Cell cycle and storage related gene expression in potato tubers

John Verhees

Promotor: prof. dr. L.H.W. van der Plas
Hoogleraar in de Plantenfysiologie
Wageningen Universiteit

Co-Promotoren: dr. ir. A.R. van der Krol
Universitair docent
Laboratorium voor Plantenfysiologie
Wageningen Universiteit

dr. D. Vreugdenhil
Universitair hoofddocent
Laboratorium voor Plantenfysiologie
Wageningen Universiteit

Promotiecommissie: prof. dr. D. Bartels, Vrije Universiteit Amsterdam
prof. dr. P. du Jardin, University of Gembloux, België
prof. dr. ir. P.C. Struik, Wageningen Univeristeit
prof. dr. ir. R.G.F. Visser, Wageningen Universiteit

John Verhees

Cell cycle and storage related gene expression in potato tubers

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
prof. dr. ir. L. Speelman
in het openbaar te verdedigen
op woensdag 19 juni 2002
des namiddags te vier uur in de aula

CIP-data Koninklijke Bibliotheek, Den Haag
Cell cycle and storage related gene expression in potato tubers
J.A. Verhees.
Thesis Wageningen University.
ISBN 90 5808 658 5

Stellingen

1. De exacte relatie tussen genexpressie en de verschillende aspecten van (knol)ontwikkeling kan alleen bepaald worden door de expressie te analyseren in meerdere individuele monsters gedurende de gehele ontwikkeling (dit proefschrift).

2. Het antagonistische effect van ABA op de GA-signalering in aardappelknollen is alleen aanwezig in het oog terwijl in de knol deze relatie afwezig is (dit proefschrift).

3. Het effect van ethanol op de genexpressie in aardappelknollen wijst op een belangrijke rol voor de alcoholdehydrogenase-activiteit met een vermoedelijk signaleringsmechanisme zoals beschreven voor hexokinase tijdens de suiker-signalering (dit proefschrift).

4. Reductionists are remarkably good at believing that their particular part of the whole is of major, if not unique, importance (Farrar J.F., Sink strength: What is it and how do we measure it? (1993) Plant Cell and Environment 16: 1015).

5. Het humane genoom-project heeft ons een boek gegeven met alle antwoorden omtrent de functie van genen tijdens de ontwikkeling van de mens; nu nog leren lezen!

6. Dat in Nederland meer mensen lid zijn van organisaties die de belangen behartigen van bedreigde diersoorten dan van organisaties die opkomen voor mensen in nood, geeft aan dat voor de gemiddelde Nederlander de aaibaarheidsfactor van dieren groter is dan die van mensen.

7. Een verplichting voor de Nederlandse landbouw om biologisch te produceren zal, met het huidige consumentengedrag, resulteren in een verhoogde import van buitenlandse niet-biologische landbouwproducten.

8. De veel besproken afstand tussen politieke partijen en de kiezer komt voort uit individualisering van de samenleving waardoor mensen zich niet meer kunnen vinden in de collectieve gedachten die de basis vormen voor de politieke partijen.

Stellingen behorende bij het proefschrift: 'Cell cycle and storage related gene expression in potato tubers'

John Verhees
19 juni 2002

Voorwoord

Na vijf jaar onderzoek aan aardappelknollen en na vele uren meten met de luminometer kan ik persoonlijk concluderen dat de ontwikkeling van een aardappelknol bestaat uit een groot aantal facetten die onderzoek vaak zo interessant en leuk maken maar ook reden zijn voor de nodige kopzorgen. In het hier beschreven onderzoek hebben we geprobeerd een nieuw licht te werpen op die factoren en processen die een belangrijke rol spelen tijdens de ontwikkeling en groei van aardappelknollen. Dit alles is tot stand gekomen dankzij de hulp en inzet van een groot aantal mensen die ik hier graag wil bedanken.

Allereerst wil ik mijn co-promotoren Sander van der Krol en Dick Vreugdenhil bedanken. Jullie diversiteit in meningen over hoe een knol zich ontwikkelt heeft er voor gezorgd dat ik de resultaten vanuit verschillende uitgangsposities heb leren bekijken, wat een goede basis was voor het schrijven van dit proefschrift. Verder wil ik Linus van der Plas als promotor danken voor de snelle, doelgerichte en stimulerende begeleiding tijdens het schrijven van dit proefschrift.

Verder wil ik mijn kamergenoot Margo Claassens bedanken. Margo, je hebt niet alleen een bijdrage geleverd aan de inhoud van het hier beschreven onderzoek maar vooral je aanstekelijke lach kon ik zeer waarderen. Ook Wessel van Leeuwen en Patrick Spoelstra hebben een grote bijdrage geleverd aan dit proefschrift. Zonder jullie kennis en vaardigheden met de luminometer en de daaraan verbonden computers, was dit proefschrift niet tot stand gekomen.

Ook wil ik Diaan, Jan, Lydia en Tijmen hartelijk danken voor de goede en prettige discussies binnen de knollengroep. Leen en Aart wil ik danken voor het verzorgen van de aardappelplanten en hun tolerantie ten aanzien van de stofwolken die regelmatig in de kelder hingen.

Verder wil ik iedereen van de vakgroep plantenfysiologie bedanken die er voor gezorgd hebben dat ik de afgelopen vijf jaar met veel plezier heb kunnen werken. Vooral de mensen van de koffiehoek wil ik bedanken. Ronny, Janhendrik, Juul, Marc, Hans, Wim, Folkert, Mark, Maarten, Tanja en wie ik zoal nog meer de laatste jaren voorbij heb zien komen. Bedankt voor de gezelligheid tijdens de pauzes en voor de nodige uurtjes squash, die me de nodige rackets hebben gekost.

Sinds enige tijd ben ik werkzaam in Utrecht. Echter toen ik daar begon was dit proefschrift nog niet voltooid. Ik wil dan ook hier graag de mensen van het Hubrecht lab danken voor hun geduld als ik weer eens afwezig was vanwege het schrijven van dit proefschrift.

Verder wil ik hier mijn ouders, broers, zus en vrienden danken voor hun belangstelling in wat ik zoal uitvoerde de afgelopen jaren. In het bijzonder wil ik Gerwin en Nanda danken voor de assistentie tijdens het samenstellen van dit proefschrift en mijn paranimfen Gerwin en Frank. Ter afsluiting: lief, je hebt een grote bijdrage geleverd aan twee hoofdstukken in dit proefschrift maar je was ook een grote steun en goede afleiding tijdens de laatste fase van het schrijven van dit proefschrift.

John.

Contents

List of abbreviations

ABA	abscisic acid
ADH	alcohol dehydrogenase
AGPaseS	large subunit ADP-glucose pyrophosphorylase
CaMV35S	Cauliflower Mosaic Virus
CDC2a	A-type cyclin-dependent kinase
CCC	2-chloroethyltrimethyl-ammonium chloride
CycB1;1	mitotic B-type cyclin
EtOH	ethanol
GA	gibberellins
GR	growth rate (mm^2*day)
λPat21	class I patatin
luc	luciferase gene
LUC	luciferase activity
4MP	4-methylpyrazole
rlu	relative light units
SD	short day

Chapter 1

General introduction

Potato (*Solanum tuberosum* L.) originates from the high Andes of Peru and Bolivia. Climate conditions in those regions made that potato became the major crop for food production especially because other crops like maize do not grow at an altitude of 3500 m or more. At the time of the conquest of South America by the Spaniards in the early sixteenth century, cultivation of potato for food production had spread over a region what is now Colombia, Ecuador, Peru, Bolivia and Chile.

Around the year 1570 the potato was introduced in Europe via Spain and England. Initially potato was grown as a botanical curiosity and not for the production of tubers; the South-American varieties need short days for tuber induction and tubers were not formed under European long-day conditions. Propagating plants from true seeds resulted in segregants from which earliness could be selected, resulting in new varieties adapted to the long summer days of Northern and Western Europe. The breeding of varieties with suitable properties in the mid-eighteenth century resulted in the introduction of potato as a general field crop (Hawkes, 1992). Nowadays, potato is the world's fourth crop produced for human consumption and for industrial processing with an annual production of 280 million tons (FAO, 1993). In The Netherlands the annual production is about 8 million tons (CBS, 1999). Understanding the processes involved in the formation, growth and dormancy of potato tubers is of crucial importance to understand and improve tuber yield.

Structural development of stolon and tuber

Tuberization in potato starts with the outgrowth of below ground axillary buds into lateral shoots (stolons) growing diageotropically, usually shortly after plant emergence. Stolons are characterized by elongated internodes, bearing spirally arranged scale leaves and a recurved apical hook. Stolon initiation starts at the most basal node and proceeds acropetally (Cutter, 1992; Plaisted, 1957). Elongation and branching of the stolons will continue till tuber initiation starts and is stimulated by long-day conditions, high temperatures and high endogenous gibberellin concentrations, conditions that prevent tuber formation (Lovell and Booth, 1969; Vreugdenhil and Struik; 1989). During stolon growth transverse cell divisions occur mainly in the bud apex and the subapical region of the stolon tip (Xu *et al.* 1998). Tuber formation is preceded by cessation of stolon elongation.

A potato tuber is in fact a modified stem with a radially expanded axis, short internodes, reduced leaves and axillary buds (Artschwager, 1924; Cutter, 1992). Radial expansion starts in the sub-apical region of the stolon by transversal cell enlargement and longitudinal cell divisions during the initial stages of tuber development (Sanz *et al.*, 1996; Xu *et al.*, 1998). The reorientation in cell enlargement and cell division during the onset of tuber formation is caused by a reorientation of the cortical microtubules, controlling the deposition of cellulose microfibres in the cell wall, from a transversal to a longitudinal direction (Sanz *et al.*, 1996). During the initial stages of tuber formation, cells in the pith and cortex contribute mostly to the growth. Later on, randomly orientated cell divisions and cell enlargements occur in the perimedullary region resulting in further growth of the tuber (Xu *et al.*, 1998).

In vitro tuber formation

Studying the developmental program of tubers and investigating the relation between morphological, biochemical and molecular changes during development is hampered by the large variation in the developmental status between individual tubers growing on a single plant. To overcome these problems alternative methods have been developed resulting in synchronised tuber formation: leaf-bud cuttings transferred to soil, single-node cuttings transferred to tuber-inducing medium, and subculturing stolons on tuber-inducing medium (Ewing and Struik and references therein, 1992). Individual axillary buds have the capacity to develop into tubers without the application of growth regulators by transferring cuttings to medium with a relatively high level of sucrose (Hussey and Stacey, 1984; Garner and Blake, 1989). Analysing *in vitro* tubers indicate that the formation of these tubers resembles the initial stages of tuber development of soil-grown grown plants (Hendriks *et al.*, 1991; Visser *et al.*, 1994; Appeldoorn *et al.*, 1997; Vreugdenhil *et al.*, 1999; Veramendi *et al.*, 1999). However, analysing the metabolites in both in *in vitro*-grown and soil-grown tubers indicates a generally higher level of amino acids in *in vitro* tubers. Furthermore, *in vitro* tubers contain higher levels of glycerol, mannitol, inositol. The increase in these metabolites points to the occurrence of osmotic stress during *in vitro* tuber development due to the relatively high level of carbohydrates in the medium (Roessner *et al.*, 2000).

Using *in vitro* methods, as described for the variety 'Bintje' by Hendriks *et al.*, (1991), tuber formation is synchronous resulting in tubers with a well-defined developmental stage. The system is based on axillary buds from soil-grown plants growing under short day conditions, followed by transfer to tuber-inducing medium with a high level of sucrose and low level of nitrogen. Despite surface sterilising of the stem cuttings derived from soil-grown plants, long-term observation is hindered by microbial contamination.

As an alternative for cuttings from soil-grown plants, single-node cuttings can be used derived from *in vitro* grown plantlets sub-cultured under sterile conditions. When grown on the proper medium they easily form *in vitro* tubers with a diameter of 3-5 mm.

Non-destructive methods to study tuber formation on intact plants

The possibilities to study the different events that occur during tuber development on soil-grown plants are limited because the formation of stolons and tubers occurs below-ground. To analyse tuber development by a series of observations, complete stolons should be lifted from the soil repeatedly. Digging up the entire stolon system results in disturbance of the stolon, tuber and root growth and might interfere with the ongoing tuber development. The use of a hydroponics system allows to separate the zone that forms stolons and tubers from the zone that forms roots by growing the tuber forming zone in a separate compartment filled with a sand/perlite mixture and the root forming zone in a nutrient solution (figure 1). This method enables frequent observation of the stolons and growing tubers because the sand/perlite mixture can be removed easily with a vacuum cleaner without disturbing the growth of the tubers (Struik and Van Voorst, 1986).

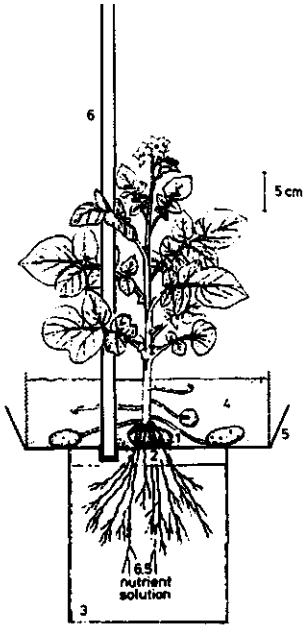


Figure 1
Illustration of the hydroponics system used to observe tuber development in potato plants. Stolons and tubers are growing in a container filled with a removable sand/perlite mixture while the zone that forms roots is growing in a container filled with nutrient solution. 1. Mother tuber, 2. Gauze, 3. Container with nutrient solution and roots, 4. Stolon chamber filled with sand/perlite mixture, 5. Saucer with ring, 6. Stick to support the shoot. Source: Struik and Van Voorst (1986).

Cell division and cell cycle regulation during tuber formation

Tuber formation starts with longitudinal cell divisions in the sub-apical region of the stolon which, together with cell enlargement, result in radial expansion of the stolon. During this period cell division and cell enlargement occur both in the pith and cortex resulting in a tuber with a diameter of approximately 0.8 cm. After this period soil-grown tubers continue to grow due to randomly orientated cell division and - enlargement in the perimedullary region whereas cells in the pith and cortex region only enlarge during this period of tuber development. In contrast to soil-grown tubers, *in vitro*-grown tubers do not develop a perimedullary region resulting in relatively small tubers (Xu *et al.*, 1998; Vreugdenhil *et al.*, 1999).

It is clear that cell division is an important process during plant growth and development and cell cycle mechanisms, which control cell division, are an interesting target for studying plant development. Four different phases can be distinguished during the cell cycle in the order G1, S, G2 and M in which the stage of DNA replication (S-phase) is separated from the cell division stage (M-phase). The progression through the cell cycle is controlled at the G1-S and G2-M transition by two major classes of cyclin-dependent kinases (CDK-A and CDK-B) in combination with regulatory subunits, called cyclins (Shaul *et al.*, 1996; Meijer and Murray, 2001). The A-type CDKs have been well characterised for plants and contain the conserved PSTAIRE motif in the cyclin-binding domain (Mironov *et al.*, 2000). Thus far several members of the A-type CDKs have been identified including CDC2a from *Arabidopsis thaliana* that is continuously expressed during the cell cycle, although the kinase activity peaks at the G1-S and G2-M phase (Ferreira *et al.*, 1991; Hemerly *et al.*, 1993). Cyclins are also encoded by a multigene family, A, B and D type cyclins, classified by the sequences of the conserved cyclin box. Expression analysis showed that the activity is under transcriptional control during the different phases of the cell cycle (Ferreira *et al.*, 1994; Mironov *et al.*, 2000). Analysing cycB1;1 expression in *Arabidopsis thaliana* indicates a specific expression during G2-M phase transition (Ferreira *et al.*, 1994; Hemerly *et al.*, 1993). Besides by cyclins, CDK activity is regulated by CDK inhibitor proteins (CKIs) that bind to CDK, cyclin or the CDK-cyclin complex. Thus far, only one CKI (ICK1) is studied in detail showing a transcriptional regulation by abscisic acid in *Arabidopsis thaliana* (Wang *et al.*, 1998).

Carbohydrates during tuber formation

Multiple changes have been observed in the potato plant at the time of tuberization, some of which are directly related to carbohydrate synthesis and transport:

1. An enhanced CO_2 assimilation in the photosynthetically active part of the plant.
2. An increase in the transport of carbohydrates towards the stolons and developing tubers.
3. An increase in sucrose and a strong reduction in fructose and glucose concentration in the region of the stolon that will develop into a tuber.
4. The synthesis of starch and storage proteins such as patatin in the tuber.

Carbohydrates play a central role in the regulation of the development and growth of root, shoot and storage organs including potato tubers (Farrar, 1992). For potato, carbohydrates stimulate tuber formation and the synthesis of storage products, in interaction with hormones that affect the transport, processing and perception of the sugar signal (see section on hormonal regulation). Tuber formation by *in vitro* based methods allows the study of the role of sugars during tuberization. A high exogenous sucrose concentration (8%) in the medium stimulates tuber formation whereas a low sucrose concentration induces the formation of a stolon as shown in figure 2 (Hussey and Stacey, 1984; Garner and Blake, 1989; Xu *et al.*, 1998). Combining gibberellins with high sucrose also results in the formation of a stolon indicating that gibberellins counteract the effects of sucrose on tuber development or affect the distribution of sucrose (Vreugdenhil *et al.*, 1998). Glucose and fructose also induce tuber formation although the efficiency is lower than for sucrose (Vreugdenhil and Helder, 1992).

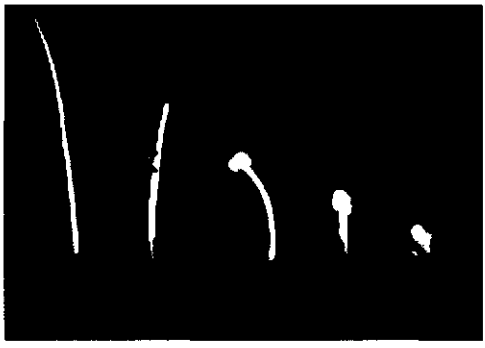


Figure 2
Effect of the sucrose level on stolon and/or tuber development of axillary buds, grown *in vitro* for 10 days, on medium with different sucrose levels ranging from 1% (left), via 2%, 4% and 6% to 8% (right).

During the initial stages of tuber formation the sucrose concentration increases at the site of the tuber followed by a decline during tuber outgrowth (Ross *et al.*, 1994; Morrel and Ap Rees, 1986). During tuber formation the endogenous glucose and fructose levels decline after start of the visible swelling. Although both glucose and fructose decline during tuber formation, the decrease of fructose is much stronger than that of glucose. Quantification of the endogenous sugar levels in the stolon, subtending the developing tuber, shows either an increase in sucrose content that equals the sucrose level in the tuber (Ross *et al.*, 1994) or a gradual decline from the tuber site towards the stolon (Viola *et al.*, 2001) while there is no large difference in the level of glucose and fructose between tuber and stolon (Vreugdenhil *et al.*, 1998; Viola *et al.*, 2001).

Sucrose unloading and starch synthesis

During growth of the stolon sucrose unloading occurs predominantly via an apoplastic route by specialised sucrose and/or hexose transporters (Oparka, 1991; Viola *et al.*, 2001). During this period, sucrose degradation is mainly catalysed by invertases localised in either the apoplast and vacuole (acid invertases) or cytosol (neutral invertase) (Ross *et al.*, 1994 Appeldoorn *et al.*, 1997). Especially apoplastic invertase is active in the apex of the growing stolon (Appeldoorn et al., 1997).

During tuber formation a switch in sucrose unloading occurs from apoplastic to symplastic unloading (Oparka, 1996; Viola *et al.*, 2001), accompanied by an increase in sucrose synthase activity whereas cytosolic invertase activity declines (Sung *et al.*,. 1989; Appeldoorn et al., 1997). Sucrose synthase (susy) is localised in the cytosol and catalyses the reversible conversion of sucrose and UDP into UDPglucose and fructose. Concomitantly with the increase in susy activity, fructokinase activity increases resulting in a drastic decline in fructose (Ross *et al.*, 1994; Appeldoorn *et al.*, 1997; Vreugdenhil *et al.*, 1998). A low cytosolic fructose concentration is required because fructose is an effective inhibitor of sucrose degradation by susy (Geigenberger and Stitt, 1993). The strong increase in fructokinase activity that coincides with the increase in sucrose synthase activity and results in further metabolisation of fructose, makes the glucose/fructose ratio a good indicator for the switch from invertase towards sucrose synthase mediated sucrose hydrolysis (Davies, 1984; Vreugdenhil *et al.*, 1998). Although an enhanced susy activity is observed during tuber formation, it is not an absolute prerequisite for tuber induction as appeared from transgenic potato lines with a reduced susy activity. These plants show no differences in tuber production in comparison with wild-type plants. However, tubers from these transgenic plants contained lower amounts of starch and storage proteins, indicating the importance of susy activity for storage processes (Zrenner *et al.*, 1995).

A small fraction of the UDPglucose produced by sucrose synthase is directly used for the synthesis of cell-wall polysaccharides but the majority is converted into glucose-1-P by UDPglucose pyrophosphorylase (UGPase) (ap Rees and Morrell, 1990). In the amyloplasts the phosphorylated glucose is converted into starch by the successive action of ADPglucose pyrophosporylase (AGPase), starch synthases and branching enzymes (Prat *et al.*, 1990). An upregulation in AGPase activity is observed during the initial stages of tuber development coinciding with an increase in starch synthesis (Sowokinos, 1997; Ross *et al.*, 1994; Visser *et al.*, 1994).

The AGPase enzyme is a heterotetramer composed of two large (51-kD) and two small (50-kD) subunits, respectively AGPaseS and AGPaseB (Okita *et al.*, 1990). In potato three copies of the genes encoding different isoforms of the large subunit and one gene encoding the small subunit were cloned (La Cognata *et al.*, 1995). During the initial stages of tuber development the expression of both AGPaseS and AGPaseB increases although the expression dynamics during tuber development differs for the two sub-units (Müller-Röber *et al.*, 1990; Visser *et al.*, 1994; Nakata and Okita, 1995). Analysing the activity of various AGPaseS promoter derivatives indicated that within the promoter sequence different regions are responsible for the specific expression of AGPaseS in petioles, stems, tubers and guard cells (Müller-Röber *et al.*, 1994).

An increase in starch synthesis is observed during very early stages of tuber development resulting in a massive accumulation of starch indicating that starch synthesis is linked to tuber formation. However, reducing the expression of starch synthesis related genes indicated that starch synthesis is not essential during tuber formation (Müller-Röber *et al.*, 1992; Zrenner *et al.*, 1995). Reducing starch synthesis resulted in the massive accumulation of sugars in tuber and stolon and a change in the size distribution indicating that starch synthesis affects the tuber sink strength distribution.

Storage proteins

During tuber formation, not only starch but also proteins, like patatin and proteinase inhibitors accumulate (Hendriks *et al.*, 1991). Patatin is the major storage protein which accounts for about 40% of the soluble protein content in potato tubers. Besides a storage function, patatin protein possesses a lipid acyl hydrolase activity cleaving fatty acids from membrane lipids (Racusen, 1986; Rosahl *et al.*, 1986). Patatin is encoded by a large family of genes with about 64-72 copies present in the cultivar Desiree (Twell and Ooms, 1988). The multiple copies of the patatin gene are divided into two classes based on the presence (class II) or absence (class I) of a 22 bp sequence in the 5' untranslated region of the gene (Pikaard *et al.*, 1987).

Expression analyses indicated a predominant expression of the class I patatin genes in tubers and tuberizing stolons whereas the transcripts of class II genes are 50-100 fold less abundant in these regions of the potato plant (Mignery *et al.*, 1988). Class I patatin expression is also induced in stem and leaves of explants when incubated on a high sucrose medium. The expression in leaves is also affected by the concentration of amino acids in the medium (Wenzler *et al.*, 1989; Rocha-Sosa *et al.*, 1989; Perl *et al.*, 1991).

Transgenic potato plants with a reduced activity of sucrose synthase (susy) or ADP-glucose pyrophosphorylase (AGPase), and as a result a reduced starch level, also have a low amount of patatin although the sugar level of these tuber is high (Müller-Röber *et al.*, 1992; Zrenner *et al.*, 1995).
These findings indicate that not the steady state level of carbohydrates in the tuber, but rather the flux of carbohydrates into starch synthesis functions as a signal that triggers the synthesis of patatin. Analysis of the patatin promoter indeed indicated two independent mechanisms that regulate the expression of patatin (class I): one mechanism connected with a specific expression in tubers and one connected with the carbohydrate flux (Jefferson *et al.*, 1990; Grierson *et al.*, 1994).

Hormonal regulation of tuber formation and growth
Gibberellins
Gibberellins stimulate stolon formation and inhibit tuber formation (Lovell and Booth, 1969; Kumar and Wareing, 1972; Hammes and Nel, 1974; Xu *et al.*, 1998). It has been suggested that environmental conditions like high nitrogen concentrations, high temperatures and long-day conditions inhibit tuber formation by stimulating the synthesis of gibberellins (Kraus and Marschner, 1982; Jackson, 1999). Quantifying the endogenous gibberellin concentrations during *in vitro* tuber formation show a decline in GA_1 one day before visible swelling whereas under non-inducing conditions (low sucrose), endogenous GA_1 remained high. The authors suggest that the exogenous sucrose concentration affect the endogenous gibberellin concentration in the stolon (Xu *et al.*, 1998).

When gibberellins are applied to growing tubers, sucrose concentrations decline and the reducing sugar level increases. The sugar status of tubers after GA-treatment equals the sugar status observed in growing stolons. GA-treatment of tubers is accompanied by a decline in ADP-glucose pyrophosphorylase (AGPase) activity, indicating a reduction in starch synthesis (Mares *et al.*, 1981). Besides affecting starch synthesis, GA-treatment also results in a reduced synthesis of patatin (Hannapel *et al.*, 1985).

Gibberellins do not only affect the synthesis of storage products but also affect the orientation of the cortical microtubules and thereby the orientation of cell division and enlargement in stolon and tuber. A high endogenous gibberellin concentration prevents the reorientation of the cortical microtubules from transversal into longitudinal, a change observed during tuber formation (Fujino *et al.*, 1995; Sanz *et al.*, 1996). After tuber formation has started, the application of gibberellins results in the inhibition of tuber growth (primary growth) and it stimulates bud growth, a phenomenon called second growth (Lippert *et al.*, 1958; Bodlaender *et al.*, 1964; Vreugdenhil and Sergeeva, 1999).

Abscisic acid

Endogenous ABA stimulates tuber formation and inhibits stolon elongation indicating that it counteracts the effect of gibberellins (Menzel, 1980; Marschner *et al.*, 1984; Xu *et al.*, 1988; Xu *et al.*, 1998). The stimulating effect of ABA on tuberization is concentration dependent with an optimal concentration that varies for different potato cultivars (Hussey and Stacey, 1984). Although exogenous ABA stimulates tuber formation a difference in endogenous ABA level was not observed between inducing and non-inducing conditions. Moreover, tuber formation also occurs in an ABA-deficient potato mutant indicating that ABA is probably not the main regulator during tuber formation, but affects tuber formation by acting as a negative regulator of gibberellin activity (Kraus, 1981; Quarrie, 1981; Xu *et al.*, 1998).

During tuber growth a negative correlation is observed between tuber growth rate and endogenous ABA concentration indicating a retarding effect of ABA on tuber growth. However, the retarding effect of ABA on tuber growth seems to correlate with the endogenous IAA concentration in the tubers (Marschner *et al.*, 1984). Reducing endogenous ABA in tubers results in the outgrowth of the apical meristem (second growth) indicating an important role for ABA in maintaining tuber dormancy (Kraus, 1985; Van den Berg *et al.*, 1991; Suttle and Hulstrand, 1994).

Other hormones

Contrasting results have been reported about the stimulating effect of **cytokinins** on tuberization (Palmer and Smith, 1969; Hussey and Stacey; 1984; Xu *et al.*, 1998). Tuber-inducing conditions result in an increase in endogenous zeatin riboside in the stolon tips prior to swelling (Mauk and Langille, 1978). In contrast, transgenic lines with a high endogenous cytokinin level showed no tuber development but starch synthesis occurred in the short shoots developing from the axillary buds whereas transgenics with a relatively small increase in endogenous cytokinin showed an increase in tuber formation.

These results indicate that cytokinins are involved in the morphological development of a tuber but do not affect starch synthesis (Sergeeva *et al.*, 2000).

A stimulation of tuber formation and growth is observed by applying low concentrations of the **auxin** indolylacetic acid (IAA) while high concentrations inhibit tuber formation (Kumar and Wareing, 1974; Xu *et al.*, 1998; Aksenova *et al.*, 2000). Removing fast growing tubers causes an increase in endogenous IAA in the remaining tubers coinciding with an increased growth rate possibly due to the promoting effect of IAA on cell expansion (Marschner *et al.*, 1984).

The effect observed with high concentrations of auxin is possibly mediated by a stimulation of **ethylene** synthesis that inhibits stolon elongation, as observed after applying IAA (Vreugdenhil and Struik, 1989). The effect of ethylene on tuberization is dual: a reduction in stolon growth indirectly resulting in a stimulation of tuber formation, whereas on the other hand ethylene inhibits tuberization (Mingo-Castel *et al.,* 1974; Vreugdenhil and Van Dijk; 1989). Applying ethylene antagonists during tuber formation results in second growth indicating that endogenous ethylene is essential for the initial period of endodormancy in potato tubers (Suttle, 1998).

Extracts from potato leaves grown under short – and long-day conditions showed a difference in tuber-inducing activity (Struik *et al.*, 1987; Koda *et al.*, 1988). Later this tuber-inducing compound was identified as 11- and 12-hydroxy-**jasmonic acid** (tuberonic acid) (Yoshihara *et al.*, 1989; Helder *et al.*, 1993). In potato leaves jasmonic acid is metabolised to tuberonic acid followed by a glycosylation resulting in tuberonic acid glucoside that is exported towards the stolons (Yoshihara *et al.*, 1996) causing cessation of stolon elongation and an induction of cell expansion at the site of tuber development (Takahashi *et al.*, 1994).

Interaction between tubers growing on a single plant

Developmental events like stolon induction, stolon growth, cessation of longitudinal growth and tuber initiation may occur simultaneously within a single potato plant (Vreugdenhil and Struik, 1989). However, it is described that tuber setting in soil-grown plants is usually completed within a short period (Helder et al., 1993; O'Brien et al., 1998). When tuber formation is initiated not all stolon tips will develop into a tuber. These differences are possibly caused by differences in the perception of the tuber-inducing signal between the stolon tips or differences in the internal stolon conditions that inhibit tuber formation like the endogenous gibberellin concentration and ethylene production (Vreugdenhil and Struik, 1998).

Prior to tuber formation there is a large difference between stolon tips in the rate of assimilate import, suggesting a difference in the distribution of carbohydrates between the different stolon tips resulting in differences in tuber growth (Oparka and Davies, 1985).

After the start of tuber formation there is a large variation in the growth pattern between the individual tubers. As a result the largest tubers at a certain moment are not necessarily the largest ones at a later point of development (Moorby, 1968; Wurr, 1977; Ahmed and Sagar, 1981). In general it is reported that during the growth of multiple tubers on a single stem fast growing tubers restrict the growth of other tubers. Removing fast growing tubers results in an enhanced growth of the remaining tubers, indicating that the growth of these tubers is not limited by factors inside the tuber but is related to the hierarchy between tubers, competing for the available nutrients (sink strength) (Moorby, 1968; Engels and Marschner, 1986; Sattelmacher and Laidig, 1991). The sink hierarchy between tubers can be determined by quantifying the import rate of carbohydrates and the activity of certain enzymes involved in the processing of carbohydrates. However, studying sink strength hierarchy between tuber growing on the same plant is difficult because this relation may change with time. Factors that possibly affect differences in growth and competition between tubers are: 1. The position of the tuber and the characteristics of the stolon, 2. The initial tuber inducing signal, 3. Other internal tuber characteristics like hormone levels and enzyme activities, 4. Differences in the environmental conditions between the tubers (Struik *et al.*, 1991, and references therein).

Reporter genes

Analysis of gene expression mostly takes place by quantifying the steady state levels of the gene product (mRNA or protein). However, this method is destructive for the tissue and the expression is mostly based on pooled samples resulting in a low spatial and temporal resolution. An alternative method is therefore desirable to study the dynamics of gene expression during development.

Transformation techniques make it possible to introduce an extra copy of the gene under study, in which the coding sequence of the gene is replaced by a "reporter" gene. By using a reporter gene that encodes an enzyme not present in the organism under study, the activity of the chimaeric gene can be studied by quantifying the activity of the reporter enzyme. The application of reporter genes is limited by the properties of the reporter enzyme, the characteristics of the plant tissue and the quality of the available assays used for detecting reporter activity.

One of the initial reporters used in plants was β-glucuronidase (GUS). The low background activity in most plants and the high stability of the enzyme made GUS a useful reporter for quantifying and localising gene expression in plant tissue (Jefferson *et al.*, 1987). However, studying the dynamics in gene induction with the GUS reporter is limited due to the high stability of the GUS-protein and because most assays for detecting the activity are destructive for the sampled material. Studying genes involved in bioluminescence, resulted in a new generation reporter genes like the Green Fluorescent Protein from jellyfish (GFP) (Chalfie *et al.*, 1994) and the luciferase gene (*luc*) from North American firefly (Ow *et al.*, 1986) that can be used as reporter genes in a non-destructive way.

Luciferase reporter system

Luciferase catalyses the oxidative decarboxylation of the substrate luciferin resulting in the release of a photon at 562 nm in 90% of the catalytic cycli. Besides luciferin, the reaction requires Mg^{2+}, ATP and oxygen (DeLuca *et al.*, 1974). After the reaction the luciferase enzyme is inactive for a prolonged time due to the slow regeneration of the luciferase enzyme from the Luciferase*Oxyluciferin complex. The half-life of the luciferase enzyme is relatively short due to a high turnover of both luciferase mRNA and protein. In Petunia cell suspensions the half-life of the LUC protein is about 150 minutes and in the presence of luciferin the apparent half-life is about 15 minutes (Van Leeuwen *et al.*, 2000). The slow regeneration and the instability of the luciferase enzyme makes that luciferase activity observed *in vivo* in the continuous presence of luciferin depends on newly synthesised luciferase. As a consequence, *in vivo* luciferase activity is closely linked to on-going transcriptional activity of the luciferase reporter gene. The luciferase reporter system is therefore useful to study the dynamics in gene expression because changes in the promoter activity can be monitored within minutes resulting in a relatively high temporal resolution in contrast to many other reporter systems like GUS and GFP. Luciferase can be detected and quantified *in planta* even on a cellular level as well as on the plant level, which allows to observe gene expression with a relatively high spatial resolution.

The research and outline of this thesis

Because cell division and the synthesis of storage products are two important events during tuber formation we analysed the expression of two sets of genes: two cell cycle genes (cycB1;1 and CDC2a) and two storage related genes (AGPaseS and λPat21).

The dynamics in gene expression during development may be different between individual samples but these differences may be obscured when samples are pooled. Only the analysis of multiple individual samples allows to determine the exact relationship between changes in gene expression in time and the different events that occur during a developmental program like tuber formation. Therefore, we analysed gene expression not by extracting mRNA but we used the luciferase reporter system to study promoter activity. *In vivo* luciferase activity can be observed non-destructively and quantified in individual tubers. This makes it possible to determine the dynamics in gene expression over prolonged period of time during tuber development and growth.

To study cell cycle related gene expression, the promoter sequence of the cyclin-dependent kinase: CDC2a and the promoter sequence of the cyclin: cycB1;1 were used, both cloned from *Arabidopsis thaliana* L. Starch synthesis related gene expression was studied by using the promoter sequence of the gene that encodes the large subunit of ADP-glucose pyrophosphorylase: AGPaseS. For studying protein storage related gene expression we used the promoter from the patatin gene: λPat21. The promoters of the storage related genes had been cloned from *Solanum tuberosum* L..

In **chapter 2** we describe the regulation of cell cycle and storage related genes in relation to the moment of visible swelling which is used as a morphological marker for the onset of tuber formation. With the obtained results we will discuss the relation between the expression of cell cycle and storage related genes and the tuber developmental program resulting in the conclusion that swelling during tuber formation and the expression of storage genes are two independent processes. **Chapter 3 and 4** describe the dynamic behaviour of cell cycle and storage related gene expression during second growth in *in vitro* tubers induced with gibberellins (chapter 2) or ethanol (chapter 3). The effect of the various treatments on the expression in tuber and bud will be discussed separately indicating a different response between tuber and bud during the switch from tuber growth (primary growth) into growth of the bud meristem (second growth). **Chapter 5** describes the relation between the sink strength of developing tubers and differences in the induction of cell cycle and storage related genes.

The variation in gene expression between individual tubers will be discussed in relation to sink strength hierarchy during tuber development indicating that the gene expression can be used as a marker for sink strength. **Chapter 6** will discuss the obtained results and describe the possible physiological and molecular mechanisms that regulate the expression of cell cycle and storage genes during tuber development, tuber competition and second growth.

Chapter 2

Characterising gene expression during potato tuber development in individuals and populations using the luciferase reporter system

John Verhees, Alexander R. van der Krol, Dick Vreugdenhil and
Linus H. W. van der Plas

Analysis of gene expression and enzyme activity in pooled potato tuber samples has previously indicated different developmental events occurring in a fixed sequential order during tuber development, starting with the upregulation of starch synthesis, then induction of protein storage followed by cell division and cell enlargement. In this report we analysed *in vivo* promoter activity of genes related to cell division and storage of reserves during tuber development in individual *in vitro* tubers, using the non invasive firefly luciferase reporter system. The average activity of the storage related promoters (AGPaseS and λPat21) was upregulated prior to visible swelling, while the average activity of both cell cycle genes (cycB1;1 and CDC2a) showed an upregulation after the onset of swelling. However this novel system allowed expression analysis in individual tubers which showed a variable upregulation of both storage genes in relation to the moment of swelling, from 4 days before to 10 days after the onset of swelling. We conclude that during the first stages of tuber development, the moment of storage gene induction is independent from swelling. These results indicate that the developmental program of potato tubers does not consist of a fixed sequential order of events, but consists of independent developmental programs (storage and swelling), together resulting in the formation of a potato tuber. It is concluded that analysis of developmental programs by studying individuals may result in new insights, possibly obscured when using pooled samples.

INTRODUCTION

Gene expression is usually quantified by measuring the steady state level of gene products (mRNA or protein), using pooled samples and destructive methods in which spatial and temporal resolution of gene expression is lost. Alternatively transcription may be analysed by fusing the promoter of the gene under study to the coding sequence of a reporter gene which activity can be detected *in planta*. In developmental studies, like the formation of potato tubers, the application of a non-invasive reporter is desirable because transcription of a gene can be studied in individual samples during development, revealing spatial and temporal regulation in gene expression in relation to the different morphological changes during development. Non-invasive analysis of gene expression has been enabled by the introduction of reporter genes like the Green Fluorescent Protein (GFP) (Niedz *et al.*, 1995) and firefly luciferase (*luc*) (Ow *et al.*, 1986; Millar *et al.*, 1992; Kost *et al.*, 1995). Luciferase catalyses the oxidative decarboxylation of luciferin and requires Mg^{2+}, ATP and oxygen. After the reaction the luciferase is inactive for an extended period because the product oxyluciferin is only slowly released from the AMP-oxyluciferin-luciferase complex. Therefore, *in vivo* luciferase (LUC) activity depends on newly synthesised luciferase in the presence of excess levels of the substrate luciferin (Van Leeuwen *et al.*, 2000). As a consequence of the short half-life of ~3 hours (Nguyen *et al.*, 1989; Thompson *et al.*, 1991; Van Leeuwen *et al.*, 2000), *in vivo* LUC activity is closely linked to on-going transcriptional activity of the luciferase reporter gene. We therefore used the luciferase reporter gene in combination with promoters from genes that are representative for the different events occurring during tuber development.

Tuber formation in potato (*Solanum tuberosum* L.) comprises the inhibition of stolon elongation, thickening of the sub-apical region by cell enlargement and cell division, and increased storage of proteins and carbohydrates at the site of tuber development (Ewing & Struik, 1992; Vreugdenhil & Struik 1989; Jackson, 1999). Morphological and molecular analysis of mRNA steady state levels showed repeatedly that tuber formation involves several processes that seem to occur in a fixed sequential order. First an upregulation is observed in starch synthesis followed by induction of storage proteins subsequently followed by cell division and cell enlargement (Visser *et al.*, 1994; Xu *et al.*, 1998). However, as most of these observations are the result from the average activity in pooled samples, it is not known whether such a sequential order is an absolute requirement for the development of individual tubers.

