Carbohydrate metabolism during potato tuber dormancy and sprouting

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op gezag van de rector magnificus
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Stellingen

1. De verhoogde AGPase-activiteit gedurende het uitlopen van aardappelen wijst op zetmeelcycling tijdens het breken van de kiemrust.
(dit proefschrift)

2. De veranderde verhouding glucose-6-fosfaat : fructose-6-fosfaat : glucose-1-fosfaat in het voordeel van glucose-1-fosfaat, tijdens de kiemrustbreking van aardappelen, is waarschijnlijk een gevolg van een veranderde PGM-regulatie.
(dit proefschrift; Fernie *et al.*, Planta 213: 418 – 426 (2001); 214: 510 – 520 (2002))

3. Zowel de ethanol-geïnduceerde kiemrustbreking bij de aardappel als het type secundaire structuur wat ontstaat, worden niet gereguleerd door veranderde GA-gehaltes, waardoor een grotere rol toebedeeld kan worden aan ethanol of alcoholdehydrogenase bij deze processen.
(dit proefschrift)

4. Rotsvast vertrouwen in de Biochemical Pathways (Boehringer Mannheim GmbH, 1993, 3rd edition, part I) is niet op zijn plaats bij onderzoek aan zetmeelmetabolisme.

5. Het nauwkeurig bedenken, opzetten, uitvoeren en interpreteren van controles is een van de meest belangrijke en meest vergeten onderdelen van onderzoek.

6. Een verplichte cursus in onderzoeksmanagment en –planning zal niet de meest geliefde bezigheid van AiO's zijn, maar wellicht noodzakelijk om tegemoet te komen aan de strenger wordende eisen van de betrokken uitkeringsinstantie USZO.

7. Het feit dat een trein soms nog gehaald kan worden ten gevolge van vertragingen, kan niet gebruikt worden als positief argument bij een imago-verbetering van de NS.

8. Wanneer de regering in het najaar nadelige maatregelen voor akkerbouwers neemt is de kans op protestacties in Den Haag het kleinst.

Stellingen behorend bij het proefschrift:
'Carbohydrate metabolism during potato tuber dormancy and sprouting'

Margo Claassens, Wageningen, 1 oktober 2002

Voorwoord

Tadáááá! Hier ligt het dan! Mijn boekje. Ik mag het nu wel *mijn* boekje noemen, maar het is natuurlijk tot stand gekomen met de hulp van velen. Daarom dit voorwoord om iedereen die geholpen heeft te bedanken.

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Wageningen, 3 augustus 2002

Margo

Contents

Abbreviations

ABA	abscisic acid
ADH	alcohol dehydrogenase
ADP-glc	adenosine-5'-diphospho-glucose (ADP-glucose)
AGPase	ADP-glucose pyrophosphorylase
AGPPase	ADP-glucose pyrophosphatase
ALDH	aldehyde dehydrogenase
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
BAP	benzyl-aminopurine
BSA	bovine serum albumin
CA	controlled atmosphere
CCC	chlorocholine chloride
CRC	catabolic redox charge
CWI	cell wall-bound invertase
DTT	dithiothreitol
DW	dry weight
EDTA	ethylenediamine tetraacetic acid
EGTA	(ethylene-bis[oxy-ethylenenitrilo]) tetraacetic acid
EtOH	ethanol
FLD	fluridone
FK	fructokinase
frc-6-P	fructose-6-phosphate
FW	fresh weight
GA	gibberellic acid
GK	glucokinase
glc-1-P	glucose-1-phosphate
glc-6-P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
HK	hexokinase
IAA	indolylacetic acid
LD	long day
4MP	4-methyl pyrazole
NAA	naphtaleneacetic acid
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NBD	2,5-norbornadiene
NBT	nitroblue tetrazolium
OPPP	oxidative pentose phosphate pathway
PDC	pyruvate decarboxylase
3PGA	3-phosphoglycerate
6PGDH	6-phosphogluconate dehydrogenase
PGI	phosphogluco-isomerase
PGM	phosphoglucomutase
Pi	inorganic phosphate
PPi	pyrophosphate
PVP	polyvinylpyrrolidone
RH	relative humidity
SD	short day
SPP	sucrose phosphate phosphatase
SPS	sucrose phosphate synthase
STP	starch phosphorylase
susy	sucrose synthase
UDP-glc	uridine-5'-diphospho-glucose (UDP-glucose)
UGPase	UDP-glucose pyrophosphorylase
Z	zeatin
ZR	zeatin riboside

1

General introduction

The potato crop

Potato (*Solanum tuberosum* L.) originates from the high Andes in South America and was introduced into Europe around the year 1570. Since then, it has been extensively cultivated all over the world and became one of the major food crops. In terms of total production, the potato ranks amongst the top four food crops, together with wheat, rice and maize. The potato is grown in more than 70% of all countries and constitutes a major source of human nutrition. Global annual production of potatoes exceeds 290 million tons (FAO estimate) and the annual production in the Netherlands is about 8 million tons (CBS, 1999).

The potato tuber is produced not only for human consumption; a major part is used for industrial processing. Especially the large starch and protein quantities make the potato tuber an ideal basis for such divergent industries, as food, feed, paper, textile, pharmaceutical and even the building industry (http://www.avebe.nl). Next to these objectives for potato production, also the production of seed potatoes is important, especially in the Netherlands. Potato tubers produced for all different purposes are placed in short- to long-term storage for a stable year-round supply. During this storage period, both physiological and pathological processes in the tuber can cause severe losses of the total potato harvest. One of the most important physiological processes affecting the quality of stored potatoes is sprouting (Suttle, 2000).

Potato life cycle

Sprouting is a part of the vegetative life cycle of the potato. The tuber sprouts and forms a new plant. As the sprout is not self-supporting, it uses the storage carbohydrates and other constituents of the so-called 'mother tuber' until it reaches the above-ground level and can start photosynthesis. The plant grows and after it has reached a certain stage, the induction of the formation of new tubers occurs. This induction is regulated by various external factors, *e.g.* length of photoperiod, temperature or nitrogen limitation. Potato tubers are formed underground at so called stolons, which develop as lateral shoots at the base of the stems of the potato plant (figure 1.1). Tuber induction leads to tuber initiation, which is defined as a swelling of the stolon to twice its original diameter (Ewing and Struik, 1992). Tuber initiation is followed by tuber growth and maturation.

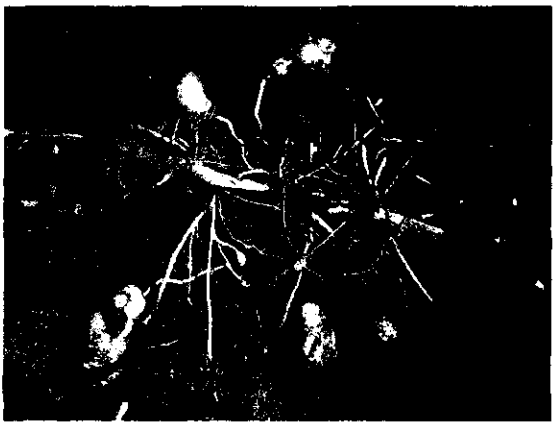

Figure 1.1 Tuberization in the potato plant.

