtPIN3          AP-NPEFSSTTTSTANKSV----NKNPKDVNTNQQTTLPTGGKSNSHDAKELHMFVWSSN 364
AtPIN7          AP-NPEFS-----TGNKTG----SKAPKENHH-------HVGKSNSNDAKELHMFVWGSN 355
AtPIN4          AP-NPEFSTG---TGVSTKP---NKIPKENQQQLQ----EKDSKASHDAKELHMFVWSSS 356
StPINVII        PP-NPMFSGQRKKEVGSGS-----GVPTPVSVPVPVPIPVPMSNSNNNSKELHMFVWSSS 392
AtPIN2          PP-NPMFTG----STSGAS-----GVKKKESGGGGSGGGVGVGGQN---KEMNMFVWSSS 391
StPINII         ------------------------------------------------------------
AtPIN8          ------------------------------------------------------------
StPINVI         TPRGSNFNELDTITVTTSGN-----------------------------TPMWVMSPV 295
AtPIN6          TPRASNFNELD---VNGNG-----------------------------TPVWMKSPA 258
StPINI          ------------------------------------------------------------
StPINVb         ------------------------------------------------------------
AtPIN5          ------------------------------------------------------------
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StPINIII        NSPVSD----VFGGHDYTANLDQP---AAPNKDVRVPISPGKVEGQ-------------- 406
AtPIN1          ASPVSD----VFGGGGGNHHADYS---TATN-DHQKDVKISVPQGN-------------- 413
StPINXa         ASPVSEGGIHVFRGG-DFGNELGI---GHHSKDYDDFGREEFSLGD-------------- 382
StPINXb         ASPVSEGGIHVFRGGGDYGNELGV---GAHPKDYDEFGREEFTFGN-------------- 391
StPINIVa        ASPVSEAAGLHVFGGTDFSANEQS---CQSDGAKEIRMLVSDHPQSGDNK--VISQD--F 407
StPINV          NSPVSEAGGLHVFGGNDFSANEQS---GRSDGAKEIRMLVSDHTQNGDSK--AIPQTGEF 430
AtPIN3          GSPVSDRAGLNVFGGAPDNDQGGR---SDQG-AKEIRMLVPDQSHNGETKAVAHPASGDF 420
AtPIN7          GSPVSDRAGLQVDNGA--NEQVGK---SDQGGAKEIRMLISDHTQN------AGPMNGDY 404
AtPIN4          ASPVSDVFG----GGAGDNVATEQ---SEQG-AKEIRMVVSDQPRK--------SGGDDI 400
StPINVII        ASPTSEGNHKHAINRGDSSELG-----VLDASKAVLQQEIAAAREN-------------- 433
AtPIN2          ASPVSEANAKNAMTRGSSTDVSTDPKVSIPPHDNLATKAMQNLIEN-------------- 437
StPINII         ------------------------------------------------------------
AtPIN8          ------------------------------------------------------------