The accumulation of starch is one of the early indicators of metabolic changes that accompany the morphological development of the tuber followed by the accumulation of storage proteins (Bradburry, 1953; Duncan and Ewing, 1984). Although tuber development coincides with an increase in starch and storage proteins, accumulation of reserves may also occur in other organs, viz. petioles, stolons and stems when adequate alternative sinks (tubers) are absent (Paiva *et al.*, 1983; Sergeeva *et al.*, 2000). The induction of starch synthesis during tuber formation can be studied by monitoring the promoter activity of the gene encoding the large subunit of ADP-glucose pyrophosphorylase (AGPaseS) which is described as a regulating enzyme during starch synthesis. The AGPaseS promoter is active in tubers, starch-containing parenchyma sheath cells of stems and petioles, pollen grains, ovaries and guard cells (Müller-Röber *et al.*, 1994). During tuber development, mRNA steady state levels indicated an upregulation in AGPaseS expression one day before the onset of stolon swelling (Visser *et al*, 1994; Bachem *et al*, 1996). These results indicate that induction of genes involved in the starch synthesis precedes swelling of the stolon during tuber development.

To monitor the induction of protein storage genes during tuber formation, the promoter from the λPat21 (class I) patatin gene was used (Bevan *et al.*, 1986). Transcription of this class I patatin gene is high in tubers and is induced in stems and leaves in response to high levels of exogenous sucrose (Paiva *et al.*, 1983; Wenzler *et al.*, 1989; Pikaard *et al.*, 1987). Observations by Grierson *et al.* (1994) indicate a possible interaction between starch synthesis and transcriptional regulation of patatin in potato tubers. For the class I patatin genes an upregulation in expression has been reported at the onset of visible swelling. (Hendriks *et al.*, 1991; Visser *et al.*, 1994; Bachem *et al.*, 1996).

Longitudinal cell division and cell enlargement starting in the sub-apical region of the stolon results in radial expansion, marking the morphological development of the tuber. It has been reported that cell division does not precede cell enlargement during swelling (Xu *et al.*, 1998; Vreugdenhil *et al.*, 1999). The progression through the cell cycle is regulated by cyclin-dependent kinases (CDKs) in combination with regulatory subunits, called cyclins (Shaul *et al.*, 1996). The A-type CDKs are well characterised for plants and contain the conserved PSTAIRE motif in the cyclin-binding domain (Mironov *et al*, 2000). Thus far five members of the A-type CDKs were identified including CDC2a (A-type cyclin-dependent kinase) from *Arabidopsis thaliana* (Ferreira *et al*, 1991; Hemerly *et al*, 1993). Cyclins are also encoded by a multigene family and classified by the sequences of the conserved cyclin box (Ferreira *et al*, 1994).

Of all CDK's and cyclins cloned from plant systems, only CDC2aAt, CDC2abAt and cycB1;1At (mitotic B-type cyclin) have been shown to be directly involved in the regulation of cell cycle (Mironov *et al*, 2000). Analysing cycB1;1At and CDC2aAt promoter activity with the GUS reporter in *Arabidopsis thaliana* (L.) (Ferreira *et al*, 1994; Hemerly *et al*, 1993) and in poplar (*Populus tremula* L.) (Rohde *et al.*, 1997) showed that the activity of these chimeric genes mark regions with cell cycle activity, both in homologous and heterologous plant species.

To analyse cell cycle and storage related gene activity in relation to tuber development, *Solanum tuberosum* cv 'Desiree' was transformed with reporter gene constructs containing firefly luciferase (*luc*) as a reporter fused to the promoter sequences of either cell cycle related genes (cycB1;1, CDC2a), a starch synthesis relate gene (AGP-S) or to the promoter from a storage protein gene (λPat21). As a control we used the Cauliflower Mosaic Virus (CaMV) 35S-*luc* reporter construct. LUC activity was imaged *in vivo* in two independent transgenic lines for each of the reporter constructs. The results presented here confirm that the *in planta* LUC activity coincides with the *luc* mRNA steady state levels, as well as with the mRNA steady state levels of the endogenous genes indicating that the reporter constructs reflect the expression of the authentic genes during tuber development.

Analysis of LUC activity in multiple individual *in vitro* tubers shows a variable timing in the induction of storage genes, occurring either before or after visible swelling of the stolon. This indicates that tuber formation does not occur by a fixed sequential order of events, as reported before, but consists of two independent processes (storage and swelling) with an independent timing of regulation. The signal(s) inducing the swelling are therefore different from the signal(s) inducing storage gene expression during tuber development.

MATERIALS AND METHODS

Reporter constructs and plant transformation
For *Agrobacterium tumefaciens* mediated transformation we constructed the following promoter-*luc* constructs:

- CaMV35S-*luc*: (pGM111) Fragment containing the sequence –343 to +8 of the CaMV35S promoter fused at the initiation codon of the luciferase gene (Promega, Sherf and Wood, 1994) with a N-terminal SV40 Nuclear Localisation Signal (NLS) (Van der Krol and Chua, 1991).
- CycB1;1-*luc*: (pGM139) cycB1;1 promoter (1.2 kB) from *Arabidopsis thaliana* (Ferreira *et al.*, 1994) fused at the initiation codon of *luc*.
- CDC2a-*luc*: (pGM 133) CDC2a promoter (1.6 kB) from *Arabidopsis thaliana* (Ferreira *et al.*, 1991) fused at the initiation codon of *luc*.
- AGPaseS-*luc*: (pGM137) AGPaseS promoter (3.2 kB) from *Solanum tuberosum* (Müller-Röber *et al.*, 1992) fused to the initiation codon of the *luc* gene with a N-terminal SV40 Nuclear Localisation Signal (NLS).
- λPat21-luc (pGM135) patatin promoter (3,5 kB) cloned from *Solanum tuberosum* (Bevan *et al.*, 1986) fused to the initiation codon of the *luc* gene with a N-terminal SV40 Nuclear Localisation Signal (NLS).

The constructs were subcloned into the binary vector pMON721 containing the 3' E9 terminator and transferred to *Agrobacterium tumefaciens* strain ABI. This strain was used for stem transformation of *Solanum tuberosum* cv 'Desiree'. Transgenic plants were selected on MS medium containing kanamycin (100 $\mu g^{*}ml^{-1}$). Per reporter construct, several primary transgenic lines were regenerated successfully from which two lines were selected per promoter for monitoring promoter activity during tuber development. No differences were observed in the upregulation of promoter activity during the onset of tuber formation between the two transgenic lines.

In vitro tuber development
Primary transformants, grown *in vitro* with a 16 h light period (50 W m^{-2}, 20°C) for 5-6 weeks, were cut into single-node cuttings containing one axillary bud per explant. Explants were placed on modified Murashige and Skoog (1962) medium containing 1/10 part of the standard amount of KNO_3 and NH_4NO_3, 8% (w/v) sucrose, 5 μM benzyl-aminopurine (BAP), 0.8% (w/v) agar with a final pH 5.8 (tuber–inducing medium). On this medium the axillary buds developed into a sessile tuber when placed in the dark at 20°C. In order to obtain non-sessile tubers, stolon formation was induced prior to tuber induction. Explants were first placed on tuber-inducing medium supplemented with 0.5 μM $GA_{4/7}$ and incubated at 20°C in the dark for 3 days. The gibberellin within the medium inhibits tuber formation and induces stolon formation. During this period, the axillary buds developed into stolons of 5-15 mm. After this treatment with gibberellin, explants were placed on tuber-inducing medium and incubated in the dark at 20°C. During this incubation tuber formation was induced, resulting in the formation of non-sessile tubers. During incubation on tuber-inducing medium, *in vivo* luciferase measurements were done on a daily base for a period of 25 days.

Measurement of LUC activity *in vivo*
The substrate for luciferase, luciferin was added every day by spraying with a 1 mM luciferin (D-luciferin, sodium salt) solution at least 3 hours prior to measuring LUC activity. For monitoring the light emitting reaction of luciferase, explants were placed under an intensified CCD camera (C2400-77, Hamamatsu Photonics, Japan). The integration period that was used to capture photons varied per reporter construct (5 to 15 min) depending on the *vivo* LUC activity.
Relative luciferase activity was depicted by an image showing the amount of relative light units per pixel (rlu $pixel^{-1}$) captured during integration time by a false colour scale for a specific bit range (blue: low activity, red: high activity).

These images were used to quantify the relative LUC activity during tuber formation for each individual tuber per day by using the Argus-50 Image Processor (Hamamatsu Photonics, Japan). Relative LUC activity was quantified in a fixed area, equal to the final size of the tuber. The same fixed area was used for quantification during the complete period of tuber development because it was as accurate as fitting the area to the exact size of the tuber every day during the period of tuber growth.

Reverse transcriptase PCR

Luciferase mRNA levels were quantified by semi-quantitative RT-PCR in *in vitro* tubers (1-5 days after onset visible swelling) in order to analyse whether the distribution of LUC activity as measured *in vivo* reflected the distribution of steady state *luc* mRNA levels. In addition, steady state mRNA levels of the endogenous *AGPaseS* and *λPat21* gene were semi-quantified by RT-PCT to determine whether the LUC activity measured *in vivo* when driven by the AGPaseS or λPat21 promoter reflected the transcriptional level of the authentic gene.

Primers used for the RT-PCR were based on the published cDNA sequences of *luc* (M15077) from firefly and *cyclophilin* (AF126551), *AGPaseS* (X61187) and *λPat21* (X03956.1) from potato.
cyclophilin primers:

- cyclophilin primers:
  cycl-f TTC GCC GAT ACC ACT CCC AAA ACC
  cycl-r GAC CCA ACA AGA CAC AAG CAG GAA
- luciferase primers:
  SK333 ATG GAA GAC GCC AAA AAC ATA AAG
  SK305 GGC GGA TCC TAT ATG AGG ATC TCT CTG ATT TTT C
- AGPaseS primers:
  AGP-S-f CCT GGG GTT GCT TAC TCT GTG A
  AGP-S-r AAA ATC TGA TGC TCG GCT GTC CTC
- λPat21 primers:
  Pat21-f TCC CTT AAT TCC ACC TCC ACC AA
  Pat21-r ACC CCA TTT AGC TGC CTC TTC TG

Cyclophilin mRNA levels showed little variation between the different samples and the mRNA of the other genes was quantified relatively to the level of *cyclophilin* (Chengappa *et al*, 1999). The primers were tested on the cDNA from potato. For each gene the primer combination resulted in a single amplified DNA fragment. The fragments were isolated and the identity of the fragments was confirmed by sequence analysis. These cloned fragments were used as a probe for hybridisation.

In vivo LUC activity was measured in individual tubers of either AGPaseS-*luc* or λPat21-*luc* reporter plants. For each reporter plant, tissue samples of high and low *in vivo* LUC activity were pooled (3 tubers per sample). Total RNA was extracted by using guanidinthiocyanate (GuSCN) extraction buffer and Rnaid MATRIX glass beads (BIO 101) as described by Van Leeuwen *et al.* (2001). After Dnase treatment, 2.5 μg of total RNA was used for first strand cDNA synthesis using oligo(dT)primers (Superscript™ Preamplification System, Gibco BRL). From the produced cDNA 1μl (of 20μl) was used for RT-PCR using the specific primer combination. During RT-PCR, samples were taken (10 μl) after 18, 20, 22 and 24 cycles (*luc* and *AGPaseS*) or after 24, 26, 28 and 30 cycles (*λPat21*). Samples were fractionated on a 1% agarose gel (1x TAE) and blotted to a nylon membrane (Genescreen Plus, NEN™ Life Science Products). After blotting and hybridisation with the appropriate labelled cDNA fragment, the intensity of the signals was quantified using a Phosphor Imager (Molecular Dynamics).

RESULTS

The luciferase reporter gene allows analysis of the transcriptional regulation of genes during organ development and because the method is non-destructive, the temporal expression profile could be observed in individual samples. We fused cell cycle and storage related promoters to the luciferase reporter gene and monitored the activity during potato tuber development. The various reporter constructs were inserted into the binary vector pMON721 which was transferred to *Agrobacterium tumefaciens* strain ABI and used to transform *Solanum tuberosum* cv 'Desiree'. Placing single-node cuttings on tuber-inducing medium resulted in the development of sessile tubers from the axillary buds. In order to monitor the activity of the promoters within the stolon before tuber formation, stolon formation was induced by giving the explants a pre-treatment with gibberellin for 3 days (0.5 μM $GA_{4/7}$). After pre-treatment, explants were transferred to tuber-inducing medium and tuber formation started 7-8 days after transfer to gibberellin-free medium. The presence of luciferin (1mM) in the medium, needed to monitor luciferase expression, did not affect rate or timing of tuber formation (data not shown).

Imaging of LUC activity during tuber development

Promoter activity in each explant was imaged daily during the complete period of tuber formation starting from the first day of incubation on tuber-inducing medium. All promoters tested in this study were active during the growth of both stolon and tuber. Figure 1A (page 23) shows for each promoter an image of the LUC activity in explants before and after tuber formation. Figure 1B (page 23) shows per promoter a representative series of selected images of LUC activity during tuber development, synchronised for the first day that a swelling was observed (day 0).

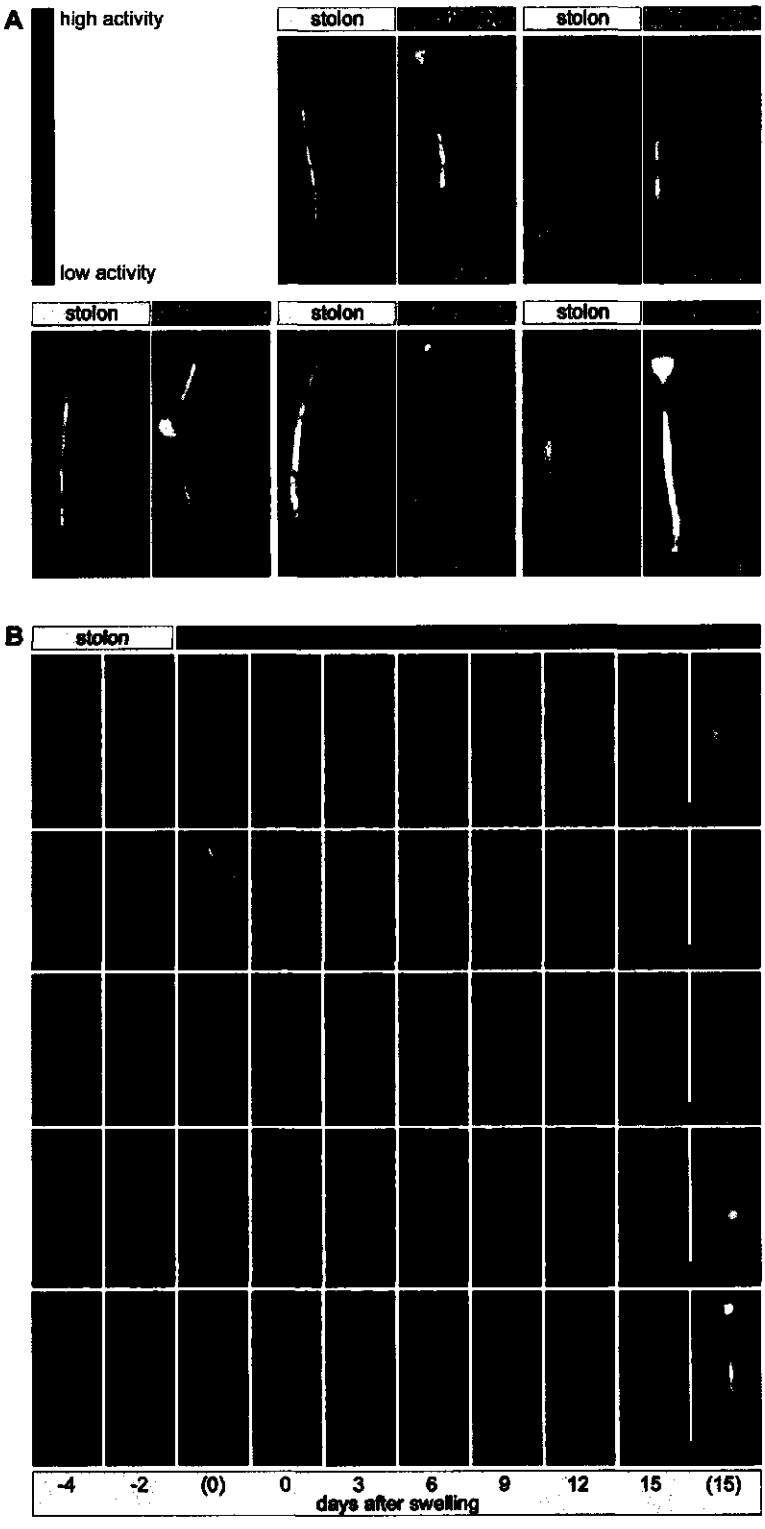
The CaMV35S promoter was active in stolon, tuber and stem region of the explants, although with a variegated pattern (figure 1A/B). After tuber formation the highest LUC activity was present in the stem and tuber. Both cell cycle promoters were active within the stolon prior to tuber formation (figure 1A/B). CycB1;1 promoter activity was mainly observed in the apical part of the stolon, presumably marking cell division resulting in longitudinal growth of the stolon. CDC2a promoter activity was found throughout the stolon prior to tuber formation. However, the highest activity was present in the sub-apical region of the longitudinally growing stolon. The differences between cycB1;1 and CDC2a promoter activity confirm that CDC2a is not only expressed in actively dividing cells but also in other cells which may mark their potential for cell division (Hemerly *et al.*, 1993).

Figure 1A

Localisation of luciferase activity in stolons and tubers. For each promoter-*luc* construct an example is shown of the activity in the stolon before the onset of tuber formation and the activity during tuber growth with a light image (left) and the luciferase activity (right). The luciferase activity (rlu $pixel^{-1}$) is displayed in pseudo colours as shown in the colour bar at the upper left corner, ranging from blue (low activity) via green and yellow to red (high activity). The relation between the colour scale for each promoter and the absolute level of luciferase activity was: CaMV35S 30 min. photon counting, colour scale 0-32 counts per pixel, CycB1;1 10 min. photon counting, colour scale 0-32 counts per pixel, CDC2a 5 min. photon counting, colour scale 0-128 counts per pixel, AGPaseS 30 min. photon counting, colour scale 0-32 counts per pixel, λPat21 10 min. photon counting, colour scale 0-128 counts per pixel.

Figure 1B

Changes in the localisation of LUC activity during tuber formation. Series of explants are shown prior to and during tuber development synchronised relatively to the day when swelling was observed (day 0). The numbers indicate the days prior to and after swelling. Besides images showing the luciferase activity, light images are shown at the first day of swelling (0) and at the last day of monitoring (15). The relation between the color scale for each promoter and the absolute level of luciferase activity was: **CaMV35S** promoter activity 10 min. photon counting, colour scale 0-16 counts per pixel, **CycB1;1** promoter activity 5 min. photon counting, colour scale 0-64 counts per pixel. **CDC2a** promoter activity 5 min. photon counting, colour scale 0-64 counts per pixel, **AGPaseS** promoter activity 10 min. photon counting, colour scale 0-16 counts per pixel, **λPat21** promoter activity 10 min. photon counting, colour scale 0-128 counts per pixel


A
high activity
low activity
stolon
stolon
stolon
stolon
stolon
B
stolon
-4
-2
(0)
0
3
6
9
12
15
(15)
days after swelling

During tuber formation radial expansion of the stolon takes place at the subapical region, due to cell enlargement and longitudinal cell divisions in pith and cortex (Duncan and Ewing, 1984; Vreugdenhil *et al.*, 1999). This increased cell division is marked by an enhanced activity of the cell cycle promoters (figure 1B). Upon tuber formation the CDC2a promoter activity decreased in the rest of the stolon marking the reduction in the potential for cell division and possibly the inhibition of other meristems to form new tubers.

The storage related promoters, AGPaseS and λPat21 showed a variegated pattern of activity in stolons before tuber formation (figure 1A/B). The regions with enhanced LUC activity were not specifically associated with the apical meristem or lateral buds, as observed for the cell cycle promoters. This indicates that prior to tuber formation storage gene activity can be locally induced in longitudinally growing stolons, independently of the regions that have the potential to develop into a tuber. During the onset of swelling, storage promoter activity increased at the site of the tuber. As for the cell cycle promoters, the activity of the storage related promoters decreased after swelling in the remaining part of the stolon (figure 1A/B).

***In vivo* LUC activity relates to steady state *luc* mRNA levels and expression of endogenous storage genes**

To verify if LUC activity as measured *in vivo* is a reflection of local *luc* mRNA levels, a semi-quantitative RT-PCR was performed. LUC activity was imaged in individual tubers from AGPaseS-*luc* and λPat21-*luc* reporter plants and sampled by high and low activity (3 tubers per sample). Total RNA was isolated from complete tubers and semi-quantitative RT-PCR was performed with specific primers for the *luc* gene. Figure 2 shows that there is a good correlation between the average LUC activity in the tissue samples and the *luc* mRNA level both for the AGPaseS-*luc* (figure 2A) and λPat21-*luc* reporter (figure 2B). Therefore we assume that changes in LUC activity reflect changes in the promoter activity that drives expression of the luciferase reporter gene.

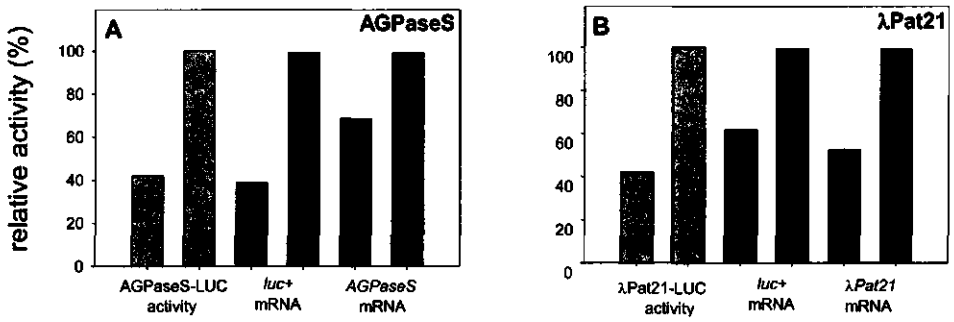


Figure 2
The relation between *in vivo* luciferase activity in potato tubers and the relative mRNA steady state levels of the *luc+* gene and the corresponding endogenous storage genes. Both LUC activity and mRNA levels are expressed as percentage of the maximum. Luciferase activity was measured in tubers (5 days after onset visible swelling), 15 min. photon counting in AGPaseS-*luc* (**A**) or λPat21-*luc* (**B**) reporter plants. At this stage tubers from genetically identical plants may show either high or low LUC activity. Per sample three tubers with a low (L) or high (H) LUC activity were pooled for RNA extraction. First strand cDNA was used as a template for RT-PCR, using *luc*, *AGPaseS*, *λPat21* and *cyclophilin* specific primers. The signal of the RT-PCR fragments were normalised for the expression level of cyclophilin mRNA in each sample.

Besides the correlation between LUC activity *in vivo* and transgene mRNA levels, it is also important to know whether the activity of the reporter construct reflects the expression of the endogenous gene. To verify if local AGPaseS-*luc* (figure 2A) and λPat21-*luc* (figure 2B) activity measured *in vivo* reflects mRNA levels of the endogenous *AGPaseS* or *λPat21* gene, a semi-quantitative RT-PCR was performed using *AGPaseS* or *λPat21* specific primers. Figure 2 shows the *AGPaseS* and *λPat21* mRNA levels in tubers with high and low AGPaseS-*luc* (figure 2A) or λPat21-*luc* (figure 2B) activity. For both reporter plants the LUC activity measured *in vivo* reflects the mRNA levels of the endogenous *AGPaseS* or *λPat21* gene. Thus, changes in LUC activity are likely to reflect changes in the expression of the endogenous storage gene.

Average promoter activity in relation to tuber formation

Photon emission caused by the LUC activity was quantified daily during tuber formation over a period of 25 days after placing the explants on tuber-inducing medium. The LUC activity profile can be related to the onset of visible swelling of the stolon, indicating the formation of a tuber. LUC activity (rlu pixel^{-1}) was quantified in the region that develops into a tuber and is expressed as a percentage of the maximum LUC activity observed for that tuber during its development. Figure 3A shows an example of the LUC activity profile in two explants containing AGPaseS-*luc*, placed on tuber-inducing medium at day 0. Tuber formation was observed at day 11 for explant A and at day 17 for explant B. In both tubers the LUC activity increased reaching a maximum at day 15 for explant A and day 20 for explant B. This figure shows that the upregulation of AGPaseS activity is related to the formation of a tuber.

However, there is a variation between the individual explants for the moment at which the tuber formation started. To compare the relation between onset of visible swelling and the upregulation in promoter activity, synchronisation of the data for the onset of swelling is required. A doubling of the stolon diameter was used as a morphological marker for onset of tuber formation and designated as day 0 in each individual sample. After synchronisation for the day of visible swelling, the average LUC activity of 28-69 tubers was calculated per promoter from five days before swelling (day –5) up to 20 days after the onset of tuber formation. Figure 3 B-F shows for each reporter gene construct the average LUC activity expressed as percentage of the maximum activity, in relation to the day of swelling (day 0). The upregulation of the average LUC activity varies between the different promoters: upregulation started clearly before visible swelling for AGPaseS followed by λPat21 (figure 3E/F) while upregulation of cycB1;1 and CDC2a (figure 3C/D) took place after visible swelling. All promoters showed an increasing LUC activity at the site where the tuber is formed during the first six days of tuber development reaching a maximum between day four and six after the onset of tuber development. This maximum activity was about five times the initial average activity within the stolon prior to tuber formation. In contrast, the average CaMV35S-*luc* activity did not change significantly after the start of tuber formation compared to the (initial) activity in the stolon before tuber formation (figure 3B).

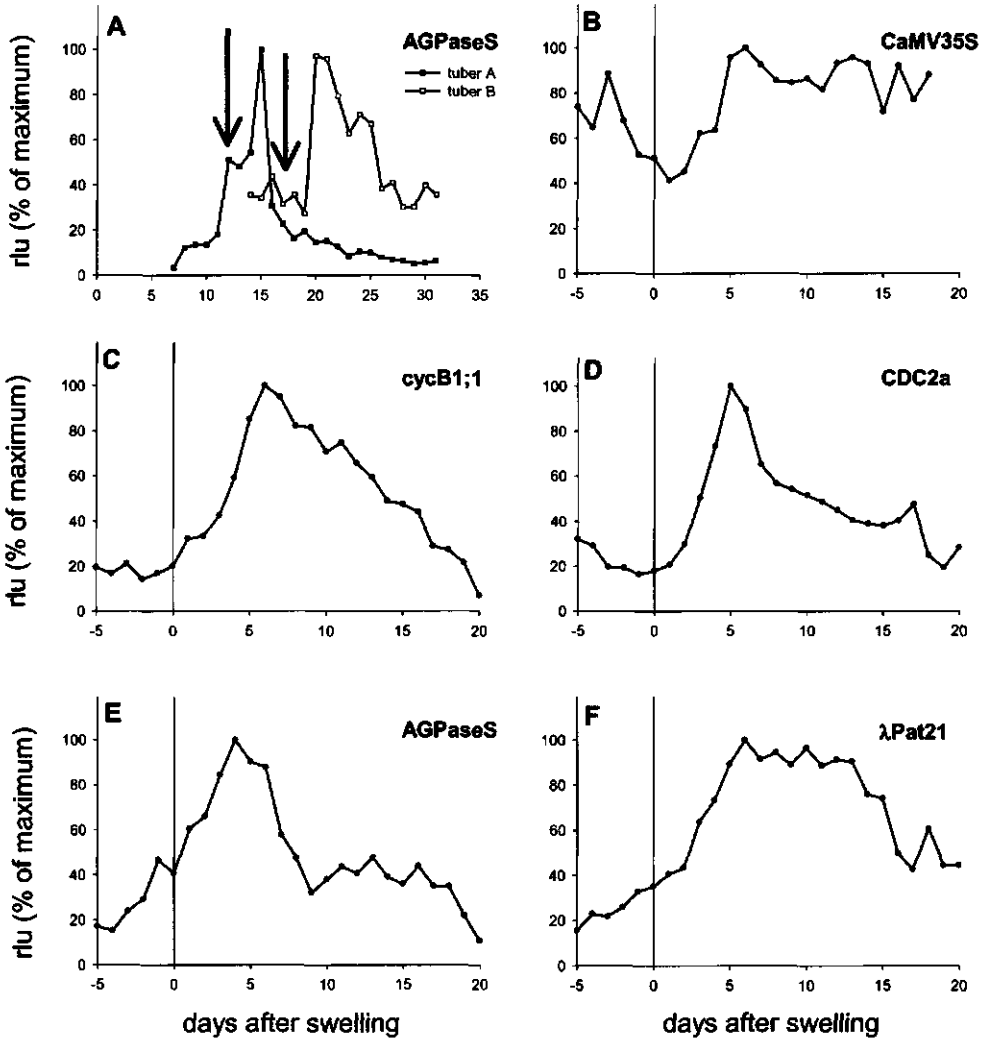


Figure 3
A. AGPaseS-*luc* activity profile during tuber formation in two individual explants. Luciferase activity measured daily after placing the explants on tuber-inducing medium at day 0, expressed as percentage of the maximum activity. Tuber formation was initiated after 11 days and 17 days respectively (arrows) leading to an enhanced induction of the AGPaseS promoter within the tubers. **B.-F.** Average luciferase activity during tuber formation synchronised for the first day of visible swelling (day 0). Activity of the various promoters was measured daily and expressed as percentage of the maximum activity. The LUC activity is based on the average of (**B**) 35 tubers for **CaMV35S**, (**C**) 46 tubers for **cycB1;1**, (**D**) 45 tubers for **CDC2a**, (**E**) 28 tubers for **AGPaseS**, (**F**) 69 tubers for **λpat21**.

Promoter activity in relation to formation of individual tubers

The average LUC activity profile for the cell cycle and storage related promoters suggested a fixed sequential order in the upregulation for each gene relative to tuber formation, with an upregulation in the activity for AGPaseS followed by that of λPat21 before swelling and the upregulation of the activity of the cell cycle promoters after swelling. However, for individual tubers the timing of the upregulation in promoter activity in relation to swelling can be highly variable. Figure 4A-D shows the LUC activity profile for three individual tubers per reporter gene construct, synchronised for the day of swelling but differing in the day upregulation of promoter activity started. The CycB1;1-*luc* (figure 4A) and CDC2a-*luc* (figure 4B) tubers showed a variable upregulation of LUC activity but the increase in LUC activity always occurred after the onset of tuber formation. The onset of AGPaseS and λPat21 also showed a variable induction of activity between the individual tubers in relation to the swelling (figure 4C/D) but in contrast to the cell cycle promoters, an upregulation in AGPaseS and λPat21 was also observed before visible swelling.

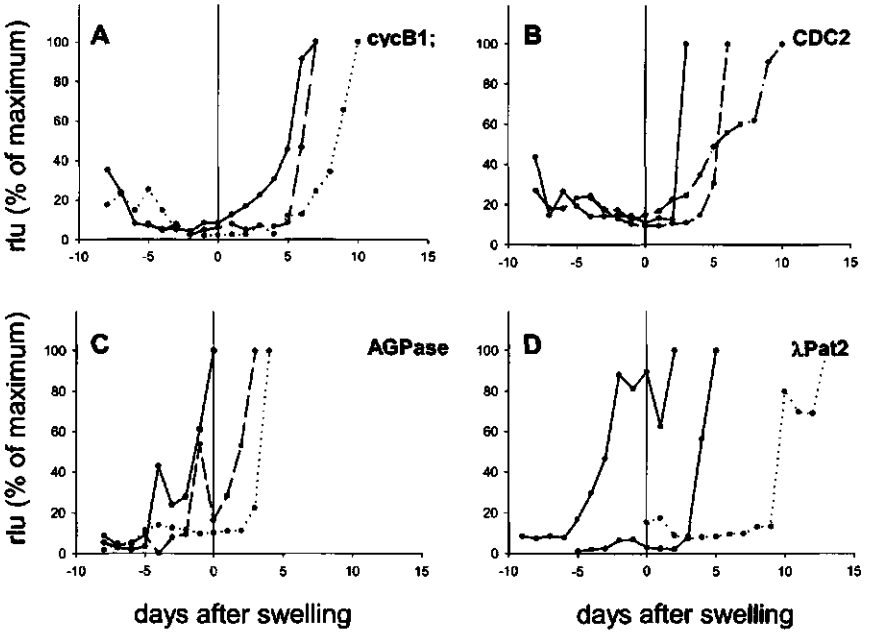


Figure 4

Luciferase activity during the development of three individual tubers synchronised for the day of swelling but differing in the upregulation of luciferase activity relatively to the day of swelling. Activity was measured daily and expressed as percentage of the maximum activity during tuber formation; (**A**) CycB1;1, (**B**) CDC2a, (**C**) AGPaseS and (**D**) λPat21.

The data for all individually monitored tubers are summarised in figure 5 A-B, which show the frequency distribution of the day at which upregulation was observed for each promoter, in relation to the day of visible swelling (day 0). The cell cycle promoters (figure 5A) show a preferential upregulation after visible swelling with the majority of tubers showing an upregulation that started 1-2 days after swelling. AGPaseS and λPat21 (figure 5B) show a start of upregulation in LUC activity both before and after visible swelling.
The earliest upregulation of the AGPaseS and the λPat21 gene expression was seen five days prior to the onset of visible swelling. The latest start of upregulation in activity was observed three days after the onset of swelling for the AGPaseS and ten days after swelling for λPat21. Although the start of upregulation for the storage promoter activity is observed both before and after swelling, the majority (76%) of the tubers with the AGPaseS-*luc* construct, showed a start of upregulation in activity prior to swelling. In contrast, for the λpat21 promoter only 36% of the tubers showed a start of upregulation in activity prior to visible swelling. Also the moment of the maximum LUC activity in the tuber differed between individual tubers. This variation is shown for each promoter in figure 5C-D. For the cell cycle promoters the moment for reaching the maximum activity, varied from day 3-12 for the cycB1;1 promoter and day 3-10 for the CDC2a promoter (figure 5C). For the storage promoters, the moment of reaching the maximum activity varied for individual tubers from day 0-8 for the AGPaseS promoter and day 1-15 for the λPat21 promoter.

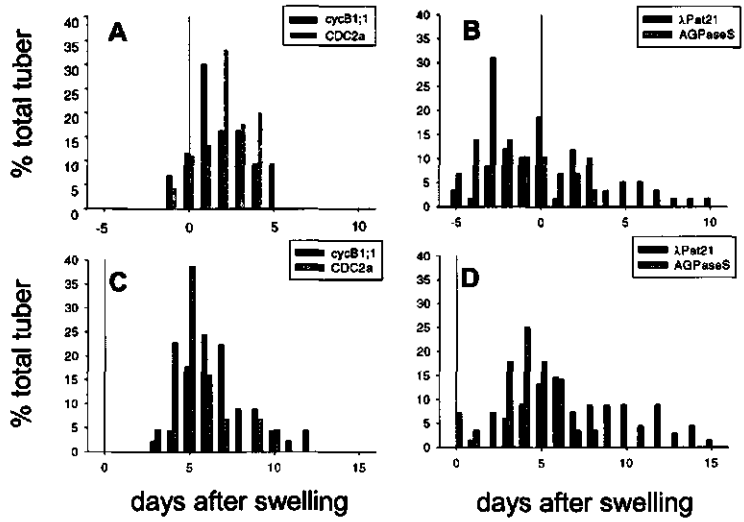


Figure 5
Frequency distribution for the day that individual tubers start upregulation of LUC activity (**A/B**) or show maximum LUC activity (**C/D**) during tuber development. The distribution is shown for each promoter relatively to the day of swelling (day 0); (**A/C**) cycB1;1 (dark bars) and CDC2a (gray bars), (**B/D**) λPat21 (dark bars) and AGPaseS (gray bars).

Quenching effect on monitoring LUC activity *in vivo*

In this study the activity of the various promoters that drive the luciferase reporter gene, is based on imaging the LUC activity in intact *in vitro* tubers. The photons produced by the LUC activity in the inner cell layers of the stolon or tuber have to pass several cell layers and may therefore have been quenched by surrounding cell layers preventing detection by the camera.

To determine the possible quenching effect, LUC activity in CaMV35S-*luc* tubers was measured before and after covering with non-transgenic tuber tissue. Covering with peridermal tissue (peel) resulted in an average reduction of 25%. When covered with tuber cortex tissue the average reduction was ~10 % per mm of tissue. The maximum diameter of the *in vitro* tubers used in this study did not exceed 5 mm (data not shown). This leads to a maximum reduction of 35% of the photons released by cells in the centre of the tuber.

DISCUSSION

The application of the firefly luciferase reporter system allows imaging and quantification of promoter activity *in planta* in individual samples over a prolonged period of time. This technique benefits gene expression analysis in relation to plant development because gene expression can be studied in individual samples in relation to the morphological changes like swelling during tuber formation. Due to the very slow regeneration of active luciferase enzyme after reacting with luciferin, in the continuous presence of this substrate, the *in vivo* LUC activity reflects transcriptional activity of the luciferase reporter gene as shown for petunia leaves (*Petunia hybrida* Vilm.) by Van Leeuwen *et al.* (2000) and for potato tubers (*Solanum tuberosum* L.) (Figure 2). Moreover, *in vivo* LUC activity reflected the expression of the storage genes under study (Figure 2) indicating that the activity of the reporter constructs reflected the expression of the endogenous genes.

Promoter activity prior to tuber formation

The cell cycle promoters used in this studied were isolated from *Arabidopsis thaliana* (L.). However, the expression pattern of the cycB1;1-*luc* and CDC2a-*luc* in *in vitro* potato plants closely resembled the pattern observed in *Arabidopsis thaliana* (Hemerley *et al.*, 1993; Ferreira *et al.*, 1994), indicating that also in potato these promoters mark regions with cell cycle activity. The cell cycle promoters cycB1;1 and CDC2a were highly active in the apical meristem of the stolon prior to tuber formation. This activity marks the transversal cell division within the apical region of the stolon during elongation (Xu *et al.*, 1998). However, CDC2a promoter activity was also observed in other parts of the stolon (figure 1). In *Arabidopsis thaliana* a CDC2a-*GUS* reporter gene showed an enhanced activity in stem segments associated with local regions of thickening and elongation (Hemerley *et al.*, 1993). After visible swelling the activity of both cell cycle related promoters increased at the tuber site (figure 1). This enhanced activity is thought to be related to longitudinal cell divisions which accompany tuber formation. The CDC2a-*luc* activity declined during tuber swelling in the rest of the stolon, indicating a loss of potential cell division in this tissue and possibly reducing the potential for the formation of new tubers.

Imaging of the distribution of λPat21-*luc* and AGPaseS-*luc* activity in *in vitro* stolons, showed a transient expression at multiple sites within a stolon before tuberization. For both reporters this local activity in growing stolons could be as high as the activity observed in developing tubers (data not shown). Thus the induction of storage genes within stolons is not strictly related to structural development of a tuber (swelling). Müller-Röber *et al.* (1994) described a rather low and non-uniform AGPaseS-*GUS* activity within stolons of soil-grown plants.

Histochemical localisation of AGPase enzyme activity confirmed that besides the transcriptional variegation, AGPase enzyme activity is also non-uniform within stolons (L. Sergeeva, personal communication). Expression of the patatin gene in stolons before tuber formation has been demonstrated before, using the patatin promoter fused to the GUS reporter (Wenzler *et al.*, 1989). However, this study showed that maximum GUS activity in developing tubers was about 3000-fold higher than the GUS activity in stolon tips before tuberization (Wenzler *et al.*, 1989). During tuberization Pat-GUS activity in stolons increased up to 10-30% of the activity observed in growing tubers (Wenzler *et al.*, 1989). In contrast, our results show that the expression of genes related to the synthesis of starch and storage proteins (as indicated by the activity of the reporter gene) can be as high in stolons prior to tuber formation as in developing tubers.

The results with the luciferase reporter gene may differ from results with similar promoters in combination with GUS reporter gene due to the greater stability of the GUS mRNA and especially of the GUS protein. The high stability of the GUS reporter results in a much longer timeframe of transgene promoter activity as compared to measurements with the luciferase reporter. Once a tuber was formed, the λPat21-*luc* and AGPaseS-*luc* activity in stolons declined. Apparently, the continued expression of storage genes at these sites depends on the continuous localised sink function within the stolon.