Then the tuber (figure 1.2A) enters a period of dormancy, which presumably is already initiated during tuber development (Burton, 1978). The length and depth of this dormancy period is dependent on cultivar and environmental factors occurring during tuber development and storage. At the end of this period, the dormancy is broken, which is followed by sprouting (figure 1.2B and 1.2C) and a new plant is formed again, thus completing the potato plant's life cycle.

Figure 1.2 Potato tubers at different developmental stages. A: tuber just after harvest, dormant; B: tuber that just starts to sprout; C: sprouting tuber.

Dormancy in general is described as 'a temporary suspension of visible growth of any plant structure containing a meristem' (Lang *et al.*, 1987). A dormancy period, which probably evolved as a survival strategy, has advantages and disadvantages. It is advantageous for the plant to survive a period unfavourable for growth (survival) or for man to store dormant plant material (*e.g.* seeds or vegetative storage/propagation organs like potato tubers) for a certain period of time. And

it is disadvantageous when growth is necessary soon after harvest of these seeds or tubers.

A lot of research is done on tuber development (Ewing and Struik, 1992 and references therein; Appeldoorn *et al.*, 1997; -1999), also because of economic interest, as insight in the regulation of tuber initiation may lead to the development of methods controlling the time-point of initiation and the number of tubers per plant. Tuber size and total tuber yield are economically relevant for producing different kind of tubers, *e.g.* seed potatoes, tubers for consumption or tubers for industrial processing. As sprouting is important either because it may cause losses during (long term) storage or because sometimes sprout growth is already necessary soon after harvest, also fundamental research on the physiological regulation of tuber dormancy, dormancy breaking and sprouting may be of interest for economic purposes. In the past, research was mainly focused on storage conditions (reviewed by Van Es and Hartmans, 1987), although several research groups also have focused on endogenous hormonal control of potato tuber dormancy (Koda, 1988; Suttle, 2000) or carbohydrate metabolism within this period (Van der Plas, 1987; Hajirezaei, 1999; Biemelt *et al.*, 2000; Sonnewald, 2001; Fernie and Willmitzer, 2001).

In vitro tuberization

Appeldoorn *et al.* (1997 and 1999) used an *in vitro* tuberization system (figure 1.3), based on a method described by Hendriks *et al.* (1991) to obtain the plant material for their research on carbohydrate metabolism in developing tubers. One of the main reasons to use tubers, produced with such an *in vitro* system, was that tuberization was highly synchronous; tuber formation started on the 5th or 6th day after placing explants on tuber-inducing medium (figure 1.3A). Another way of producing microtubers (described as type II) is shown in figure 1.3B. The most important reason for using type II microtubers was the fact that mass production is possible when a lot of tubers of one batch are necessary for experiments, as described in chapter 3.

Either system is very synchronous and advantageous when investigating developmental programmes. Because the *in vitro* system (type I) was highly synchronous in tuber formation, it was also expected to be quite synchronous in sprouting too. However, a 4-week period elapsed between the sprouting of the first tubers in a batch and the time at which 80% of the tubers had sprouted (figure 1.4). Moreover, the starting point of sprouting of different batches varies up to 4 weeks (figure 1.4).

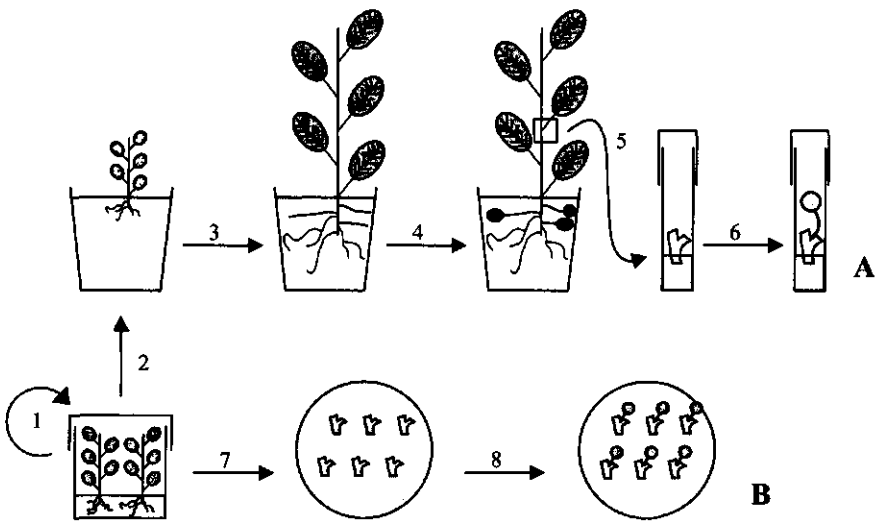

Figure 1.3 Schematic drawing of the *in vitro* tuberization system based on the method described by Hendriks *et al.* (1991).
A. type I microtubers: *in vitro* grown and propagated (1) plants are transferred to soil (2) and grown for four weeks under long day conditions in a climate chamber (3). To induce these plants for tuberization, the plants are placed under short day conditions for three more weeks (4). Single node cuttings containing an axillary bud from the main stem of these soil-grown plants are placed on tuber-inducing medium (5) and cultured in the dark (6). Tuber formation started on the 5th or 6th day.
B: type II microtubers: single node cuttings containing an axillary bud from the main stem of the *in vitro* grown plants are placed on tuber-inducing medium in a petridish (7) and cultured in the dark (8). Tuber formation started on the 6th or 7th day.

Thus, tuber sprouting was a-synchronous within a batch and between batches. The a-synchronicity within a tuber batch was approximately the same as found in field-grown tubers (Jan van Loon, personal communication). Despite the lack of synchronicity, another reason to choose for an *in vitro* system was the fact that in this way, our experiments are not depending on one harvest a year and not on the weather conditions during that year. Moreover, *in vitro* tubers are more homogeneous than field- or pot-grown tubers.

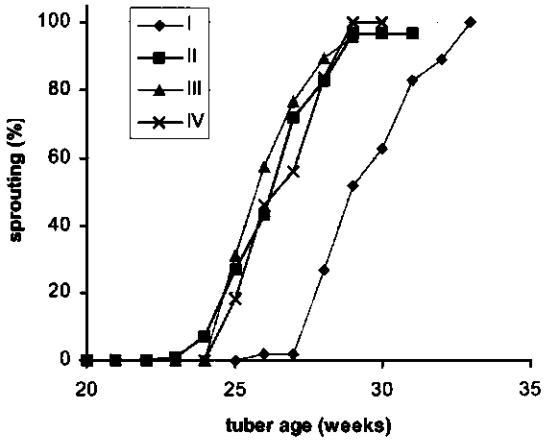

Figure 1.4 Sprouting curves of four different batches of type I microtubers, all dried at a RH > 80% and stored afterwards at 10°C.