StPINVI         AGKVFK------QASPSSKMAWES-----SCLNGERQGYRDDVGEK-------------- 330
AtPIN6          AGRIYR------QSSP--KMMWES-----GQRHAAKD-INGSVPEK-------------- 290
StPINI          ------------------------------------------------------------
StPINVb         ------------------------------------------------------------
AtPIN5          ------------------------------------------------------------

StPINIII        ---------------RNNNQENYMERDDFSFANRDGADQMNNQDGEKAGENK---AKVM 447
AtPIN1          ---------------SNDNQ--YVEREEFSFGNKDDDSKVLATDGGNNISNKTTQAKVM 455
StPINXa         ---------------KNNSNGCHREEPVLKKLGSSSTAELFP-----NTAN-ETKATAM 420
StPINXb         ---------------KQNLNGNDREGPVVR---SSSTTELRP-----KIAQEETKATAM 427
StPINIVa        GGEEFSFGGDVGGG-DGKNKDEKKEEKEGLTGLNTGAT-----------GVQDSGTGKQM 455
StPINV          GGEDFTFGGANGGGKDGDEEKGEKEGPTGLTKLGSSSTSELHPKLA---GGQDAGMGKQM 487
AtPIN3          GGEQQFSF-----AGKEEEAERPKDAENGLNKLAPNSTAALQSKTGL--GGAEASQRKNM 473
AtPIN7          GGE-------------EESERVKEVPNGLHKLRCNSTAELNPKEAI--ETGETVPVKHM 448
AtPIN4          GGLDS-----------GEGEREIEKATAGLNKMGSNSTAELEAAGGD--GGGNNG--THM 445
StPINVII        ----------------ASSVSKGNVEKEIEIEDGSK---------NMEDG---EKKSQM 464
AtPIN2          ----------------MSPGRKGHVEMDQDGNNGGKSPYMGKKGSDVEDGGPGPRKQQM 480
StPINII         ------------------------------------------------------------
AtPIN8          ------------------------------------------------------VAIV 200
StPINVI         ----------------EISFRDISNFPVQEVGAA-----------DSLNTNDIIKQEM 361
AtPIN6          ----------------EISFRDALKAAPQATAAGGGASMEEGAAGKDTTPVAAIGKQEM 333
StPINI          ----------------------------------------------------------RNI 186
StPINVb         ------------------------------------------------------------
AtPIN5          ------------------------------------------------------------TV 182

StPINIII        PPTSVMTRLILIMVWRKLIRNPNTYSSLFGLTWSLVSFRWNLKMPAIIAQSISILSDAGL 507
AtPIN1          PPTSVMTRLILIMVWRKLIRNPNSYSSLFGITWSLISFKWNIEMPALIAKSISILSDAGL 515
StPINXa         PAASVMTRLILIMVWRKLIRNPNTYSSLLGLAWSLISFRWNIQMPLIFAKSISILSDAGL 480