Promoter activity during tuber formation

Both the cell cycle and storage promoters showed a strong upregulation during tuber formation. Under control of the cell cycle promoters the average LUC activities showed an upregulation coinciding with visible swelling (figure 3C/D), indicating that the swelling is coupled to cell division. In individual tubers, the start of the upregulation varied for cell cycle promoter activity from one day prior to five days after the onset of tuber formation (figure 5A). Only a small fraction (4-7%) of the individual tubers showed an upregulation in cell cycle activity starting one day before visible swelling (figure 5A). However, scoring accuracy for tuber formation (carried out by visible observation) may vary plus or minus one day. Reports by Sanz *et al.* (1996) and Xu *et al.* (1998) show that during the first stages of tuber development, swelling can be accomplished by cell enlargement only. Results presented here indicate that during the first stages of tuber formation, swelling of the stolon is possible without an upregulation in the expression of cell cycle genes.

Storage promoters were also highly upregulated during tuber formation at the tuber site. The average LUC activity in multiple AGPaseS-*luc* and λPat21-*luc* tubers showed an upregulation in activity that started well before visible swelling at day -4 for AGPaseS and at day -3 for λPat21 (figure 3E/F). In a similar *in vitro* tuber system, transcriptional analysis of pooled tuber samples revealed an upregulation of the AGPaseS expression starting one day before visible swelling while for patatin an upregulation was observed at the onset of tuber formation (Hendriks *et al.*, 1991; Visser *et al.*,1994; Bachem *et al.*, 1996). The results reported here show that the LUC reporter activity may be measured more sensitively than mRNA or protein levels of the endogenous genes. Combined, these different studies all using pooled samples, suggest that tuber formation is the result of several processes that occur in a fixed sequential order during the developmental program of every individual tuber: tuber formation is initiated by an upregulation of starch synthesis followed by induction of storage proteins subsequently followed by swelling and cell division. However, by using the non-invasive luciferase reporter system, we were able to determine the relation between the expression in individual tubers and the average activity in multiple tubers. When related to the onset of tuber formation, the expression profiles observed in individual tubers indicate a far more flexible control of the developmental program then suggested by the average LUC activity profiles. LUC activity in individual tubers showed a large variation in the timing of the upregulation, starting from day –5 to +3 days after the onset of tuber formation for AGPaseS and from day –5 to day +10 after the onset of tuber formation for the λPat21 promoter (figure 5B). This shows that the upregulation of storage genes during the first stages of tuber development is highly variable and not directly related to swelling. Moreover, also the different frequency distribution for AGPaseS and λPat21 for the moment of upregulation in individual tubers suggest an independent regulation of starch and protein storage activity during the first stages of tuber development (figure 5B). Accumulation of storage product without tuber formation is described by Paiva *et al.* (1983) and in transgenic potato plants containing the *ipt* gene from *Agrobacterium tumefaciens* involved in the cytokinin biosynthesis (Sergeeva *et al.*, 2000). Both methods showed an accumulation of starch and patatin in the stolon under tuber-inducing conditions, but only when an adequate sink (tuber) was absent. Although these results indicate that the induction of storage activity is possible independently from the morphological changes (swelling) associated with tuberization, in both methods tuber formation was prevented.

The results presented here indicate that during natural tuber formation the timing of the upregulation in expression of genes involved in carbohydrate and protein storage, is not strictly synchronized to the onset of visible swelling and an upregulation of the storage genes is not required for the morphological development of tubers.

Concluding remarks

The use of the luciferase reporter gene allowed (semi)-continuous analysis of gene expression during plant development in individual samples. The results of this study during tuber development in potato lead to the following conclusion: The **average** LUC activity profile suggests a fixed sequential order of events during the developmental program of tuber formation i.e. (*i*) the induction of starch synthesis, (*ii*) storage protein synthesis, (*iii*) swelling and (*iv*) induction of cell cycle. This confirms earlier reports that showed an upregulation of transcription of storage genes prior to tuber formation. However the analysis of **individual** tubers reveals a different picture. Induction of cell cycle related genes always occurs coinciding with, or shortly after visible swelling (but never before). The upregulation of storage gene expression is not fixed in relation to the moment of swelling and can occur before and after swelling. This indicates an uncoupling of storage activity from the structural development of the tuber. We infer that the signal(s) inducing swelling are not the same as those activating the different storage genes. The results demonstrate that analysis of developmental programs by pooled samples may obscure independently operating regulating mechanisms. Only the analysis of multiple individual samples allows to study the exact relationship between gene expression and the different aspects of a developmental program.

Acknowledgements

The authors thank Dr. Dirk Inzé from the University of Gent for kindly providing the cycB1;1 and CDC2a promoters, Dr. Michael Bevan from John Innes Centre for providing the λPat21 promoter and Dr. Bernd Müller-Röber from the Max Planck Institute of Molecular Plant Physiology for providing the AGPaseS promoter.

Chapter 3

Differential hormone responses in tuber and bud of potato tubers during second growth

John Verhees, Marieke van Hulten, Alexander R.van der Krol, Dick Vreugdenhil and Linus H.W. van der Plas

Inhibition of tuber growth and outgrowth of the apical bud during potato tuber development (*Solanum tuberosum* L.) may occur when tuber-inducing conditions become adverse. This phenomenon is called second growth and can be mimicked by transferring developing *in vitro* tubers to medium with GA_{4+7} (10 μM). This method of synchronized second growth induction was used to study the causal relation between tuber development (primary growth) and an induced growth of the bud (second growth). Different aspects of tuber development were studied by analysing the induction of cell cycle - (cycB1;1, CDC2a) and storage related genes (AGPaseS, λPat21), using the firefly (*Photinus pyralis*) luciferase reporter system. Transferring young *in vitro* tubers to medium with GA_{4+7} resulted in a decline in the activity of the cycB1;1, AGPaseS and λPat21 promoter in the tuber, preceding visible growth of the apical bud. In contrast, GA_{4+7} had no effect on the activity of the CaMV35S and CDC2a promoter in the tubers. Although the decline in gene expression preceded second growth there was no causal relation, as shown in tubers where second growth, induced by GA, was prevented by combining GA with ABA. There was a significant difference *in cell cycle gene expression* (cycB1;1) and *starch synthesis related gene expression* (AGPaseS) between bud and tuber region in response to GA. Expression in the tuber region declined whereas expression in the bud region remained high or increased during second growth. Combining ABA with GA, prevented second growth and affected the differential cycB1;1 and AGPaseS expression in bud and tuber region indicating that these two regions of the potato tuber respond differently upon hormones.

INTRODUCTION

In potato plants, environmental conditions e.g. lengthening of the photoperiod, increasing nitrogen supply, heat or drought stress may lead to inhibition of tuber growth and induced growth of the apical meristem in the tuber bud. This phenomenon is called second growth (Van den Berg *et al.*, 1990; Ewing and Struik, 1992). Whether such conditions result in second growth depends on the duration of these conditions and the developmental stage of the tubers (Van den Berg *et al.*, 1991). The type of second growth in soil-grown potato tubers depends on the inducing conditions resulting in a stolon, a stolon directly followed by a tuber (tuber chains) or a secondary tuber on the primary tuber (bottle necks or knobby tubers) (Lugt *et al.*, 1964; Hiller *et al.*, 1985; Bodlaender *et al.*, 1964).

Heat treatment of tubers results in second growth and a concomitant decline in endogenous ABA (Van den Berg *et al.*, 1991). GA-treatment of tubers also results in second growth, both in *in vitro* tubers and tubers on soil-grown plants (Lippert *et al.*, 1958; Bodlaender *et al.*, 1964; Claassens and Vreugdenhil, 2000). The amount of GA required to induce second growth increases with the maturation of the tuber (Claassens and Vreugdenhil, 2000). The results suggest that the GA/ABA ratio is a key factor in the balance between tuber growth (primary growth) and second growth (Kraus, 1985; Van den Berg *et al.*, 1991).

At the cellular and molecular level the induction of second growth in tubers is characterised by a re-orientation of the growth, from a sub-apical radial expansion of the tuber towards longitudinal growth of the apical bud. Simultaneously, the synthesis of starch and storage proteins in the tuber declines. Extracts from tubers treated with GA show a decline in starch biosynthesis but not in the starch content. This decline in starch synthesis can be attributed to a strong decrease in ADPglucose-pyrophosphorylase (AGPase) activity, a key-enzyme in starch biosynthesis (Mares *et al.*, 1981). In contrast, there is no immediate change in the activity of other enzymes involved in starch biosynthesis, like starch phosphorylase and starch synthase (Mares *et al.*, 1981). The decrease in starch biosynthesis coincides with an increase in the reducing sugar concentration and a decline in sucrose concentration (Mares *et al.*, 1981). Besides a reduction in starch biosynthesis, there is also a strong decline in the accumulation of patatin, one of the major storage proteins. GA-treatment also results in a reduced accumulation of two other storage proteins (22 kD and 15 kD) but it does not affect the level of most other tuber proteins (Hannapel *et al.*, 1985).

Although GA-treatment has been shown to reduce tuber growth and the synthesis of starch and storage proteins in potato tubers and to induce growth of the bud meristem, it is not known whether these changes are regulated at the protein level or at the transcriptional level. The aim of these experiments is to describe the dynamic behaviour of specific gene expression during transition from tuber development (primary growth) to second growth. We analysed the induction of cell cycle (cycB1;1 and CDC2a) and storage related genes (AGPaseS and λPat21) in the tuber and bud region, using transformed potato plants containing the promoter of these genes fused to the coding sequence of the luciferase reporter gene. Non-invasive detection of LUC activity in tubers enabled us to relate spatial and temporal changes in gene expression to second growth. We have demonstrated before that the luciferase gene from firefly (*Photinus pyralis*) is an useful reporter to study promoter activity during tuber development, showing a high correlation between *in vivo* LUC activity and steady state mRNA levels of the reporter and the endogenous genes under study (Chapter 2). In the presence of saturating amounts of the substrate luciferin, these reporter plants can reveal changes in promoter activity within minutes (Van Leeuwen *et al.*, 2000). Furthermore, the activity of the luciferase reporter can be observed non-invasively, allowing comparison of transcriptional induction both in populations and in individual tubers (Chapter 2).

Our analysis revealed a switch in gene expression during second growth: the cell cycle and storage related gene expression in the tuber (primary growth) are switched off whereas cycB1;1 and AGPaseS expression remain present in the bud region coinciding with the growth of the apical meristem (second growth). We show that during second growth transcription of the same gene(s) is differentially regulated by hormones in tuber and bud.

MATERIALS AND METHODS

Plant material
Transgenic potato plants (*Solanum tuberosum* cv 'Desiree') with the various reporter gene constructs were grown *in vitro* with 16 hours light period (50 W m^{-2} at 20°C) for 4-5 weeks. To produce *in vitro* tubers, single node cuttings containing one axillary bud per explant were placed on Murashige and Skoog (1962) medium containing 1/10 part of the standard amount of KNO_3 and NH_4NO_3, 8% (w/v) sucrose, 5 µM benzyl-aminopurine (BAP) and 0.8% (w/v) agar with a final pH of 5.8 (tuber–inducing medium) (Hendriks *et al.*, 1991; Verhees *et al.*, 2002). After 7 days of incubation in the dark at 20°C the axillary buds started to develop into a sessile tuber with a final diameter between 4 and 6 mm. For most experiments tubers were used 1-5 days after visible swelling (young tubers). During some of the experiments mature tubers were used, 25-30 days after visible swelling.

Reporter gene constructs
To monitor transcriptional dynamics of cell cycle and storage genes during second growth, we constructed various reporters between the promoter of the gene under study and the coding sequence of the luciferase+ reporter gene (Promega, Sherf and Wood, 1994; Verhees *et al.*, 2002). The cell cycle related promoters used for this study were cycB1;1 (Ferreira *et al.*, 1994) and CDC2a (Hemerley *et al.*, 1993) from *Arabidopsis thaliana* L. As representatives of starch and protein storage genes in potato tubers, two reporter constructs were made containing the luciferase gene fused to the AGPaseS promoter of the large subunit of the ADP-glucose pyrophosphorylase (Müller-Röber *et al.*, 1992) or the promoter sequence of the λPat21 patatin gene (Bevan *et al.*, 1986) from *Solanum tuberosum* L. As a control we used reporter plants containing the CaMV35S promoter fused to the luciferase reporter gene. *Agrobacterium tumefaciens* mediated transformation of *Solanum tuberosum* cv 'Desiree' resulted in several primary transformants. Gene induction was analysed in two independent transformed lines per promoter during GA-induced second growth.

Induction of second growth and in vivo measurements of luciferase activity
One day before second growth induction by gibberellin treatment, *in vitro* tubers were pre-treated with the substrate luciferin. The stem segments bearing the tuber were placed on tuber-inducing medium containing 0.2 mM luciferin (D-luciferin, sodium salt) followed by additional spraying with a 1mM luciferin solution. To ensure substrate uptake from the medium a thin slice was removed from the basal part of the stem segment before pre-treatment with luciferin.

After pre-treatment, *in vitro* tubers were transferred to medium containing 8% sucrose supplemented with 10 µM GA_{4+7}, applied to the medium from a stock solution by filter sterilisation, and 0.2 mM luciferin. To determine a possible effect of transferring tubers to fresh medium, control tubers were transferred to fresh tuber-inducing medium + 0.2 mM luciferin, but lacking GA_{4+7}.
When required, abscisic acid was applied to the medium from a stock solution by filter sterilization at a final concentration of 10 µM or 100 µM in combination with 10 µM GA_{4+7}.

Measurement of luciferase activity
Directly after transferring the in vitro tubers to medium with GA_{4+7} (t=0), in vivo luciferase (LUC) activity was measured during 30 minutes intervals, using a cryogenic cooled CCD camera for low light imaging (VersArray ™, Roper Scientific). Relative luciferase activities were depicted in images showing the amount of relative light units per pixel (rlu $pixel^{-1}$). Relative LUC activity was quantified in the tuber and bud region of each individual tuber after transfer to the various conditions. Digital image analyses were performed by using MetaMorph™ version 4.5 (Universal Imaging Corporation). Differences in the relative luciferase activities between the bud and tuber region during the various treatments were statistically analysed by applying a F-test at the given time-point (hours after starting the treatment) and specified as significant when $P<0.05$.

RESULTS

Induction of second growth by GA_{4+7} treatment is dependent on GA/ABA concentration and tuber age

Transferring *in vitro* tubers to medium with GA_{4+7} resulted in the formation of secondary stolons. Second growth was scored visually each day after transfer by counting the number of tubers that had formed a secondary stolon with a length of >3 mm. The induction of second growth in young tubers (1-5 days after swelling) at different concentrations of GA_{4+7} is shown in figure 1A. Although all three concentrations of GA_{4+7} were effective in inducing second growth, the most synchronous second growth was observed with the highest GA concentration (10 µM) with >90% of the tubers showing second growth after 2 days.

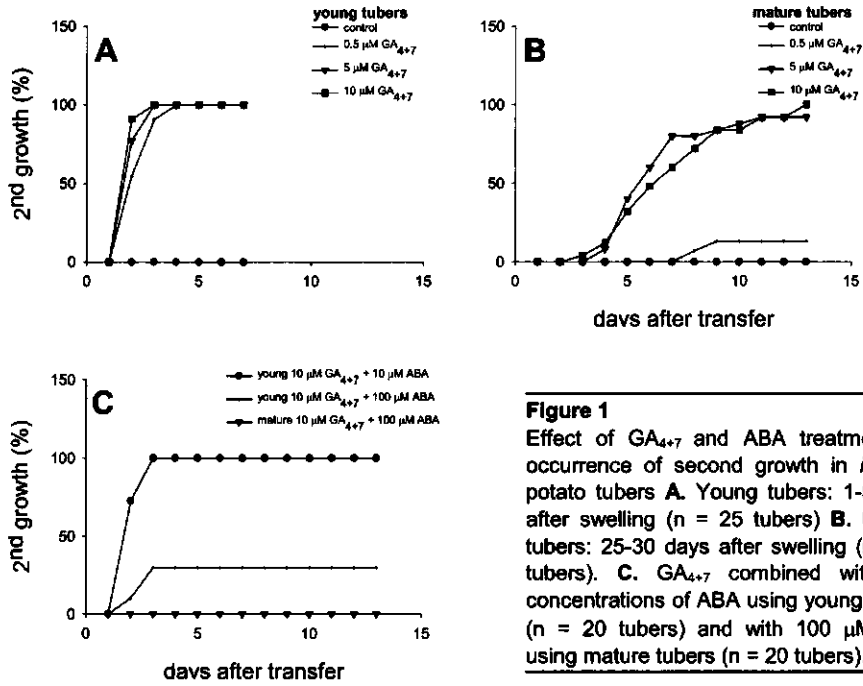


Figure 1
Effect of GA_{4+7} and ABA treatment on occurrence of second growth in *in vitro* potato tubers **A.** Young tubers: 1-5 days after swelling (n = 25 tubers) **B.** Mature tubers: 25-30 days after swelling (n = 25 tubers). **C.** GA_{4+7} combined with two concentrations of ABA using young tubers (n = 20 tubers) and with 100 µM ABA using mature tubers (n = 20 tubers).

To determine if the sensitivity to GA changes during tuber development, mature tubers (25-30 days after swelling) were transferred to medium with different GA_{4+7} concentrations. In mature tubers, induction of second growth by GA_{4+7} was delayed (day 4-10) and less effective (15-90 %) as compared to young tubers (compare figures 1B and 1A).

The delay in second growth in mature tubers could be caused by a lower uptake rate of GA from the medium compared to young tubers. To determine whether there is still transport of compounds from the medium into the tuber, young and mature tubers were placed on medium containing eosine. After 24 hours of incubation the eosine was observed in the complete vascular tissue of both young and mature tubers including the apical bud (results not shown).

The difference in response between young and mature tubers to GA_{4+7} might also be due to differences in internal ABA content. Therefore, we tested the effect of GA_{4+7} in combination with two concentrations of ABA. ABA in the medium at a concentration of 10 µM did not prevent second growth in young tubers when combined with 10 µM GA_{4+7} but 100 µM ABA combined with GA_{4+7} largely prevented second growth (compare figures 1C and 1A). The efficiency of second growth induced by GA_{4+7} in young tubers was lower in combination with 100 µM ABA (30 %) but second growth was not delayed. Transferring mature tubers to medium with 10 µM GA_{4+7} and 100 µM ABA completely prevented second growth (compare figures 1C and 1B).

Differential gene expression in the tuber precedes second growth

To determine the dynamics in cell cycle and storage related gene expression in relation to second growth, young *in vitro* tubers with the various reporter constructs were transferred to medium with GA_{4+7}. For each reporter construct, two independent lines were used for analysing gene expression during GA-treatment. However, no differences were observed in the responses between the two lines during second growth.

LUC activity was quantified (rlu $pixel^{-1}$) per tuber and the results were combined to calculate, for each reporter construct, the average LUC activity of 25 tubers. Figure 2 shows this average LUC activity for each promoter, expressed as percentage of the LUC activity at t=0. The treatment of young tubers with GA_{4+7} did not result in a significant change (t = 50 h) in CaMV35S (figure 2A) or CDC2a (figure 2C) activity as compared to the control tubers. For cycB1;1, AGPaseS and λPat21 a significant decline was observed (t = 50 h) in LUC activity relative to the control tubers after transfer to medium with GA_{4+7}. However, the moment at which average LUC activity in GA-treated tubers differed from the activity in the control tubers varied for the various promoters, starting with AGPaseS (figure 2D) and λPat21 (figure 2E) followed by cycB1;1 (figure 2B). LUC activity was also quantified during second growth induction in mature tubers to see if the response on GA was related to the developmental stage of the tuber.

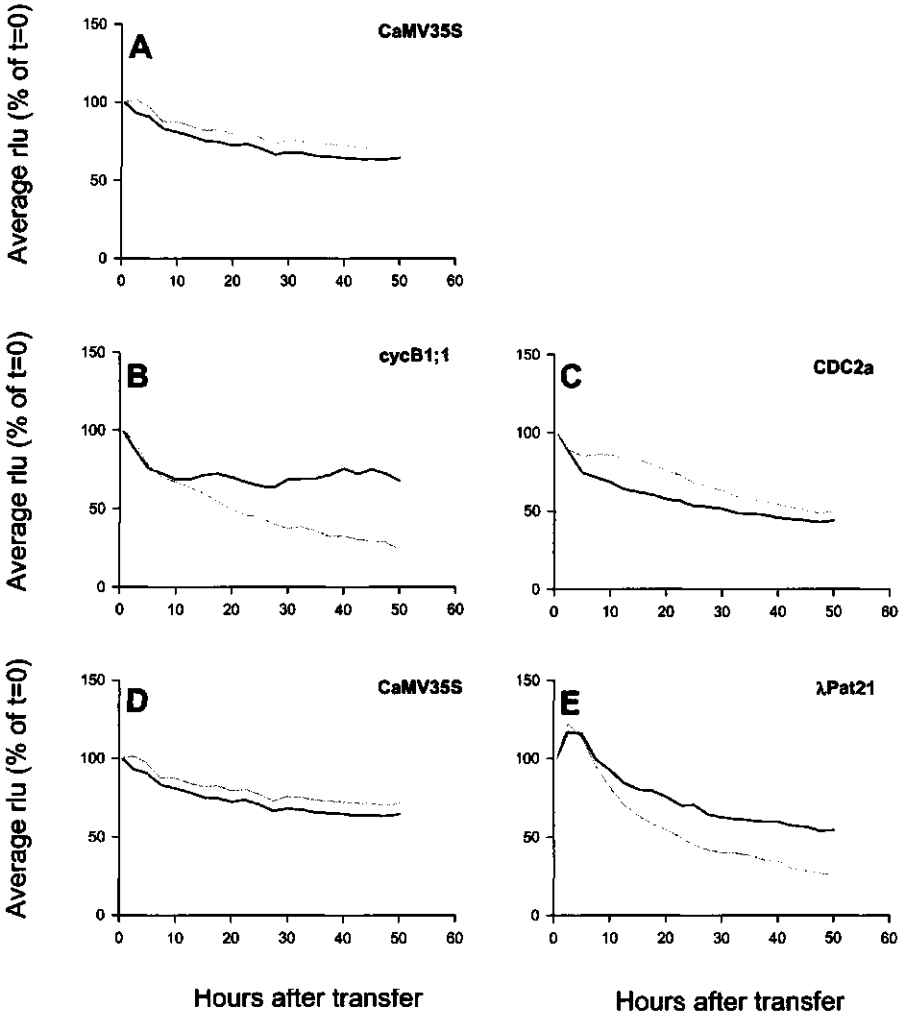
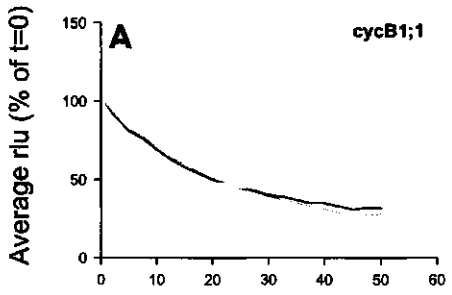
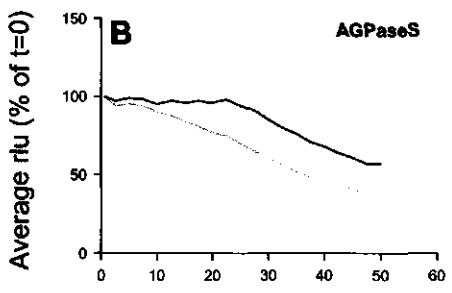


Figure 2
Average luciferase activity of 25 young tubers (1-5 days after swelling) after transfer to medium with 10 µM GA_{4+7} (grey) and in tubers on control medium without GA_{4+7} (black). For each promoter luciferase activity is expressed as percentage of the initial activity at the moment of transfer; **(A)** CaMV35S, **(B)** cycB1;1, **(C)** CDC2a, **(D)** AGPaseS, **(E)** λPat21.

Transferring mature tubers to medium with GA_{4+7} did not result in a significant difference (t = 50 h) in cycB1;1 promoter activity compared to tubers transferred to medium without GA_{4+7} (figure 3A). In contrast, storage related promoters (figure 3 B-C) showed a decline in promoter activity like observed in young tubers. As the second growth is delayed by several days in mature tubers (figure 1B), the immediate decline in storage promoter activity in the tuber is not directly related to the onset of second growth.





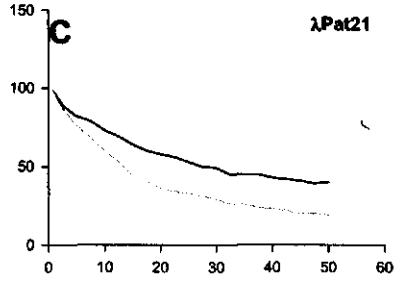


Figure 3
Average luciferase activity of 20 mature tubers (25-30 days after swelling) after transfer to medium with 10 µM GA_{4+7} (grey) and in tubers on control medium (black). For each promoter luciferase activity is expressed as percentage of the initial activity (**A**) cycB1;1, (**B**) AGPaseS, (**C**) λPat21.

That storage gene induction in the tuber and outgrowth of the bud are two independent processes, was confirmed by transferring young tubers to medium with GA_{4+7} combined with 100 µM ABA. Although ABA largely prevented second growth (figure 1C), AGPaseS-*luc* activity in the tuber declined similarly to that in tubers transferred to medium with only GA_{4+7} (figure 4). Average AGPaseS-*luc* activity was only calculated for those tubers that did not have second growth. No response was observed in the activity of the AGPaseS promoter in tubers transferred to medium with only ABA compared to the control (figure 4). For both the cycB1;1 and λPat21 promoter, ABA had also no effect on the decline in promoter activity induced by GA_{4+7} (results not shown).

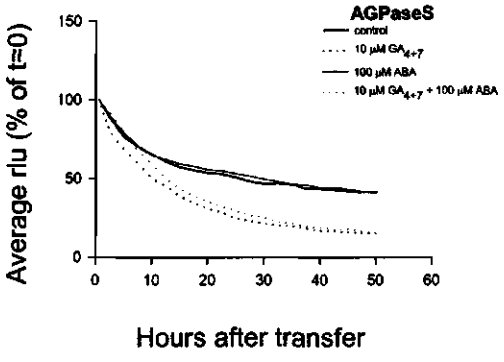


Figure 4
Average AGPaseS-*luc* activity profiles of 20 young tubers (1-5 days after swelling) after transfer to medium with 10 µM GA_{4+7} and 100 µM ABA. Luciferase activity, during the combined treatment, was only quantified in tubers without second growth. For comparison, activity profiles of control tubers (tuber-inducing medium), and of tuber transferred to medium with 10 µM GA_{4+7} or 100 µM ABA are also depicted. Luciferase activity is expressed as percentage of the initial activity at the moment of transfer.

Induction of second growth affects promoter activity in the bud region preceding second growth

Besides the decline in cell cycle and storage related gene expression in the tuber, spatial variation in promoter activity was observed between the tuber and bud region during second growth. Figure 5 (page 45) shows representative series of images of the LUC activity, in young tubers after transfer to medium with GA_{4+7}.

CaMV35S-*luc* activity varied between stem segment and tuber and showed an enhanced activity in the bud compared to the tuber during bud extension (figure 5A). The cycB1;1 promoter activity showed a decline in the tuber, whereas the activity in the bud remained high (figure 5B). During growth of the secondary stolon, enhanced cycB1;1 activity was observed in the second meristem (arrow). For the CDC2a promoter the maximum activity was not observed in the stolon but in the tuber (figure 5C). For both storage promoters (AGPaseS, λPat21) activity declined in the tuber (figure 5 D-E). However, for the AGPaseS promoter the activity remained high at or just below the apical bud, during growth of the secondary stolon (figure 5D). In contrast, the λPat21 promoter activity declined both in tuber and bud region during second growth (figure 5E).

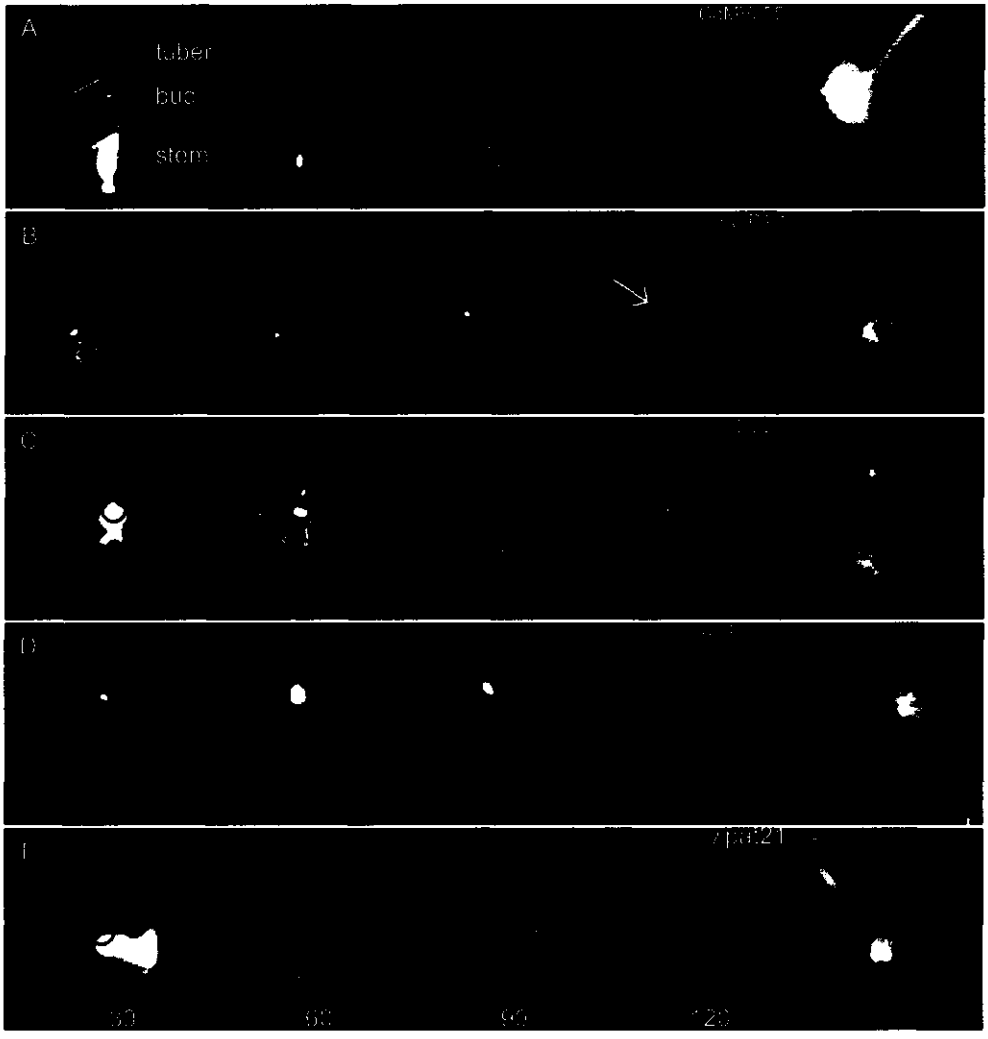
Figure 5
Spatial distribution of luciferase activity in young tubers (1-5 days after swelling) during second growth induced by gibberellin treatment (10 μM GA_{4+7}) resulting in tubers with a secondary stolon (right panels). The luciferase activity is depicted in pseudo colours as shown in the bar (lower left) ranging from blue (low activity) via green, yellow and red to white (high activity). **A.** CaMV35S, **B.** cycB1;1, **C.** CDC2a, **D.** AGPaseS and **E.** λPat21. In the first images of each promoter series a dotted circle marks the bud region. The changes in the luciferase activity are shown in sequential order after transfer to medium with GA at the given time (30-120 hours, lower panel) after transfer

Promoter activity was also imaged in mature tubers transferred to medium with GA_{4+7}, exhibiting delayed second growth. Figure 6 shows the distribution in the activity of the cycB1;1 (figure 6A-B) and AGPaseS (figure 6C-D) promoter, in mature tubers imaged 90 hours after transfer to medium with GA_{4+7}. Because the timing of second growth varied between the individual mature tubers, for each promoter two images are shown of two tubers 90 hours after transfer, one of a tuber with early second growth (2^{nd} growth at $t < 90$ hours, figure 6A and 6C) and of a tuber with late second growth (2^{nd} growth at $t > 90$ hours, figure 6B and 6D). Mature tubers with early second growth (figure 6A) showed an enhanced activity of cycB1;1 in the bud region. In contrast, no specific cycB1;1 activity was observed in the bud region of tubers with late second growth (figure 6B). AGPaseS promoter activity remained high in the bud region while the activity in the tuber declined both in tubers with early (figure 6C) and late (figure 6D) second growth. This suggests that the enhanced AGPaseS activity in the bud region is not strictly related to the time of second growth.

Figure 6 (left)
Spatial distribution of the luciferase activity in mature tubers (25-30 days after swelling) 90 hours after transfer to medium with 10μM GA_{4+7}. A,B: cycB1;1-*luc* activity in representative mature tubers with early (A, during the first 90 hours) and late (B, after the first 90 hours) second growth. C,D: AGPaseS-*luc* activity in representative mature tubers with early (C) and late (D) second growth 90 hours after transfer. A dotted circle marks the bud region of the tubers without second growth.

Figure 7 (right)
Spatial distribution of the luciferase activity in young tubers (1-5 days after swelling) 60 hours after transfer to medium with 10μM GA_{4+7} (A,C) or 10μM GA_{4+7}+100μM ABA (B,D). **A,B:** cycB1;1-*luc* activity in representative tubers with (A) and without second growth (B). **C,D:** AGPaseS-*luc* activity in representative tubers with (C) and without second growth (D). A dotted circle marks the bud region of the tubers without second growth.

Differential hormone responses in tuber and bud during second growth



To test if the enhanced cycB1;1 and AGPaseS promoter activities in the apical bud were specifically induced by gibberellins or related to actual growth of the apical bud, the distribution of LUC activity was imaged in young tubers after transfer to medium with 10 μM GA_{4+7} and 100 μM ABA. Figure 7 (page 45) shows the distribution in the activity of the cycB1;1 and AGPaseS promoter, in young tubers imaged 60 hours after transfer to medium with GA_{4+7} (figure 7A and 7C) or GA_{4+7} +ABA (figure 7B and 7D). Although second growth was absent in tuber transferred to medium with GA_{4+7} +ABA, an enhanced cycB1;1 activity was observed in the bud region of these tubers (figure 7B), similar to that observed in tubers transferred to medium with GA_{4+7} (figure 7A). For the AGPaseS promoter a difference was observed: in tubers transferred to medium with GA_{4+7} + ABA no enhanced activity of the AGPaseS promoter was observed in the bud region (figure 7D). In some tubers transferred to medium with GA_{4+7} + ABA an enhanced AGPaseS promoter activity was observed in the apical part of the tuber, below the bud region.

Besides imaging cycB1;1 and AGPaseS promoter activity during second growth, we quantified the LUC activity in the bud and tuber region of several individual tubers after transfer to medium with GA_{4+7}. Figure 8A (cycB1;1) and 8C (AGPaseS) show the average LUC activity of 15 individual young tubers in bud and tuber region after transfer to medium with GA_{4+7}. Horizontal bars indicate the variation in timing of bud extension for the pooled tuber samples. The LUC activity is expressed as a percentage of the activity at t=15 hours and shown until bud extension was observed. To illustrate the variation in LUC activity between individual tubers, figure 8B (cycB1;1) and figure 8D (AGPaseS) show the LUC activity in the bud region of two individual young tubers after transfer to medium with GA_{4+7}. The arrows indicate the start of bud extension in the individual tubers.

The average activity profile of the cycB1;1 promoter shows a strong upregulation in the bud region preceding second growth which was observed about 40 hours after transfer to medium with GA_{4+7}. However, analysis of cycB1;1-*luc* activity in individual tubers (figure 8B) shows no direct relation between the timing of second growth and the cycB1;1-*luc* activity profile. The average AGPaseS-*luc* activity shows no significant difference (t = 50 h) between bud and tuber region after transfer to medium with GA_{4+7} (figure 8C). However, a higher AGPaseS promoter activity was observed in the bud region of some individual tubers preceding second growth (figure 8D). In the other tubers, AGPaseS promoter activity declined in both bud and tuber during second growth.

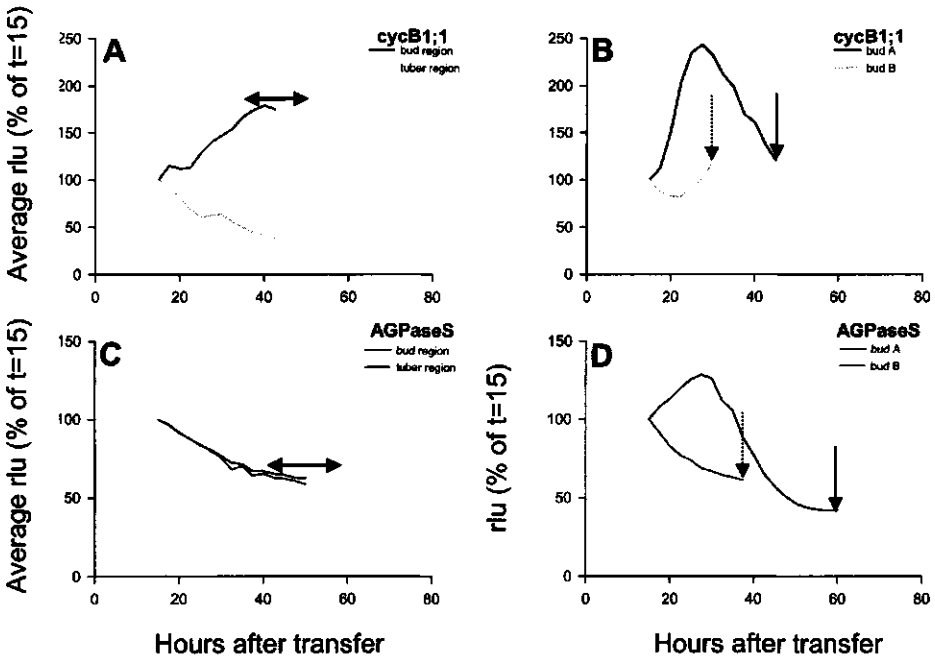


Figure 8

Promoter activity profiles of cycB1;1 and AGPaseS during second growth quantified in bud and tuber region of young tubers (1-5 days after swelling) after transfer to medium with 10 µM GA_{4+7}. Luciferase activity is plotted as percentage of the activity at t =15 hours. Both average activity profiles (A,C) of the pooled tubers and activity profiles of individual tubers (B,D) are depicted. Horizontal bars indicate the variation in the timing of second growth of the pooled tubers. Arrows indicate the timing of bud extension for the individual tubers. **A.** Average cycB1;1-*luc* activity profile of 15 tubers in bud region (black) and tuber (grey) region. **B.** cycB1;1-*luc* activity in the bud region of two individual tubers, differing in the time of bud extension. **C.** Average AGPaseS-*luc* activity profile of 15 tubers in bud region (black) and tuber region (grey). **D.** AGPaseS-*luc* activity in the bud region of two individual tubers, differing in the time of bud extension.

CycB1;I and AGPaseS promoter activities in tuber and bud region in relation to second growth were further investigated by quantifying the LUC activity in young and mature tubers transferred to medium with GA_{4+7} + ABA. Figure 9 shows the average LUC activity in bud and tuber region of young and mature tubers after transfer to control medium (tuber-inducing medium) and to medium with GA_{4+7} or GA_{4+7} + ABA. Average LUC activity is expressed as percentage of the activities at t = 15 hours. LUC activity was quantified until second growth was observed for those tubers transferred to medium with GA. The LUC activity in young tubers transferred to control medium and in young tubers transferred to medium with GA_{4+7} + ABA, was quantified 40 hours after transfer. In mature control tubers, mature tubers on medium with GA with late second growth (> 90 hours) and in mature tubers transferred to medium with GA_{4+7} + ABA, LUC activity was quantified 90 hours after transfer.

	young tubers			mature tubers			
cycB1;1	80	175*	59*	70	122*	34	
	80	38	35	72	34	34	ND
	n=20	n=15	n=12	n=20	n=11	n=9	
	control	10μM GA_{4+7}	10μM GA_{4+7} 100μM ABA	control	10μM GA_{4+7}	10μM GA_{4+7}	10μM GA_{4+7} 100μM ABA
AGPaseS	82	65	50	75	118*	58*	39
	80	58	49	72	35	26	37
	n=20	n=15	n=12	n=20	n=8	n=12	n=20
	control	10μM GA_{4+7}	10μM GA_{4+7} 100μM ABA	control	10μM GA_{4+7}	10μM GA_{4+7}	10μM GA_{4+7} 100μM ABA

Figure 9

Average luciferase activities for cycB1;1 and AGPaseS in bud and tuber region of young (1-5 days after swelling) and mature tubers (25-30 days after swelling) after transfer to tuber-inducing medium (control) and to medium with 10 μM GA_{4+7} or 10 μM GA_{4+7} + 100 μM ABA. Luciferase activity is shown as observed at the moment of bud extension in young and mature tubers, transferred to medium with GA. For mature tubers, transferred to medium with GA, calculations were made separately for tubers with and without second growth at t = 90 h (3rd and 4th row respectively). The average activity in the controls and in tubers on medium with GA_{4+7} + ABA, both without second growth, was quantified respectively 40 hours (young) and 90 hours (mature) after transfer. The relative luciferase activities of the given number of tubers were used to calculate the average activity and expressed as percentage of the activity at t = 15 h. Differences in the relative luciferase activities between bud and tuber region were statistically analysed by applying a F-test and indicated with an asterisk as significant when $P<0.05$ (asterisk). ND = not determined.