Scope of this thesis

A wide range of factors, both external and internal, affect or are involved in the initiation of dormancy, maintenance of dormancy and in dormancy breakage and subsequent sprouting. External factors during plant growth, *e.g.* photoperiod, available nitrogen and temperature influence the time-point of tuber induction and initiation, but supposedly these factors also affect dormancy later in the tuber's lifecycle (Burton, 1989; Suttle, 1998). Also, the external factors during storage, *e.g.* O_2, CO_2, temperature and humidity affect potato tuber physiology and dormancy. For example, when tubers are stored at a temperature below 8°C, neutral sugars accumulate in the tuber. This phenomenon is called cold-sweetening or low temperature sweetening. When these tubers are then transferred to a temperature above 10°C, the reverse, *i.e.* formation of starch from sugar occurs. Also precocious sprouting is then observed (figure 1.5).

This thesis concentrates on the analysis of the role of endogenous components in the sprouting process; the whole dormancy period, including the period of dormancy breaking and (early) sprouting was investigated. The role of both endogenous and exogenously applied plant hormones, is reviewed in chapter 2, together with carbohydrate metabolism, its levels of intermediates and the activities of enzymes responsible for their conversions. Moreover, the initiation of dormancy (or the tuber developmental stage, at which initiation of dormancy

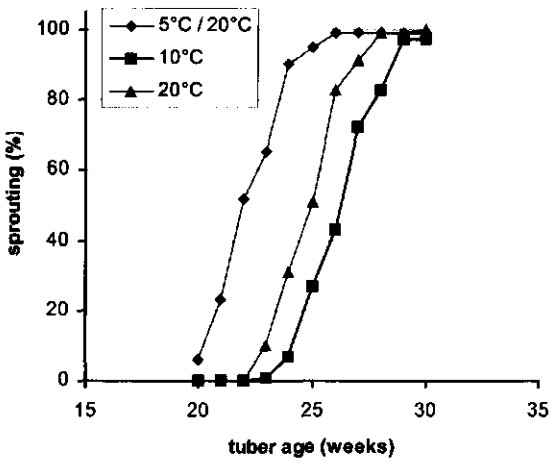

Figure 1.5 Sprouting curves of one batch of tubers, divided in several groups, which are stored at different temperatures.

is supposed to occur) and dormancy breaking are compared, highlighting differences and similarities of internal factors, *e.g.* hormones, sugars, starch and some enzymes involved in the metabolism of sugars and starch. The research presented in this thesis focuses on induced sprouting (chapter 3) and spontaneous breaking of dormancy and sprouting (chapter 4 and 5).

A well-known compound, able to induce sprouting in potato tubers, is gibberellin. As gibberellins affect all kind of processes within the tuber, it is not easy to decide whether changes observed in carbohydrate metabolism after GA-application are the result of 'dormancy breaking' or a direct effect of the hormone. Therefore, another way of inducing sprouting in potato tubers, and thus dormancy breaking, was investigated. Alcohols are known to break dormancy in seeds of various plant species, and therefore ethanol was chosen to investigate its dormancy breaking capacity in potato tubers. The putative mode of action (via ADH) of ethanol as a dormancy breaking chemical is investigated in chapter 3. During the formation of potato tubers, unfavourable conditions, *e.g.* heat, can cause so called second growth. Different forms of secondary structures can be distinguished: deformation of tubers, formation of secondary tubers, formation of aerial sprouts from tubers and formation of stolons (Van den Berg *et al.*, 1990). Assuming that dormancy gradually develops in the tuber, second growth could also be seen as breaking of (early) dormancy. Different secondary structures developed also during ethanol treatment; 1% sucrose in the medium resulted in formation of a sprout and 8% sucrose resulted in a secondary tuber.

The regulation of the formation of these secondary structures, and the possible involvement of endogenous gibberellins, was investigated and discussed in chapter 3.

The spontaneous breakage of dormancy and the subsequent sprouting was the main objective of this thesis. Starch metabolism during the whole period of dormancy and the start of sprouting is followed and discussed in chapter 4. This is followed by a discussion of sucrose metabolism during that same period in chapter 5. Against this background of sucrose metabolism, the possibility of starch cycling is again discussed at the end of chapter 5, presenting also a model for carbohydrate metabolism in dormant and sprouting tubers.

In chapter 6 (general discussion), the use of *in vitro* tubers as a model system for soil-grown tubers is discussed first. Then a comparison is made of carbohydrate metabolism during spontaneous sprouting and during ethanol-induced or GA-induced sprouting. Finally, we elaborate further on the comparison of internal factors during tuber development and during dormancy breaking, reviewed in chapter 2, on the basis of studies on tuber development (Appeldoorn *et al.*, 1997; -1999) and on dormancy breaking (this thesis), using the same model system.

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2

Is dormancy breaking of potato tubers the reverse of tuber initiation?

Margo M.J. Claassens and Dick Vreugdenhil

Potato Research 43 (2000) 347-369.

Abstract

Tuber formation is a well orchestrated physiological event that involves many metabolic changes. Dormancy gradually develops in potato tubers from the moment cell division in the stolon tip has stopped and the tuber starts to develop. Dormancy breakage may be the reverse of dormancy initiation suggesting that there may be similarities between tuber induction and dormancy development.

Based on a literature review it is concluded that, when comparing tuber induction and the breaking of dormancy, hormonal activities are only partly the reverse, whereas for carbohydrates and enzyme activities some clearly reverse trends are found.

For more definite conclusions more research should be done to assess precisely the moment of dormancy breaking. Moreover, measurements on events associated with tuber induction and dormancy breaking should be carried out using the same techniques and the same material.

Molecular genetic analysis may provide well-defined markers for the timing of breaking of dormancy.

Tuber development and initiation of dormancy

Potato plants are grown from seed potatoes to produce tubers, both for fresh consumption, industrial processing or vegetative reproduction. At the base of the main stem of the plants a number of lateral shoots are formed, which usually grow below soil level. Unlike the aboveground stems, these underground stems, or stolons, grow diageotropically (Van Es and Hartmans, 1987a). When the conditions are favourable (*e.g.* depending on length of photoperiod, temperature, nitrogen limitation), tubers are formed at the stolons. A potato tuber is in fact a modified stem with a shortened and broadened axis (Van Es and Hartmans, 1987a, and references therein). At the onset of tuber formation, the elongation of stolons stops and cells in pith and cortex enlarge and divide longitudinally, which results in the swelling of the subapical part of the stolon. When these young tubers reach a certain size, the longitudinal divisions stop and randomly oriented divisions and cell enlargement occur in the perimedullary region and continue until the tubers reach their final diameter (Xu *et al.*, 1998a). During the development of the tubers on the plant, the buds in the eyes of the tuber successively become dormant, starting at the stolon end. The apical eye is the last to become dormant (Van Es and Hartmans, 1987a). Tuber initiation is accompanied by two major biochemical changes, the accumulation of starch and the production of storage proteins (Visser *et al.*, 1994).