StPINXb         PPASVMTRLILIMVWRKLIRNPNTYSSLIGLTWSLVSFKWNVQMPAIIAKSISILSDAGL 487
StPINIVa        PPASVMTRLILIMVWRKLIRNPNTYSSLIGLIWSLISYRWHVHMPKIIEKSISILSDAGL 515
StPINV          PPASVMTRLILIMVWRKLIRNPNTYSSLIGLIWSLISFRWHVHMPKIIEKSISILSDAGL 547
AtPIN3          PPASVMTRLILIMVWRKLIRNPNTYSSLIGLIWALVAFRWHVAMPKIIQQSISILSDAGL 533
AtPIN7          PPASVMTRLILIMVWRKLIRNPNTYSSLIGLIWALVAFRWDVAMPKIIQQSISILSDAGL 508
AtPIN4          PPTSVMTRLILIMVWRKLIRNPNTYSSLIGLIWALVAYRWHVAMPKILQQSISILSDAGL 505
StPINVII        PPASVMTRLILIMVWRKLIRNPNTYASLIGLIWSLVSFRWNIQMPSIVKGSISILSDAGL 524
AtPIN2          PPASVMTRLILIMVWRKLIRNPNTYSSLFGLAWSLVSFKWNIKMPTIMSGSISILSDAGL 540
StPINII         ------------------------------------------------------------
AtPIN8          RTRSVGTMKILLKAWRKLIINPNTYATLIGIIWATLHFRLGWNLPEMIDKSIHLLSDGGL 260
StPINVI         PNALVMLRLIIVMVGRKLSRNPNTYSSILGLLWSLISFKWNVGMPSLVKYSIKIISDAGL 421
AtPIN6          PSAIVMMRLILTVVGRKLSRNPNTYSSLLGLVWSLISFKWNIPMPNIVDFSIKIISDAGL 393
StPINI          NNAELAFWPLMKAVSTKLAKNPNSYACFLGLFWALVASRWHFRMPSIIEGSILIMSKAGS 246
StPINVb         KNTTPSLGSVMTIVWTKLSKNPNFYACFLGIMWSLVADRWHFVLPNIVKECISIMSKAGS 240
AtPIN5          VVGEKSFLEVMSLVWLKLATNPNCYSCILGIAWAFISNRWHLELPGILEGSILIMSKAGT 242

StPINIII        GMAMFSLGLFMALQPRIIACGNSVASFAMAVRFLTGPAVMAAASIAVGLRGTLLHVAIVQ 567
AtPIN1          GMAMFSLGLFMALNPRIIACGNRRAAFAAAMRFVVGPAVMLVASYAVGLRGVLLHVAIIQ 575
StPINXa         GMAMFSLGLFMALQPKMISCGKTIAAFSMAVRFISGPAVMAAASFAIGLRGVLLHIAIVQ 540
StPINXb         GMAMFSLGLFMALSPRIIACGKTIALFSMGVRFLTGPAVMAAASIAVGLRGVLLHIAIVQ 547
StPINIVa        GMAMFSLGLFMALQPKIIACGNTVATFAMAVRFLTGPAVMAAASIIVGLRGTLLHVAIVQ 575
StPINV          GMAMFSLGLFMALQPKIIACGNTVATFAMAVRFLTGPAVMAAASIAVGLRGTLLHVAIVQ 607
AtPIN3          GMAMFSLGLFMALQPKLIACGNSVATFAMAVRFLTGPAVMAVAAIAIGLRGDLLRVAIVQ 593
AtPIN7          GMAMFSLG---------------ESSFYSVSFFR------------------------ 527
AtPIN4          GMAMFSLGLFMALQPKIIACGNSVATFAMAVRFITGPAIMAVAGIAIGLHGDLLRIAIVQ 565
StPINVII        GMAMFSLGLFMALQPKIIACGKSVATFSMAVRFLTGPAVIAATSIAIGLRGVLLHVAIVQ 584
AtPIN2          GMAMFSLGLFMALQPKIIACGKSVAGFAMAVRFLTGPAVIAATSIAIGIRGDLLHIAIVQ 600