For both the cycB1;1 and AGPaseS promoter no significant difference was observed between bud and tuber region of young and mature tubers transferred to control medium (figure 9). Compared to the control tubers, cycB1;1 promoter activity in the tuber region declined while cycB1;1 expression in the bud region increased of tubers transferred to medium with GA_{4+7} (figure 9)

When comparing the bud and tuber region of young tubers transferred to medium with GA_{4+7}+ABA, a significant difference in cycB1;1 expression was observed between these regions despite the absence of second growth (figure 9). This result shows that a higher cycB1;1 promoter activity in the bud region, is not strictly related to actual growth of the bud. In mature tubers with early second growth an enhanced cycB1;1 promoter activity was observed in the bud region, while in mature tubers with late second growth no difference was observed in cycB1;1 promoter activity between bud and tuber region.

Although an increase in AGPaseS promoter activity was observed in some individual young tubers after transfer to medium with GA_{4+7}, statistical analysis showed no significant difference in AGPaseS promoter activity between bud and tuber region. In contrast, mature tubers with early second growth showed a significantly higher AGPaseS expression in the bud region compared to the tuber region. However, a significant higher AGPaseS promoter activity was also observed in the bud region of mature tubers with late second growth when compared to the activity in the tuber region (figure 9). These results indicate that a higher AGPaseS promoter activity in the bud region of mature tubers compared to the tuber region is not strictly related to the timing of second growth but probably a direct consequence of the GA-treatment. Preventing second growth by combining GA with ABA resulted in a low and not significantly different AGPaseS promoter activity both in the tuber and bud region (figure 9), indicating that ABA prevents the development of a difference in AGPaseS promoter activity between tuber and bud region.

DISCUSSION

In our experiments we studied the responses of *in vitro* tubers during conditions that may induce second growth. By analysing the expression of cell cycle and storage genes separately in tuber and bud region we could study whether both regions respond independently or not upon GA and ABA.

The effect of GA_{4+7} on cell cycle and storage related gene expression in the tuber is not related to second growth

Transferring young tubers (1-5 days after swelling) to medium supplemented with 10 μM GA_{4+7} resulted in a rapid and synchronised formation of secondary stolons by outgrowth of the apical bud (figure 1A). Transferring mature tubers (25-30 days after swelling) to medium with GA_{4+7} resulted in second growth that was delayed by 4-10 days (figure 1B), indicating that the sensitivity for exogenous applied gibberellins is related to the developmental stage of the tuber.

The age dependent GA-responsiveness of tubers may be related to a decrease in the GA/ABA ratio during tuber development due to an increase in ABA content during tuber maturation (Koda, 1988). However, combining GA_{4+7} with ABA in young tubers resulted in a lower efficiency (30%) but not in a delay in second growth as in mature tubers (compare figure 1B and figure 1C). Moreover, young tubers transferred to medium with GA and ABA showed an higher cycB1;1 expression in the bud region than in the tuber region like observed in young tubers with only GA (figure 9). Mature tubers transferred to medium with GA with late second growth showed no difference in cycB1;1 expression between bud region and tuber region while tubers with early second growth showed an higher cycB;1 expression in the bud region (figure 9). These results indicate that the timing of second growth, in response to GA, is dependent on the developmental stage of the tuber and most likely not the result of an increase in ABA content during tuber maturation.

There is a preferential order in the response of the different promoters, preceding second growth. The average LUC activity of the different reporter constructs, shows first a decline relative to the activity in control tubers in AGPaseS and λPat21 activity, followed by a decline in cycB1;1 activity (figure 2). In contrast, CaMV35S and CDC2a promoter activity does not differ when compared to the control tubers in response to GA-treatment. The decline in cycB1;1, AGPaseS and λPat21 expression in the tuber was not affected by combining GA_{4+7} with ABA although second growth was prevented (figure 4). This indicates that there is no direct causal relation between bud growth and the down-regulation of cycB1;1, AGPaseS and λPat21 expression in the tuber.

The transcriptional activity in the tuber is not affected by the GA/ABA ratio but mainly by the GA level whereas bud growth is controlled by the GA/ABA ratio.

The decline in cycB1;1 activity in the tuber (figure 2B), relative to the controls, suggests that tuber growth may be arrested at the G_2-M transition. However, experiments by Campbell *et al.* (1996) indicated that growth arrest in potato tubers is not at the G_2-M transition but at the G_1-S transition, controlled by A-type Cyclin Dependent Kinases (CDK's). No effect was observed of gibberellins on the CDC2a expression within the tuber. CDC2a activity may therefore be modulated at a post-transcriptional level by D-type cyclins (Riou-Khamlichi *et al.*, 2000) or Cyclin Kinase Inhibiters (CKI) (Wang *et al.*, 1998).

A relatively high cycB1;1 gene expression in tuber buds is necessary but not sufficient for bud growth

Second growth always coincided with an enhanced cycB1;1 expression in the bud region of both young and mature tubers (figure 9). In response to GA_{4+7}+ABA the cycB1;1 promoter showed a significant difference in activity between bud and tuber region, although in none of these tubers second growth was observed (figure 9). This indicates that a specific cycB1;1 expression in the bud region is not sufficient for actual outgrowth of the bud. The changes in cycB1;1 promoter activity in tubers indicate that this promoter from *Arabidopsis thaliana* is affected by GA-signalling in potato tubers. In contrast, the same promoter in *Arabidopsis thaliana* is not directly affected by GA-signalling (Ferreira *et al.*, 1994).

Possible role of AGPase expression in the bud region during second growth

We unexpectedly found a higher expression of the starch biosynthesis related AGPaseS gene in the bud when compared to the tuber region, in some of the young tubers (figure 8D) and in all mature tubers (figure 9), after transfer to medium with GA_{4+7}. However, in tubers transferred to medium with GA_{4+7} +ABA, AGPaseS promoter activity in tuber and bud showed a comparable decline (figure 9). The results indicate that the decline in AGPaseS promoter activity in the complete tuber (figure 2D) and the significant difference in AGPaseS expression between bud and tuber region (figure 8-9) during second growth is a response to GA-treatment. ABA is able to counteract the GA response in the bud region (figure 9) whereas ABA does not affect the GA response in the tuber (figure 4), indicating that bud and tuber region respond differently upon GA and ABA.

The results lead to the question: why is AGPaseS expressed in the bud region during second growth and what is the possible role of ABA in tuber and bud region in relation to this AGPaseS expression? A similar enhanced activity of the AGPaseS promoter in the bud region and a simultaneous increase in AGPase enzyme activity in the bud region and the vascular tissue has been observed in sprouting potato tubers (Claassens; personal communication).

The secondary stolon formed after GA-treatment resembles a stolon tip prior to tuberization, with respect to carbohydrate composition (Mares *et al.* 1981). Invertase mediated hydrolysis of sucrose is the predominant pathway of sucrose utilisation in growing stolons prior tuberization (Appeldoorn *et al.*, 1997) and in the growing bud during sprouting (Claassens, personal communication). The enhanced invertase activity, especially apoplastic invertase, in the growing bud indicates an enhanced partitioning of sugars into the bud meristem. The increased import of sugars into the bud meristem tissue possibly surpasses the amount of sugars that is necessary for the supply of energy and building blocks during growth of the bud meristem, resulting in an increase in local sugar levels which may act as a signal for induction of AGPaseS expression (Müller-Röber *et al.*, 1990).

The AGPase activity within the bud region can lead to a temporary redirection of sugars that are not directly used, into starch. Due to such a temporary transition to starch, accumulation of excess sugars within the bud is prevented. This might prevent osmotic inhibition of cell proliferation during bud extension as described by Burssens *et al.* (2000) for *Arabidopsis thaliana*. We tested the effect of osmotic stress on bud extension by placing explants on 1% sucrose + 7% sorbitol. Sorbitol indeed caused inhibition of axillary bud extension (results not shown).

Growing tuber buds require the import of carbohydrates. When this supply is restricted the growth of the bud meristem is inhibited (Hajirezaei *et al.*, 1999). Possibly ABA prevents GA-induced second growth and counteracts the difference in AGPaseS expression between bud and tuber region of mature tubers by preventing the mobilisation of assimilates towards the bud, in contrast to GA, which stimulates the transport of assimilates to the bud region. A comparable mechanism is reported for seedling growth in which ABA prevents growth due to a low invertase activity whereas a high invertase activity was observed in growing seedlings without ABA (Garciarrubio *et al.*, 1997; Finkelstein and Lynch, 2000).

In conclusion, the expression of cycB1;1 (cell cycle) and AGPaseS and λPat21 (storage) in the tuber was affected by gibberellins whereas ABA did not counteract the decline in the expression of these genes although it prevented second growth. The results indicate that cell cycle and storage related gene expression in the tuber region responds to GA and not to ABA. In contrast, growth of the bud meristem and AGPaseS promoter activity in the bud region responds both upon GA and ABA.

Chapter 4

Alcohol dehydrogenase activity mediates ethanol-induced second growth in potato tubers

John Verhees, Margo Claassens, Marieke van Hulten, Alexander R. van der Krol, Dick Vreugdenhil and Linus H.W. van der Plas

Ethanol inhibits tuber growth (primary growth) and breaks the dormancy of the bud (second growth). To investigate the mode of action of ethanol on tuber growth and - dormancy we describe the effect of ethanol on *in vitro* potato tubers resulting in second growth and affecting the expression of cell cycle and storage related genes. The activities of both cell cycle (cycB1;1, CDC2a) and storage genes (AGPaseS, λPat21) were analyzed using the firefly (*Photinus pyralis*) luciferase reporter system. Transferring *in vitro* tubers to medium with ethanol (0.5% v/v) resulted in an induction of second growth and a decline in cell cycle and storage related gene expression in the tuber. In contrast to the tuber region, gene expression did not decline in the bud region resulting in a differential gene expression between bud and tuber region preceding visible growth of the bud. The CaMV35S promoter activity in tuber and bud was not affected by ethanol, indicating that the effect of ethanol on gene expression is not caused by general toxicity of ethanol. Combining ethanol with CCC, an inhibitor of the gibberellin synthesis, did not prevent second growth or the effects of ethanol on gene expression. Tubers transferred to medium with ethanol and ABA showed no second growth but the changes in gene expression in the tuber and bud region were similar, as in tubers transferred to medium with ethanol only. These results indicate that ethanol-induced second growth in potato tubers does not act via changes in the GA and ABA contents. 4-methyl pyrazole, an inhibitor of alcohol dehydrogenase activity, prevented ethanol-induced second growth as well as the changes in gene expression. Furthermore, second growth and changes in gene expression were also observed with other alcohols that can be used by ADH as a substrate. ADH converts ethanol into acetaldehyde. However, no second growth and changes in gene expression were observed in tubers transferred to medium with acetaldehyde. These results indicate that ADH activity but not the conversion products affects gene expression in tuber and bud preceding ethanol-induced second growth.

INTRODUCTION

In vitro grown tubers, transferred to medium supplemented with ethanol show a reduced dormancy, indicating that ethanol affects tuber dormancy in potato (Claassens *et al.*, 2002). Several reports showed that ethanol and other anaesthetics like ethyl ether, chloroform, methanol and acetone also break dormancy in various seeds and in artichoke tubers (Taylorson and Hendricks, 1979; Cohn *et al.*, 1989; Pétel *et al.*, 1993). The mechanism of dormancy breaking by ethanol is still unknown, but changes in fluidity of the plasmalemmma, cytoplasmic ATP content, cytoplasmic pH and activity of glycolysis or endogenous phytohormone concentrations have all been suggested to be related to the observed effect of ethanol treatment both in seeds and artichoke tubers (Cohn *et al.*, 1989; Pétel *et al.*, 1993).

Blocking the activity of alcohol dehydrogenase (ADH) by 4-methyl pyrazole (4MP) prevents ethanol-induced germination of oat seeds (Corbineau *et al.*, 1991) and ethanol-induced sprouting of potato tubers (Claassens *et al.*, 2002) , indicating that ADH enzyme activity is required for the ethanol effect. ADH catalyses the reversible conversion of ethanol to acetaldehyde. However, products of the ADH mediated conversion of ethanol, acetaldehyde and acetic acid have no effect on tuber dormancy in potato (Claassens *et al.*, 2002). Both ethanol and acetaldehyde vapour results in an upsurge in respiration in dormant potato tubers and seeds (Rychter *et al.*, 1979; Adkins *et al.*, 1984; Corbineau *et al.*, 1991), but only one of them (ethanol) results in dormancy breaking in potato tubers, indicating that the effect on respiration is not directly linked to dormancy.

Recently it has been suggested that following low oxygen conditions in potato tubers sprouting may be induced by ethanol produced by fermentation (Claassens *et al.*, 2002). Tubers are dense bulky organs that lack circulatory systems for oxygen exchange resulting in low oxygen concentrations in the centre of growing tubers even when tubers are grown in well-aerated soil (Geigenberger *et al.*, 2000). Under field conditions the oxygen concentration can decrease further due to flooding or wetted soil, resulting in hypoxic conditions (<1% O_2) (Pfister-Sieber and Brändle, 1995). As a response to low oxygen conditions, tubers exhibit a reduced respiration and a mixed fermentation to regenerate NAD^+ resulting in the production of lactate by lactate dehydrogenase activity and ethanol by alcohol dehydrogenase activity (Pfister-Sieber and Brändle, 1995). Re-aeration results in the oxidation of the accumulated ethanol into acetaldehyde by ADH activity (Pfister-Sieber and Brändle, 1995; Geigenberger *et al.*, 2000).

Second growth in potato tubers comprises a reduction in tuber growth (primary growth) and an increased growth of the meristematic tissue in the bud (Van den Berg *et al.*, 1990, 1991; Ewing and Struik, 1992). Preliminary experiments show that ethanol not only reduces bud dormancy but induces also second growth in young developing *in vitro* tubers. Conditions that affect the endogenous gibberellin and abscisic acid levels can also result in second growth e.g.: irregular nitrogen supply, heat and drought stress and mechanical damage (Lippert *et al.*, 1958; Bodlaender *et al.*, 1964; Van den Berg *et al.*, 1991). Therefore, it is suggested that the internal GA/ABA ratio is a key factor determining the balance between tuber growth (primary growth) and second growth (Kraus, 1985; Van den Berg *et al.*, 1991).

Ethanol-treatment results in a decline in endogenous ABA in barley seeds (Wang *et al.*, 1998b), while exogenous ABA applied to potato tubers prevents ethanol-induced sprouting (Claassens, *et al.*, 2002) indicating that ethanol possibly affects the endogenous ABA contents. Applying CCC, an inhibitor of GA-synthesis, blocks ethanol-induced germination in oat seeds (Adkins *et al.*, 1984) indicating that ethanol induced germination includes an enhanced synthesis of GA. However in potato tubers, CCC does not prevent ethanol-induced sprouting and quantifying endogenous GA concentrations in tubers showed no increase in response to ethanol-treatment (Claassens *et al.*, 2002).

In order to further characterise the mode of action of ethanol, we studied the effects of ethanol on specific gene expression during induction of second growth. We analysed the dynamics in cell cycle and storage related gene expression during ethanol-induced second growth and studied the role of ADH activity. Moreover, we studied the possible role of GA and ABA during ethanol-induced second growth.

Expression was studied in transgenic *in vitro* tubers containing heterologous promoter reporter fusions with the promoter sequences of cell cycle (cycB1;1, CDC2a) or storage related genes (AGPaseS, λPat21) and the luciferase reporter gene from firefly *(Photinus pyralis)* (Chapter 2). This method allowed the monitoring of spatial differences in gene expression between tuber and bud and the changes in gene expression in the same sample over a time-frame of 3 days during ethanol treatment. Although we have shown that GA can induce second growth and differentially affect gene expression in bud and tuber region (Chapter 3), ethanol-induced second growth seems to be independent of GA signalling. The results indicate an essential role for ADH-activity during ethanol-induced second growth.

MATERIALS AND METHODS

Plant material
Transgenic potato plants *Solanum tuberosum* cv 'Desiree' with the various reporter constructs were grown in vitro with 16 hours of light (50 W m^{-2}, 20°C) for 4-5 weeks. *In vitro* tubers were produced by transferring single node cuttings to tuber-inducing medium as described previously, resulting in the formation of tubers starting 7 days after transfer with a final diameter that varied between 4-8 mm (Hendriks *et al.*, 1991; Chapter 2). For most experiments tubers were used 1-5 days after visible swelling (young tubers). During some of the experiments mature tubers were used, 25-30 days after visible swelling.

Reporter constructs
Cell cycle and storage related gene expression was studied using fusions between the promoter of cell cycle (cycB1;1 and CDC2a) and storage related genes (AGPaseS and λPat21) and the codon sequence of the luciferase reporter gene (Sherf and Wood, 1994), as described before (Chapter 2). Cell cycle related gene expression was studied by monitoring promoter activity of the chimeric *Arabidopsis* genes cycB1;1 (Ferreira *et al.*, 1994) and CDC2a (Hemerley *et al.*, 1993) fused to the luciferase reporter gene. To study storage related gene expression, two luciferase reporter constructs were used under control of the AGPaseS promoter (Müller-Röber *et al.*, 1992) or the λPat21 promoter (Bevan *et al.*, 1986) from *Solanum tuberosum* L. As a control we used transgenic plants with the CaMV35S promoter fused to the luciferase reporter.

Ethanol treatments
24 hours before ethanol-treatment, the *in vitro* tubers were pre-treated with the luciferase substrate luciferin by placing stem segments bearing the tuber on tuber-inducing medium containing 0.2 mM luciferin (D-luciferin, sodium salt) followed by additional spraying with a 1 mM luciferin solution. To ensure luciferin uptake from the medium a thin slice was removed from the basal part of the stem segment bearing the tuber. After pre-treatment tubers were transferred to medium containing either 1% or 8% sucrose, supplemented with 0.5% (v/v) ethanol (87 mM) and 0.2 mM luciferin.

To test whether GA-biosynthesis was induced during second growth, ethanol (0.5%) was combined with the inhibitor of GA-synthesis 2-chloroethyltrimethyl-ammonium chloride (CCC, BDH chemical Ltd.) at 500 mg/L. To test the role of ABA, ethanol (0.5%) was combined with ABA (5, 10 and 100 μM). Before starting the combined treatment of CCC or ABA with ethanol, tubers were pre-treated with CCC or ABA 24 hours before adding ethanol. CCC and ABA were added to the medium by filter sterilization from a stock solution.

To test if the second growth induction and the observed changes in gene expression were related to the type of alcohol, tubers were transferred to medium supplemented with 1-propanol or 2-propanol (87 mM) and 8% sucrose. To study the function of alcohol dehydrogenase (ADH) during ethanol-induced second growth, 4-methylpyrazole (4MP, ICN Pharmaceuticals Inc.) was applied to the medium (1 mM) in combination with ethanol (0.5%) and 8% sucrose. Pyrazoles inhibit the activity of alcohol dehydrogenase (ADH) by forming a complex between ADH and NAD^+ (Blomstrand *et al.*, 1979). 24 hours before transferring tubers to medium with 4MP + ethanol, tubers were transferred to medium with only 4MP (1mM). To investigate whether acetaldehyde, produced via ADH activity, was inducing second growth, tubers were transferred to new plates and acetaldehyde was applied on a piece of filter paper lying on top of the medium. The final pH of the medium was set to 5.8 for all treatments.

***In vivo* measurements of LUC activity**

Directly after transfer of the *in vitro* tubers to medium with the various conditions, *in vivo* luciferase (LUC) activity was measured during 30 minutes intervals, using a cryogenic cooled CCD camera for low light imaging (VersArray ™, Roper Scientific). Relative LUC activities as measured during an integration period of 30 minutes, were depicted in an image showing the relative light units per pixel (rlu $pixel^{-1}$). Relative LUC activity was quantified in different regions of individual tubers by digital image analysis using MetaMorph™ (Universal Imaging Corporation) and is given in relative light units per pixel * $minute^{-1}$ (rlu $pixel^{-1}$). Differences in the relative LUC activities during the various treatments were statistically analysed by using a F-test at the given time-point (hours after starting the treatment) and specified as significant when $P<0.05$.

RESULTS

Induction of second growth by ethanol treatment is related to ethanol concentration and developmental stage of the tuber

Transferring young or mature *in vitro* tubers to medium with 0.5 % ethanol resulted in the breaking of dormancy and on medium with 1% sucrose in the formation of a secondary stolon whereas ethanol combined with 8% sucrose resulted in the formation of a secondary tuber. No difference was observed in the timing of second growth between the two sucrose concentrations (data not shown).

Figure 1A shows second growth in young tubers (1-5 days after swelling) at different concentrations of ethanol combined with 1% sucrose. Second growth was scored each day by counting the number of tubers that formed a stolon with a length of >3 mm. Second growth induction in young tubers was most effective at the highest ethanol concentration (0.5%) with >90% of the tubers showing second growth 9 days after transfer. Only 10% of the young tubers showed second growth when transferred to medium with 1% sucrose but without ethanol.

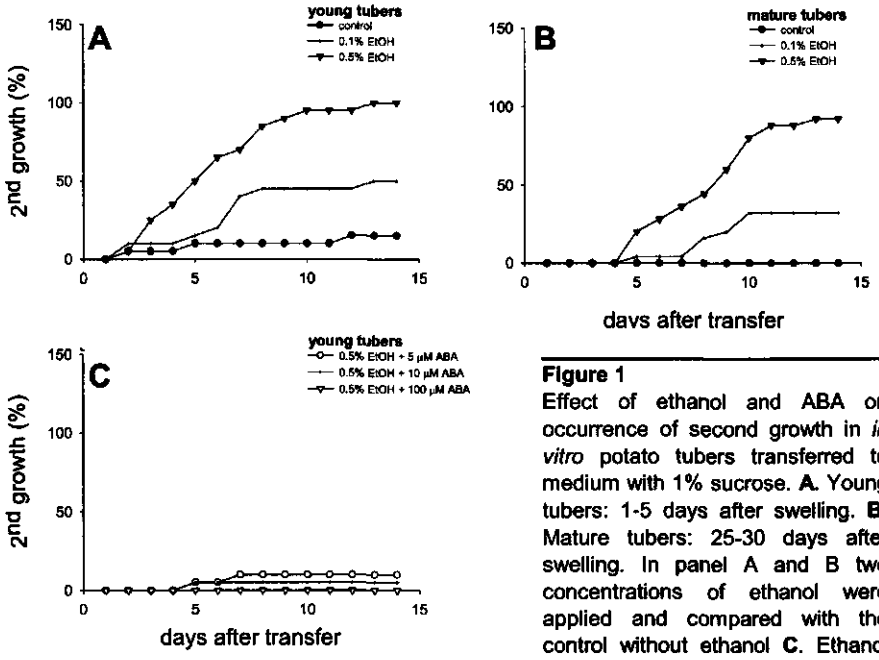


Figure 1
Effect of ethanol and ABA on occurrence of second growth in *in vitro* potato tubers transferred to medium with 1% sucrose. **A.** Young tubers: 1-5 days after swelling. **B.** Mature tubers: 25-30 days after swelling. In panel A and B two concentrations of ethanol were applied and compared with the control without ethanol **C.** Ethanol (0.5%) combined with three concentrations of ABA using young tubers. Data are average of 25 tubers per treatment.

To study the relation between developmental stage of the tubers and the response to ethanol, mature tubers (25-30 days after swelling) were also transferred to medium with different concentrations of ethanol (figure 1B). In mature tubers the efficiency of ethanol to induce second growth was similar to the efficiency found in young tubers, although the start of second growth was delayed by about 3 days (compare figure 1A and figure 1B).

Ethanol possibly breaks dormancy by reducing the endogenous ABA concentration, like observed in barley seeds (Wang *et al.*, 1998b). Therefore, we transferred young tubers to medium with ethanol (0.5%) and different concentrations of ABA (figure 1C). ABA applied to the medium completely prevented second growth induced by ethanol (compare figure 1A and figure 1C). It has been reported that CCC, an inhibitor of GA-synthesis (Hammes and Nel, 1975), counteracts ethanol-induced germination in seeds (Adkins *et al.*, 1984). However, second growth was not prevented when young tubers were transferred to medium with ethanol (0.5%) combined with CCC (500 mg/L) (data not shown). Although CCC did not prevent second growth, it affected the type of the second growth: in the presence of CCC a tuber rather than a stolon was formed, even with 1% sucrose in the medium.

Ethanol affects cell cycle and storage related gene expression in the tuber prior to second growth

To study changes in cell cycle and storage related gene expression during ethanol-induced second growth, reporter plants were made containing the promoter of cell cycle (cycB1;1 and CDC2a) or storage related genes (AGPaseS and λPat21) fused to the coding sequence of the luciferase reporter gene. Per reporter construct two representative lines were selected for expression analysis during ethanol-treatment. No major differences were observed between the two independent transformed lines.

LUC activity (rlu pixel^{-1}) was quantified in young tubers after transfer to medium with ethanol (0.5%) combined with 1% or 8% sucrose. LUC activities quantified in whole tubers were combined and averaged for 25 tubers per treatment. As a control tubers were transferred to medium without ethanol. Figure 2 (page 62) shows the average LUC activities expressed as percentage of the initial activity at t=0 for each promoter during the various treatments. The control treatment transfer (8% sucrose) lead to a gradual decline in LUC activity while the transfer to medium with 1% sucrose lead to a transient increase between 10 and 30 hours for cycB1;1, CDC2a and λPat21. AGPaseS activity declined after transfer to medium with 1% sucrose indicating that a reduction in sucrose supply affects AGPaseS gene expression.

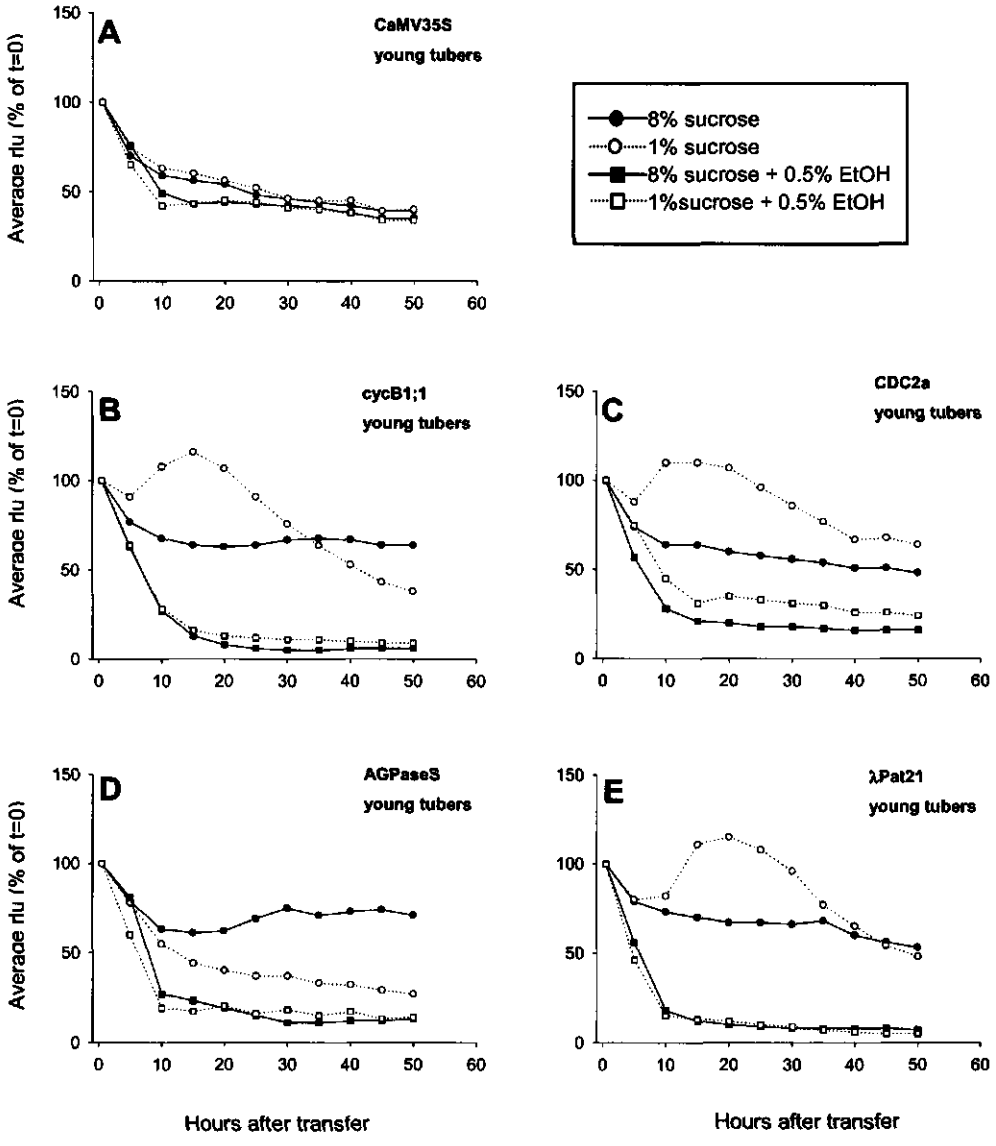
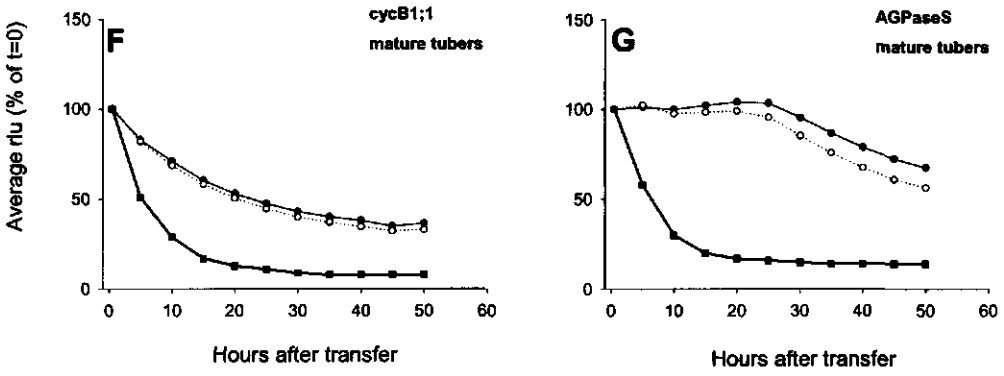


Figure 2
Average luciferase activity in 25 young tubers (**A-E**, 1-5 days after swelling) and mature tubers (**F-G**, 25-30 days after swelling) after transfer to medium with 0.5% ethanol combined with 1% or 8% sucrose. As a control tubers were transferred to medium with 1% or 8% sucrose but without ethanol. For each promoter the luciferase activity is expressed as percentage of the initial activity at the moment of transfer; (**A**) CaMV35S, (**B**) cycB1;1, (**C**) CDC2a, (**D**) AGPaseS, (**E**) λPat21, (**F**) cycB1;1, (**G**) AGPaseS.



No major effects were observed in the activity of the CaMV35S promoter after transfer to medium with ethanol (figure 2A), whereas a strong decline was observed in the activity of cell cycle (figure 2B-C) and storage related promoters (figure 2D-E) almost directly after transfer to medium supplemented with ethanol. In the presence of ethanol, the activity profiles did not differ between tubers transferred to medium with 1% and 8% of sucrose. In only a small number of the tubers (5-10%) second growth was already observed at 50 hours after transfer. These results indicate that the decline in cell cycle and storage related gene expression preceded visible growth of the bud.

Second growth after transfer to medium with ethanol is delayed in mature tubers (compare figure 1A and 1B). However, cycB1;1 and AGPaseS promoter activity in mature tubers declined immediately after transfer to medium with ethanol as observed for young tubers, although in the control treatment (transfer to medium with 1% and 8% sucrose) the decline in AGPaseS promoter activity was clearly delayed for mature tubers (figure 2F-G).

Ethanol affects cell cycle and storage related gene expression in tubers even when second growth is prevented

To study if responses in gene expression during ethanol-induced second growth are related to the GA/ABA ratio, young tubers were transferred to medium with 8% sucrose and 0.5% ethanol combined with ABA (10 μM) or CCC (0.5 g/L). Figure 3 (page 64) shows the average λPat21-*luc* activity calculated for 25 tubers after transfer to the various conditions.

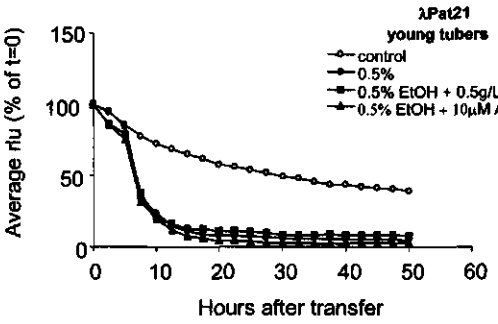


Figure 3
Average λPat21-*luc*+ activity profiles in 25 young tubers (1-5 days after swelling) after transfer to medium with 8% sucrose combined with 0.5% ethanol, 0.5 g/L CCC or 10 μM ABA and to control medium with 8% sucrose without ethanol. Average luciferase activity is expressed as percentage of the initial activity at the moment of transfer.

Combining ethanol with ABA prevented second growth (figure 1C) while CCC did not prevent second growth but did affect the type of second growth (tubers instead of stolons). Combining ethanol with ABA or CCC resulted in a decline in λPat21 expression as observed after transfer to medium with ethanol only. A similar response was observed for cycB1;1, CDC2a and AGPaseS (data not shown). Transferring tubers to medium with ABA or CCC without ethanol did not affect cell cycle and storage related gene expression in the tuber (Chapter 2).

To test a possible relation between changes in gene expression and the oxidation of the applied ethanol by alcohol dehydrogenase (ADH), young tubers were transferred to medium with different types of alcohols that differed in their ability to act as a substrate for ADH-mediated oxidation. Figure 4 shows the average λPat21-*luc* activity calculated of 25 tubers after transfer to the various conditions. Transferring tubers to medium supplemented with 1-propanol (87 mM) resulted in a decline in λPat21 activity as observed after transfer to medium with ethanol (87 mM = 0.5%) while 2-propanol in the same concentration (87mM) did not affect λPat21 activity (figure 4A). Similar differences in response were obtained for cycB1;1, CDC2a and AGPaseS (data not shown). Besides a difference in effect on gene expression between 1-propanol and 2-propanol, it appeared that second growth was induced after transfer to medium with 1-propanol but not in tubers transferred to medium with 2-propanol. These results indicate that both second growth and changes in cell cycle and storage related gene expression, can only be triggered by alcohols that can be used as a substrate by ADH. Alternatively, the observed differences could be caused by a difference in the lipid solubility between the different alcohols.

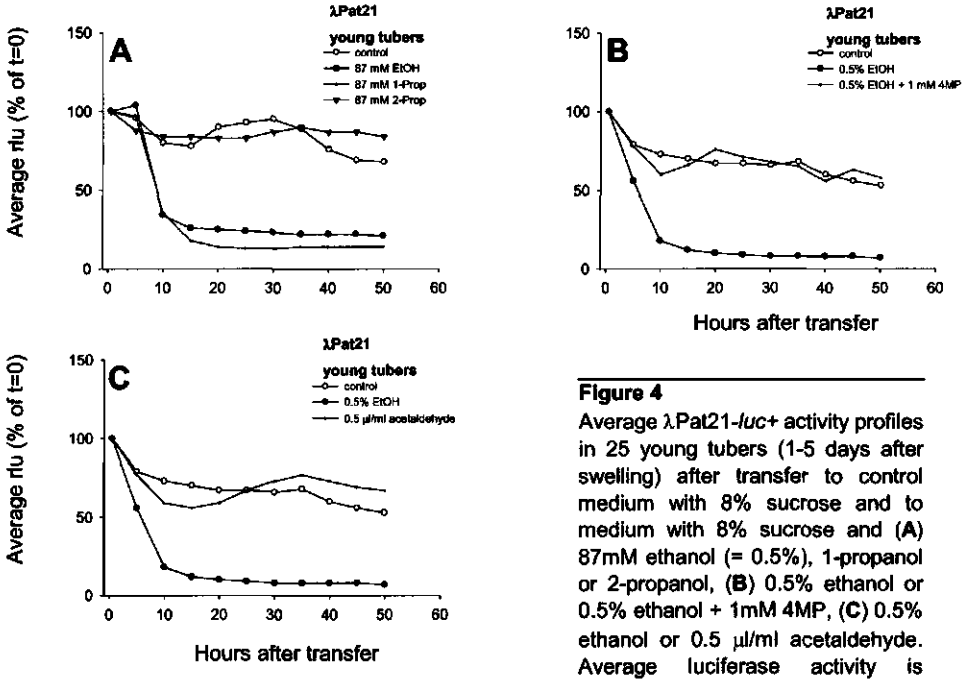


Figure 4
Average λPat21-*luc+* activity profiles in 25 young tubers (1-5 days after swelling) after transfer to control medium with 8% sucrose and to medium with 8% sucrose and (**A**) 87mM ethanol (= 0.5%), 1-propanol or 2-propanol, (**B**) 0.5% ethanol or 0.5% ethanol + 1mM 4MP, (**C**) 0.5% ethanol or 0.5 µl/ml acetaldehyde. Average luciferase activity is expressed as percentage of the initial activity at the moment of transfer.

To investigate whether second growth and suppression in gene expression is mediated by the activity of ADH, 4-methyl pyrazole (1mM) was combined with ethanol. No decline was observed in activity of the λPat21 promoter in tubers transferred to medium with ethanol + 4MP (figure 4B) indicating that ADH activity is required for the alcohol effect. Both ethanol and 1-propanol can be converted by ADH, yielding acetaldehyde and propanal, respectively.

Acetaldehyde was applied to the medium (0.05%) to study the effect of this product on gene expression and second growth. Only a minor and transient decline in λPat21 activity was observed after transfer and no significant difference was observed at t = 50 h (figure 4C). Besides the absence of an effect on gene expression, second growth was not observed in tubers transferred to medium with acetaldehyde.

Ethanol-induced second growth affects cell cycle and storage related gene expression in the bud region preceding second growth

Since the responses upon ethanol-treatment might be differentially regulated in the different parts of the tuber, as observed for GA-induced second growth (Chapter 3), LUC activities were quantified separately in bud and tuber region, as specified in figure 5A. During the initial 20 hours after transfer to medium with ethanol, cell cycle and storage related gene expression declined both in tuber and bud. Forty hours after transfer, cycB1;1-*luc* activity increased in the bud region whereas the LUC activity in the tuber region remained low. When the LUC activity was quantified in the complete tuber (region I, figure 5A-B) including the bud region and expressed as percentage of the activity at t = 0, the increase in cycB1;1 expression in the bud region was obscured because the bud region is a relatively small part of the selected region and the increase in LUC activity in the bud region is relatively small compared to the activity in the tuber before transfer. A similar differential response between bud and tuber region during ethanol-induced second growth was observed for the other cell cycle (CDC2a) and storage related promoters (AGPaseS and λPat21).

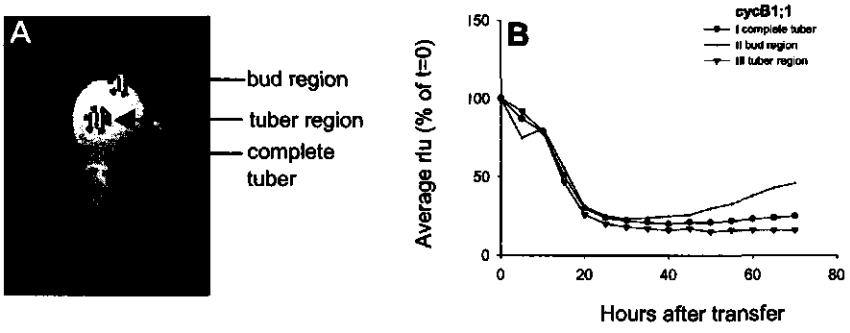


Figure 5

A. Regions selected for quantifying luciferase activities in individual tubers during second growth: I. complete tuber, II. bud region and III. tuber region.