To grow tubers *in vitro*, some of the favourable conditions mentioned above are mimicked. In the *in vitro* system used in our lab, single-node cuttings from soil-grown plants (grown under short day (SD) conditions) are placed on a so called tuber-inducing medium, which contains a low amount of nitrogen and a high amount of sucrose (Hendriks *et al.*, 1991). The initiation and further development of the microtubers (called so because of their size, which almost never exceeds 0.8 cm in diameter) is quite synchronous. Like the *in planta* grown tubers, also here pith and cortex are involved in the tuberization process. The small size of the microtubers is due to the absence of development of the perimedullary region (Xu *et al.*, 1998a). The described *in vitro* system, used in our lab, may differ from *in vitro* systems used in other laboratories.

One of the remarkable aspects of tuber formation is the switching off of activity of the apical meristem in the stolon apex. This apex becomes the apical end of the tuber, which only resumes activity after prolonged storage of the mature tuber. Thus the question arises: when does tuber dormancy start? For a long time, the time of harvest was seen as the starting point for dormancy. However, the date of harvest, although used as a practical tool, has no physiological

significance (Burton, 1978 in Van Es and Hartmans, 1987b). The end of tuber growth may then be the time point when dormancy starts. Alternatively, Burton (1978, in Van Es and Hartmans, 1987b) suggested that dormancy start at tuber initiation. This implies that dormancy as well as tuber initiation, via the aboveground parts of the plant, might be affected by environmental factors during growth. Van Ittersum and Struik (1992) showed some correlations between the duration of dormancy and some stolon and tuber variables, *e.g.*, tuber weight, tuber initiation and stolon initiation (correlation coefficients are respectively –0.82, 0.51 and 0.41, all statistically significant at $P<0.001$ and $n=355$). They concluded that tuber weight was the variable by far the best related to the duration of dormancy and that tuber weight is also related to some other variables.

Figure 2.1 Effect of GA on development of *in vitro* tubers. Tubers are approximately 0.7-0.8 cm in diameter. Single-node cuttings were grown on tuber-inducing medium (Hendriks *et al.*, 1991) and then transferred to the same medium supplemented with GA_{4+7}.
A. transferred 6 days after tuber induction to medium with 0.5 μM GA_{4+7}, resulting in formation of a new stolon at the apical bud;
B. transferred 16 days after tuber induction to medium with 10 μM GA_{4+7}, resulting in formation of a sprout at the apical bud.

Concerning plant hormones, Suttle (1998a) showed that ethylene is produced throughout tuber development. Exposing developing tubers to the ethylene antagonist silver nitrate or 2,5-norbornadiene (NBD) resulted in premature sprouting, indicating that endogenous ethylene is essential for the full-expression of potato microtuber dormancy. Also, our experiments showed that treatment with gibberellins (GAs) in the very early stages of (*in vitro*) tuber development results in stolon formation at the apical bud of the tuber (figure 2.1A). After this

GA-treatment the young tuber itself is behaving more as a stolon than as a tuber, in terms of metabolites, indicating that tuber formation in the very early stages of development seems to be reversible. This may be dependent on cell division in the stolon apical meristem. As soon as these cell divisions have stopped, tuber formation is not reversible anymore (Lian Yung, personal communication). Treatment with GA_{4+7} in a later stage of tuber development causes sprouting (figure 2.1B). The concentration of GA required to induce sprouting is higher in older, more developed tubers. This suggests that dormancy gradually develops in the tuber from the moment cell division in the stolon tip has stopped and the tuber starts to develop.

Dormancy breaking and sprouting

The length of the dormancy period depends not only on cultivar, but also on conditions during development (as discussed above) and during storage. Day length during development has an effect on the dormancy period; potatoes cultivated under short-day conditions have a shorter dormancy than those cultivated under long days (Emilsson, 1949, quoted by Hemberg, 1985). Different storage conditions, such as temperature, humidity of the storage atmosphere, light, oxygen concentration, carbon dioxide concentration and the possible presence of volatile substances from the potato (ethylene) also affect the dormancy period (Van Es and Hartmans, 1987b). But, even if a batch of tubers was grown under the same conditions and was stored under the same conditions, sprouting in individual tubers does not occur synchronously. The starting moment of tuber formation is of importance for the length of the sprouting period (Van Es and Hartmans, 1987b), only not that important as expected. In the field, tubers are formed asynchronously. For field grown seed potatoes, a sprouting period of four weeks is found (Jan van Loon, personal communication). Using an *in vitro* system, in which tuber formation is far more synchronous than in the field, a sprouting period of four weeks is also found (both cv. Bintje).

This asynchronicity in sprouting is not only a problem for investigating the breakage of dormancy. When we consider sprouting as the visible effect of the breaking of dormancy and the topic of interest is dormancy breaking, than one should be very careful not to measure parameters of the sprouting process. This means that determination of relevant parameters should take place before visible sprouting, but even then it is hard to identify the parameters as dormancy breaking parameters or sprouting parameters. Discussing dormancy of true seeds, Cohn (1996) stated: 'if one desires to increase the chances of successfully

identifying events associated with the dormancy breaking process, it is essential to conduct time course studies using chemical pulse treatments applied to a uniform and vigorous seed sample'. Markers for the progression from dormancy to germination processes, *i.e.* those occurring prior to visible embryo growth, are almost non-existent. For seeds of red rice he reported that most of the cellular responses occurred within 4 hours after application of a dormancy breaking chemical. These events were taking place at least 16 – 24 hours before the first indications of the visible germination event.

In potato research, such a quick and synchronous system is not yet known. Spontaneous sprouting occurs over a too long period (four weeks, see above) to allow collection of well-defined stages of dormancy breaking, before visible sprouting occurs. Dormancy breaking treatments may be the solution to overcome this problem. Sprouting can be stimulated by chemicals, by damaging the tuber, by increasing the humidity of the storage atmosphere, by temperature variation, etc. (Van Es and Hartmans, 1987b). Rindite (7:3:1 anhydrous ethylene chlorohydrin : ethylene dichloride : carbon tetrachloride), carbon disulphide and gibberellic acid have been used to break potato tuber dormancy. The main disadvantage of the first two compounds is their toxicity to human. When using GA, tubers are immersed in or sprayed with a solution. GA is usually absorbed more efficiently by the tubers after wounding. The wounding itself also stimulates sprouting, presumably by synthesis of gibberellins in the tuber (Rappaport and Sachs, 1967). Furthermore, it is known that near the end of dormancy the concentration needed to break dormancy is less than during the middle period of storage (deep dormancy) or as Bruinsma (1967) stated: 'the concentration of GA can be increased if dormancy is suspected to be very intense'. Guzman (1963) describes the necessity for treating freshly harvested seed tubers with GA when used for fall planting. For spring planting with seed tubers stored for four months, no GA treatment is needed. Thus, during tuber development, GA-concentrations needed to break dormancy increase and near the end of dormancy, the concentration decreases again.