StPINII         -------GVFMASQASIIACGTKKAILAMALKFVLGPVLMAISSIAVGLRGQLFRLAIVQ 236
AtPIN8          GMAMFSLGLFMASQSSIIACGTKMAIITMLLKFVLGPALMIASAYCIRLKSTLFKVAILQ 320
StPINVI         GMAMFSLGLFMALQPRIIACGTKMATIGMAIRFIGGPLVMSAASIAVGLKGVRLHTAIVQ 481
AtPIN6          GMAMFSLGLFMALQPKMIPCGAKKATMGMLIRFISGPLFMAGASLLVGLRGSRLHAAIVQ 453
StPINI          GVAMFSMGLFMALQGKIIACGAALTIYAMILRFVVGPATMALGCVVLGLRGNVLRIAIIQ 306
StPINVb         GIGMFTIGVFVAMQQKVMAGGTGVIVFGLFLRFFIGPATMTIGSFVVGLHGNVLRASILQ 300
AtPIN5          GTAMFNMGIFMALQEKLIVCGTSLTVMGMVLKFIAGPAAMAIGSIVLGLHGDVLRVAIIQ 302
                       *                    : *.
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StPINIII        AALPQGIVPFVFAKEYNVHPDILSTGVIFGMLIALPITLVYYIFMGL-- 614
AtPIN1          AALPQGIVPFVFAKEYNVHPDILSTAVIFGMLIALPITLLYYILLGL-- 622
StPINXa         AALPQGIVPFVFAKEYSLHPDILSTGVIFGMLIALPITLVYYILLGL-- 587
StPINXb         AALPQGIVPFVFAKEYGVHPDILSTGVIFGMLVALPITLVYYILLGL-- 594
StPINIVa        AALPQGIVPFVFAKEYNVHPAILSTX---------------------- 601
StPINV          AALPQGIVPFVFAKEYNVHPAILSTAVIFGMLIALPITLVYYIILGL-- 654
AtPIN3          AALPQGIVPFVFAKEYNVHPAILSTGVIFGMLIALPITLVYYILLGL-- 640
AtPIN7          ---------------------------------------------
AtPIN4          AALPQGIVPFVFAKEYNVHPTILSTGVIFGMLIALPITLVYYILLGL-- 612
StPINVII        AALPQGIVPFVFAKEYNLHPDILSTAVIFGMLVALPITILYYVLLGV-- 631
AtPIN2          AALPQGIVPFVFAKEYNVHPDILSTAVIFGMLVALPVTVLYYVLLGL-- 647
StPINII         AALPQGIVPFVFAKEYNIHPTILSTGVIFGMLIAIPIALAYYFLLEI-- 283
AtPIN8          AALPQGVVPFVFAKEYNLHPEIISTGVIFGMLIALPTTLAYYFLLDL-- 367
StPINVI         AALPQGIVPFVFAREYGLHPDILSTGVIFGMLVSLPVTLLYYVLLGL-- 528
AtPIN6          AALPQGIVPFVFAREYNLHPDLLSTLVIFGMIVSLPVTILYYVLLGL-- 500
StPINI          AALPQAVTSFVYAQEYGLHADVLSTAVIVGTIISLPLLIAYYAILDIMP 355
StPINVb         VILSPYSLSFLNHTEY--------IVIII------------------- 321
AtPIN5          AALPQSITSFIFAKEYGLHADVLSTAVIFGMLVSLPVLVAYYAALEFIH 351
```
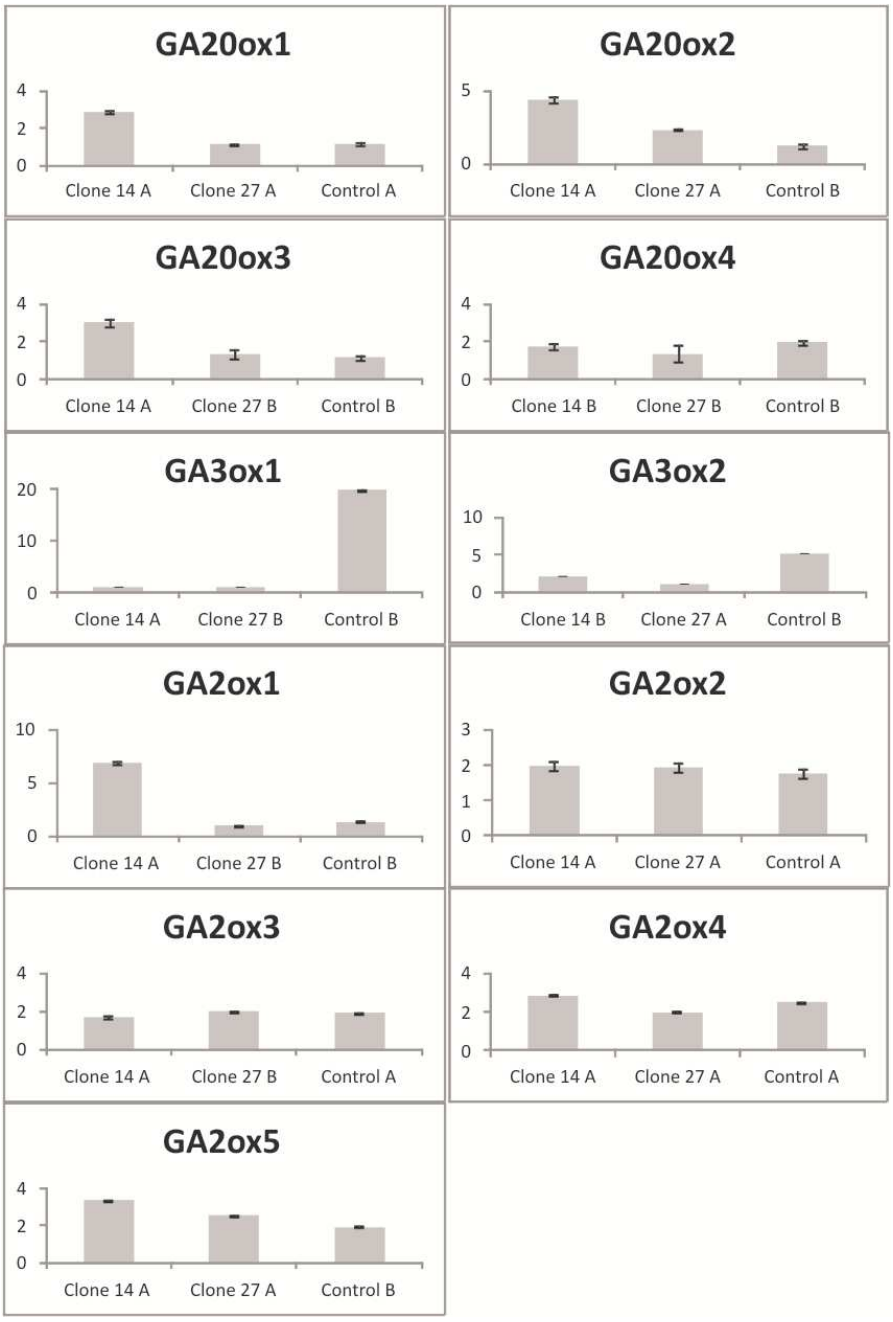

Chapter 4

Supplementary Table 4.1. PGSC transcript number and primer pair for the genes used in this study.