B. cycB1;1-*luc* activity quantified in an individual tuber after transfer to medium with 0.5% ethanol and 8% sucrose. Relative luciferase activities were quantified for the three regions and expressed as percentage of the initial activity at the moment of transfer

To investigate the relation between second growth and gene expression in the bud region in more detail, we further analysed cycB1;1 and AGPaseS expression in the bud region during second growth. The relative LUC activities in 20 young tubers were used to calculate the average LUC activity and expressed as percentage of the activity at t = 15 h (figure 6).

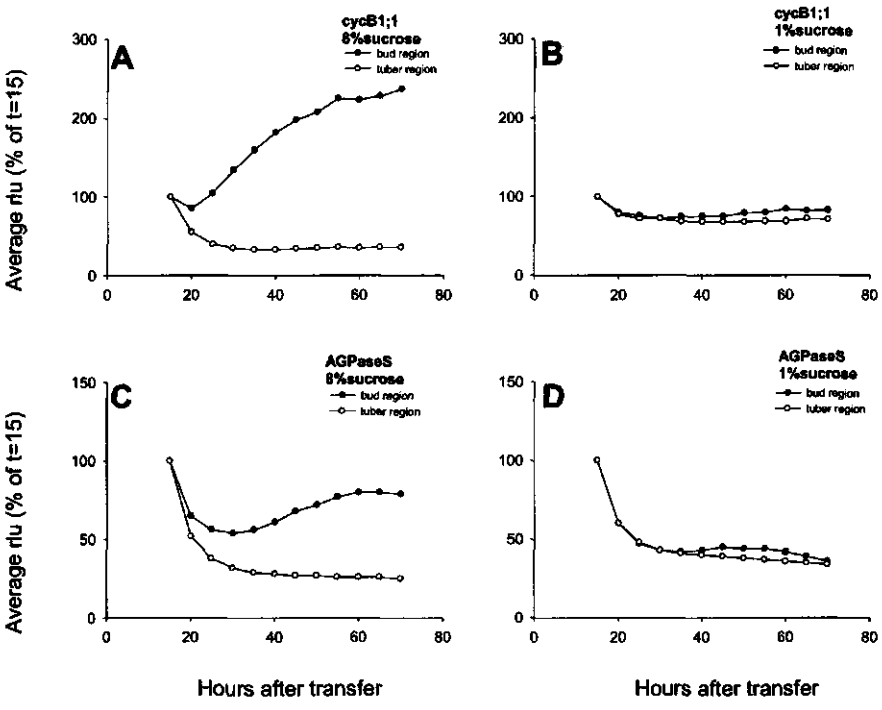


Figure 6
Activity profiles of cycB1;1-*luc* (**A/B**) and AGPaseS-*luc* (**C/D**) during second growth quantified in bud and tuber region of young tubers (1-5 days after swelling) after transfer to medium with 0.5% ethanol combined with 1% (**B/D**) or 8% (**A/C**) sucrose. Average luciferase activity (n = 20 tubers) is plotted as percentage of the activity at t = 15 hours.

Young tubers transferred to medium with ethanol (0.5%) and 8% sucrose showed a strong upregulation in cycB1;1 activity preceding visible growth of the bud (figure 6A) while tubers transferred to ethanol and 1% sucrose only showed a slightly higher cycB1;1 activity in the bud region as compared to the activity in the tuber (figure 6B). Young tubers transferred to medium with ethanol and 8% sucrose showed an enhanced activity for AGPaseS in the bud region relative to the tuber region (figure 6C).

Young tubers transferred to medium with ethanol and 1% sucrose showed no significant difference (t = 70h) in AGPaseS activity between bud and tuber region (figure 6D), indicating that an enhanced AGPaseS activity in the bud region of growing tubers requires a high sucrose level in the medium. During the first 70 hours after transfer, second growth was observed in 10-25% of the tubers. However, the dynamics in cycB1;1 and AGPaseS activity as measured in the bud region of individual tubers, was not linked to the time of second growth.

To study the relation between the dynamics in cycB1;1 and AGPaseS expression in the bud region and the outgrowth of the bud, LUC activities were quantified in bud and tuber region of young and mature tubers under various conditions resulting in a delay (mature tubers) or an inhibition (ABA and 4MP) of second growth during ethanol-treatment. Figure 7 shows the average LUC activities for cycB1;1 (figure 7A) and AGPaseS (figure 7B) as measured in bud and tuber region at 70 hours after transfer of 20 tubers per treatment expressed as percentage of the activity at t = 15 h. As a control transfer, young and mature tubers were transferred to tuber-inducing medium. These controls showed no difference in cycB1;1 and AGPaseS activity between bud and tuber region (figure 7A/B).

In mature tubers transferred to medium with ethanol no second growth was observed at t = 70 h but second growth was observed starting 5-6 days after transfer. Although second growth was absent in mature tubers 70 hours after transfer, a significant difference in cycB1;1 promoter activity was observed between bud and tuber region, independently of the sucrose concentration in the medium (figure 7A). This significant difference was especially caused by the decline in the tuber region compared to the control tuber. However, at t = 70 h cycB1;1-*luc* expression in the bud region was about 3-fold higher in young tubers than in mature tubers after transfer to medium with ethanol and 8% sucrose. Transferring mature tubers to medium supplemented with ethanol also resulted in a significantly higher AGPaseS activity in the bud region relative to the activity in the tuber region (t = 70 h), independently of the sucrose concentration (figure 7B). These results indicate that a difference in cycB1;1 and AGPaseS promoter activity between bud and tuber region of mature tubers is not strictly related to the time of second growth.

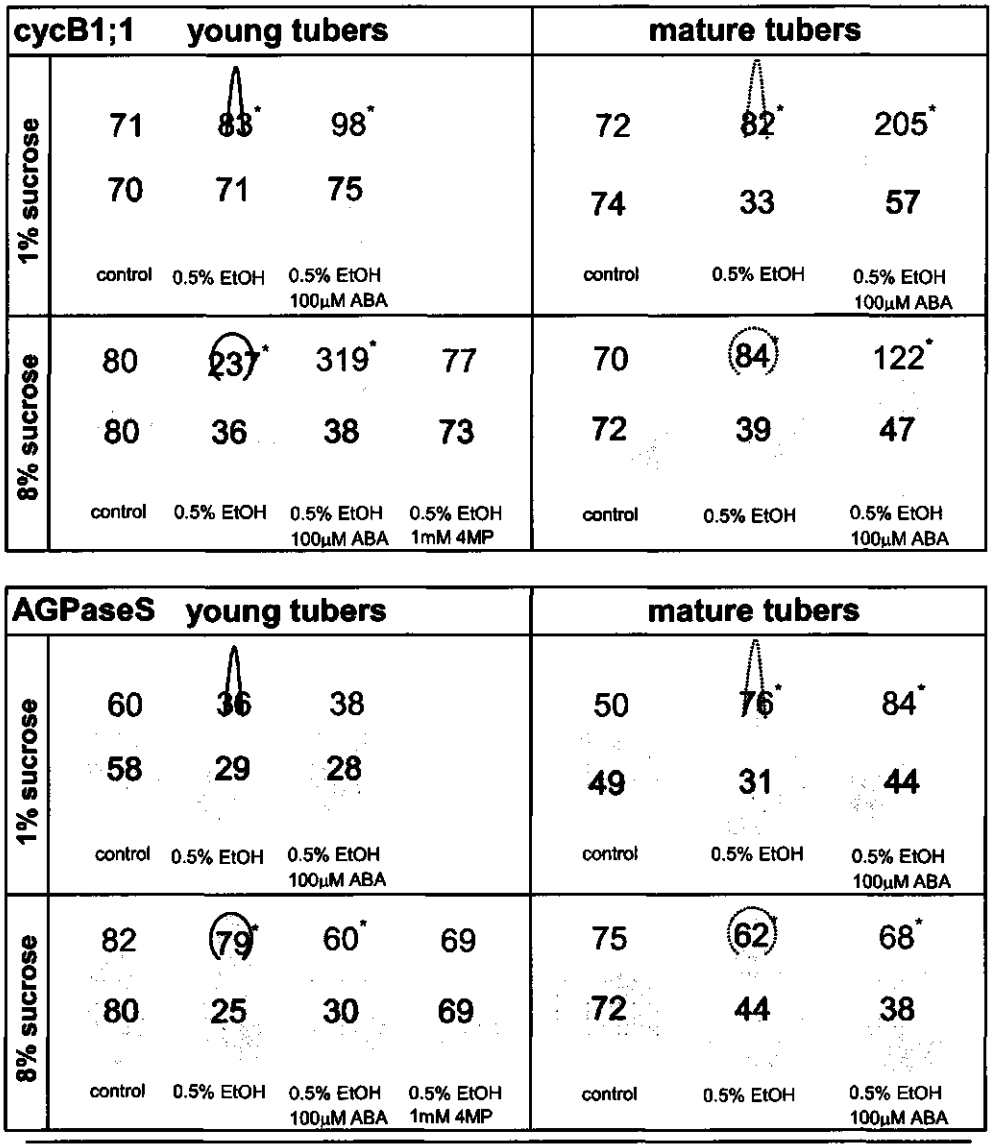


Figure 7

Average luciferase activity (n= 20 tubers) in bud and tuber region of young (1-5 days after swelling) and mature tubers (25-30 days after swelling) quantified 70 hours after transfer as percentage of the activity at t =15 hours with various combinations of 0.5% ethanol, 100 µM ABA and 1 mM 4MP with 1% or 8% sucrose and in tubers transferred to medium without ethanol (control). The presence or absence and the type of second growth (tuber or stolon) for the various treatments is schematically indicated in the drawings with a solid line when second growth was observed during the first 70 hours after transfer (young) and a dashed line when the start of second growth occurred later than 70 hours after transfer (mature). Differences in the relative luciferase activities between bud and tuber region were statistically analyzed by applying a F-test and marked (asterisk) as significant when $P<0.05$. cycB1;1 (upper panel) , AGPaseS (lower panel).

In all cases where ethanol leads to a differential cycB1;1 and AGPaseS activity between bud and tuber region, the same effect was also observed in the presence of 100µM ABA although second growth was not observed (figure 7A-B). These results indicate that ABA prevents second growth during ethanol-treatment but it does not counteract the differential cycB1;1 and AGPaseS promoter activity between bud and tuber region.

To test if this difference in cycB1;1 and AGPaseS activity between bud and tuber region relates to ADH activity, young tubers were transferred to medium with 8% sucrose, ethanol (0.5%) and 4MP (1mM) (figure 7). A significant difference was not longer observed in cycB1;1 and AGPaseS promoter activity between the bud and tuber region (t = 70 h) after this transfer.

DISCUSSION

Ethanol-induced second growth specifically affects cell cycle and storage related gene expression

After transferring young *in vitro* tubers to medium supplemented with ethanol, second growth was induced (figure 1) and cell cycle and storage related gene expression declined relative to that in the control tubers (figure 2B-E). Expression profiles observed in mature tubers transferred to medium with ethanol equalled the expression profiles observed in young tubers (figure 2F-G), although second growth in mature tubers started approximately 3 days later. These results indicate that the decline in the expression of the cell cycle and storage genes in the tuber is not related to the timing of second growth. The type of second growth observed during ethanol treatment was either a tuber when combined with 8% sucrose or a stolon when combined with 1% sucrose. However, no difference was observed in the expression profiles of cell cycle and storage related gene expression in the complete tuber (figure 2B-E), indicating that the observed decline did not correlate with the type of second growth. CaMV35S-*luc* activity was not affected by ethanol (figure 2A), showing that the decline in gene expression was not caused by a lower viability of the tuber cells due to a general toxicity of ethanol.

Ethanol-induced second growth was preceded by an increase in cycB1;1 activity in the bud region, relative to the tuber region (figure 6A-B), which resembles the pattern observed during GA-induced second growth (Chapter 3). A delay in second growth, as observed in mature tubers (3 days) did not affect the cycB1;1 expression profile in the bud region (figure 7A).
These results differ from cycB1;1 induction in the bud region during GA-induced second growth where an enhanced cycB1;1 was absent in the bud region of tubers with a delay in second growth (Chapter 3).

The outgrowth of a tuber bud requires the import of sugars into the bud region (Hajirezaei and Sonnewald, 1999). During ethanol-treatment AGPaseS expression declined in the tuber indicating a decline in starch synthesis. This transition possibly results in an enhanced transport of sugars to the bud region causing an enhanced AGPaseS expression in this region of the tuber because the expression of AGPaseS strongly responds on sucrose (Müller-Röber *et al.*, 1990; Chapter 3). Ethanol-treatment combined with high sucrose in the medium resulted in an enhanced AGPaseS expression in the bud region of tubers (figure 6C).

This increase in AGPaseS expression preceded an enhanced AGPase enzyme activity in this region of the tuber as observed by *in situ* enzyme staining (unpublished results), corresponding to an enhanced partitioning of sugars into the bud region during ethanol-treatment combined with 8% sucrose. Young tubers transferred to ethanol and 1% sucrose showed no enhanced AGPaseS expression in the bud region (figure 6D). These results indicate that the type of second growth and the enhanced AGPaseS expression in the bud region of young tubers probably are related to the amount of sugars available for the bud.

In contrast, mature tubers transferred to medium with ethanol and 1% sucrose showed a significantly higher AGPaseS expression in the bud region (figure 7B). The enhanced import of sugars into the bud region of these tubers with a delay in bud growth possibly surpasses the amount of sugars necessary for supply of energy and building blocks, resulting in an increase in local sugar levels, even on 1% sucrose. This results in a relatively higher AGPaseS expression in the bud region in comparison to the tuber region.

The effect of ethanol on specific gene expression does not relate to changes in the GA/ABA ratio

Although gibberellins and ethanol induce second growth in potato tubers, several differences were observed in the effect on gene expression between the two treatments, such as a strong decline in CDC2a expression in the tuber during ethanol-treatment whereas GA-induced second growth did not affect CDC2a expression. Furthermore, cycB1;1, AGPaseS and λPat21 expression declined more gradually during GA-induced second growth (Chapter 3) than observed during ethanol-induced second growth.

CCC, an inhibitor of GA-synthesis, did not counteract the decline in cell cycle and storage related gene expression during ethanol-treatment (figure 3) and it did not prevent second growth. These results indicate that second growth and the concomitant decline in cell cycle and storage related gene expression during ethanol-treatment, is not mediated by an enhanced synthesis of gibberellins. These conclusions are supported by the findings of Claassens *et al.* (2002) that showed no increase in the endogenous gibberellin levels after ethanol-treatment of potato tubers. This suggests that the signalling pathway(s) during GA-induced and ethanol-induced sprouting are different. Differences between ethanol-induction and GA-induction have also been reported for induction of germination of oat seeds (Gallais *et al.*, 1998).

Combining ethanol with ABA resulted in a similar decline in cell cycle and storage related gene expression in the complete tuber as observed with ethanol only (figure 3) although second growth was prevented. These results indicate that the effect of ethanol on cell cycle and storage related gene expression was not caused by an ethanol-induced decline in endogenous ABA content. Moreover, the results show that the suppression in cell cycle and storage related gene expression in the tuber is not directly linked to outgrowth of the bud. A similar relation between tuber and bud was found for GA-induced second growth (Chapter 3),

Although ABA prevented second growth during ethanol-treatment (compare figure 1A and figure 1C), it did not prevent the characteristic difference in cycB1;1 expression between bud and tuber region (figure 7A) indicating that a relatively higher cycB1;1 expression in the bud is not sufficient for bud growth as also observed during GA-induced second growth (Chapter 3). These results indicate that growth arrest of tuber buds is not controlled by cycB1;1. Alternatively, growth arrest in the tuber bud may be controlled by D-type cyclins that respond to carbohydrates (Riou-Khamlichi *et al.*, 2000) or Cyclin Kinase Inhibitors (CKI) affected by abscisic acid (Wang *et al.*, 1998b).

GA combined with ABA results in lower cycB1;1 expression in the bud region, than observed with only GA (Chapter 3). In contrast, ethanol combined with ABA generally resulted in a higher cycB1;1 expression than with only ethanol in the medium (figure 7A). Macnicol and Jacobsen (2001) reported an ABA induced expression and activity of ADH in the aleurone layers of barley seeds. The enhanced cycB1;1 activity in the bud region of tubers transferred to medium with ethanol + ABA might be related to an enhanced ADH activity as a result of ABA, resulting in an enhanced oxidation of ethanol.

ADH activity is essential during ethanol-induced second growth

Tubers transferred to medium with 2-propanol (no substrate for ADH) or to medium with ethanol combined with 4MP (inhibitor of ADH activity) showed no second growth and no major changes in cell cycle and storage related gene expression (figure 4A-B and figure 7). These results indicate that ADH-mediated oxidation of ethanol is part of the signalling pathway during ethanol-induced second growth. ADH mediates the reversible conversion between acetaldehyde and ethanol.

It has been shown that ethanol-treatment can have a deleterious effect on cell growth and seed vigor through its conversion into acetaldehyde mediated by ADH (Perata and Alpi, 1991; Zhang *et al.*, 1995). Inhibiting ADH enzyme activity with 4MP prevents these deleterious effects of ethanol.

Although these reports indicate that acetaldehyde is the major compound to cause deterioration, the results presented here show that the product of ADH-activity, acetaldehyde, did not induce second growth and did not affect cell cycle and storage related gene expression. Combining these observations, we conclude that ADH activity and/or the increase in NADH as a result of the conversion of ethanol into acetaldehyde, might be the key-factor in ethanol-induced second growth and in the changes in cell cycle and storage related gene expression preceding second growth. This leads to a model in which ADH has a dual function similar to that of some hexokinases in sugar sensing: a catalytic conversion and a signaling function coupled to the catabolic redox charge ($NADH/NADH+NAD^+$) which affects tuber development as well as the expression of cell cycle and storage related genes in potato tubers.

Chapter 5

The dynamic relation between gene expression and tuber sink strength in potato (Solanum tuberosum L.)

John Verhees, Alexander R. van der Krol, Dick Vreugdenhil and Linus H.W. van der Plas

The flux of nutrients into a storage organs like potato (*Solanum tuberosum* L.) tubers (sink strength) is controlled by the size and the activity of the sink (sink strength = sink activity * sink size). In this report we deduced the sink strength of tubers during development by determining the growth rate and tested whether there was a relation between sink strength and cell cycle (cycB1;1 and CDC2a) or storage related gene expression (AGPaseS and λPat21), using the non-invasive firefly luciferase reporter system. Analysis of growth of individual tubers showed that directly after tuber initiation, tuber growth rate and size varied between the tubers that were initiated on the same potato plant. During tuber development, three different stages of growth were distinguished viz., initiation, linear tuber growth and maturation. During tuber initiation the differences in sink strength were especially affected by differences in tuber size and did not relate to differences in the expression level of cell cycle and storage related genes in the tubers. During the period of linear tuber growth there was a good correlation between the activity of the λPat21 promoter and sink strength while during maturation the activity of cycB1;1, AGPaseS and λPat21 correlated well with the sink strength (i.e. growth rate of the tubers). These results indicate that among the cell cycle and storage related genes described here, λPat21 is the most predictive marker for sink strength during tuber development.

INTRODUCTION

Potato plants have multiple tubers (sinks) that compete for the available amount of photosynthates supplied by the aerial parts of the plant (Farrar, 1986). Besides for photosynthates, tubers compete for mineral nutrients, hormones and water (Struik *et al.*, 1991). The ability of a tuber to compete for these nutrients is defined as the sink strength and depends on the size and the activity of the tuber (sink activity) and on the sink strength of the other tubers on the same plant competing for the same nutrients (Engels and Marschner, 1986a, 1986b). The sink activity is defined as the amount of nutrients utilised per unit tuber tissue and is determined by the amount of assimilates used for growth, synthesis of storage products and respiration (Doehlert, 1996). All these processes occur simultaneously during tuber growth; as a consequence the differences in the sink activity between tubers are related to differences in growth, in the synthesis of storage products and in respiration (Engels and Marschner, 1986b; Plaisted, 1957).

The timing of tuber initiation, their growth rate and growth duration differ between individual tubers growing on a single plant resulting in tubers that differ in size (Ahmed and Sagar, 1981; Engels and Marschner, 1986a). Possibly these differences in growth between tubers relate to differences in the import and utilisation of assimilates (O'Brein *et al.*, 1998; Herbers *et al.*, 1998). Analysis of the import of carbohydrates showed a high correlation with the growth rate of tubers indicating that growth rate can be used as a measure for tuber sink strength. In young tubers (initiation phase) the sink strength significantly correlates with the fresh weight of the individual tubers attached to the same plant (Oparka, 1985, Engels and Marschner, 1986b and 1986c). At this stage of tuber development, starch concentration is negatively correlated with tuber size (Engels and Marschner, 1986c) indicating that the enhanced import of assimilates into larger tubers is possibly utilised for the synthesis of structural components (cell walls). Later during tuber development the relation between import rate and tuber size weakens, indicating that the competition between individual tubers no longer depends on sink size only, but also on sink activity (Oparka, 1985).

Internal tuber characteristics that affect sink activity are possibly related to sucrose hydrolysis and the processing of sugars used for growth, storage or respiration, which is controlled by multiple enzymes (Engels and Marschner, 1986b and c).

Experiments by Merlo *et al.* (1993) and Sung *et al.* (1989) indicated that the internal tuber characteristics like the activities of sucrose synthase and ADP-glucose pyrophosphorylase (AGPase) become more important for tuber sink strength during later stages of development, although the correlation with tuber size was always low. However, reducing AGPase activity resulted in a strong effect on tuber size distribution indicating that AGPase activity affects the sink hierarchy between tubers (Müller-Röber *et al.*, 1992).

Many authors reported about the relation between sink strength and the competition between sinks by studying the import rate of photosynthate and the activities of enzymes involved in the processing of photosynthate. We have studied the expression level of two sets of genes related to cell cycle regulation (cycB1;1 and CDC2a) and to the formation of storage products (AGPaseS and λPat21) to determine whether the expression level of these genes, measured per tuber, could be used as markers for sink strength and the expression per unit tuber area could be used as markers for the sink activity.

Analysis of gene expression during tuber development is usually done by quantifying steady state mRNA levels, using pooled samples in a destructive method. Because sink strength hierarchy between tubers may change with time, it is important to do a time-based study when studying the relation between sink strength and gene expression (Struik *et al.*, 1990; Marcelis, 1996; Black 1996). A non destructive method allows studying of gene expression with a high temporal resolution resulting in a better understanding of the interaction between gene expression and sink strength for tubers growing on a single plant. In previous reports we described a method to study gene expression non-invasively during *in vitro* tuber development by using heterologous promoter fusions between promoters cloned from cell cycle or storage related genes and the luciferase reporter gene cloned from firefly (*Photinus pyralis*) (Chapter 2). The results presented before showed that *in vivo* luciferase activity reflects both the transcriptional level of the reporter gene as well as the expression level of the endogenous genes.

It appeared that λPat21 promoter activity is a good indicator for the tuber sink strength, as determined by the tuber growth rate, during linear tuber growth and tuber maturation, whereas the correlation between growth rate and expression level was low for the other promoters.

MATERIALS AND METHODS

Plant material

Transgenic reporter plants (*Solanum tuberosum* cv 'Desiree') were propagated *in-vitro* and grown with a light period of 16 hours (50 W m^{-2}, 20°C). After four weeks the *in vitro* plants were transferred to soil and placed in growth chambers with a 16 hour light period (80 W m^{-2}, 20°C and 70% RH). After a period of 2-3 weeks the potato plants were transferred to pot assemblies with stolons and tubers growing in a separate compartment filled with a mixture of sand and perlite (ratio 1:1, v/v). These pot assemblies allow to observe stolons and tubers non-destructively by removing the sand and perlite mixture with a vacuum cleaner. Roots were growing in a continuously aerated nutrient solution (pH 6.0) as described by Struik et al. (1989). During the first 4 weeks the plants were growing with a light period of 16 hours after which the light period was reduced to 8.5 hours (80 W m^{-2}, 20°C and 70% RH). After transfer to short-day conditions (day 0) tuber formation and luciferase activity was monitored every 3-4 days for a period of ~55 days.

Reporter plants

Cell cycle and storage related gene expression was monitored during tuber development by using transgenic potato plants containing heterologous promoter fusions with the luciferase reporter gene (Photinus pyralis) (Chapter 2). As a marker for cell cycle related gene induction the reporters cycB1;1-luc and CDC2a-luc were used. Both cell cycle related promoters have been cloned from Arabidopsis thaliana (Ferreira et al., 1991; Ferreira et al., 1994). As a marker for storage related gene induction we used the reporters AGPaseS-luc (starch) and λPat21-luc (protein storage). Storage related promoters have been cloned from potato (*Solanum tuberosum*) (Bevan et al., 1986; Müller-Röber et al., 1992).

In vivo measurements of luciferase activity

After removing the sand-perlite mixture, stolons and tubers were sprayed with a 0.2 mM luciferin solution (D-luciferin, sodium salt, 0.01% Tween 80). Luciferin treatment was given 3 hours before measuring luciferase activity, allowing the accumulated luciferase enzyme to react with the substrate. Between pre-treatment and measuring luciferase activity, stolon and tubers were covered with wet cheesecloth to prevent dehydration and placed in the growth chamber. To monitor the *in planta* luciferase (LUC) activity, plants were placed under a cryogenic cooled CCD camera (VersArray ™, Roper Scientific) allowing low light detection. The integration period for measuring luciferase activity differed between the transgenic lines (15 or 30 minutes) depending on the level of luciferase activity. During this integration period, photons were captured by the camera and reconstructed in a single image showing the relative light units per pixel (rlu $pixel^{-1}$). Image analysis was performed by MetaMorph™ version 4.5 (Universal Imaging Corparation). Individual tubers were analysed by selecting the tuber region, resulting in the tuber size (mm^2), luciferase activity per tuber (rlu*$tuber^{-1}$) and luciferase activity per tuber area (rlu $pixel^{-1}$). The changes in tuber size during development were used to calculate the growth rate (mm^2 day^{-1}) as an indication of sink strength. We studied the competitive strength between tubers by studying the sink strength in relation to the tuber size hierarchy and tuber growth rate hierarchy. The term sink strength and competitive strength are synonymously used in this chapter.

Reverse transcriptase PCR

In order to analyse whether *in planta* luciferase activities reflect steady state *luc* mRNA levels we determined the steady state *luc* mRNA levels by semi-quantitative RT-PCR in tubers that differed in *in planta* luciferase activities. *In vivo* luciferase activity was measured in three individual tubers attached to the same potato plant with the λPat21-*luc* reporter construct, 55 days after transfer to short day conditions.

Material for RNA extraction was harvested by cutting tuber discs using a cylinder (1 cm diameter) using the first 0.5 cm from the outside of the tuber. RNA extraction and cDNA preparation was performed as reported before (Chapter 2). From the produced cDNA 1µl was used for RT-PCR using specific primer combinations based on the cDNA sequence of *luc* and the *cyclophilin* gene (Chengappa *et al.*, 1999). *Cyclophilin* was quantified and used as an internal standard for amount of cDNA loaded per sample. During RT-PCR, samples (10 µl) were taken after 16, 18, 20, 22 and 24 cycles. Samples were fractioned on a 1% agarose gel, blotted to a nylon membrane (Genescreen Plus, NEN™ Life Sciences Products) and hybridised with the probe. The intensity of the signals was quantified using a Phosphor Imager (Molecular Dynamics)

RESULTS

During tuber initiation potato plants formed multiple tubers with a variable growth rate resulting in tubers that differ in size. These differences in growth rate between tubers indicate a difference in sink strength.

Growth pattern of potato tubers

Potato plants were transferred to short day conditions (8.5 h of light) at day 0. During the first 10 days after transfer, only elongated stolon growth was observed; no tuber development was observed till 12-19 days after transfer. The number of tubers initiated varied between 5 and 44 tubers per plant. The average tuber weight per plant, quantified 55 days after transfer to short-day conditions, was inversely related with the number of tubers per plant, for plants bearing less than 20 tubers (figure 1A), indicating that a lower number of tubers per plant resulted in larger tubers. In contrast, no relation was observed between total tuber yield per plant and tuber number per plant (data not shown). Tuber development was monitored every 3-4 days after transfer to short day conditions. Analysing the light images made by the CCD camera resulted in a growth curve for every individual tuber. Figure 1B shows the growth curve of 5 tubers growing on a single plant. The tuber sizes of tubers growing on the same plant differed considerably already 1 week after initiation.

To determine when the tuber size hierarchy was fixed during tuber development, the correlation coefficient (r) was calculated between the final tuber sizes (55 days after transfer to short-day conditions) and the tuber sizes at a given time during development for all tubers attached to the same plant (figure 1C). The low correlation during the initial stages of tuber development indicates that tuber size hierarchy was not fixed during this period of tuber development. This indicates that the largest tubers at this stage of tuber development were not necessarily the largest tubers during later stages of plant development. The linear correlation coefficient increased during plant development reaching a maximum of $r > 0.9$ around 40 days after transfer to short-day conditions, indicating a fixed tuber size hierarchy there after.

Both the growth curve (figure 1B) as well as the tuber size hierarchy (figure 1C), indicated three different stages of tuber development: initiation period (<20 days), linear tuber growth period or tuber bulking period (20-40 days) and subsequently a maturation period (>40 days). The average growth rate, used as an indicator for tuber sink strength, (figure 1D) differed during the three stages of tuber development with a maximum growth rate (sink strength) during linear tuber growth (20-40 days).

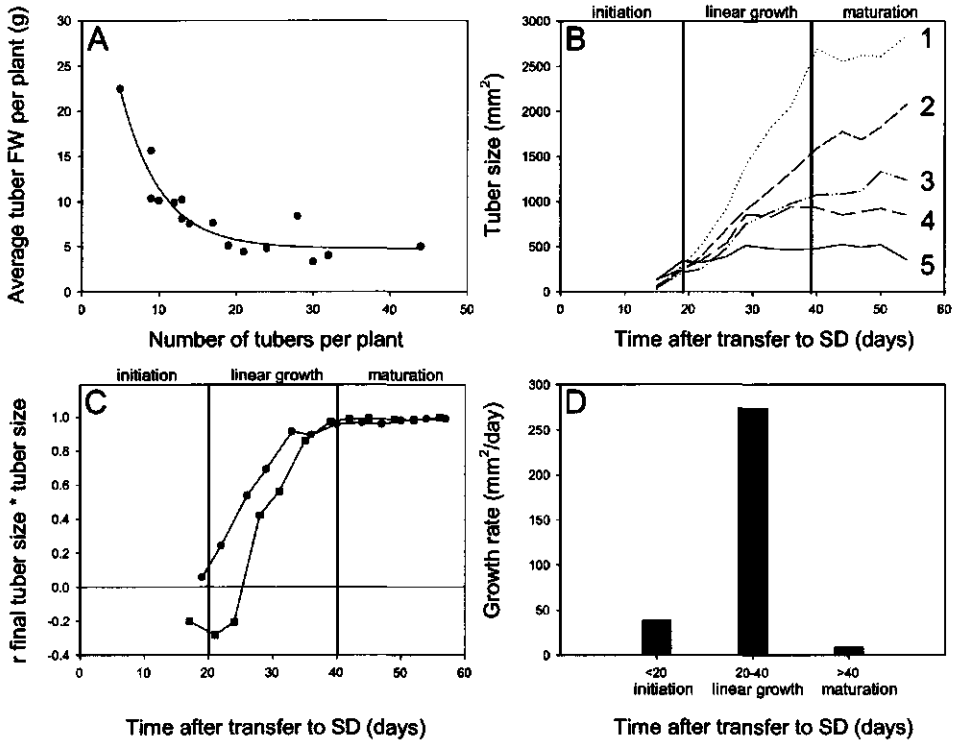


Figure 1

A. Relation between number of tubers per plant and average tuber fresh weight, 55 days after transfer to short day conditions (n ≈ 16 plants).

B. Growth pattern of 5 individual tubers attached to the same plant.

C. The linear correlation (r) between the final tuber size and the tuber size at a given time during tuber development for two individual plants with 13 and 16 tubers per plant, respectively.

D. The average growth rate (measured as mm^2/day) during the three different stages of tuber development; initiation, linear growth and maturation. The average growth rate was calculated using 15 tubers, growing on a single plant.

In planta LUC activity in complete potato tubers

To verify if the observed in vivo luciferase activities (LUC) reflect the actual luc mRNA level, we performed a semi-quantitative RT-PCR. Total RNA was isolated from three tubers attached to the same plant with the λPat21-luc reporter. RT-PCR with luc specific primer combinations indicated a good correlation between in vivo LUC activities and local luc mRNA levels (figure 2). Therefore, we conclude that quantifying LUC activity in planta is a reliable method to quantify promoter activity during tuber development. We recently described the relation between the activity of the storage related reporters and the expression of the authentic storage genes (AGPaseS and λPat21) in potato tubers, indicating a direct relation between the LUC activity measured in planta and the steady state mRNA level of the authentic storage genes (Chapter 2).

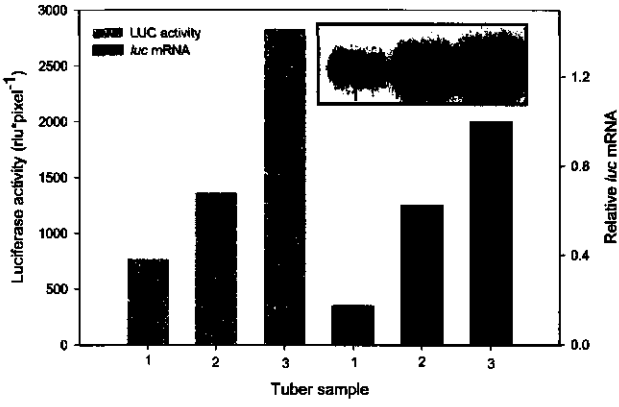


Figure 2
Relation between the *in vivo* luciferase activity (gray bars) and the relative luciferase mRNA steady state levels (black bars) in potato tubers. Luciferase activity (rlu*pixel^{-1}) was measured in three tubers attached to the same plant with the λPat21-*luc* construct. Tubers were harvested and total RNA was extracted to make first strand cDNA which was used as a template for RT-PCR using *luc* specific primers. The hybridised blot shows the RT-PCR products after 22 cycles for the three tubers.

We measured promoter activity in tubers by imaging the LUC activity non-invasively. Photons produced by the LUC activity in the inner cell layers of the tuber may be quenched because they have to pass several cell layers before they can be detected by the camera. To determine the possible quenching effect, LUC activity in CaMV35S-*luc* tubers was measured before and after covering with non-transgenic tuber tissue. Peridermal tissue (peel) resulted in an average reduction of ~50 %. When covered with tuber cortex tissue the average reduction was ~10 % per mm tissue. The diameter of the tubers used in this study varied, ranging from a few mm during the tuber initiation period up to 4-5 cm during tuber maturation. Thus, not all photons released by the inner cell layers of the tuber will be detected.

To further study the quenching effect, CaMV35S-*luc* activity was quantified in tubers that reached the maximum size (maturation). The tuber size was quantified by image analysis resulting in a two-dimensional value for the tuber size. Assuming a spherical tuber, an increase in tuber size results in an exponential increase in tuber volume (4/3r*tuber area). Quantifying the CaMV35S-*luc* activity in a population of tubers that varied in size shows a linear correlation between LUC activity per tuber and tuber size (figure 3A) and not an exponential correlation like expected for this constitutive promoter if all photons released inside the tuber were captured by the camera.

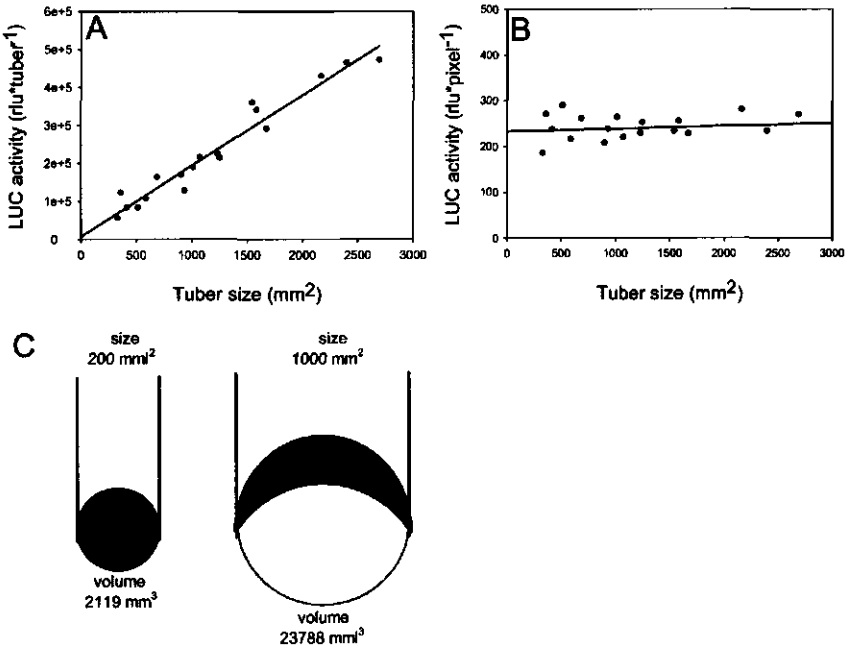


Figure 3

A. Relation between CaMV35S-*luc* activity per tuber (rlu*tuber^{-1}) and tuber size (mm^2) in a tuber population attached to a single plant (18 tubers of 1 plant).

B. Relation between CaMV35S-*luc* activity per unit tuber area (rlu*pixel^{-1}) and tuber size (mm^2) in a tuber population attached to a single plant.

C. Relation between CaMV35-*luc* activity quantified per tuber (rlu*tuber^{-1}; value inside tubers), tuber size (mm^2) and tuber volume (mm^3) of two tubers that differ in size. Shaded area indicate the tuber region of which the LUC activity will be detected while the photons from the lower region will be quenched.

Measuring the LUC activity per unit tuber area (rlu*pixel^{-1}), indicates indeed that the LUC activity did not increase with the increase in tuber volume (figure 3B) although LUC activity within the tuber was equally distributed (data not shown).

Figure 3C shows the most likely explanation of this effect for two tubers that differ in size. Tuber size quantified by image-analysis showed a difference in tuber size by a factor 5 and a difference in tuber volume by a factor 11. Quantifying the LUC activity per tuber (rlu*tuber^{-1}) shows a difference between the two tubers that differs by a factor 5.5. These results indicate that an increase in LUC activity per tuber seems to correlate more with the tuber size than with the tuber volume; apparently only the LUC activity of the outer cell layers will be detected. Thus to study the relation between gene expression and tuber growth, differences in LUC activities should be compared with the differences in tuber sizes (tuber area) and not with differences in tuber volumes (as e.g. tuber FW).

Promoter activity in tuber populations during tuber development

LUC activity was quantified during tuber development and the average activities were calculated, based on all tubers of a single plant, for the three different periods of tuber development. Average LUC activity (rlu*tuber^{-1}) is shown in figure 4 for the five different promoters, indicating a relatively constant activity for the CaMV35S, CDC2a and λPat21 (figure 4A, C, E) promoter during tuber development whereas cycB1;1 and especially AGPaseS (figure 4B, D) activities declined during tuber development.

In figure 5 (page 86) the average LUC activity per unit tuber area (rlu*pixel^{-1}) is shown indicating a maximum activity during tuber initiation followed by a decline during linear tuber growth and tuber maturation. This indicates that the promoter activities per tuber remained high due to an increase in sink size while the activity per unit tuber area declined.