This finding and a few others gave rise to the idea that dormancy breakage may be the reverse of dormancy initiation. Above we already presented evidence that dormancy initiation takes place during tuber initiation. Hereafter we will discuss changes in hormones, carbohydrates and enzyme activities during tuber formation and during dormancy breakage in order to try to answer the question whether breakage of dormancy might be considered as the reverse of tuber initiation. Different authors have reviewed physiological aspects of either tuber formation (Ewing and Struik, 1992) or tuber storage (Van der Plas, 1987). By our knowledge, we are the first to compare tuber initiation and dormancy breaking.

Hormones

In the following part the effects of exogenous and endogenous phytohormones on the processes of tuber development and dormancy breaking / sprouting are discussed.

Gibberellins

Gibberellins are well known to have an inhibitory effect on tuberization and tuber growth. When using tuberizing conditions, such as SD, tuberization can be prevented by the application of GA (Kumar and Wareing, 1974). On the other hand, under long day (LD), non-inducing conditions, tuberization can be induced by blocking the GA synthesis with CCC (chlorocholine chloride) (Hammes and Nel, 1975). Railton and Wareing (1973) obtained evidence that the level of gibberellins in the potato plant decreases upon transfer from LD to SD conditions. Vreugdenhil and Sergeeva (2000) reviewed GAs and their role in tuberization, discussing in more detail the data on endogenous GAs and the effects of exogenously applied GAs on cellular events, starch accumulation, etc.

Xu *et al.* (1998b) quantified endogenous GAs during various stages of stolon elongation and tuber formation in an *in vitro* tuberizing system. They found significant variation of the GA_1 level during the development of stolons. The GA_1 content was high during the elongation of stolons and became very low during the development of tubers. Moreover, the decrease of GA_1 occurred well before visible swelling, consistent with the findings of Koda (1983b). Furthermore, during the whole process of tuber growth, the GA_1 content stayed very low. These experiments strongly suggest a (negative) regulating role of GA_1 on tuber formation as well as on tuber growth. The observed decrease in GA_1 before visible swelling not only suggests a regulating role in tuber induction, but also in cessation of stolon elongation. Although tuber induction and stolon cessation are different steps in the process of tuber formation and their regulation is not the same (Vreugdenhil and Struik, 1989), they are closely linked in time and observed changes in hormone levels can often not be attributed to only one of these processes.

Furthermore, a supposed interaction between sucrose and GA during tuber development is discussed by Xu *et al.* (1998b). They concluded that presumably sucrose affects the endogenous GA levels and not vice versa (see section on carbohydrates and enzyme activities).

It can be summarized that endogenous GA decreases before tuber formation and the level remains low during tuber growth. Tuberization can be inhibited or initiated by applying GAs or inhibiting GA synthesis, respectively.

During storage, dormancy can be broken by using gibberellins. In fact, the use of GA to stimulate early sprouting of potato seed tubers is nowadays an approved commercial treatment. Several reports in the late fifties and early sixties describe the potential use of GA to break dormancy for early planting instead of Rindite or its main component ethylene chlorohydrin (Kato and Ito, 1961; Boo, 1961; Rappaport *et al.*, 1957). A series of reports describe the effect of different gibberellin treatments on percentage of sprouting, average sprout length, number of sprouts, mean total sprout length per eyepiece, etc., to find an optimal treatment in terms of GA concentration and duration of the treatment. In spite of the research on GA treatment to break dormancy Duda *et al.* (1971) reported that gibberellin had not been introduced into practice in Russia because of the unreliability of the sprouting of tubers and weakened plant growth. They explained that treatment with a high concentration of gibberellin causes the appearance of fine, filamentary sprouts and also root formation is weakened. Indeed this was also reported by other authors (Bruinsma, 1967), who discusses the appearance of excessive stem elongation at the cost of leaf development at larger doses of GA. But when using lower doses of GA, most varieties give normally growing plants; only when a slender growing variety with a superficial dormancy is subjected to a GA treatment, excessive stem elongation appears. Bruinsma (1967) resolves this problem by counteracting GA effects using a growth retarding substance like N-dimethylaminosuccinamic acid (B9). This proves that different varieties react differently to a GA treatment, mostly depending on the depth and duration of dormancy (Guzman, 1963).

Furthermore, GA treatments seem to have an effect on endogenous carbohydrate levels. Clegg and Rappaport (1970) reported that applied GA_3 stimulated the formation of reducing sugars. But, they also found that neither α-amylase nor invertase activities increased significantly prior to the increase in reducing sugars. Bailey *et al.* (1978) found an increase in reducing sugar content in untreated tubers prior to visible sprouting. When these tubers were disbudded the increase in reducing sugar content was not as high as in untreated tubers or even not appearing. In turn, when these disbudded tubers were treated with GA_3, the sugar content rose to a similar or higher level than was found in tubers with buds, suggesting that buds give a signal to the storage tissue to mobilize reserve material, which could also be accomplished by GA. More interactions between GA and carbohydrates or enzyme activities will be described below. It is also described (Boo, 1961) that the content of inhibitor-β (or ABA, see section on abscisic acid) in dormant tubers decreases upon treatment with GA.

Endogenous levels of GA during dormancy are described in different ways. Smith and Rappaport (1961) sampled tubers throughout dormancy at 12-day

intervals until sprouts appeared. Peel layers, including buds, were extracted and GA activity was determined using a bioassay. The level of endogenous GAs remained low during the rest period and increased near the onset of sprouting. Unfortunately, it was not clear from these experiments whether the increase in the level of gibberellin-like substances precedes or is a consequence of sprouting. As the authors themselves explain, this may be due to the difficulty in determining the exact time of sprouting. Also Kato and Ito (1961) determined endogenous GAs by means of a bioassay (rice seedling test). They found no endogenous GAs in freshly harvested tubers, after a few weeks GA appeared and gradually increased thereafter. They also found that the endogenous level of GAs in the apical eyes was enriched by applying GA. This exogenous GA could not break dormancy, but eventually promoted the growth of the buds. Bialek and Bielinska-Czarnecka (1975) used a dwarf pea test to determine gibberellin-like substances in the extracts of tubers during growth, ripening, dormancy and sprouting. They found two periods of high GA activity: one at the time of rapid tuber growth and the second shortly before the beginning of sprouting. This first peak contradicts the findings of Xu *et al.* (1998b). We assume, as the authors themselves also suggested, that the GAs of this first peak are bound or altered (*e.g.* methylated, glycosylated), in this way being a stored or transportable form of GA. From these three studies, it seems that endogenous GA levels increase just before or at the time of sprouting. Rappaport and Sachs (1967) found that endogenous gibberellin levels in potato tubers rose only shortly after visible bud growth had occurred. It remains unclear whether the increasing levels are preceding sprouting or are a consequence of sprouting, thus the behavior of GA in dormancy breakage is still unclear.