Gene	PGSC transcript number	Forward primer	Reverse primer	NCBI Accession number (when applicable)
StGA2ox 1	PGSC0003DMT400054348	AGGCACAGAGTGATCGCAGAT	TGGTGGCCCTCCAAAGTAAA	EU003995
StGA2ox2	PGSC0003DMT400034057	GACGAACGGAAGGTTTTTGA	ACAAGCTATCCTGGCATTGG	NM_001247936
StGA2ox3	PGSC0003DMT400054848	GGTGATGACAAATGGGAGGT	GGTGGCCCTCCAAAATAAAT	n/a
StGA2ox4	PGSC0003DMT400071043	CCAACAACACTTCCGGTCTT	CCTGTAATGAGTCGCCAACA	n/a
StGA2ox5	PGSC0003DMT400071094	ACTCTTGCAAATCCCCCTCT	CGATGAGGTTCTTGGAATCG	n/a
StGA3ox1	PGSC0003DMT400014555	CGAAATTGCTGGACCATAGG	TGGCCTTAGTGCCCAAATAC	FJ792644
StGA3ox2	PGSC0003DMT400054354	TATTTTCCCTTCCGATGCAG	GAAATTCGAGCAACCCCATA	FJ792643
StGA20ox1	PGSC0003DMT400062314	CGGCCCAACAAGCATCTAAG	AAGCCATGACTCCGACACG	AJ291453
StGA20ox2	PGSC0003DMT400071866	CTACAAGGCTCGTCCCACAT	TCGCATTGCAGTATTCTTGG	AJ291454
StGA20ox3	PGSC0003DMT400038382	TGACGAGAAGCCTTGTGTTG	GCAGGGTCACCAGAAAAGAA	AJ291455
StGA20ox4	PGSC0003DMT400000036	ATGGCCAGATGATGAAAAGC	CCGGAGAGATAGCCATTCAA	n/a
eIF3e	PGSC0003DMT400076704	GGAGCACAGGAGGAAGATGAAGGAG	CGTTGGTGAATGCGGCAGGAAGGAG	n/a

Supplementary Figure 4.1. Expression levels of StGA20ox, StGA3ox and StGA2ox genes in the two transgenic clones and the control.



Chapter 5

Supplementary Table 5.1. Sequences of the primers used in this study.

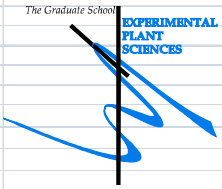
Primer Name	**sequence (in 5'----> 3' order)**
eIF3e F	GGAGCACAGGAGGAAGATGAAGGAG
eIF3e R	CGTTGGTGAATGCGGCAGGAAGGAG
StYUC-like1 F	TGTTTTGGACATTGGTGCAT
StYUC-like1 R	AACGGTGCCACATGAAAACT
StCCD7 F	TAATTTCCCCAACACCCTCA
StCCD7 R	GTCGAGCCACAAAGAATGGT
StCCD8 F	CCAACACCCTCACCAAGATT
StCCD8 R	ATCATCTTCCTCGGTTGCAC

Supplementary Table 5.2. Annotation numbers of the genes used in this study.