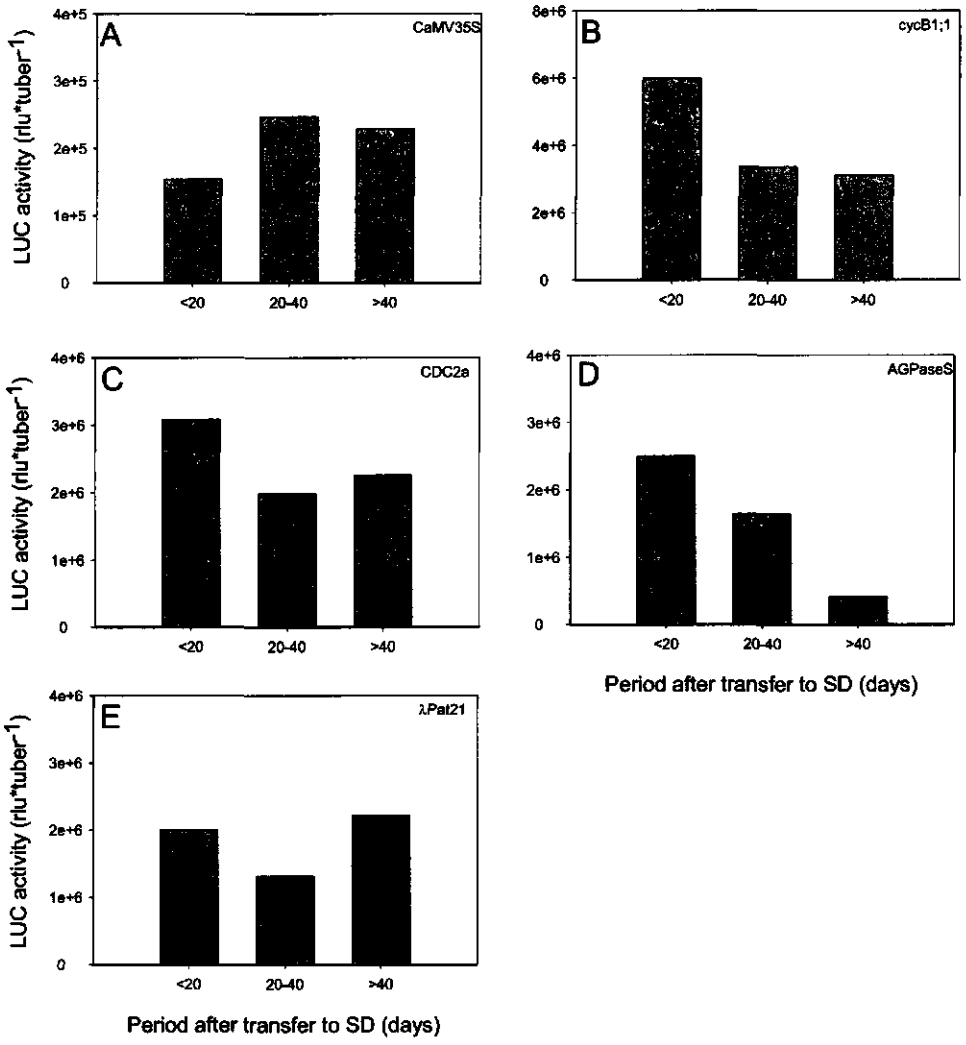


Figure 4
Average luciferase activity per tuber (rlu*tuber^{-1}) based on all tubers of a single plant during the three different stages of tuber development based on the average of (A) 8 tubers for CaMV35S, (B) 9 tubers for cycB1;1, (C) 7 tubers for CDC2a, (D) 8 tubers for AGPaseS, (E) 7 tubers for λPat21.

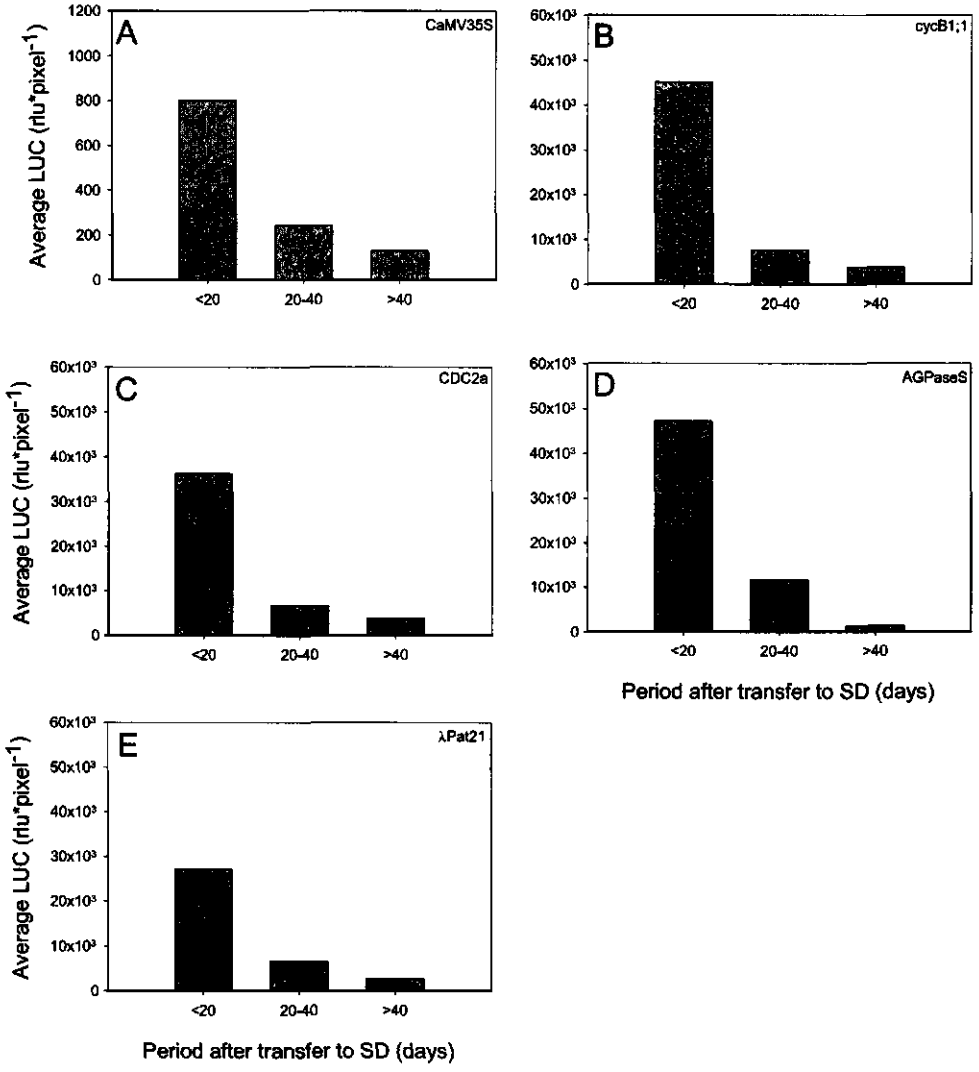


Figure 5
Average luciferase activity per unit tuber area (rlu* pixel^{-1}) based on all tubers of a single plant during the three different stages of tuber development based on the average of (A) 8 tubers for CaMV35S, (B) 9 tubers for cycB1;1, (C) 7 tubers for CDC2a, (D) 8 tubers for AGPaseS, (E) 7 tubers for λPat21.

Relation between promoter activity and tuber growth rate

Gene expression varied considerably between tubers that differed in growth rate. To investigate whether promoter activity can be used as a marker for sink strength, we studied the differences in the linear correlation between LUC activity per tuber (rlu*tuber^{-1}) and the growth rate of the tuber. Figure 6 (page 88) shows the results for the analysis of the λPat21 promoter. The growth rate per tuber (mm^2*day^{-1}) was used as a measure for sink strength (see introduction). We also studied the correlation between LUC activity per unit tuber area (rlu*pixel^{-1}) and growth rate to determine whether this parameter can be used as a measure for the sink activity. The results indicate a relatively low correlation (r = 0.36 and 0.45, respectively) between tuber growth rate and λPat21 promoter activity during the initial phase with a considerable scattering whereas a high linear correlation (r ranges from 0.85 to 0.96) was observed during linear tuber growth and maturation. The results indicate a high correlation between sink strength, quantified by determining the growth rate and λPat21 promoter activity for these specific stages of tuber development. The increase in the linear correlation was observed for both the LUC activity quantified per tuber as well as per unit tuber area indicating that not only sink size but also sink activity is important for determining sink strength during these later stages of tuber development.

In a similar way as for the λPat21 promoter, we determined the relation between promoter activities and the distribution in tuber growth rates for the other promoters. The correlation between these two parameters is expressed as the linear correlation coefficient (r) between LUC activity (both per tuber and per pixel) and tuber growth rate (sink strength, figure 7, page 89).

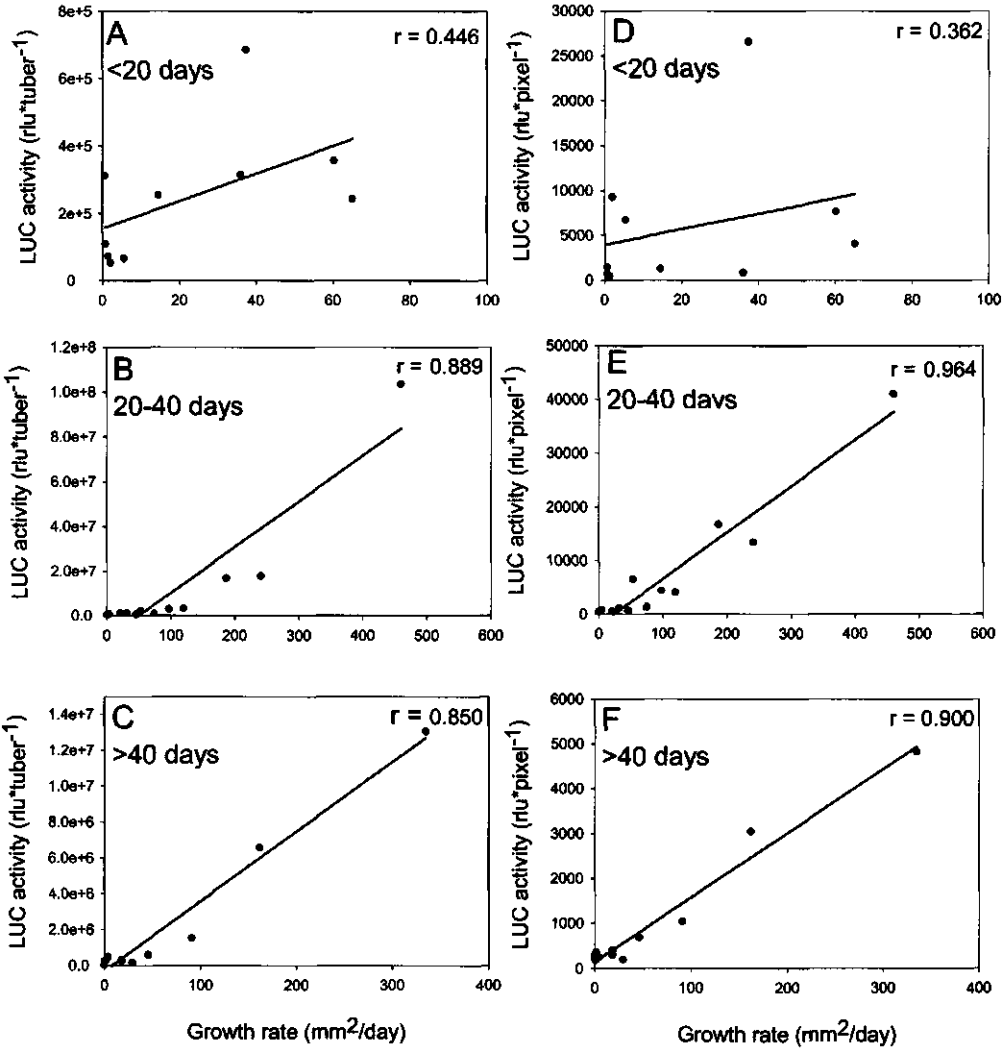


Figure 6

Relation between λPat21-*luc* activity per tuber (rlu*tuber^{-1}) and tuber growth rate (mm^2/day) (**A-C**) and between λPat21-*luc* activity per unit tuber area (rlu*pixel^{-1}) and tuber growth rate (mm^2/day) (**D-F**) of a population of tubers growing on a single plant, during tuber initiation (**A, D**), linear tuber growth (**B, E**) and tuber maturation (**C, F**), respectively.

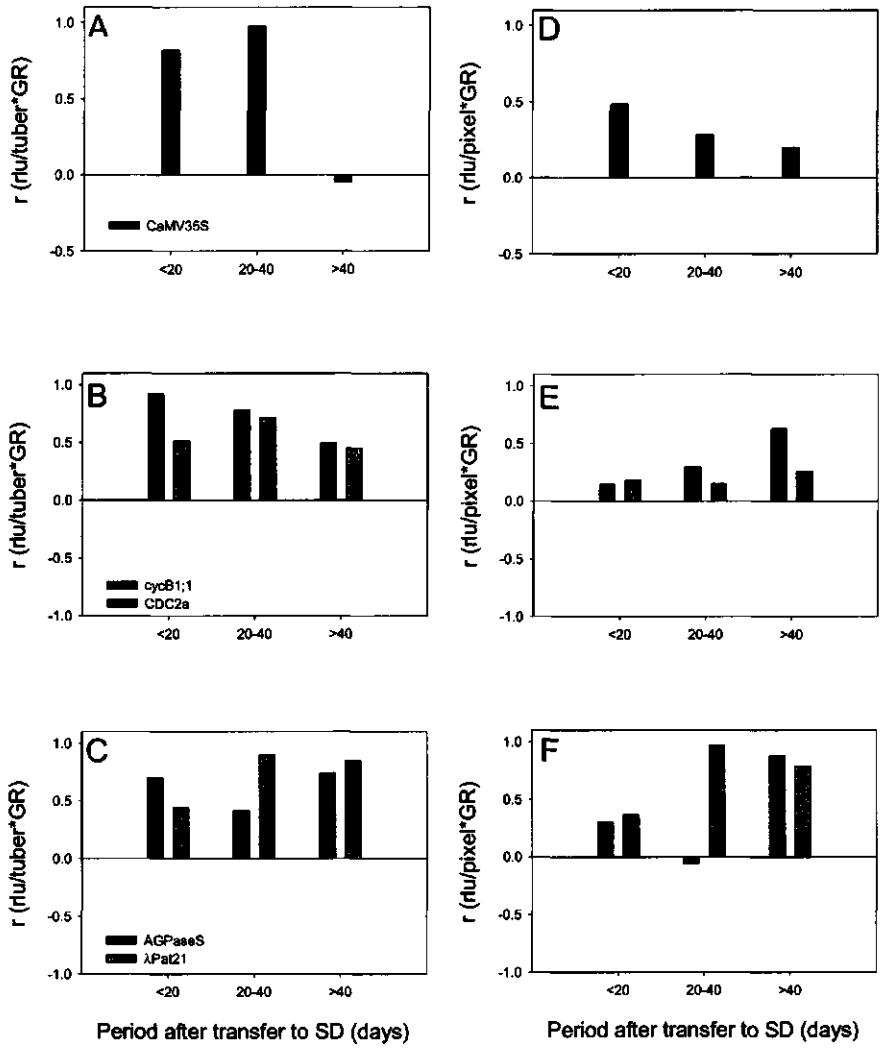


Figure 7
Linear correlation coefficient (r) between luciferase activity per tuber (rlu*tuber^{-1}) and tuber growth rate (mm^2/day) (**A-C**) and between luciferase activity per unit tuber area (rlu* pixel^{-1}) and tuber growth rate (mm^2/day) (**D-F**) during the different stages of tuber development. The linear correlation between the activity per pixel and per tuber is based on (**A**) 8 tubers for CaMV35S, (**B**) 9 tubers for cycB1;1, (**C**) 7 tubers for CDC2a, (**D**) 8 tubers for AGPaseS, (**E**) 10 tubers for λPat21, growing on a single plant.

The relation between CaMV35S activity per tuber and tuber growth rate showed a high correlation during initiation and linear tuber growth (figure 7A) while the correlation between CaMV35S activity per unit tuber area and tuber growth rate was low during all stages of tuber development (figure 7D). The cycB1;1 activity per tuber was rather well correlated ($r > 0.7$) with tuber growth rate during tuber initiation and linear tuber growth (figure 7B), while for CDC2a this was only clear during linear growth. However, the correlation between both cycB1;1 activity and CDC2a activity per unit tuber area and tuber growth rate was generally (very) low. Only during maturation a relatively high correlation was observed for cycB1;1 (figure 7E). These results indicate that during initiation and linear tuber growth the positive correlation between cycB1;1 activity per tuber and growth rate is related to differences in tuber size and not to differences in the activity per unit tuber area. For the AGPaseS promoter, a high correlation was observed between the LUC activity per tuber and tuber growth rate during initiation and maturation (figure 7C), while only during maturation a high correlation was observed when measuring the LUC activity per unit tuber area (figure 7F). With the λPat21 promoter a high correlation was observed during linear tuber growth and maturation both when LUC activity was quantified per tuber and per unit tuber area (figure 7C and 7F; cf. figure 6).

Plasticity in tuber growth and gene induction

To determine the plasticity in tuber growth and to investigate if this plasticity also occurred in gene expression, three tubers with the highest level of promoter activity were removed from two plants 26 days after transfer to short-day conditions. Figure 8 shows the effect of this tuber removal (arrow) on the growth of tubers that remained attached to the plant. The growth pattern of the remaining tubers indicates that tuber removal resulted in a growth response in these tubers.

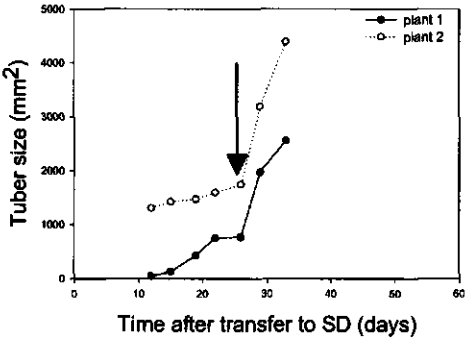


Figure 8
Growth pattern (mm^2) of two individual tubers growing on two separate plants after removal of the three tubers with the highest luciferase activity per tuber from these plants, 26 days after transfer to short-day conditions (arrow).

The enhanced growth of the remaining tubers after tuber removal was accompanied by a response in activity of cell cycle and storage related promoters. Figure 9 shows the average LUC activity as quantified per tuber (rlu* tuber^{-1}) and per unit tuber area (rlu*pixel^{-1}) based on 8-20 tubers before (day 26) and after (day 29 and 33) tuber removal. As a consequence of tuber removal the sink strength of the remaining tubers increased resulting in both a stimulation of tuber growth rate (cf. figure 8) as well as an enhanced activity of especially CDC2a and λPat21 promoter activities. These responses indicate that the remaining tubers were restricted in growth and the expression of cell cycle and storage related genes by the competitive strength of the removed tubers.

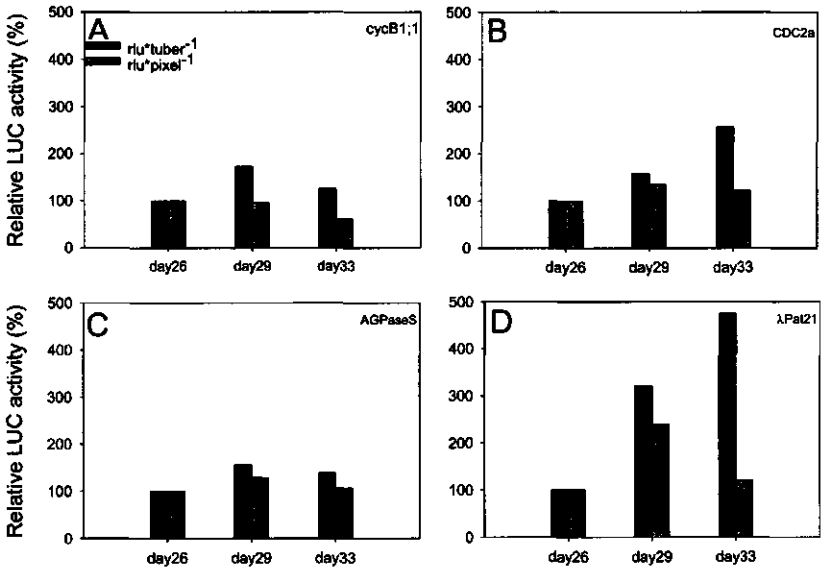


Figure 9
Relative luciferase activity per tuber (rlu*tuber^{-1}, black bars) and per unit tuber area (rlu*pixel^{-1}, gray bars) in tubers remaining on a potato plant after removal of three tubers with the highest luciferase activity per tuber, 26 days after transfer to short-day conditions. The activity was measured in all remaining tubers and the average values were calculated based on (**A**) 12 tubers for cycB1;1, (**B**) 14 tubers for CDC2a, (**C**) 8 tubers for AGPaseS, (**D**) 20 tubers for λPat21. The average activities were expressed as percentage of the activity measured at the day of tuber removal (day 26).

Although the increase in cycB1;1 promoter activity was limited when the LUC activity was averaged, a variable response was observed for cycB1;1 activity when the activity was quantified in individual tubers. An example of these differences in the response between tubers is shown in figure 10, before and after tuber removal, in nine individual tubers attached to a single stolon. Tuber removal resulted in an increase of the growth rate of all remaining tubers (figure 10A) which coincided with an enhanced LUC activity per tuber (figure 10B) and an enhanced LUC activity per unit tuber area (figure 10C) except for tuber 1, 3 and 6. The increase in tuber growth in these tubers was most likely the result of enhanced cell enlargement rather than enhanced cell division because cycB1;1 expression declined.

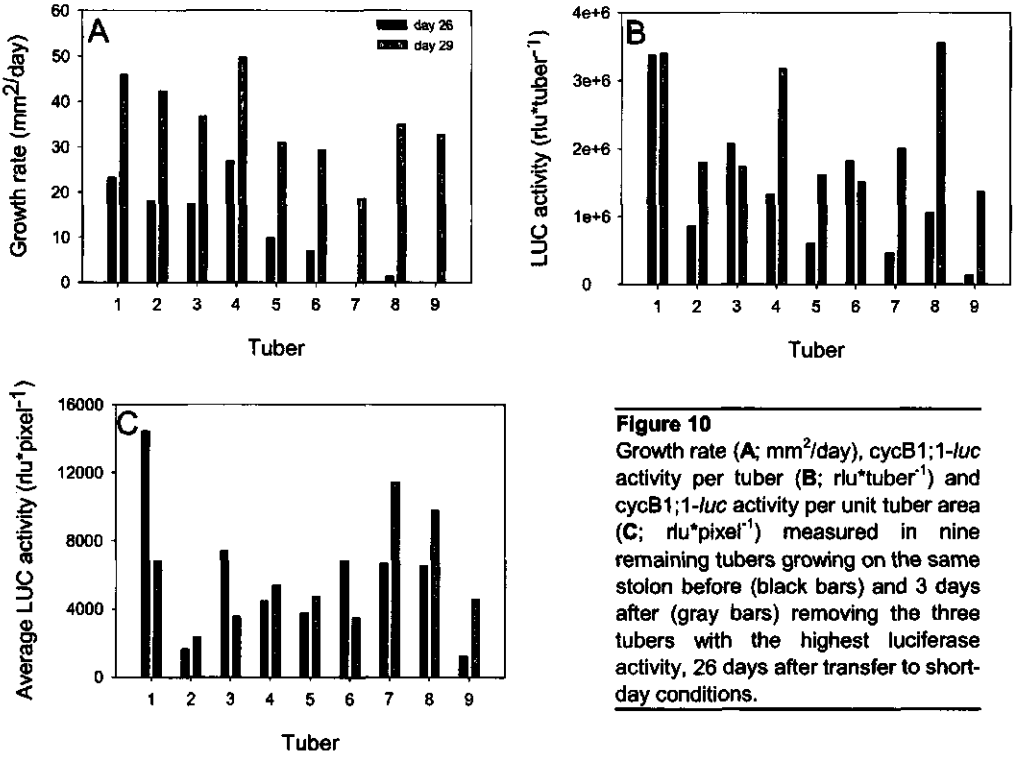


Figure 10
Growth rate (**A**; mm^2/day), cycB1;1-*luc* activity per tuber (**B**; $rlu*tuber^{-1}$) and cycB1;1-*luc* activity per unit tuber area (**C**; $rlu*pixel^{-1}$) measured in nine remaining tubers growing on the same stolon before (black bars) and 3 days after (gray bars) removing the three tubers with the highest luciferase activity, 26 days after transfer to short-day conditions.

DISCUSSION

Sink strength has been described as the ability of plant organs to import nutrients required for growth, the synthesis of storage products and respiration (Engels and Marschner, 1986b; Doehlert, 1996). In potato tubers a close correlation has been found between the growth rate and the import of photosynthates (Engels and Marschner, 1986b), indicating that the growth rate is a reliable maker for tuber sink strength although it is only defined in the context of competition for photosynthates. Studying properties like the activities of specific enzymes related to starch synthesis and differences in the level of plant growth regulators showed a correlation between these parameters and the sink strength of potato tubers (Mares *et al.*, 1981; Sung *et al.*, 1989, Merlo *et al.*, 1993). In the present study the expression of cell cycle and storage related genes was quantified per tuber and per unit tuber area to determine whether the expression of these genes can be used as a marker for the sink strength and sink activity in potato tubers.

Non-invasive detection of luciferase activities in developing tubers

The activity of the different reporter constructs was quantified by measuring the LUC activity *in planta* during tuber development. Tubers with a greater volume were expected to exhibit a higher LUC activity per unit tuber tissue ($rlu*pixel^{-1}$) because the number of cell layers increases. However, the activity of the constitutive promoter CaMV35S indicated that the LUC activity per unit tuber area did not differ in tubers that differed in tuber volume (figure 3B). This indicates that from a certain size an increase in tuber volume does not lead to an increase in the LUC activity per unit tuber area, most likely because the photons released by the inner cell layers do not reach the surface of the tuber (figure 3C).

Although only the LUC activity from the outer cell layers is detected, a direct relation was observed between the LUC activities, measured *in planta*, and the steady state *luc* mRNA levels (figure 2). This indicates that although the measured LUC activity represents the outer cell layers of the tubers, this LUC activity reflected also the promoter activity in the rest of the tuber.

Gene expression profiles show a decline in sink activity during tuber development

Tuber size increased by a factor 5-40 during tuber development (figure 1B). However, average LUC activity per tuber ($rlu*tuber^{-1}$) did not increase but was relatively constant during the different stages of development except for AGPaseS (figure 4A-E).

Because average LUC activity per tuber did not increase proportionally with tuber size, the activity per unit tuber area must decline, as indeed observed for the average LUC activity per tuber area (rlu* pixel^{-1}) (figure 5A-E).

Analysis of carbohydrate contents during tuber development showed a decline in the sucrose concentration when the tuber size increased (Morrell and ap Rees, 1986) and during the successive stages of development (Oparka, 1985; Merlo *et al.*,1993). Besides a decline in carbohydrates, a decline was described in the activity of enzymes involved in starch synthesis, viz., sucrose synthase, AGPase and fructokinase when quantified per gram of tuber tissue (Sowokinos, 1976; Morrell and ap Rees, 1986; Merlo *et al.*, 1993). LUC activity per unit tuber area also showed a decline, like reported for enzyme activities and carbohydrate content. This decline might be linked to a decline in the sugar level during tuber development. Alternatively, the LUC activities per tuber area possibly declined due to cell enlargement resulting in a lower cell number per tuber area (dilution effect) although the expression level per cell may be fairly constant during tuber development (Sowokinos *et al.*, 1976). For AGPaseS, the activity per tuber clearly declined in contrast to the other promoters. As tuber development proceeds a decreasing turnover of sucrose into starch is observed (Oparka, 1985) indicating that at later stages of development the contribution of starch synthesis to the sink strength of tubers decreases.

Plasticity of tuber growth relates to plasticity in sink strength

Tuber removal resulted in an enhanced growth of the remaining tubers (figure 8). Tuber removal also resulted in enhanced expression of cell cycle and storage related genes in the remaining tubers indicating that both growth as well as gene expression in these tubers was not limited by internal tuber conditions but by the competitive interaction between the removed and remaining tubers (figure 9). The enhanced gene expression was observed both by monitoring LUC activity per unit tuber area as well as the LUC activity per tuber (figure 9). However, the observed response after tuber removal varied between the individual tubers as shown for cycB1;1 in figure 10.

Interaction between sink size and sink activity changes during tuber development

Transfer of potato plants to tuber-inducing conditions, resulted in the formation of tubers that differed in size already 1 week after initiation (figure 1B). Many mechanisms affect tuber size distribution, some of which are fixed before tuber initiation like the number of stolons, the position of the stolons and the duration of tuber initiation (Struik *et al.*, 1991).

These and other mechanisms, active after tuber setting, e.g., internal tuber characteristics, may result in differences in sink strength between tubers and consequently in differences in tuber sizes (Ahmed and Sagar, 1981; Struik *et al.*, 1990).

Tuber size distribution during early stages of tuber development was not fixed (figure 1C) indicating that the competitive strength was also not fixed during these stages of tuber development. These results agree with the findings of Moorby (1968) and Ahmed and Sagar (1981), showing that individual tubers differ in growth rate during development. As a result, the largest tuber at any time is not necessarily the largest one during later stages of tuber development. Thus, it is difficult to determine the exact sink hierarchy between tubers at an early stage because this hierarchy may change in time (Struik *et al.*, 1990). We determined the growth rate (mm^2/day) as a measure for the sink strength of all tubers growing on the same plant during the different stages of development.

Besides a variation in growth rate, differences were also observed in the expression of cell cycle and storage related genes between tubers. The determine whether gene expression could be used as a maker for sink strength, we compared the growth rate as a measure for sink strength with the activity of the various promoters, quantified per tuber and per unit tuber area.

During tuber development (especially during initiation and linear tuber growth) a rather high correlation was observed between cycB1;1 activity measured per tuber and tuber growth rate (figure 7B), while for CDC2a this was only clear during linear growth. For both storage related promoters, a high correlation was observed between the activity per tuber and tuber growth rate except for AGPaseS during linear tuber growth and for λPat21 during the initiation period (Figure 7A-C). Therefor, we indeed conclude that promoter activity per tuber can be a good / reasonable marker for sink strength.

However, between LUC activity per unit tuber area and growth rate only a rather high correlation was observed during linear tuber growth for λPat21 and during maturation for cycB1;1 and both storage related promoters (figure 7D-F). This indicates that, among the genes studied here, only λPat21 expression reflects the competitive strength during linear tuber growth. These differences between λPat21 and the other genes might be explained when the λPat21 expression level is controlled by the flux of carbohydrates (Grierson *et al.*, 1994) into the tuber while the expression of cycB1;1, CDC2a and AGPaseS depends not only on the flux of carbohydrates into the tuber but also upon conversion of the carbohydrates, like described during sugar modulated expression of the AGPaseS gene (Sonnewald and Herber, 1999).

Alternatively, the difference might be caused by the fact that during patatin expression, tubers not only compete for the available carbohydrates but also for the available nitrogen, affecting the expression of patatin (Peña-Cortés *et al.*, 1992).

During tuber maturation, the correlation between cell cycle and storage related gene expression and tuber growth rate was high, both per tuber and per unit tuber area. When tubers reach this stage of development, tuber sink hierarchy is fixed and as a results tuber growth distribution is also fixed (figure 1C). During tuber initiation, a relatively high correlation was observed between cell cycle and storage related expression per tuber and tuber growth rate, while the correlation between the LUC activity per unit tuber area and tuber growth rate was (very) low during this phase suggesting that during initiation the differences in sink activity between tubers is of minor importance.

Our results indicate that AGPaseS and λPat21 expression can both be used as a marker for tuber sink strength in potato tubers. However, for AGPaseS the differences in expression are only evident in the maturation phase when the sink hierarchy is fixed. In contrast, the expression of the λPat21 gene is a good marker for sink strength both during linear tuber growth (tuber bulking) and during tuber maturation.

Chapter 6

General discussion

Potato (*Solanum tuberosum* L.) is used by many researchers to study the development of storage organs and to study the interaction between source (assimilates producing organs) and sinks (assimilates importing organs). Most of these studies focus on structural development (changes in cell number and cell size), carbohydrate metabolism (changes in sugar level and the accumulation of starch) or the processing of carbohydrates (changes in the activity of enzymes). In this thesis the different stages of tuber development are described in relation to temporal and spatial changes in the expression of genes which are involved in cell cycle regulation or the accumulation of storage products during tuber development. Essential results will be repeated briefly in this chapter to give a good overview of the obtained results.

Tuber development: many ways to form a tuber

Upon tuber induction cessation of stolon growth occurs followed by longitudinal cell division and cell enlargement starting in the sub-apical region of the stolon (Xu *et al.*, 1998; Vreugdenhil *et al.*, 1999). During stolon growth an enhanced cycB1;1 and CDC2a promoter activity was observed in the apical meristem of the stolon (Chapter 2, figure 1), marking the transversal cell divisions within this region of the stolon. During stolon growth, carbohydrates (mainly sucrose) are transported towards the stolon tips where they are unloaded into the apoplast and imported into the cytosol by sucrose - and hexose transporters (Viola *et al.*, 2001). Sucrose unloading in the apical part of the stolon is required for the supply of energy and building blocks for the formation of new cells in the apex. Apoplastic invertase activity is observed in the apical region of the stolon whereas cytosolic invertase activity is more abundant in the sub-apical stolon region (Appeldoorn *et al.*, 1997; Viola *et al.*, 2001). Several publications suggest that glucose generated by apoplastic invertase contributes to the control of cell division by creating apoplastic conditions favourable for mitotic activity (Weber *et al.*, 1996; Borisjuk *et al.*, 1995; Herbers and Sonnewald, 1998). The activity of cell wall acid invertase seems to be strictly correlated to hexokinase activity during stolon growth (Appeldoorn *et al.*, 1997). Hexokinase activity is described as an integrated part of the sugar-signaling mechanism leading to the modulation of sugar responsive genes including cell cycle genes (Koch, 1996; Weber *et al.*, 1996 and 1997; Jang and Sheen, 1997).

Cell cycle related gene expression during tuber development
During tuber formation cell cycle related promoter activity increased at the tuber site and declined in the apical part of the stolon, indicating a transition in cell division from the apical stolon region towards the site of tuber formation (Chapter 2, figure 1). No change was observed in both apoplastic and cytosolic invertase activity in the apical part of the stolon upon tuberization while the sucrose and glucose levels remained high in the apical part of the stolon (Viola *et al.*, 2001). Thus, the decline in cycB1;1 and CDC2a promoter activity observed in the apical part during tuberization is not directly linked to apoplastic invertase activity and the level of sucrose and glucose within this region. The activity of hexokinase declines which might be correlated with the decline in cycB1;1 and CDC2a expression. However, during tuberization both cell cycle genes were upregulated at the site of tuber development although apoplastic and cytosolic invertase and hexokinase activity remained low at the tuber site. This indicates that also the activity of hexokinase is not directly related to cycB1;1 and CDC2a expression.

Storage related gene expression during tuber development
Imaging AGPaseS and λPat21 promoter activity during stolon growth before tuberization, in explants incubated on 8% sucrose, showed a transient expression pattern at various sites within the stolon that can be as high as observed in developing tubers. However, these sites were not strictly related to those sites that eventually develop into a tuber (Chapter 2, figure 1). Upon tuberization the expression of these storage genes in the stolon declined while in the tuber the expression increased.

However, the start of upregulation of AGPaseS and λPat21 expression at the tuber site took place both before and after the start of visible swelling (Chapter 2; figure 5) while the start of upregulation in cell cycle related gene expression at the tuber site starts at the moment of visible swelling or shortly after (but never before) (Chapter 2, figure 5), indicating that storage related gene expression is not related to a tuber-specific (swelling-inducing) transcriptional signal.

The expression of storage genes like AGPaseS and λPat21 is strongly affected by the level of sucrose (Müller-Röber *et al.*, 1990; Perl *et al.*, 1991; Visser *et al.*, 1994). When the sucrose supply is interrupted the expression of storage related genes and the accumulation of starch and storage proteins declines (chapter 4, figure 2) (Geigenberger *et al.*, 1998; Geiger *et al.*, 1998).

However, in growing stolons there is no significant difference in the activity of susy, fructokinase, AGPase and starch phosphorylase between explants incubated on 1% sucrose or 8% sucrose although the internal sucrose levels differ (Appeldoorn *et al.*, 1997 and 1999; Vreugdenhil *et al.*, 1998). This indicates that the expression and the activities of storage related genes in stolons do not relate to the level of sucrose.

Tuber development coincides with a decline in the level of glucose and fructose in stolon and tuber, whereas sucrose levels increase at the tuber site (Ross *et al.*, 1994; Hajirezaei *et al.*, 2000). There is no consensus about the sucrose level in the stolon subtending the developing tuber, showing either an increase in sucrose content that equals the sucrose level in the tuber (Ross *et al.*, 1994) or a gradual decline from the tuber site towards the stolon (Viola *et al.*, 2001), both quantified in stolons from soil grown plants. However, analysing the sucrose levels during *in vitro* tuber formation shows a higher level in the stolon in comparison with the tuber (unpublished results). From these observations we may conclude that the enhanced storage related gene expression in the tuber is not related to the absolute sucrose level. Possibly an enhanced expression of storage related genes during tuber development is related to an enhanced influx of sucrose into the tuber (Viola *et al.*, 2001).

Sensing such an influx of sucrose may involve different sugar-sensing mechanisms like hexokinases or SNF1 related protein kinases. However, no significant changes were observed in the starch level of tubers of transgenic potato plants when hexokinase activity was specifically reduced in tubers, indicating that the hexokinase mediated signalling is not important for the storage function of potato tubers (Veramendi *et al.*, 1999). In contrast, a reduced susy expression was observed in transgenic potato plants with a reduced activity of the SNF1-related protein (PKIN1) (Purcell *et al.*, 1998). Besides, these plants showed a reduced sucrose-inducible susy expression in leaves indicating an important role for this sucrose signalling mechanism in sensing the influx of sucrose and the expression of storage related gene expression. Whether these lines, with a reduced SNF1-activity, also have a reduced λPat21 or AGPaseS expression is not described. However, a reduced susy expression in tubers resulted in a reduced AGPaseS and λPat21 expression (Zrenner *et al.*, 1995). The differences in timing between swelling and the start of the upregulation of storage related gene expression indicate a possible variation in the moment of sucrose influx between the individual tubers which is not related to the moment of swelling.

Second growth: Effect of gibberellins and ethanol on cell cycle and storage related gene expression

Transferring developing *in vitro* tubers to medium with gibberellins or ethanol results in a decline in tuber growth and the outgrowth of the apical meristem, a process called second growth (Chapter 3 and 4). Analysing gene expression in *in vitro* tubers after transfer to medium with gibberellins or ethanol showed a decline in the expression of cycB1;1, AGPaseS and λPat21, whereas CDC2a expression only declined during EtOH-induced second growth (Chapter 3, figure 2 and Chapter 4, figure 2). The type of second growth differed between GA- and EtOH-induced second growth: GA-induced second growth resulted in a secondary stolon (Chapter 3), whereas ethanol combined with high sucrose results in the formation of a secondary tuber, while ethanol combined with low sucrose results in the formation of a secondary stolon (Chapter 4). These results indicate that gibberellins and ethanol both affect tuber development and gene expression by different signal-transduction pathways (Chapter 4 and Claassens *et al.* 2002). Combining gibberellins or ethanol with ABA prevented bud growth but it did not overcome the decline in cell cycle and storage related gene expression in the tuber (Chapter 3, figure 4 and Chapter 4, figure 3), indicating that the decline in gene expression in the tuber is directly affected by GA and ethanol, independently from ABA whereas the growth of the bud meristem is controlled by an interaction between GA or EtOH and ABA.

Gibberellins reduce ADP-glucose-pyrophosphorylase activity (AGPase) and the expression of genes encoding patatin and other tuber-specific storage proteins resulting in a lower synthesis of starch and storage proteins (Mares *et al.*, 1981; Hannapel *et al.*, 1985; Park, 1990). Besides, the level of hexoses increases while the sucrose levels decline resulting in a sugar status resembling that in growing stolons.
Furthermore, a reduced import of carbohydrates into the tuber was observed (Mares *et al.*, 1981) indicating that gibberellins reduce the sink strength of the tubers.

The expression of the storage related genes and the accumulation of starch and storage proteins are induced by sucrose (Visser *et al.*, 1994; Appeldoorn *et al.*, 1997, 1999). However, when *in vitro*-grown explants are incubated on medium with high sucrose (8%) in combination with GA, the induction of storage related genes and the accumulation of starch and storage proteins is not observed despite the high level of sucrose in the medium (Appeldoorn *et al.*, 1997; Vreugdenhil *et al.*, 1998). Vreugdenhil *et al.* (1999) concluded that the changes in enzyme activities during tuber formation and the regulation by GA are likely to be the result of an altered morphogenetic pattern (no tuber formation) rather than a result of GA itself.

However, the results presented in chapter 3 indicate that the expression of AGPaseS and λPat21 in the tuber can be modulated directly by GA, even without growth of the bud.

During germination of barley seeds the expression of α-amylase, involved in the hydrolysis of starch, appears to be controlled by the sugar level and by GA. However, when studied in more detail it appeared that in the aleurone layer GA stimulates α-amylase expression while sugars seem to have no effect on the expression. In the embryos α-amylase expression is controlled both by GA and sugars (Perata *et al.*, 1997). These results indicate that both in potato tubers as well as in the aleurone layer of seeds GA prevents sugar induced gene expression.