Abscisic acid

As mentioned before, tuberization can occur when the endogenous GA concentration is low. A logical consequence of these observations was to hypothesize that the tuberization stimulus is a natural inhibitor or antagonist of gibberellin. The inhibitor-β complex, which is identical to abscisic acid (ABA) (Bialek, 1973) or at least ABA is an important part of the β-complex of inhibitors (Hemberg, 1985), was one of the possible candidates. Wareing and Jennings (1980) found that a cutting from a SD-induced plant will form a tuber either when the leaf remains attached to the cutting or when ABA was applied to the petiole stump. Although ABA seemed to have a tuberizing effect, experiments with cuttings from non-induced plants, done by the same authors, showed that tuberization can not simply be induced by ABA.

Koda and Okazawa (1988) and Koda *et al.* (1988a) detected and isolated a compound, which was transported from the leaf and which caused tuberization. This compound is called tuberonic acid and resembles jasmonic acid. The latter is known to have an inhibitory effect on plant growth, such as stimulation of leaf senescence and inhibition of cell division. Tuberonic acid on the other hand did not show an effect on leaf senescence and it had only a slight inhibitory effect on cell division in soybean callus (Koda *et al.*, 1988b).

So, exogenous ABA did not have a tuberizing effect on non-induced cuttings, but only promoted tuberization in induced cuttings. Xu *et al.* (1998b) did some similar experiments in an *in vitro* system; when ABA was added to tuberizing medium, tuber formation occurred one day earlier than in the absence of ABA. Wareing and Jennings (1980), found that addition of ABA to non-inducing medium (low sucrose) resulted in tuber formation, although much delayed as compared to inducing medium (high sucrose). Furthermore, experiments with both GA and ABA showed that ABA could partly overcome the inhibiting effect of GA.

Kumar and Wareing (1974) showed that there is no significant difference in the levels of endogenous ABA under LD or SD conditions (in *Solanum andigena*). Koda (1988) determined endogenous ABA levels via a bioassay and found increasing levels of an ABA-like substance during tuberization. Xu *et al.* (1998b) also determined endogenous levels of ABA during stolon elongation, tuber initiation and tuber growth. In both inducing and non-inducing conditions, the ABA levels decreased during the first two days of *in vitro* culture and remained low thereafter. This in contrast to the findings of Koda (1988). Because no differences between inducing and non-inducing conditions were detected, the conclusion was drawn that ABA is not likely to be the main regulator of tuber formation (Xu *et al.*, 1998b).

On the other hand, ABA seems to play a major role in maintaining dormancy. Suttle and Hultstrand (1994) described some experiments, in which developing tubers were treated with fluridone (FLD). Upon treatment with this herbicide, ABA content declined and sprouting occurred. The effect of FLD treatment could be reversed by applying exogenous ABA; endogenous ABA increased and precocious sprouting was abolished. Furthermore, application of FLD to fully dormant microtubers resulted in a decrease of endogenous ABA and sprouting occurred for 56% in comparison to 13% in the control experiment. From these and other experiments, the conclusion was drawn that endogenous ABA is directly involved in the initiation and possibly the maintenance of potato microtuber dormancy.

ABA levels decline during postharvest storage, but there appears to be no threshold concentration that endogenous ABA levels must fall below in order for dormancy to be lost and sprouting to commence. Furthermore, the postharvest decline in endogenous ABA levels does not appear to be the result of an enhanced rate of breakdown, but more likely the result of a reduction in *de novo* synthesis of ABA coupled with a constant rate of turn-over (Suttle, 1995).

Simko *et al.* (1997) did a QTL (quantitative trait loci) -study on dormancy and ABA. They found at least 8 loci associated with tuber dormancy and three of them were also associated with variation in ABA content, indicating a relationship between tuber dormancy and ABA content.

Cytokinins

Cytokinins are believed to have a stimulating effect on tuberization (Melis and van Staden, 1984). Palmer and Smith (1969) found induction of tuber formation of isolated stolons in cultures containing cytokinins, whereas the isolated stolons in cultures devoid of cytokinins showed no signs of tuber initiation. Furthermore, when they stained longitudinal sections of these stolons with IKI, they found starch accumulation in the apical regions of stolons treated with cytokinins and none in the controls. Moreover, starch accumulation was detectable before any visible signs of tuber formation. As Palmer and Smith (1969) also suggested, cytokinins may be acting by mobilizing metabolites to the sites of tuber formation and by creating a metabolic sink. Also Mauk and Langille (1978) found a stimulation of tuberization when using zeatin riboside (ZR) in the medium of an *in vitro* tuberization system. Cytokinins are also involved in regulating enzyme activities of carbohydrate metabolism (Palmer and Barker, 1973).

On the other hand, not all literature data points to a promoting role of cytokinins in tuber formation. Kumar and Wareing (1974) showed inhibition of tuber development by high concentrations of kinetin and Woolley and Wareing (1972) showed that kinetin application to a stolon tip resulted in the formation of a leafy shoot. Also McGrady *et al.* (1986) showed that cytokinin applications impede tuberization rather than promoting it. They noted that the effects of cytokinin in their experiments were more like reported effects of gibberellins (*e.g.* stimulation of stolon development, delay or inhibition of tuberization, stimulation of starch hydrolysis or inhibition of starch synthesis, etc.). Because cytokinins on the one hand seem to stimulate tuber initiation and on the other hand can convert a stolon into a leafy shoot, Vreugdenhil and Struik (1989) suggested that the response of a stolon to elevated cytokinin levels depends on the interaction with other hormones. If the level of gibberellins is high, then conversion to a leafy

shoot will occur; if the elongation of the stolon has stopped (high ethylene, low gibberellin level), cytokinins can promote tuber initiation.

Mauk and Langille (1978) found a higher level of ZR in plants grown under tuber-inducing conditions as compared to plants grown under non-inducing conditions. The level of endogenous cytokinins during tuber initiation and formation is further described by Koda (1982a). During extraction the cytokinins were divided into butanol- and water-soluble cytokinins and activity was determined by measuring soybean callus growth, a cytokinin bioassay. The butanol-soluble cytokinin, consisting mainly of zeatin-riboside, was found in the elongating stolon tips and concomitant with the swelling of the tips, its level increased rapidly. The level of water-soluble cytokinin (zeatin ribotide) decreased at the same time, which makes it plausible that the zeatin ribotide is converted into zeatin riboside. Furthermore, the high level of zeatin ribotide during the elongation of stolons, when no tubers are formed, suggests that this cytokinin is less active than its corresponding free base and the riboside. So, zeatin ribotide may be seen as a temporary storage form. Also, Koda (1982a) makes it clear that the possibility exists that the increased level of cytokinin is not responsible for the initiation of tuber formation, but only for the subsequent thickening. Turnbull and Hanke (1985b) also measured endogenous (zeatin-type) cytokinins and they found a very high level of cytokinins in the early stages of tuber formation. During subsequent tuber growth the level decreased.