Arabidopsis gene	**Accession number**
AtYUC1	NM_119406.2
AtYUC2	NM_117399.3
AtYUC3	NM_100340.3
AtYUC4	NM_121170.2
AtYUC5	NM_123756.1
AtYUC6	NM_122473.2
AtYUC7	NM_128882.1
AtCCD7	NP_182026
AtCCD8	NP_195007
Potato gene	Transcript number or genomic region
StYUC-like1	PGSC0003DMT200038139
StYUC-like2	PGSC0003DMT200051713
StYUC-like3	PGSC0003DMT200030255
StYUC-like4	PGSC0003DMT200092778
StCCD7	PGSC0003DMB000000095: 705616..701464
StCCD8	PGSC0003DMB000000306:358370..362690
Tomato gene	Accession number
flavin monooxygenase-like protein	ACX33888.1
SlCCD7	NP_001234433
Petunia gene	Accession number
Dad/CCD8	AAW33596

Education statement

Education Statement of the Graduate School Experimental Plant Sciences



Issued to: Efstathios Roumeliotis
Date: 5 October 2012
Group: Plant Breeding, Wageningen University & Research Centre

1) Start-up phase		*date*
▶	**First presentation of your project**	
	Auxin interaction on potato tyber development (general)	Nov 2008
▶	**Writing or rewriting a project proposal**	
	Tuber development: a gene expression based approach to unraveling the prerequisites for tuber yield and quality	Jan 2009
▶	**Writing a review or book chapter**	
	list title if applicable	
▶	**MSc courses**	
	PBR 30306: Breeding for Resistance and Quality	Feb-Mar 2010
▶	**Laboratory use of isotopes**	
	Subtotal Start-up Phase	*13,5 credits**

2) Scientific Exposure		*date*
▶	**EPS PhD student days**	
	EPS PhD student day, Leiden University	Feb 26, 2009
	EPS PhD student day, Utrecht University	Jun 01, 2010
▶	**EPS theme symposia**	
	EPS Theme 1 symposium 'Developmental Biology of Plants', Leiden University	Jan 30, 2009
	EPS Theme 1 symposium 'Developmental Biology of Plants', Wageningen University	Jan 28, 2010
	EPS Theme 1 symposium 'Developmental Biology of Plants', Leiden University	Jan 20, 2011
▶	**NWO Lunteren days and other National Platforms**	
	ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 07-08, 2008
	ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 24-25, 2009
	ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19-20, 2010
	Plant Breeding Research Day	Feb 08, 2010
▶	**Seminars (series), workshops and symposia**	
	Ecology and EPS II	Sep 22, 2009
	Plant Sciences Seminar Climate change	Feb 08, 2011
▶	**Seminar plus**	
▶	**International symposia and congresses**	
	EUSOL meeting Toledo	Oct 05-08, 2009
	7th Solanacea conference, SOL 2010	Sep 05-08, 2010
	EUSOL meeting Natal	Nov 14-17, 2010
	Sol& IGuGI 2011meeting, Kobe, Japan	Nov 28-Dec 02, 2011
▶	**Presentations**	
	Poster presentation in EUSOL meeting	Oct 07, 2009
	Poster for Plant Breeding Research Day	Feb 08, 2010
	Poster ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19, 2010
	Oral presentation EUSOL meeting Natal	Nov 14-17, 2010
	Oral presentation SOL & ICuCI 2011	Nov 30, 2011
▶	**IAB interview**	Dec 04, 2009
▶	**Excursions**	
	Subtotal Scientific Exposure	*14,9 credits**

3) In-Depth Studies		*date*
▶	**EPS courses or other PhD courses**	
	Bootcamp Enterpreneuship	Jul 20-25, 2008
	Gateway to Gateway technology	Nov 17-22, 2008
	Basic statistics	Dec 14-22, 2009
▶	**Journal club**	
	Member of the literature discussion group at the Plant Breeding Group	2008-2011
▶	**Individual research training**	
	Subtotal In-Depth Studies	*7,2 credits**

4) Personal development		*date*
▶	**Skill training courses**	
	Cource: Personal efficiency	Apr 24 & May 22, 2008
	Cource: Scientific writing	May 09-Jun 24, 2010
▶	**Organisation of PhD students day, course or conference**	
▶	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*2,5 credits**

TOTAL NUMBER OF CREDIT POINTS*	**38,1**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

Layout: Efstathios Roumeliotis

Cover pictures: Gus expression driven by the StPINV promoter is detected in flowers (a), stolon tip (b), root tip (c) swelling tuber (d) and StPINVII expression was found the in flower bud (e), swelling stolon (f), root tip (g) and in swelling tuber (h). Pictures (a) to (d) taken by Bjorn Kloosterman. Pictures (e) to (h) taken by Efstathios Roumeliotis.