During stolon development sucrose unloading occurs predominantly via an apoplastic route whereas during tuber development a bulk flow of sucrose is observed from the phloem into the sink cells predominantly via a symplastic route (Viola *et al.*, 2001). The size exclusion limit (SEL) of plasmodesmata is therefore an important factor controlling the bulk flow of assimilates into the tuber. It has been described that SEL is modulated by differences in the turgor pressure between cells and by the cellular Ca^{2+} level (Oparka and Roberts, 2001). GA might trigger the closure of plasmodesmata by increasing the intracellular osmolarity or by elevating the cellular Ca^{2+} level as described in aleuron protoplasts (Lovegrove and Hooley, 2000 and references therein), thereby inducing a switch from symplastic to apoplastic loading. The reduced import via the symplast, resulting from the lower SEL, would possibly lead to a reduced import of sugars into storage cells and a reduction in storage related gene expression.

Although ABA stimulates tuber initiation (Xu *et al.*, 1998) and despite the significant correlation between tuber growth rate and ABA content (Marschner *et al.*, 1984), our results did not show a direct effect of ABA on the expression of cell cycle and storage related genes in *in vitro* tubers, after transfer of developing tubers to medium with ABA or fluridone (an inhibitor of ABA biosynthesis; results not shown).

Second growth: What induces and controls bud growth?

During second growth induction in potato by transferring tubers to medium with gibberellins or ethanol, the outgrowth of the bud was accompanied by changes in gene expression both in tuber and bud region. Depending on the treatment, gene expression in the bud and tuber region varied. Transferring *in vitro* tubers to medium with GA or EtOH resulted in a significantly higher cycB1;1 promoter activity in the bud region compared to the tuber region which could not be prevented by combining GA or EtOH with ABA although ABA prevented bud growth. Besides this significant difference in the activity of the cycB1;1 promoter between bud and tuber region, higher activity of the AGPaseS promoter was observed in the bud region of young tubers (1-5 days after visible swelling) transferred to medium with ethanol and high sucrose and in all mature tubers (25-30 days after visible swelling) transferred to medium with GA or EtOH. AGPaseS expression in the bud region could be prevented by combining GA with ABA whereas EtOH combined with ABA resulted in a similar pattern like observed in tubers transferred to medium with EtOH only. The results of the different treatments are summarized in table 1 for the cycB1;1 promoter (table 1A-B) and for the AGPaseS promoter (table 1C-D).

A

Young tubers Treatment	CycB1	CycB1;1 ABA
Control	-	-
GA_{4+7}	+	+
EtOH 1%	+	+
EtOH 8%	+	+

B

Mature tubers Treatment	CycB1	CycB1;1 ABA
Control	-	-
GA_{4+7} (early)	+	ND
GA_{4+7} (late)	-	ND
EtOH 1%	+	+
EtOH 8%	+	+

C

Young tubers Treatment	AGPaseS	AGPase ABA
Control	-	-
GA_{4+7}	-	-
EtOH 1%	-	-
EtOH 8%	+	+

D

Mature tubers Treatment	AGPaseS	AGPase ABA
Control	-	-
GA_{4+7} (early)	+	-
GA_{4+7} (late)	+	-
EtOH 1%	+	+
EtOH 8%	+	+

Table 1

Differences in the activity of the cycB1;1 (**A-B**) and AGPaseS (**C-D**) promoter between bud and tuber region of young (**A/C**: 1-5 days after visible swelling) and mature (**B/D**: 25-30 days after visible swelling) *in vitro* tubers after transfer to medium with 10 μM GA_{4+7} or 0.5 % ethanol combined with 1% or 8% sucrose and in the presence or absence of 100 μM ABA. The relative luciferase activities were measured in the bud and tuber region of a series of individual tubers per treatment and statistically analysed. A significantly higher activity in the bud region is indicated by +, the absence of a significant difference, by -. For mature tubers, calculations were made separately for tubers with early (during the first 90 hours after transfer) and late second growth while second growth was never observed in mature tubers transferred to medium with GA+ABA (rectangle). ND = not determined. For more detailed information on cycB1;1 and AGPaseS expression in bud and tuber region see chapter 3, figure 9 (GA) and chapter 4, figure 7 (ethanol).

Cell cycle genes and cell division during bud growth

Transferring *in vitro* tubers to medium with GA or EtOH resulted in a significant higher cycB1;1 promoter activity in the bud than in tuber region, when growth of the bud meristem is induced (table 1A-B). Gibberellins induce internode elongation in deepwater rice (*Oryza sativa*) which is accompanied by an increased expression of histone H3 and the mitotic cyclin, cycOs1, indicating that gibberellins can directly affect cell cycle related gene expression (Sauter *et al.*, 1997).

In mature tubers (25-30 days after visible swelling) the timing of second growth varies. Tubers with early second growth showed a significantly higher cycB1;1 expression in the bud region in comparison with the tuber region when transferred to medium with GA whereas tubers with late second growth showed no significant difference between bud and tuber region (table 1B). A delay in second growth and differences in cycB1;1 promoter activity between mature tubers with early and late second growth might relate to differences in the ABA level between these tubers. However, preventing second growth by ABA did not prevent the significantly higher cycB1;1 promoter activity in the bud region both during GA- and EtOH-treatment of young tubers, indicating that ABA levels have no direct effect on cycB1;1 expression. CycB1;1 expression has been observed in axillary buds of *Arabidopsis thaliana*, *Nicotiana tabacum* and *Populus tremula* even when morphological changes (growth) were not observed (Ferreira *et al.*, 1994; Rohde *et al.*, 1997). It has been described that in synchronised BY-2 cells exogenous ABA inhibits the G1-S transition (Swiatek *et al*, 2002). A possible mechanism underlying the ABA effect on bud growth is a reduction in CDC2a expression and stimulation in expression of the cyclin-dependent kinase inhibitor ICK1 both involved in controlling the G1-S transition phase (Hemerley *et al.*, 1983; Wang *et al.*, 1998).

Sucrose unloading and AGPaseS expression during bud growth

An enhanced invertase activity was observed in the bud region when second growth was induced with GA whereas this enhanced invertase activity was absent in the bud region of tubers transferred to medium with ethanol and 8% sucrose (unpublished results). Because a histochemical method was used to study invertase activity in the bud region, we could not discriminate between apoplastic and cytosolic invertase. Exogenous GA stimulates stem elongation in pea seedlings (*Pisum sativum*) which is preceded by an enhanced invertase activity followed by an increase in the hexose level (Miyamoto *et al.*, 1993; Kördel and Kutschera, 1998).

The role of hexoses during elongation growth was studied before by Broughton and McComb (1971), indicating that injecting glucose directly into pea stem cells could mimic the effect of GA. Actinomycin D inhibited the effects of GA on invertase activity, indicating that the GA-response may be mediated by an enhanced invertase gene expression (Miyamoto *et al.*, 1993).

These results indicate that during GA-induced second growth, apoplastic sucrose unloading in the bud region might be stimulated by an enhanced invertase activity resulting in a high hexose to sucrose ratio in the apoplast that seems to induce stolon / shoot growth. During EtOH-induced second growth high sucrose in the medium results in a low hexose to sucrose ratio which promotes the development of a secondary tuber; here unloading occurs most likely via a symplastic route. Figure 1 shows schematically the difference in sucrose unloading and sucrose utilisation in the bud meristem during second growth after transferring young tubers to medium with GA_{4+7} or ethanol combined with 8% sucrose, or ethanol combined with 1% sucrose.

During second growth induced by GA (figure 1A) or by ethanol with low sucrose (figure 1B), the sucrose unloading occurs via the apoplast probably leading to the formation of a secondary stolon. Ethanol combined with high sucrose resulted in a secondary tuber with symplastic sucrose unloading and a relatively high AGPaseS expression due to a bulk flow of sugars into the cytosol (figure 1C).

Young tubers showed only a higher AGPaseS promoter activity in the bud region after transfer to medium with ethanol and 8% sucrose (table 1C). In contrast, in mature tubers all second growth-inducing conditions resulted in a significantly higher AGPaseS promoter activity in the bud region when compared to the tuber region (table 1D). The relatively high AGPaseS promoter activity in the bud region of mature tubers possibly relates to a delay in bud growth as observed in these tubers resulting in the accumulation of sugars in the bud. However, a decline in the assimilate flow towards the bud region would be expected when bud growth is delayed because the flux of phloem sap translocated to and partitioned between sinks, depends on the unloading of the major osmotic substances which are sucrose and potassium (Patrick, 1997 and references therein). Terminal storage sinks loose only minimal amounts of phloem imported water by transpiration. In such cases, the water is re-exported via the xylem (Patrick, 1997 and references therein). In dormant tuber buds continuous xylem strands are observed connecting the buds to the tuber region (Artschwager, 1924).

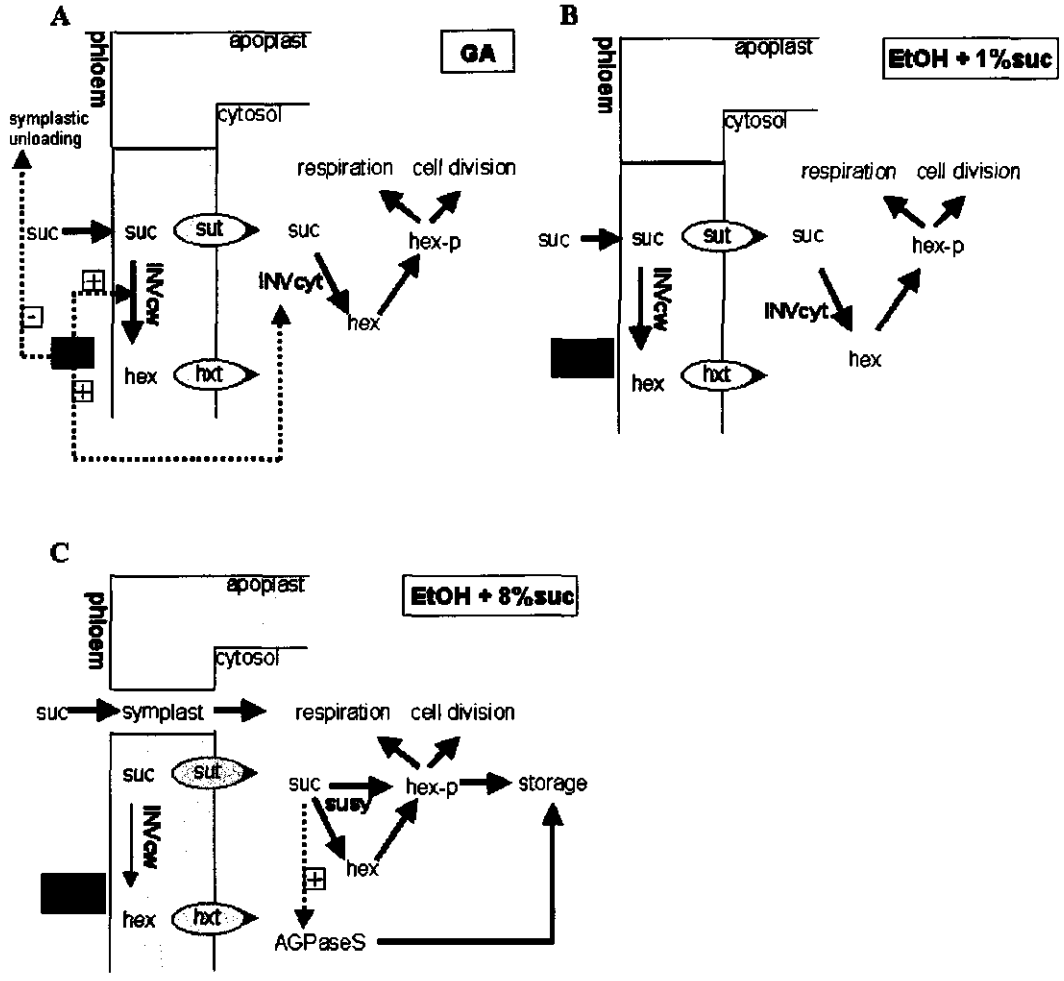


Figure 1

Schematic drawing of sucrose unloading, degradation and subsequent metabolisation and the consequence for AGPaseS gene expression in the bud meristem of young *in vitro* tubers (1-5 days after visible swelling) after transfer to medium with (**A**) GA_{4+7} or ethanol combined with (**B**) 1% or (**C**) 8% sucrose. The thick arrows reflect the major pathway for sucrose (suc), hexoses (hex) and phosphorylated hexoses (hex-p). During GA-induced second growth apoplastic sucrose unloading in the bud meristem is induced by an enhanced (+) invertase activity (INV) or by an enhanced activity of the sucrose (sut) and hexose transporters (hxt) while symplastic unloading is repressed (-). In tubers transferred to medium with ethanol sucrose unloading may be dependent of the sucrose level, leading to apoplastic unloading when ethanol is combined with low sucrose (1%) and symplastic unloading when ethanol is combined with high sucrose. Symplastic sucrose unloading results in an enhanced flux of sucrose into the cytosol causing an enhanced (+) AGPaseS expression. susy = sucrose synthase.

In vitro-grown explants with mature dormant tubers placed on medium containing eosine showed that transport from the medium into the bud region occurs although bud growth was absent (chapter 3), indicating that transport from the medium into the bud region occurs independently of bud growth.

The continuous flow of sucrose towards the bud results in the accumulation of sugars leading to osmotic inhibition of cell proliferation, similar as that described during seed germination and root growth (Bewley, 1997; Burssens *et al.*, 2000). A temporary transition of sugars into starch would prevent osmotic stress in the bud meristem, preventing a reduced cell division of the meristem.

ABA prevented the GA-induced outgrowth of the bud region and prevented enhanced AGPaseS expression in the bud region of mature tubers during GA-treatment, whereas ABA did not prevent AGPaseS expression in the bud during ethanol-treatment (table 1D). Assuming that AGPaseS expression in the bud is related to sucrose partitioning, these results indicate that sugar import in the bud region during GA-induced second growth is affected by ABA whereas the sugar import during EtOH induced second growth is not affected by ABA.

ABA prevents the germination of *Arabidopsis thaliana* seeds. This effect can be suppressed by relatively low amount of sugars (Garciarrubia *et al.*, 1997; Finkelstein and Lynch, 2000). However, when studied in more detail, it was shown that although sugars could overcome the ABA-induced blocking of germination, externally supplied sugars could not counteract inhibition of seedling growth by ABA (Finkelstein and Lynch, 2000). Seeds incubated in the presence of ABA and sugars showed no invertase activity in the root tip while the controls (no ABA) showed an enhanced invertase activity in the root tip during seedling growth. This indicates that root growth correlates with sustained invertase activity which can be modulated by ABA (Finkelstein *et al.*, 2000).

An enhanced invertase activity was observed in the bud region during GA-induced second growth whereas during ethanol treatment no increased invertase activity was observed in the bud region (unpublished results). Figure 2 shows schematically the possible effects of ABA on sucrose unloading and the subsequent effects on AGPaseS expression during second growth induced with GA (figure 2A) or with ethanol combined with 1% sucrose (figure 2B) or 8% sucrose (figure 2C) in mature tubers. An enhanced partitioning of sucrose to the bud meristem during GA-induced second growth occurs via an enhanced activity of the invertase enzyme and sucrose unloading via the apoplast. ABA prevents an enhanced partitioning of sucrose to the bud meristem, during GA-treatment, possibly by preventing an increase in invertase activity resulting in a lower import of sugars and a decline of AGPaseS expression.

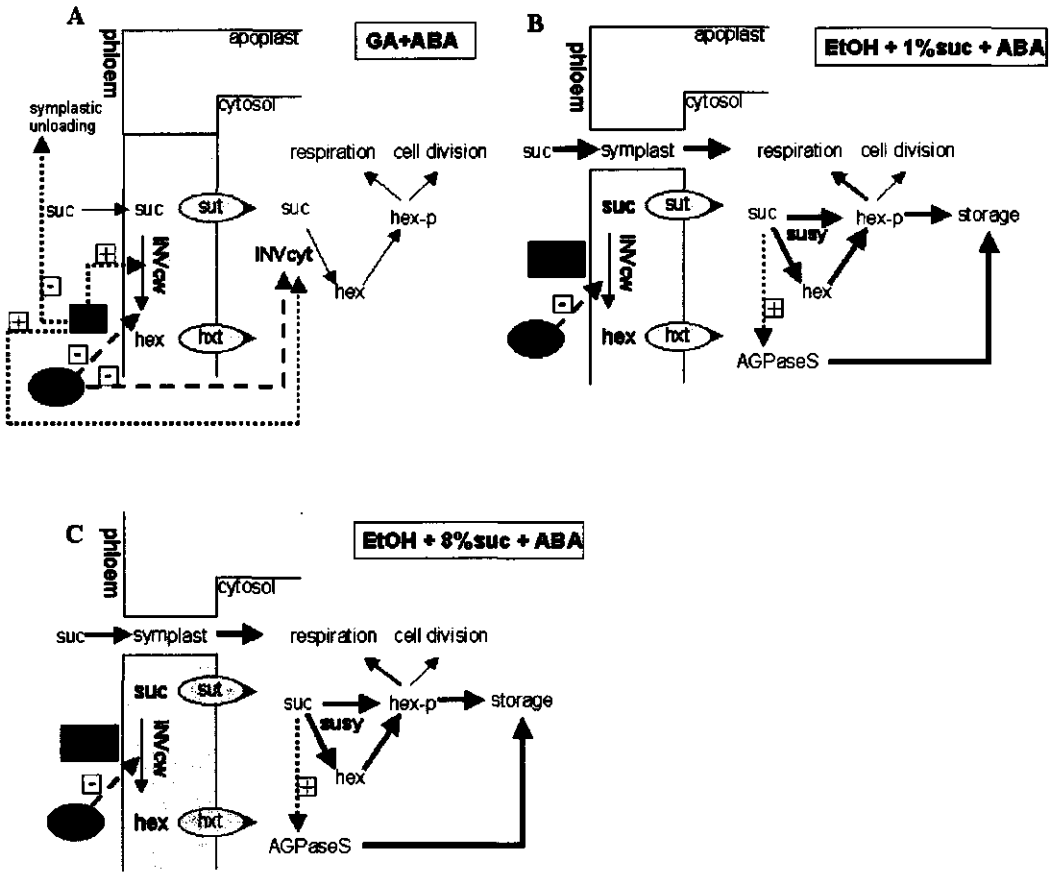


Figure 2

Schematic drawing of sucrose unloading, degradation and subsequent metabolisation and the consequence for AGPaseS gene expression in the bud meristem of mature *in vitro* tubers (25-30 days after visible swelling) after transfer to medium with (**A**) GA_{4+7} + ABA or 0.5 % ethanol + ABA combined with 1% (**B**) or 8% sucrose (**C**). The thick arrows reflect the major pathway for sucrose (suc), hexoses (hex) and phosphorylated hexoses (hex-p). In panel A ABA counteracts the enhanced apoplastic import of sugars (-) by preventing the GA-induced invertase activity (INV), and the sucrose (sut) and hexose transporters (hxt). However, ABA does not prevent the reduced symplastic unloading as a result of GA-treatment. A reduced import of sugars into the cytosol results in a reduced AGPaseS expression in comparison to mature tubers transferred to medium with only GA where a delay in bud growth leads to the accumulation of sugars. ABA prevents the development of apoplastic sucrose unloading during ethanol-treatment with 1% sucrose (figure 1B), because of its effect on invertase activity. Apparently under these conditions the symplastic import that disappeared during ethanol-treatment with low sucrose, remains present when ABA is added. Symplastic sucrose unloading results in an enhanced flux of sucrose into the cytosol causing an enhanced (+) AGPaseS expression. susy = sucrose synthase.

The effect of GA on the import of sucrose via the apoplast is counteracted by ABA whereas cycB1;1 induction is not prevented indicating an ABA-dependent GA signalling for sugar import and an ABA-independent GA signalling for cycB1;1 expression. When EtOH is combined with low sucrose (1%) a secondary stolon is formed. Sucrose unloading occurs most likely via the apoplast like observed in stolons on medium with low sucrose (figure 1B). When tubers were transferred to medium with EtOH and ABA, apoplastic unloading is prevented. However, in contrast to tubers transferred to medium with GA + ABA, unloading possibly continues via a symplastic route because ethanol does not prevent symplastic unloading like GA (figure 1B). Tubers transferred to medium with EtOH and 8% sucrose formed secondary tubers. Here sucrose unloading occurs via a symplastic route that is not affected by ABA (figure 2C).

There is no consensus about the role of abscisic acid (ABA) during tuber formation and during tuber dormancy. Lowering the ABA level in potato tubers by heat treatment or by preventing ABA synthesis by applying the herbicide fluridone (FLD) resulted in pre-mature sprouting (Van den Berg *et al.*, 1991; Suttle and Hultstrand, 1994). However, quantifying the endogenous ABA concentrations in potato tubers showed no correlation with the sprouting behaviour of the tubers (Biemelt *et al.*, 2000). The results presented here indicate that ABA possibly affects bud growth by affecting cell cycle gene expression, independently of GA and EtOH. Alternatively, ABA may prevent bud growth because it acts as an inhibitor of GA-induced sugar partitioning in the bud whereas during EtOH-induced second growth ABA did not affect sucrose partitioning to the bud.

Is gene expression an indication for sink strength?

The sink strength of individual storage organs like potato tubers, is always the result of their interaction with the source and the other sinks growing on the same plant. This indicates that the sink strength of a specific organ can not be studied on just an individual organ but has to be considered as part of the whole plant (Farrar, 1996). However, in a situation like tuber formation we can study the interaction between the sinks because they are all fed by the same source. When there is no source limitation the sink strength of a tuber is determined by the activity of the sink and by its size. When there is a source limitation, the sink strength of tubers depends on sink activity and size of that tuber but also upon the sink strength of the other tubers. Whether carbon metabolism is source- or sink limited depends on the growth conditions and may alter during development (Farrar, 1996)

The sink activity is the result of growth, synthesis of storage products in the sink (starch and storage proteins) and respiration (Doehlert, 1996). Conversion of sucrose into starch represents the major metabolic event in the tuber (starch represents 65-70% of the tuber dry matter at maturity) whereas 25% of the imported sucrose is utilized for respiration and biosynthesis of organic acids, amino acids and proteins and about 4% is used for cell-wall material (Hopff and Castro, 1969; Morrell and ap Rees, 1986). The fact that the majority of the carbon flux is used for starch synthesis may explain the relatively high correlation between sink strength and the activity of starch synthesis related enzyme activities, both in potato tubers and in grain seeds (Engels and Marschner, 1986a; Jenner and Hawker, 1993; Liang *et al.*, 2001).

There has been a lot of debate about what determines the sink strength of a plant organ and how to measure sink strength (Farrar, 1993 and accompanying papers), especially because the sink strength depends on several factors and the sink strength of developing storage organs may change during development. The advantage of the experimental system described in chapter 5 is that it is non-destructive which allowed us to study the dynamic relations between sink competition and cell cycle and storage related gene expression during tuber growth. However, we did not determine the carbon flux towards the tubers during development. Therefore, we measured the sink size every 3-4 days and determined the growth rate as an indicator for tuber sink strength (Engels and Marschner, 1986b)

Differences in the growth rate between tubers attached to the same plant were compared with differences in the expression of cell cycle and storage related genes. The results showed that of the 5 different promoters studied, the λPat21 promoter is the best candidate to study the relation between tuber sink strength and gene expression because of the relatively high correlation between growth rate and the activity of the λPat21-*luc* reporter (chapter 5, figure 7).

As mentioned before growth, storage of reserves and respiration affect the sink strength of a tuber. In chapter 5 we studied the relation between growth rate (by measuring the size) and gene expression, assuming that the growth rate corresponded with the competitive sink strength of the tubers (Engels and Marschner, 1986b). However, tubers with a relatively low growth rate possibly contain relatively more starch as observed in young developing tubers (Engels and Marschner, 1986b) and therefore their sink strength may be equal to the sink strength of tubers with a higher growth rate. Therefore, it might be better to use our results to determine what proportion of the control of growth of this tuber is related to the expression of a specific gene, instead of considering gene expression as a direct measure for sink strength.

A low correlation was observed between tuber growth rate and the expression of CycB1;1, CDC2a and AGPaseS during tuber initiation (< 20 days after transfer to short-day conditions) and during linear tuber growth (20-40 days after transfer to short-day conditions). For the λPat21 promoter, the correlation between tuber growth rate and promoter activity was relatively high in comparison with the other promoters. Whether the variable expression of the λPat21 gene relates to differences in the flux of carbohydrates between tubers or whether it relates to differences in the flux of other nutrients required for growth, is still unknown.

In conclusion, we found a high correlation between tuber growth rate and the expression of the storage gene patatin, indicating that this gene can be a good marker for studying sink strength in growing tubers. However, the relation between gene expression and sink strength may be an indirect one because the product itself, in this case a storage protein, is not affecting the size or the activity of the sink.

Concluding remarks

Tuber development has been studied intensively in the past. Although the exact mechanisms leading to the formation of tubers are still unknown, many factors have been identified that play an important role during the life cycle of the tuber. In the experiments described here we used a novel non-invasive method to study gene expression during the different stages of tuber development and growth. The use of a non-invasive method has some clear benefits in comparison with destructive methods applied previously.

The luciferase reporter system allows gene expression analysis during developmental changes in individual samples and in pooled samples. This made it possible to study the exact relation between changes in gene expression and developmental changes that may be hidden by using pooled samples in a destructive method. The high spatial resolution that can be achieved with the luciferase system makes it possible to detect differences in gene expression between the different regions of the tuber. Besides, the luciferase reporter system also allows the detection of changes in gene expression with a high temporal resolution which is desirable when changes in gene expression and developmental changes and/or changes in growth may occur in different time-frames.

Summary

Potato tubers are vegetative structures aimed at survival of the period when conditions are not optimal for the growth of the potato plant. Understanding the process of tuber formation in potatoes (*Solanum tuberosum*) is important, both from a scientific view, and for practical applications. During the initial stages of tuber formation an enhanced synthesis of starch and storage proteins is observed at the site of the stolon that develops into a tuber. Besides an enhanced synthesis of storage products, longitudinal cell division and cell enlargement is observed resulting in a radial expansion of the stolon. Because cell division and the synthesis of storage products are two important events during tuber development we analyzed the expression of two sets of genes: two cell cycle genes (cycB1;1 and CDC2a) and two storage related genes (AGPaseS and λPat21). To analyze cell cycle and storage related gene expression we fused the promoter sequences of these genes to the firefly luciferase reporter gene from North American firefly (*Photinus pyralis*). Luciferase (LUC) activity can be observed non- destructively in individual tubers and correlates with the luciferase mRNA level in both in vitro grown as well as in soil grown tubers, indicating that *in planta* LUC activity reflects the ongoing expression level of the reporter gene. More important, LUC activity correlates with the mRNA level of the endogenous genes.

In chapter 2 we describe the expression of cell cycle and storage related gene expression in *in vitro* grown explants in relation to the onset of swelling marking the morphological onset of tuber formation. The average activity quantified in multiple tubers indicated that the storage related genes were upregulated well before visible swelling, viz., four days before swelling for AGPaseS, and three days before swelling for λPat21, while the average activities of both cell cycle genes showed an upregulation after the onset of swelling. However with the ability to observe changes in gene expression in individual tubers a variable upregulation for both storage related genes could be shown in relation to the moment of visible swelling, varying between four days before till ten days after the onset of swelling. The results indicate that tuber development does not occur via a fixed sequential order of events, but consists of independent individual programs that occur in the same region of the stolon resulting in the formation of a potato tuber.

When after tuber initiation, environmental conditions become adverse for tuber induction, second growth may occur. Adverse tuber-inducing conditions may result in the outgrowth of the apical bud (second growth) and a reduced growth of the tuber (primary growth). We mimicked second growth inducing conditions by transferring developing in vitro tubers to medium with gibberellin (chapter 3) or to medium with a low level of ethanol (chapter 4).

GA-induced second growth resulted in the outgrowth of the apical bud followed by the formation of a secondary stolon. Transfer of tubers to medium supplemented with ethanol resulted in the formation of a secondary tuber when combined with 8% sucrose, whereas ethanol combined with 1% sucrose resulted in the formation of a secondary stolon. Preceding bud growth cycB1;1, AGPaseS and λPat21 expression declined in the complete tuber both in young (1-5 days after visible swelling) and in mature tubers (25-30 days after visible swelling) after transfer to medium with GA_{4+7} or medium with ethanol, whereas CDC2a activity only declined when tubers were transferred to medium with ethanol. Second growth inducing conditions did not affect the activity of the CaMV35S promoter, indicating that the decline in the cell cycle and storage related promoter activity in the tubers during second growth is not related to a general effect on gene expression. Although a decline in cell cycle and storage related gene expression is observed before second growth, there is no causal relation between this decline and the outgrowth of the bud, as concluded from analysis of tubers transferred to medium with GA or ethanol combined with ABA which prevents outgrowth of the bud. Under these conditions cell cycle and storage related gene expression also declined. Transferring tubers to medium with different alcohols and combining ethanol with an inhibitor of alcohol dehydrogenase (4-methyl pyrazole) showed that ADH activity is a key factor during ethanol-induced second growth and the changes observed in gene expression in bud and tuber region.

Preceding second growth, a higher cycB1;1 and AGPaseS expression is observed in the bud region as compared to the activity in the tuber region. CycB1;1 expression in the bud region indicates a specific induction of the cell cycle genes during second growth. However, a relatively higher cycB1;1 expression in the bud is not sufficient for bud growth both during GA or ethanol-induced second growth. These results indicate that bud growth is not controlled by cycB1;1. AGPaseS expression in the bud region during second growth possibly indicates the enhanced import of sugars into the bud region during second growth. Preventing bud outgrowth by combining GA with ABA counteracts the difference in AGPaseS expression between bud and tuber in mature tubers, indicating that tuber and bud region respond differentially upon GA and ABA.

During ethanol-induced second growth, relatively higher AGPaseS expression was observed in the bud region in young tubers when transferred to medium with ethanol and 8% sucrose whereas tubers transferred to medium with ethanol and 1% sucrose showed a decline in AGPaseS expression both in bud and tuber region during second growth.

These results indicate that the type of second growth and the enhanced AGPaseS expression in the bud region during ethanol-induced second growth are related to the amount of sugars available for the bud. Mature tubers transferred to medium with ethanol showed higher AGPaseS expression in the bud region, irrespectively of the sucrose concentration in the medium. Combining ethanol with ABA could not prevent AGPaseS expression in the bud region during ethanol-induced second growth. These results indicate that ABA affects sugar import in the bud region during GA-induced second growth whereas ABA does not affect sugar import during ethanol-induced second growth.

In chapter 5 we described the relation between the expression of the cell cycle and storage related genes in the tubers and the differences in tuber growth rate of tubers growing on complete plants, to determine if the expression of these genes can be used as a measure for differences in sink strength and sink activity between tubers. Tuber growth rate was determined during the complete period of tuber development as an indication for sink strength and the correlation was determined with the level of gene expression quantified per tuber and per unit tuber area. By using hydroponics pot assemblies we were able to observe tuber growth and to quantify gene expression non-destructively during the complete period of tuber development. Three different stages of tuber growth were distinguished viz., initiation, linear tuber growth and maturation. During initiation, a low correlation was observed between tuber growth rate and the expression level of the cell cycle and storage related genes. During the period of linear tuber growth a good correlation was observed for λPat21 while during maturation the expression of cycB1;1, AGPaseS and λPat21 correlated well with the sink strength, as reflected by the growth rate. The results presented in chapter 5 indicate that patatin gene expression is a good predictive marker for differences in sink strength between tubers initiated on the same potato plant.

It has become clear that non-invasive methods to study gene expression are useful tools for developmental studies as described in this thesis on potato tuber development. Changes in gene expression can be observed in individual tuber samples, showing the exact relation between changes in gene expression and developmental changes.

Samenvatting

Aardappelknollen zijn vegetatieve overlevingsstructuren, waarmee de plant gedurende de periode dat de condities voor de groei van de aardappelplant ongunstig zijn, kan overleven. Het bestuderen van de verschillende processen gedurende knolvorming in aardappel (*Solanum tuberosum* L.) is van groot belang zowel vanuit een wetenschappelijk, alsmede vanuit een teelttechnisch oogpunt. De ontwikkeling van aardappelknollen kenmerkt zich door celstrekking en celdeling in het apicale meristeem resulterend in de zwelling van de horizontaal groeiende stengel, die stoloon genoemd wordt. Verder gaat de ontwikkeling van knollen gepaard met de accumulatie van zetmeel en specifieke eiwitten die dienen als voeding voor de nieuwe scheut tijdens het uitlopen van de aardappel aan het einde van de kiemrustperiode. Aangezien celdeling en de opslag van reservestoffen twee belangrijke processen zijn tijdens de ontwikkeling en groei van aardappelknollen, hebben we de activiteit van genen bestudeerd, die betrokken zijn bij de celcyclus (cycB1;1 en CDC2a) of betrokken zijn bij de opslag van zetmeel (AGPaseS) en specifieke eiwitten (λPat21) in ontwikkelende knollen.

Het bestuderen van genexpressie gebeurt meestal door het gebruik van destructieve methodes waarbij het niveau van genexpressie een gemiddelde momentopname is gebaseerd op monsters, bestaande uit meerdere individuen. In het hier beschreven onderzoek hebben we echter gebruik gemaakt van een nieuwe, niet-destructieve methode, die het mogelijk maakt om genexpressie te observeren zonder dat de verdere groei en ontwikkeling wordt belemmerd. Hiervoor is het regulerende gedeelte (promoter) van de bovengenoemde genen gekoppeld aan het reportergen 'luciferase' afkomstig van de vuurvlieg (*Photinus pyralis*). Na transformatie van aardappelplanten met deze constructen kan de activiteit van de promoter vervolgens worden waargenomen via het luciferase eiwit. Dit eiwit reageert met het substraat luciferine, dat aan het medium word toegevoegd, hetgeen resulteert in de productie van licht dat kan worden waargenomen met een zeer gevoelige camera. Met behulp van een computerprogramma kunnen de beelden vervolgens geanalyseerd worden en kan de hoeveelheid op verschillende plaatsen in de knol gekwantificeerd worden. Met destructieve controle experimenten werd aangetoond dat de luciferase activiteit (hoeveelheid licht), die werd gemeten in verschillende aardappelknollen, overeenkwam met het expressie-niveau van het luciferase gen. Zoals verwacht, bleek er een goede relatie te bestaan tussen de activiteit van de transgene reporterconstructen, AGPaseS-*luc* en λPat21-*luc*, en het expressieniveau van de eigenlijke AGPaseS en λPat21 genen.

Dit gaf aan dat de gebruikte methode geschikt is om genexpressie te meten in aardappelknollen met als voordeel dat de expressie in individuele knollen kon worden gemeten gedurende de gehele periode van knolontwikkeling.

Het observeren van knolontwikkeling aan aardappelplanten wordt bemoeilijkt doordat knolvorming plaatsvindt onder het grondoppervlak. Het meermalen verwijderen van de grond kan de knolontwikkeling beïnvloeden. Verder heeft het bestuderen van knolvorming aan de plant als nadeel dat de ontwikkeling van de verschillende knollen aan een plant en tussen planten niet synchroon verloopt. Om deze problemen te vermijden hebben we tijdens onze experimenten gebruik gemaakt van een *in vitro* systeem om knollen te produceren. Hierbij werden stengelstukjes met één okselknop op medium geplaatst met een hoge (8%) concentratie aan saccharose en een lage concentratie aan stikstofhoudende mineralen. Dit *in vitro* systeem heeft als voordeel dat de knolontwikkeling synchroon verloopt en dat het effect van bepaalde stoffen, zoals groeiregulatoren, gemakkelijk getest kan worden door ze aan het medium toe te voegen.

In hoofdstuk 2 beschrijven we de verandering in de expressie van de celcylusgenen en de genen betrokken bij de synthese van zetmeel en specifieke eiwitten tijdens de ontwikkeling van aardappelknollen. Tijdens deze experimenten hebben we gebruik gemaakt van het *in vitro* systeem waarbij het moment van zichtbare zwelling als beginpunt van de knolvorming werd omschreven, aangeduid met dag nul. De gemiddelde expressie (> 20 knollen), laat een toename zien in AGPaseS expressie vier dagen voor zichtbare zwelling, terwijl een toename in λPat21 expressie werd waargenomen drie dagen voor zwelling. De expressie van de celcylusgenen werd alleen waargenomen na zichtbare zwelling. Echter, de waarnemingen aan individuele knollen laten een veel gevarieerder beeld zien, namelijk dat het moment van een toename in de expressie van AGPaseS en λPat21 kan plaatsvinden zowel vier dagen voor tot ruim tien dagen na zichtbare zwelling. Dit geeft aan dat de vorming van aardappelknollen bestaat uit twee, mogelijk onafhankelijke processen, namelijk de zwelling door middel van celdeling en celstrekking en een toename in de synthese van zetmeel en specifieke eiwitten.

Als aardappelplanten in het veld worden blootgesteld aan hoge temperaturen of een overmaat aan stikstofhoudende mineralen kan dit resulteren in het voortijdig uitlopen van aardappelknollen (doorwas). In hoofdstuk 3 en 4 beschrijven we de relatie tussen veranderingen in genexpressie en het voortijdig uitlopen van de aardappelknollen. In het *in vitro* systeem hebben we twee methodes gebruikt om dit voortijdig uitlopen te induceren: enerzijds door knollen over te zetten naar medium met de groeiregulator gibberelline (GA) (hoofdstuk 3), anderzijds door knollen over te zetten op medium met een laag percentage alcohol (ethanol) (hoofdstuk 4).

Als aardappelknollen op medium worden geplaatst met GA resulteert dit in het uitlopen van het apicale oog en de vorming van een secundaire stoloon. Het overzetten van knollen op medium met ethanol resulteert in de vorming van een secundaire knol als de ethanol wordt gecombineerd met een hoge (8%) concentratie saccharose. Als ethanol wordt gecombineerd met een lage (1%) concentratie saccharose groeit het oog ook uit maar vormt het een secundaire stoloon.

Als *in vitro* knollen werden overgezet naar medium met GA of ethanol daalde de activiteit van de cycB1;1, AGPaseS en λPat21 promoter in de gehele knol voordat uitlopen van het apicale oog zichtbaar was. De activiteit van de CDC2a promoter daalde alleen als knollen werden overgeplaatst naar medium met ethanol en niet tijdens de behandeling met GA. Zowel jonge knollen (1-5 dagen na zichtbare zwelling) als volgroeide knollen (25-30 dagen na zichtbare zwelling) vertoonden een gelijktijdige daling in de activiteit van de promoters, nadat de knollen waren overgezet, terwijl het uitlopen van volgroeide knollen enkele dagen later werd waargenomen dan in jonge knollen. De condities die voortijdig uitlopen induceren hebben geen effect op de activiteit van de CaMV35S promoter, wat impliceert dat de afname in de activiteit van de andere genen specifiek is. Ondanks dat er een daling in genexpressie werd waargenomen alvorens het apicale oog uitgroeide, is deze relatie niet causaal. Dit bleek uit waarnemingen aan knollen die werden overgezet naar medium met GA, of medium met ethanol gecombineerd met de groeiregulator ABA. ABA voorkomt het uitlopen van het oog, maar had geen invloed op de daling in de expressie van de specifieke genen. De exacte mechanismen die voortijdig uitlopen induceren tijdens de behandeling van knollen met ethanol zijn tot nu toe onbekend. Wel blijkt dat wanneer ethanol wordt gecombineerd met 4-methyl pyrazole (4-MP), een remmer van alcohol-dehydrogenase, de knollen geen uitgroei vertonen; dan is er ook geen daling in de expressie van de specifieke genen. Als knollen worden overgezet naar medium met verschillende type alcoholen, blijkt dat de daling in genexpressie en het voortijdig uitlopen alleen plaatsvindt bij alcoholen die als substraat kunnen dienen voor alcohol-dehydrogenase. Deze resultaten impliceren een belangrijke rol voor alcohol-dehydrogenase tijdens doorwas in aardappelknollen geïnduceerd door ethanol.

De activiteit van de cycB1;1 en AGPaseS promoters in het oog nam toe voordat het uitlopen van het apicale oog zichtbaar was. Deze toename in cycB1;1 activiteit in het oog suggereert een toename in celcyclusactiviteit voordat zichtbare groei optreedt. Echter, als GA of ethanol werd gecombineerd met ABA, was deze groei van het oog afwezig maar bleef de activiteit van de cycB1;1 promoter in het oog aanwezig. Deze resultaten geven aan dat cycB1;1 expressie niet de controlerende factor is voor het al of niet uitlopen van het oog.