The effect of cytokinins on cell division and enlargement in developing tubers is not well described. Sattelmacher and Marschner (1978) describe a causal relation between the increase in cytokinin activity after tuberization and the stimulation of cell division of the cortical cells. Also the change in direction of cell elongation from a lateral to a dorsiventral direction is described as a possible effect of the increase in cytokinin activity.

Furthermore, cytokinins are supposed to have a stimulating effect on ethylene production (McGrady *et al.*, 1986 and references therein). The ethylene in turn is supposed to inhibit stolon elongation (see section on ethylene), a prerequisite for tuber initiation.

Kinetin and zeatin are known to break dormancy in potato tubers (Hemberg, 1970). Moreover, in tubers, treated with one of these compounds, the level of β-inhibitors was much lower than in the controls. However, Turnbull and Hanke (1985a) found that applied cytokinins only broke dormancy in the first 6 weeks after tuber initiation and in the last 3 weeks of the dormancy period. The tubers did not react to a cytokinin treatment in the period in between and the authors suggested tissue sensitivity to be responsible for this phenomenon. In a

companion paper (Turnbull and Hanke, 1985b), they found that exogenous cytokinin could not induce sprouting in older, dormant tubers in which the level of endogenous cytokinins in the buds was low. In contrast, exogenous cytokinins induced sprouting of young tubers, even though the level of endogenous bud cytokinins was already very high. The authors concluded that the endogenous cytokinin concentration could not be used as a marker for the dormancy state or for the sensitivity to cytokinins. Furthermore, it was shown that cytokinins induced cell division and cell expansion in sprouts within 48 h of a cytokinin injection. The rates were similar to the ones of sprouts whose dormancy had ended naturally. Moreover, there was no increase in cell number in buds which did not grow out (Turnbull and Hanke, 1985a).

The endogenous levels of cytokinins during dormancy and sprouting have briefly been discussed above. Turnbull and Hanke (1985b) reported that the level of cytokinins decreased during tuber growth, both in tubers and in buds. After harvest, the cytokinin level in the cortex decreased even more and the level in the buds increased. In 1972, Engelbrecht & Bielinska-Czarnecka found an increase in the cytokinin level, especially in regions around the eyes, near the end of dormancy, which presumably coincides with the disappearance of β-inhibitors as reported by Hemberg (1967). Koda (1982b) also found a decrease of cytokinin levels (butanol-soluble) immediately after harvest and the levels were increasing again at the end of dormancy. The water-soluble cytokinins decreased at the end of dormancy suggesting again that zeatin riboside (butanol-soluble) originated from zeatin ribotide (water-soluble) (see beginning of this section). Obhlidalova *et al.* (1979) found that the content of cytokinins rapidly increased during the breaking of dormancy and decreased again as sprouting proceeded. Furthermore, the cytokinins were mainly localized in the apical part of the tuber and the maximum level was found in the sprouts. Suttle (1998b) also found increases in bioactive cytokinin content in potato tuber buds prior to the loss of dormancy. More recently, Suttle and Banowetz (2000) determined the endogenous levels of *cis*-zeatin (*cis*-Z) and *cis*-zeatin riboside (*cis*-ZR). They found that the level of *cis*-Z increased already at day 25 during the 81 days of dormancy and remained constant thereafter. The level of *cis*-ZR remained rather constant during the whole postharvest storage period. The biological activity of *cis*-Z was checked by applying exogenous *cis*-Z to dormant tubers. All concentrations of *cis*-Z tested did not show an effect on tubers after 25 days of storage. After 53 days, the highest concentration of *cis*-Z resulted in 60% sprouting compared to only 1 or 2% in the control experiment. Thereafter, the efficacy of *cis*-Z increased with increased storage time. These findings coincide with the theory of tissue sensitivity by Turnbull and Hanke (1985a), where tubers only reacted to applied cytokinins at

the last stages of dormancy. The high level of endogenous *cis*-Z from early dormancy to sprouting tubers also coincides with this theory.

Above-mentioned papers (Suttle, 1998b; Turnbull and Hanke, 1985a/b) discussed *trans*-isomeric forms of cytokinins. In contrast, Suttle and Banowetz (2000) discussed *cis*-isomers. The question raises whether both isomeric forms are biologically relevant. As Suttle and Banowetz (2000) suggest; 'it is possible that only one of the two isomeric forms is biologically important or, alternatively, it is possible that both have a role in dormancy termination either directly as cytokinin agonists or indirectly in a precursor/product relationship'.

Auxins

Although auxins are known to have a promoting effect on cell elongation / expansion, not much attention has been paid to their possible role in tuber development. Melis and van Staden (1984) stressed the importance of the auxin-cytokinin interaction for tuber growth. Indeed, Machackova *et al.* (1998) found a higher cytokinin / auxin ratio in stolons under tuber-inducing conditions than under non-inducing conditions. Melis and van Staden (1984) mentioned the possibility that auxins can be formed in leaves or shoots under inducing conditions and phloem-translocated to the tuberization site.

Xu (1998b) observed earlier tuberization when the auxin indolylacetic acid (IAA) was applied to single node cuttings in tuber-inducing medium. On the other hand, tuber formation was completely inhibited by IAA, using high concentrations and retarded when using intermediate concentrations (Kumar and Wareing, 1974). Endogenous levels of auxins were found to be high in stage 1 and 2, where stage 1 is the stolon tip just before swelling and stage 2 is the swelling stolon tip. Hereafter the auxin level decreased (Obata-Sasamoto and Suzuki, 1979). Koda and Okazawa (1983b) also found high auxin levels in these first two stages, the maximum level was found at stage 2. According to the latter authors, the swelling in stage 2 was mainly caused by cell expansion. In the third stage, initiation of active cell division was observed and in that same stage the cytokinin level reached its maximum.

Furthermore, IAA is supposed to have a stimulating effect on ethylene production, which inhibits stolon elongation (Vreugdenhil and Struik, 1989) (see also section on ethylene). On the other hand, ethylene application can reduce the levels of IAA, thus providing a feed-back mechanism (Minato and Okazawa, 1978; Thomas, 1981).

Also not much is known about auxins during dormancy. Rappaport *et al.* (1965) showed that high concentrations of IAA and naphthaleneacetic acid (NAA)

inhibited sprouting. Low concentrations of both NAA and IAA slightly stimulated sprouting. But, as the authors stated, 'These results do not negate the possibility that IAA or other endogenous auxins are involved in release from rest period'. This idea is also supported by the finding that the level of endogenous auxins increases near the end of dormancy (Hemberg, 1949). Of course, it could be argued that the increase in endogenous auxins is a consequence of renewed growth, rather than a way to induce sprouting.