De toename in AGPaseS expressie in het oog, voorafgaand aan het voortijdig uitlopen, zou kunnen samenhangen met een toename van de suikerconcentraties in het oog. Als GA werd gecombineerd met ABA, voorkwam ABA het uitlopen van het oog en werd ook een toename in AGPaseS expressie in het oog voorkomen. Echter, ABA had geen invloed op de afname in AGPaseS expressie in de rest van de knol. Deze resultaten geven aan dat de groei van het meristeem in het oog alsmede de AGPaseS promoter activiteit in het oog worden gereguleerd door zowel GA als ABA, terwijl de expressie in de knol alleen wordt beïnvloed door GA en niet door ABA. In jonge knollen werd een verhoogde AGPaseS activiteit in het oog alleen waargenomen als ethanol werd gecombineerd met een hoge (8%) saccharose concentratie in het medium terwijl ethanol gecombineerd met een lage (1%) saccharose concentratie resulteerde in een dalende AGPaseS expressie zowel in de knol als in het oog. Blijkbaar worden de structuur van de uitloper (knol of stoloon) en het niveau van AGPaseS expressie in het oog beïnvloed door de hoeveelheid saccharose beschikbaar voor het oog. ABA voorkwam het ethanol-geïnduceerde voortijdig uitlopen maar voorkwam niet de toename in AGPaseS activiteit in het oog, zoals tijdens de behandeling met GA. Dit geeft aan dat de import van suikers tijdens voortijdig uitlopen geïnduceerd met ethanol niet wordt beïnvloed door ABA.

In hoofdstuk 5 van dit proefschrift beschrijven we de activiteit van de celcyclusgenen en van genen betrokken bij de synthese van zetmeel en specifieke eiwitten in knollen aan de intacte plant. Door gebruik te maken van een speciale opstelling waarbij het wortelsysteem is gescheiden van het stengelgedeelte dat de stolonen en knollen vormt, konden we de groei van knollen en de expressie van de specifieke genen meerdere malen observeren zonder dat de knolontwikkeling gehinderd werd. De groeisnelheid werd gekwantificeerd voor de individuele knollen gedurende de gehele periode van knolontwikkeling als een maat voor de 'sink strength', oftewel de mate waarin knollen assimilaten naar zich toe trekken. Er bleken grote verschillen in groeisnelheid tussen de individuele knollen te bestaan, wat er op wijst dat er een verschil is in de 'sink-strength' tussen de individuele knollen. Gedurende diezelfde periode is de activiteit van de verschillende promoters gekwantificeerd in de individuele knollen. Tijdens knolontwikkeling werd de correlatie tussen beide factoren bepaald om te toetsen of de expressie van de verschillende genen gerelateerd is aan de verschillen in sink strength. De knolontwikkeling in aardappel kon ingedeeld worden in drie periodes: initiatie-periode, de fase van lineaire knolgroei en een fase waarin de knollen afrijpen. Gedurende de initiatie-periode is er een lage correlatie tussen het expressieniveau van de specifieke genen en de groeisnelheid van de knollen.

Tijdens de periode van lineaire knolgroei was er een relatief hoge correlatie tussen de activiteit van de λPat21 promoter en de groeisnelheid van de knollen terwijl gedurende de rijpingsperiode er een relatieve hoge correlatie bestaat tussen cycB1;1, AGPaseS en λPat21 activiteit en de groeisnelheid. Deze resultaten impliceren dat het λPat21 expressieniveau een goede indicator is voor de sink strength tijdens knolontwikkeling.

De resultaten beschreven in dit proefschrift geven aan dat het gebruik van een niet-destructieve methode om genexpressie waar te nemen, nieuwe aspecten van knolontwikkeling aan het licht kan brengen. De verandering in genexpressie kan worden waargenomen in individuele knollen gedurende de gehele periode van ontwikkeling. Dit resulteert in een completer beeld van de relatie tussen verandering in genexpressie en morfologische veranderingen tijdens groei en ontwikkeling.

References

Adkins S.W., Naylor J.M. and Simpson G.M. 1984. The physiological basis of seed dormancy in *Avena fatua* V. Action of ethanol and other organic compounds. Physiol. Plant. 62: 18-24.

Ahmed C.H.M. and Sagar G.R. 1981. Volume increase of individual tubers of potatoes grown under field conditions. Potato Res. 24: 279-288.

Aksenova N.P., Konstantinova TN, Golyanovskaya SA, Kossmann J, Willmitzer L and Romanov G.A. 2000. Transformed potato plants as a model for studying the hormonal and carbohydrate regulation of tuberization. Russ. J. Plant Physiol. 47: 370-379.

Appeldoorn N.J.G., De Bruijn S.M., Koot-Gronsveld E.A.M., Visser R.G.F., Vreugdenhil D. and Van der Plas L.H.W. 1997. Developmental changes of enzymes involved in sucrose to hexose-phosphate conversion during early tuberization of potato. Planta 202: 220-226.

Appeldoorn N.J.G., De Bruijn S.M., Koot-Gronsveld E.A.M., Visser R.G.F., Vreugdenhil D. and Van der Plas L.H.W. 1999. Developmental changes in enzymes involved in the conversion of hexose-phosphate and its subsequent metabolites during early tuberisation pf potato. Plant Cell Environ. 22: 1085-1096.

Ap Rees T. and Morrell S. 1990. Carbohydrate metabolism in developing potatoes. Amer. Potato J. 67: 835-847.

Artschwager E. 1924. Studies on the potato tuber. J. Agr. Res. 27: 809-835.

Bachem C.W.B., Van der Hoeven R.S., De Bruijn S.M., Vreugdenhil D., Zabeau M. and Visser R.G.F. 1996. Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato development. The Plant J. 9: 745-753.

Bailey K.M., Phillips I.D.J. and Pit D. 1978. The role of buds and gibberellin in dormancy and the mobilization of reserve materials in potato tubers. Ann. Bot. 42: 649-657.

Bevan M., Barker R., Goldsbrough A., Jarvis M., Kavanagh T. and Iturriaga G. 1986. The structure and transcription start site of a major potato tuber protein gene. Nucl. Acids Res. 14: 4625-4638.

Bewley J.D. 1997. Seed germination and dormancy. Plant cell 9: 1055-1066.

Biemelt S., Hajirezaei M., Hentschel E. and Sonnewald, U. 2000. Comparative analysis of abscisic acid content and starch degradation during storage of tubers harvested from different potato varieties. Potato Res. 43: 371-382.

Blomstrand R., Östling-Wintzell H.L., Löf A., McMartin K., Tolf B.R. and Hedström K.G. 1979. Pyrazoles as inhibitors of alcohol oxidation and as important tools in alcohol research: An approach to therapy against methanol poisoning. Proc. Natl. Acad. Sci. 76(7): 3499-3503.

Bodlaender K.B.A., Lugt C. and Marinus J. 1964. The induction of second-growth in potato tubers. Eur. Potato J. 7: 57-71.

Borisjuk L., Walenta S., Weber H., Mueller-Klieser W. and Wobus U. 1998. High-resolution histographical mapping of glucose concentrations in developing cotyledons on *Vicia faba* in relation to mitotic activity and storage processes: glucose as a possible developmental trigger. The Plant J. 15: 583-591.

Bradburry D. 1953. Division of starch-containing cells. Amer. J. Bot. 40: 286-288.

Broughton W.J. and McComb A.J. 1971 Changes in the pattern of enzyme development in gibberellin-treated pea internodes. Annals. of Bot. 35: 213-228.

Burssens S., Himanen K., Van de Cotte B., Beeckman T., Van Montagu M., Inzé D. and Verbruggen N. 2000. Expression of cell cycle regulatory genes and morphological alterations in response to salt stress in *Arabidopsis thaliana*. Planta 211: 632-640.

Campbell M.A., Suttle J.C. and Sell T.W. 1996. Changes in the cell cycle status and expression of $p34^{cdc2}$ kinase during potato tuber meristem dormancy. Physiol. Plant. 98: 743-752.

Claassens M.M.J. and Vreugdenhil D. 2000. Is dormancy breaking of potato tubers the reverse of tuber initiation? Potato Res. 43: 347-369.

Claassens M., Vreugdenhil D. and Van der Plas L.H.W. 2002. Ethanol and the putative mode of action in breaking dormancy in potato tubers (*Solanum tuberosum* L.). (submitted).
Chalfie M., Tu Y., Euskirchen G., Ward W.W. and Prasher D.C. 1994. Green fluorescent protein as a marker for gene expression. Science 263: 802-805.
Cutter E.G. 1992. Structure and development of the potato plant. p. 65-161. In: The potato crop. 2nd edition, Harris P.M. (ed.), Chapman and Hill, Londen.
Chengappa S., Guilleroux M., Phillips W. and Shields R. 1999. Transgenic tomato plants with decreased sucrose synthase are unaltered in starch and sugar accumulation in the fruit. Plant Mol. Biol. 40: 213-221.
Cohn M.A., Jones K.L., Chiles L.A. and Church D.F. 1989. Seed dormancy in red rice: VII. Structure-activity studies of germination stimulants. Plant Physiol. 89: 879-882.
Corbineau F., Gouble S., Lecat S. and Côme D. 1991. Stimulation of germination of dormant oat (Avena sative L.) seeds by ethanol and other alcohols. Seed Science Res. 1: 21-28.
Davies H.V. 1984. Sugar metabolism in stolon tips of potato during early tuberization. Z. Pflanzenphysiol. 113: 377-381.
DeLuca M. and McElroy W.D. 1974. Kinetics of the firefly luciferase catalyzed reactions. Biochemistry 13: 921-925.
Doehlert D.C. 1996. Sink strength: Dynamic with source strength. J. Exp.Bot. 47: 1027-1029.
Duncan D.A. and Ewing E.E. 1984. Initial anatomical changes associated with tuber formation on single-node potato (*Solanum tuberosum* L.) cuttings. Annals of Bot. 53: 607-610.
Engels C.H. and Marschner H. 1986a. Allocation of photosynthate to individual tubers of *Solanum tuberosum* L. I. Relationship between tuber growth rate and enzyme activities of the starch metabolism. J. Exp.Bot. 37: 1795-1803.
Engels C.H. and Marschner H. 1986b. Allocation of photosynthate to individual tubers of *Solanum tuberosum* L. II. Relationship between growth rate, carbohydrate concentration and ^{14}C-partitioning within tubers. J. Exp. Bot. 37: 1804-1812.
Engels C.H. and Marschner H. 1986c. Allocation of photosynthate to individual tubers of *Solanum tuberosum* L. III. Relationship between growth rate of individual tubers, tuber weight and stolon growth prior to tuber initiation. J. Exp. Bot. 37: 1813-1822.
Ewing E.E. and Struik P.C. 1992. Tuber formation in potato: induction, initiation, and growth. Horticul. Rev. 14: 89-198.
Farrar J.F. 1992. The whole plant: carbon partitioning during development. In: Carbon Partitioning. Polluck, Farrar and Gordon. (ed.), Bios Scient. Publ.
Farrar J.F. 1993. Sink strength: what is it and how do we measure it? Plant Cell Environ. 16: 1015.
Farrar J.F. 1996. Sinks-integral parts of a whole plant. J. Exp. Bot. 47: 1273-1279.
Ferreira P.C.G., Hemerley A.S., Engler J.D., Van Montagu M., Engler G. and Inzé D. 1994. Developmental expression of the *Arabidopsis* cyclin gene cyc1At. The Plant Cell 6: 1763-1774.
Ferreira P.C.G., Hemerley A.S., Villarroel R., Van Montagu M. and Inzé D. 1991. The *Arabidopsis* functional homolog of the $P34^{cdc2}$ protein kinase. The Plant Cell 3: 531-540.
Finkelstein R.R. and Lynch T.J. 2000. Abscisic acid inhibition of radicle emergence but not seedling growth is suppressed by sugars. Plant Physiol. 122: 1179-1186.
Fujino K., Koda Y. and Kikuta Y. 1995. Reorientation of cortical microtubules in the sub-apical region during tuberization in single-node stem segments of potato in culture. Plant Cell Physiol. 36: 891-895.
Gallais S., Pou de Crescenzo M.A. and Laval-Martin D.L. 1998. Pyridine nucleotides and redox charges during germination of non-dormant and dormant caryopses of *Avena sativa* L. J. of Plant Physiol. 153: 664-669.
Garciarrubio A., Legaria J.P. and Covarrubias A.A. 1997. ABA inhibits germination of mature *Arabidopsis* seeds by limiting the availability of energy and nutrients. Planta 203: 182-187.
Garner N. and Blake J. 1989. The induction and development of potato microtubers in vitro on media free of growth regulating substances. Annals of Bot. 63: 663-674.

Geigenberger P., Fernie A.R., Gibon Y., Christ M. and Stitt M. 2000. Metabolic activity decreases as an adaptive response to low internal oxygen in growing potato tubers. Biol. Chem. 381: 723-740.

Geigenberger P., Hajirezaei M., Geiger M., Deiting U., Sonnewald U. and Stitt M. 1998. Overexpression of pyrophosphatase leads to increased sucrose degradation and starch synthesis, increased activities of enzymes for sucrose-starch interconversions, and increased levels of nucleotides in growing potato tubers. Planta 205: 428-437.

Geigenberger P. and Stitt M. 1993. Sucrose synthase catalyses a readily reversible reaction in vivo in developing potato tubers and other plant tissues. Planta 189: 329-339.

Geiger M., Geigenberger P. and Stitt M. 1998. Metabolism in slices from growing potato tubers responds differentially to addition of glucose and sucrose. Planta 206: 234-244.

Grierson C., Du J.S., Zabala M.D.T., Beggs K., Smith C., Holdsworth M. and Bevan M. 1994. Separate cis sequences and trans factors direct metabolic and developmental regulation of a potato tuber storage protein gene. The Plant J. 5: 815-826.

Hajirezaei M. and Sonnewald U. 1999. Inhibition of potato tuber sprouting: Low levels of cytosolic pyrophosphate lead to non-sprouting tubers harvested from transgenic potato plants. Potato Res. 42: 353-372.

Hammes P.S. and Nel P.C. 1975. Control mechanisms in the tuberization process. Potato Res. 18: 262-272.

Hannapel D.J., Miller J.C. and Park W.D. 1985. Regulation of potato tuber protein accumulation by gibberellic acid. Plant Physiol. 78: 700-703.

Hawkes J.G. 1992. Biosystematics of the potato. p. 13-64. In: The potato crop. The scientific basis for improvement, 2nd edition, Harris PM (ed.), Chapman & Hall, London.

Helder H., Van der Maarl A., Veugdenhil D. and Struik P.C. 1993. Stolon characteristics and tuber initiation in a wild potato species (*Solanum demissum* Lindl.) Potato Res. 36: 317-326.

Hemerly A.S., Ferreira P.C.G., De Almeida-Engler J., Van Montagu M., Engler G. and Inzé D. 1993. Cdc2a expression in *Arabidopsis* is linked with competence for cell division. The Plant Cell 5: 1711-1723.

Hendriks T., Vreugdenhil D. and Stiekema W.J. 1991. Patatin and four serine inhibitors genes are differentially expressed during potato tuber development. Plant Mol. Biol. 17: 385-394.

Herbers K. and Sonnewald U. 1998. Molecular determinants of sink strength. Curr. Opinion in Plant Biol. 1: 207-216.

Hiller L.K., Koller D.C. and Thornton R.E. 1985. Physiological disorders of potato tubers. In Potato Physiology, ed. Li PH, pp: 389-445, Academic Press, Orlando, Florida.

Hoff J.E. and Castro M.D. 1969. Chemical composition of potato cell wall. J. Agric. Food Chem. 17: 1328-1331.

Hussey G. and Stacey N.J. 1984. Factors affecting the formation of *in vitro* tubers of potato (*Solanum tuberosum* L.). Annals of Bot. 53: 565-578.

Jackson S.D. 1999. Multiple signalling pathways control tuber induction in potato. Plant Physiol. 119: 1-8.

Jang J.C. and Sheen J. 1994. Sugar sensing in higher plants. The Plant Cell 6: 1665-1679.

Jefferson R., Goldbrough A. and Bevan M. 1990. Transcriptional regulation of a patatin-1 gene in potato. Plant Mol. Biol. 14: 995-1006.

Jefferson R.A., Kavanagh T.A. and Bevan M.W. 1987. GUS-fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901-3907.

Jenner C.F. and Hawker J.S. 1993. Sink strength: Soluble starch synthase as a measure of sink strength in wheat endosperm. Plant Cell Environ. 16: 1023-1024.

Koch K.E. 1996. Carbohydrate-modulation gene expression in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 509-540.

Koda Y. 1988. Factors controlling potato tuberization. Memoirs of the Faculty of Agriculture Hokkaido University 16: 82-128.

Koda Y., Omer E.A., Yoshihara T., Shibata H., Sakamura S. and Okazawa Y. 1988. Isolation of a specific potato tuber-inducing substance from potato leaves. Plant Cell Physiol. 29: 1047-1051.
Kördel B. and Kutschera U. 1998. Re-examination of the solube-import hypothesis of gibberellin action in developing pea internodes. J. of Plant Physiol. 153: 693-699.
Kost B., Schnorf M., Potrykus I. and Neuhaus G. 1995. Non-destructive detection of firefly luciferase (LUC) activity in single plant cells using a cooled, slow-scan CCD camera and an optimized assay. The Plant J. 8: 155-166.
Kraus S.A. 1985. Interaction of nitrogen nutrition, phytohormones and tuberization. In Potato Physiology, ed. Li PH, pp: 209-230, Academic Press, Orlando, Florida.
Kraus S.A. 1982. Abscisic acid and gibberellic acid in growing potato tubers. Potato Res. 24: 435-439.
Kumar D. and Wareing P.F. 1972. Factors controlling stolon development in the potato plant. New Phytologist 71: 639-648.Liang J., Zhang J. and Cao X. 2001. Grain sink strength may be related to the poor grain filling of indica-japonica rice (*Oryza sativa*) hybrids. Phys. Plant. 112: 470-477.
La Cognata U. and Willmitzer L. 1995. Molecular cloning and characterization of novel isoforms of potato ADP-glucose pyrophosphorylase. Mol. Gen. Genet. 246: 538-548.
Lippert F., Rappaport L. and Timm H. 1958. Systemic induction of sprouting in white potatoes by foliar applications of gibberellin. Plant Physiol. 33: 132-133.
Lovegrove A. and Hooley R. 2000. Gibberellin and abscisic acid singalling in aleurone. Trends in plant science 5: 102-110.
Lovell P.H. and Booth A. 1969. Stolon initiation and development in *Solanum tuberosum* L. New Phytol. 68: 1175-1185.
Lugt C., Bodlaender K.B.A. and Goodijk G. 1964. Observations on the induction of second-growth in potato tubers. Eur. Potato J. 7: 219-227.
Macnicol P.K. and Jacobsen J.V. 2001. Regulation of alcohol dehydrogenase gene expression in barley aleurone by gibberellinn and abscisic acid. Physiol. Plant. 111: 533-539.
Marcelis L.F.M. 1996. Sink strength as a determinant of dry matter partitioning in the whole plant. J. Exp. Bot. 47: 1281-1291.
Mares D.J., Marschner H. and Krauss A. 1981. Effect of gibberellic acid on growth and carbohydrate metabolism of developing tuber of potato (*Solanum tuberosum*). Physiol. Plant. 52: 267-274.
Marschner H., Sattelmacher B. and Bangerth F. 1984. Growth rate of potato tubers and endogenous contents of indolylacetic acid and abscisic acid. Physiol. Plant. 60: 16-20.
Mauk C.S. and Langille A.R. 1978. Physiology of tuberization in *Solanum tuberosum* L. Plant Physiol. 62: 438-442.
Meijer M. and Murray J.A.H. 2001. Cell cycle controls and the development of plant form. Curr. Opinion in Plant Biol. 4: 44-49.
Menzel C.M. 1980. Tuberization in potato at high temperatures: responses to gibberellin and growth inhibitors. Annals of Bot. 46: 259-265.
Merlo L., Geigenberger P., Hajirezaei M. and Stitt M. 1993. Changes of carbohydrates, metabolites and enzyme activities in potato tubers during development, and within a single tuber along a stolon-apex gradient. J. Plant Physiol. 142: 392-402.
Mignery G.A., Pikaard C.S. and Park W.D. 1988. Molecular characterization of the patatin multigene family of potato. Gene 62: 27-44.
Millar A.J., Short S.R., Chua N.H. and Kay S.A. 1992. A novel circadian phenotype based on firefly luciferase expression in transgenic plants. The Plant Cell 4: 1075-1087.
Mingo-Castel A.M., Negm F.B. and Smith O.E. 1974. Effect of carbon dioxide and ethylene on tuberization of isolated stolons cultured *in vitro*. Plant Physiol. 53: 798-801.
Mironov V., De Veylder L., Van Montagu M. and Inzé D. 2000. Cyclin-Dependent Kinases and cell division in plants-The Nexus. The Plant Cell 11: 509-521.

Miyamoto K., Ueda J. and Kamisaka S. 1993. Gibberellin-enhanced sugar accumulation in growing subhooks of etiolated *Pisum sativum* seedlings. Effect of gibberellic acid, indoleacetic acid and cycloheximide on invertase activity, sugar accumulation and gorwth. Physiol. Plant. 88: 301-306.

Moorby J. 1968. The influence of carbohydrate and mineral nutrient supply on the growth of potato tubers. Ann. Bot. 32: 57-68.

Morrel S. and ap Rees T. 1986. Sugar metabolism in developing tuber *of Solanum tuberosum.* Phytochemistry 25: 1579-1586.

Müller-Röber B., Sonnewald U. and Willmitzer L. 1992. Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. EMBO J. 11: 1229-1238.

Müller-Röber B., La-Cognata U., Sonnewald U. and Willmitzer L. 1994. A truncated version of an ADP-glucose pyrophosphorylase promoter from potato specifies guard cell-selective expression in transgenic plants. The Plant Cell 6: 601-612.

Müller-Röber B., Kossmann J., Hannah L.C., Willmitzer L. and Sonnewald U. 1990. One of two different ADP-glucose pyrophosphorylase gene from potato responds strongly to elevated levels of sucrose. Mol. Gen. Genet. 224: 136-146.

Murashige T. and Skoog F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Nakata P.A. and Okita T.W. 1995. Differential reguolation of ADP-glucose pyrophosphorylase in the sink and source tissues of potato. Plant Physiol. 108: 361-368.

Nguyen V.T., Morange M. and Bensaude O. 1989. Protein denaturation during heat shock and related stress. J. Biol. Chem. 264: 10487-10492.

Niedz R.P., Sussman M.R. and Satterlee J.S. 1995. Green fluorescent protein: An in vivo reporter of plant gene expression. Plant Cell Rep. 14: 403-406.

O'Brein E.J.A. and Firman 1998. A review of some studies into tuber initiation in potato (*Solanum tuberosum*) crops. J. of Agricultural Sci. 130: 251-270.

Okita T.W., Nakata P.A., Anderson J.M., Sowokinos J., Morell M. and Preiss J. 1990. The subunit stucture of potato tuber ADPglucose pyrophosphorylase. Plant Physiol. 93: 785-790.

Oparka K.J. 1985. Changes in partitioning of current assimilate during tuber bulking in potato (*Solanum tuberosum* L.) cv Maris Piper. Annals of Bot. 55: 705-713.

Oparka K.J. 1991. Sugar transport and the metabolism in the potato tuber. P. 91-114. In: Carbon Partitioning. Polluck, Farrar and Gordon. (ed.), Bios Scient. Publ.

Oparka K.J. and Davies H.V. 1985. Translocation of assimilates within and between potato stems. Annals of Bot. 56: 45-54.

Oparka K.J. and Roberts A.G. 2001. Plasmodesmata. A not so open-and-shut case. Plant Physiol. 125: 123-126.

Ow D.W., Wood K.V., DeLuca M., DeWey J.R., Helinski J.R. and Howell S.H. 1986. Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Proc. Natl. Acad. Sci. 84: 4870-4874.

Paiva E., Lister R.M. and Park W.D. 1983. Induction and accumulation of major tuber proteins of potato in stems and petioles. Plant Physiol. 71: 161-168.

Palmer C.E. and Smith O.E. 1969 Cytokinins and tuber initiation in the potato plant. Nature 221: 279-280.

Park W.D. 1990. Molecular approaches to tuberization in potato. p. 44-55 In: The molecular and cellular biology of the potato. Vayda M.E. and Park W.D. (ed.) Redwood Press, Melksham.

Patrick J.W. 1997. Phloem unloading: sieve element unloading and post-sieve element transport. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 191-122.

Peña-Cortés H., Liu X., Sanchez Serrano J., Schmid R. and Willmitzer L. 1992. Factors affecting gene expression of patatin and proteinase-inhibitor-II gene families in detached potato leaves: Implication for their co-expression in developing tubers. Planta 186: 495-502.

Perata P., Matsukura C., Vernieri P. and Yamaguchi J. 1997. Sugar repression of a gibberellin-dependent signaling pathway in Barley embryos. The Plant Cell 9: 2197-2208.
Perl A., Aviv D., Willmitzer L. and Galun E. 1991. In vitro tuberization in transgenic potatoes harboring beta-glucuronidase linked to a patatin promoter: effects of sucrose levels and photoperiods. Plant Sci. 73: 87-95.
Pétel G., Candelier P. and Gendraud M. 1993. Effect of ethanol on filiate tubers of Jerusalem artichoke: a new tool to study dormancy. Plant Physiol. Biochem. 31(1): 67-71.
Pfister-Sieber M. and Brändle R. 1995. Response of potato tubers to hypoxia followed by re-aeration. Potato Res. 38: 231-239.
Pikaard C.S., Brusca J.S., Hannapel D.J. and Park W.D. 1987. The two classes of genes for the major potato tuber protein, patatin, are differentially expressed in tubers and roots. Nucl. Acids Res. 15: 1979-1994.
Plaisted P.H. 1957. Growth of the potato tuber. Plant Physiol. 445-452.
Perata P. and Alpi A. 1991. Ethanol metabolism in suspension cultured carrot cells. Physiol. Plant. 82: 103-108.
Prat S., Frommer W.B., Hofgen R., Keil M., Kossmann J., Koster-topfer M., Lui X-J., Muller B., Pena-Cortes H., Rocha-Sosa M., Sanchez-Serrano J.J., Sonnewald U. and Willmitzer L. 1990. Gene expression during tuber development in potato plants. FEBS Letters 268: 334-338.
Purcell P.C., Smith A.M. and Halford N.G. 1998. Antisense expression of a sucrose non-fermneting-1-related protein kinase sequance in potato results in decreased expression of sucrose synthase in tubers and loss of sucrose-inducibility of sucrose synthase transcripts in leaves. The Plant J. 14: 195-202.
Quarrie S.A. 1981. Droopy: a wilty mutant of potato deficient in abscisic acid. Plant Cell Environ. 5: 27-30.
Racusen D. 1986. Esterase specificity of patatin from two potato cultivars. Can. J. Bot. 64: 2104-2106.
Riou-Khamlichi C., Menges M., Healy S. and Murray J.A.H. 2000. Sugar control of the plant cell cycle: differential regulation of *Arabidopsis* D-type cyclin gene expression. Mol. Cell. Biol. 20: 4513-4521.
Rhode A., Van Montagu M., Inzé D. and Boerjan W. 1997. Factors regulating the expression of cell cycle genes in individual buds of *Populus*. Planta 201: 43-52.
Rocha-Sosa M., Sonnewald U., Frommer W., Stratman M., Schell J. and Willmitzer L. 1989. Both developmental and metabolic signals activate the promoter of a class I patatin gene. EMBO J. 8: 23-29.
Roessner U., Wagner C., Kopka J., Trethewey R.N. and Willmitzer L. 2000. Simultanous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. The Plant J. 32: 131-142.
Rosahl S., Eckes P., Schell J. and Willmitzer L. 1986. Organ-specific gene expression in potato: isolation and characterization of tuber-specific cDNA sequences. Mol. Gen. Genet. 202: 368-373.
Ross H.A., Davies H.V., Burch L.R., Viola R. and Mcrae D. 1994. Developmental changes in carbohydrate content and sucrose degrading enzymes in terising stolons of potato (Solanum tuberosum). Physiol. Plant. 90: 748-756.
Rychter A., Janes H.W., Chin C.K. and Frenkel C. 1979. Effect of ethanol, acetaldehyde, Acetic acid and ethylene on changes in respiration and respiration metabolites in potato tubers. Plant Physiol. 64: 108-111.
Sanz M.J., Mingo-Castel A., Van Lammeren A.A.M. and Vreugdenhil D. 1996. Changes in the microtubular cytoskeleton precede in vitro tuber formation in potato. Protoplasma 19: 46-54.
Sattelmacher B. and Laidig R. 1991. Interrelation between growth rate of individual potato (*Solanum tuberosum* L.) tubers and their cell number. Annals of Bot. 68: 41-45.
Sauter M. 1997. Differential expression of CAK (cdc2-activating kinase)-like protein kinase, cyclins and cdc2 genes from rice during the cell cycle and in response to gibberellin. Plant J. 11: 181-190.

Sergeeva L., De Bruijn S.M., Koot-Gronsveld E.A.M., Navratil O. and Vreugdenhil D. 2000. Tuber morphology and starch acumulation are independent phenomena: Evidence from *ipt*-transgenic potato lines. Physiol. Plant. 108: 435-443.

Shaul O., Van Montagu M. and Inzé D. 1996. Regulation of cell division in *Arabidopsis*. Crit. Rev. Plant Sci. 15: 97-112.

Sherf B.A. and Wood K.V. 1994. Firefly luciferase engineered for improved genetic reporting. Promega notes 49:14-21.

Sonnewald U. and Herber K. 1999. Sugars, far more than just fuel for plant growth. p. 69-78. In: Plant Carbohydrate Biochemistry, Bryant J.A., Burrell M.M. and Kruger N.J. (ed.), Bios Scientific Publishers.

Sowokinos J.R. 1976. Pyrophosphorylases in *Solanum tuberosum*: I Changes in ADP-glucose and UDP-glucose pyrophosphorylase activities associated with starch biosynthesis during tuberization, maturation and storage of potatoes. Plant Physiol. 57: 63-68.

Struik P.C., Boon E.J. and Vreugdenhil D. 1987. Effects of extracellular extracts from leaves on the tuberization of cuttings of potato (*Solanum tuberosum* L.) Plant Physiol. 84: 214-217.

Struik P.C., Haverkort A.J., Vreugdenhil D., Bus C.B. and Dankert R. 1990. Manipulation of tuber-size distribution of a potato crop. Potato Res. 33: 417-432.

Struik P.C., Haverkort A.J., Vreugdenhil D., Bus C.B. and Dankert R. 1991. Possible mechanisms of size hierarchy among tubers on one stem of a potato (*Solanum tuberosum* L.). Potato Res. 34: 187-203.

Struik P.C. and Van Voorst G. 1986. Effects of drought on the initiation, yield, and size distribution of tubers of *Solanum tuberosum* L. cv. Bintje. Potato Res. 29: 487-500.

Sung S.J., Xu D.P. and Black C.C. 1989. Identification of active filling sucrose sinks. Plant Physiol. 89: 1117-1121.

Suttle J.C. 1998. Involvement of ethylene in potato microtuber dormancy. Plant Physiol. 118: 843-848.

Suttle J.C. and Hultstrand J.F. 1994. Role of endogenous abscisic acid in potato microtuber dormancy. Plant Physiol. 105: 891-896.

Swiatek A., Lenjou M., Van Bockstaele D., Inzé D. and Van Onckelen H. 2002. Differential effect of jasmonic acid and abscisic acid on cell cycle progression in tobacco BY-2 cells. Plant Physiol. 128: 201-211.

Takahashi K., Fujino K., Kikuta Y. and Koda Y. 1994. Expansion of potato cells in response to jasmonic acid. Plant Sci. 100: 3-8.

Taylorson R.B. and Hendricks S.B. 1979. Overcoming dormancy in seeds with ethanol and other anesthetics. Planta 145: 507-510.

Thompson J.F., Hayes L.S. and Lloyd D.B. 1991. Modulation of firefly luciferase stability and impact on studies of gene regulation. Gene 103: 171-177.

Twell D. and Ooms G. 1988. The 5' flanking DNA of a patatin gene directs tuber specific expression of a chimaeric gene in potato. Plant Mol. Biol. 9: 365-375.

Van den Berg J.H., Struik P.C. and Ewing E.E. 1990. One-leaf cuttings as a model to study second growth in the potato (*Solanum tuberosum*) plant. Annals of Bot. 66: 273-280.

Van den Berg J.H., Vreugdenhil D., Ludford P.M., Hillman L.L. and Ewing E.E. 1991. Changes in starch, sugar and abscisic acid contents associated with second growth in tubers of potato (*Solanum tuberosum* L.) one-leaf cuttings. J. Plant Physiol. 139:86-89.

Van der Krol A.R. and Chua N.H. 1991. The basic domain of plant B-Zip proteins facilitates import of a reporter protein into plant nuclei. The Plant Cell 3: 667-675.

Van Leeuwen W., Hagendoorn M.J.M., Ruttink T., van Poecke R., Van der Plas L.H.W. and Van der Krol A.R. 2000. The use of the luciferase reporter system for *in planta* gene expression studies. Plant Mol. Biol. Rep. 18: 143a-143t.

Van Leeuwen W., Ruttink T., Borst-Vrenssen A.W.M., Van der Plas L.H.W. and Van der Krol A.R. 2001. Characterization of position-induced spatial and temporal regulation of transgene promoter activity in plants. J. Exp. Bot. 52: 949-959.

Veramendi J., Roessner U., Renz A., Willmitzer L. and Trethewey R.N. 1999. Antisense repression of hexokinase 1 leads to an overaccumulation of starch in leaves and transgenic potato plants but not to significant changes in tuber carbohydrate metabolism. Plant Phys. 121: 123-133.

Veramendi J., Willmitzer L. and Trethewey R.N. 1999. In vitro grown potato microtubers are a suitable system for the study of primary carbohydrate metabolism. Plant Physiol. Biochem. 37: 693-697.

Verhees J., Van der Krol A.R., Vreugdenhil D. and Van der Plas L.H.W. 2002. Characterising gene expression during potato tuber development in individuals and populations using the luciferase reporter system. Plant Mol. Biol. (in press).

Viola R., Roberts A.G., Haupt S., Gazzani S., Hancock R.D., Marmiroli N., Marchray G.C. and Oparka K.J. 2001. Tuberization in potato involves a switch from apoplastic to symplastic phloem unloading. The Plant Cell 13: 385-398.

Visser R.G.F., Vreugdenhil D., Hendriks T. and Jacobsen E. 1994. Gene expression and carbohydrate content during stolon to tuber transition in potatoes (*Solanum tuberosum* L.). Physiol Plant. 90: 285-292.

Vreugdenhil D., Boogaard Y., Visser R.G.F. and De Bruijn S.M. 1998. Comparison of tuber and shoot formation from in vitro cultured potato explants. Plant Cell Tissue Organ. Cult. 53: 197-204.

Vreugdenhil D. and Heider H. 1992. Hormonal and metabolic control of tuber formation. p. 393-400. In: Progress in plant Growth Regulation. Karssen C.M., Van Loon L.C. and Vreugdenhil D. (ed.).

Vreugdenhil D. and Sergeeva L. 1999. Gibberellins and tuberization in potato. Potato Res. 42: 471-481.

Vreugdenhil D. and Struik P.C. 1989. An integrated view of the hormonal regulation of tuber formation in potato (*Solanum tuberosum* L.). Physiol. Plant. 75: 525-531.

Vreugdenhil D. and Van Dijk W. 1989. Effects of ethylene on the tuberization of potato (*Solanum tuberosum*) cuttings. Plant Growth Regulation 8: 31-39.

Vreugdenhil D., Xu X., Jung C.S., Van Lammeren A.A.M. and Ewing E.E. 1999. Initial anatomical changes associated with tuber formation in single-node potato (*Solanum tuberosum* L.) cuttings: a re-evaluation. Annals of Bot. 84: 675-680.

Wang H., Qi Q., Schorr P., Cutler A.J., Crosby W.L. and Fowke L.C. 1998a. ICK1, a cyclin-dependent protein kinase inhibitor from *Arabidopsis thaliana* interacts with both Cdc2a and CycD3 and its expression is induced by abscisic acid. The Plant J. 15: 501-510.

Wang M., van der Meulen R.M., Visser K., van Schaik H., van Duijn B. and De Boer A.H. 1998b. Effects of dormancy-breaking chemicals on ABA levels in barley grain embryos. Seed Sci. Res. 8: 129-137.

Weber H., Borisjuk L and Wobus U. 1997. Sugar import and metabolism during seed development. Trends in Plant Sci. 2: 169-174.

Wenzler H.C., Mignery G.A., Fisher L.M. and Park W.D. 1989. Analysis of a chimeric class-I patatin-GUS gene in transgenic potato plants: High-level expression in tubers and sucrose-inducible expression in cultured leaf and stem explants. Plant Mol. Biol. 12: 41-50.

Wurr D.C.E. 1977. Some observations of patterns of tuber formation and growth in potato. Potato Res. 20: 63-75.

Xu X., Van Lammeren A.M., Vermeer E. and Vreugdenhil D. 1998. The role of gibberellin, abscisic acid and sucrose in the regulation of potato tuber formation in vitro. Plant Physiol. 117: 575-584.

Xu X., Vreugdenhil D. and Van Lammeren A.A.M. 1998. Cell division and cell enlargement during potato tuber formation. J. Exp. Bot. 49: 573-582.

Yoshihara T., Omer E.A., Koshino H., Sakamura S., Kikuta Y. and Koda Y. 1989. Structure of a tuber-inducing stimulus from potato leaves (*Solanum tuberosum* L.) Agric. Biol. Chem. 53: 2835-2837.

Yoshihara T., Amanuma M., Tsutsumi T., Okumura Y., Matsuura H. and Ichihara A. 1996. Metabolism and transport of [2-^{14}C](±) jasmonic acid in potato plant. Plant Cell Physiol. 37: 586-590.

Zhang M., Nakamaru Y., Tsuda S., Nagashima T. and Esashi Y. 1995. Enzymatic conversion of volatile metabolites in dry seeds during storage. Plant Cell Physiol. 36: 157-164.

Zrenner R., Salanoubat M., Willmitzer L. and Sonnewald U. 1995. Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). Plant J. 7: 97-107.

Curriculum Vitae

John Verhees werd geboren op 23 april 1971 te Someren. In 1989 behaalde hij zijn HAVO diploma aan het College Asten Someren te Asten. In ditzelfde jaar begon hij een studie aan de Hogeschool Larenstein te Wageningen waar hij in 1993 zijn diploma behaalde voor de studierichting Botanische laboratoriumtechnieken. Tijdens deze studie heeft hij stage gelopen bij het toenmalige Centrum voor Plantenveredeling en Reproductieonderzoek, het huidige Plant Research International, met een onderzoek naar het gebruik van RAPD's bij lelie. Verder werd er gedurende deze periode een afstudeeropdracht voltooid bij de vakgroep Vegetatiekunde Plantenoecologie en Onkruidkunde naar de opkomst van atrazine-resistente zwarte nachtschade. In 1993 begon hij aan de studie Biologie aan de Landbouwuniversiteit te Wageningen. Binnen deze studie koos hij voor de specialisatie Cel. Het doctoraalexamen werd in 1996 behaald met een afstudeeropdracht bij de vakgroep Moleculaire Biologie getiteld: Transformatie van *Daucus carota* en real-time analyse naar promoter activiteit in somatische embryo's. Verder voltooide hij een afstudeeropdracht binnen het Department for Environmental Biology te Guelph, Canada naar expressie van proteine-kinases in *Colletotrichum gloeosporoides*. In 1997 werd hij aangesteld als AIO bij de leerstoelgroep Plantenfysiologie van Wageningen Universiteit resulterende in dit proefschrift. Vanaf november 2001 is hij werkzaam als post-doc bij het Nederlands Instituut voor Ontwikkelingsbiologie, Hubrecht Laboratorium, te Utrecht.

This work presented in this thesis was performed at:
Wageningen University
Laboratory of Plant Physiology
Arboretumlaan 4
6703 BD Wageningen
The Netherlands.

Present address of the author:
Hubrecht Laboratory
Netherlands Institute for Developmental Biology
Uppsalalaan 8
3584 CT Utrecht
The Netherlands.

Publication of this thesis was financially supported by the Funkefonds.

Coverdesign: GER-DA

Printed by Propress, Wageningen.