Ethylene

Ethylene may play an active role in the control of stolon growth. Ethylene production in plants is known to increase upon stress. Vreugdenhil and Struik (1989) suggested that potato stolons produce ethylene upon mechanical constraint in the soil. As a result, elongation will stop, provided that the level of gibberellins is sufficiently low. In 1964, Lugt *et al.* reported that when the stolon environment did not provide enough mechanical resistance, the stolon growth was extremely vigorous and a delay of tuberization occurred. Vreugdenhil and Struik (1989) also suggested that if ethylene production results from mechanical constraint in the soil, then, after elongation of the stolon has ceased, the mechanical stress will decrease and so will the production of ethylene, enabling tuber formation by other regulatory factors. Mingo-Castel *et al.* (1974, 1976) indeed concluded that ethylene inhibits tuber initiation. Some other investigators (Catchpole and Hillman 1969, Garcia-Torres and Gomes-Campo, 1973) reported that ethylene might have a positive effect on tuber initiation. Suttle (1998a) showed that single node explants produced low amounts of ethylene during the entire period of culture. The rates of ethylene production were highest during the initial period of culture and declined from the moment subapical swelling became visible. Moreover, when ethylene action was inhibited by $AgNO_3$ or NBD, premature sprouting occurred, showing that ethylene action is needed during tuber development to have a full expression of tuber dormancy.

Applying ethylene to dormant tubers can either hasten or delay tuber sprouting, depending on the concentration and duration of exposure. Rylski *et al.* (1974) treated dormant potato tubers with various concentrations of ethylene for various exposure times. Treatment for a short period (not longer than 3 days) was found to stimulate sprouting, whereas continuous treatment with ethylene completely inhibited sprouting. When the continuous treatment was stopped, sprouting ensued at a rate apparently identical to that in tubers that had received brief exposures to the gas. Rylski *et al.* (1974) also showed that ethylene had an effect on sprout growth: ethylene inhibited sprout elongation and caused thickened sprouts. Timm *et al.* (1986) reported similar results upon treatment

with ethylene; lateral branch growth increased and stem elongation declined after exposure to 1 μl l^{-1} C_2H_4 for 72 hours. Rylski *et al.* (1974) postulated the following theory: both extended and short term ethylene treatments terminate dormancy, but long term exposures to ethylene inhibit bud elongation, even though dormancy is broken.

Considering the effect of ethylene on other growth-regulating substances, it is known that ethylene treatment causes a decline in auxin activity in the apical part of the tubers (Minato and Okazawa, 1978). This shortage of auxin in the apical bud may participate in inducing abnormal sprouting of tubers. Furthermore, Stitt (1986) found that ethylene induced an increase of fructose-2,6-biphosphate, which is a regulatory metabolite for sucrose synthesis.

Carbohydrates

In 1929, Wellensiek proposed the hypothesis that carbohydrate levels in the stolon tip are the controlling factor for tuberization. Several arguments favor this hypothesis; firstly tuber formation *in vitro* occurs at high levels of sucrose in the culture medium, without addition of external phytohormones. Secondly, molecular-genetic experiments show that sucrose can induce expression of a wide variety of genes, of which patatin, a tuber-specific storage protein, is the most prominent one (Vreugdenhil and Helder, 1992 and references therein). Werner (1934) proposed that the carbohydrate/nitrogen ratio is the causal factor in tuberization. However, the results of Koda and Okazawa (1983a) contradict this hypothesis, although in their experiments sucrose still seems to have a controlling effect on tuberization. They conclude that it seems more feasible that some naturally occurring hormonal substance would be involved in the induction of tuberization. The hormones were discussed in the previous part and in this section several carbohydrates, which may be important in dormancy initiation as well as dormancy breakage, will be discussed.

Sucrose

The frequency of tuberization in an *in vitro* system becomes higher when the concentration of sucrose in the medium increases. Moreover, the final size of the tubers grown in medium containing the highest sucrose concentration (8%) is larger than of those grown in 4 or 6% sucrose medium. Also, as soon as tubers are formed, stolon elongation stops and the final length of stolons decreases with increasing sucrose level (Xu *et al.*, 1998b).

Based on these results one would expect a high endogenous level of sucrose in the stolon tip and/or the swelling tuber, both *in vitro* and *in vivo*. Vreugdenhil *et al.*

(1998) determined the sucrose levels in stolons and (swelling) tubers *in vitro*. They found no correlation between endogenous sucrose levels and the formation of the tuber. Ross *et al.* (1994) determined sugars during tuber formation *in vivo*. From just before swelling until swelling of 3 cm (maximum), they found a general increase. Also Hawker *et al.* (1979) determined sucrose in stolon tips and developing tubers and they also found an increase, whereas Vreugdenhil *et al.* (1998) found a decreasing trend starting before swelling in an *in vitro* system. According to the latter authors, this discrepancy might be due to the longer developmental period analyzed by the other authors. As mentioned before, Xu *et al.* (1998b) suggested a negative effect of sucrose on GA-levels. Comparing the results on endogenous sucrose levels of Vreugdenhil *et al.* (1998) to the results on endogenous GA levels of Xu *et al.* (1998b), we see that a high sucrose level corresponds with a low GA level (inducing conditions) and vice versa (non-inducing conditions), indicating the validity of the theory.

Dimalla and van Staden (1977) determined soluble sugars in different regions (tissue sections of the apical bud, a lateral bud and internodal regions) of dormant and sprouting tubers. In all the tissues, which all consist of mainly periderm and cortex, the sucrose level decreased during dormancy and subsequent sprouting. Ross and Davies (1992) found a constant level of sucrose during dormancy. The theory that sucrose levels affects GA levels in a negative way still holds. The decreasing levels of sucrose found at the end of dormancy would mean an increasing GA content, which is also found (see section on gibberellins). Tubers treated with GA_3 did not show any significant change in the level of sucrose (Davies and Viola, 1988). This would favor the hypothesis that sucrose affects GA and not vice versa (Xu *et al.*, 1998b).

Reducing sugars

Glucose and fructose have a tuber-inducing effect *in vitro*, although it is not clear from literature whether the reducing sugars induce tuber formation to the same extent as sucrose (Vreugdenhil and Helder, 1992 and references therein).

Endogenous levels of reducing sugars during *in vitro* tuber formation are determined by Vreugdenhil *et al.* (1998). Both glucose and fructose increase until day 7 and 6 respectively and after swelling the level of both sugars decrease. Ross *et al.* (1994) determined levels of reducing sugars during *in planta* development. Both glucose and fructose showed the same trend as Vreugdenhil *et al.* (1998) found *in vitro*. Also Hawker *et al.* (1979) found a decrease in reducing sugars after swelling of the tuber and thereafter a fluctuating line, similar to the results of Ross *et al.* (1994).

GA application to developing tubers affects the content of reducing sugars. Mares *et al.* (1981) found an increase in the level of reducing sugars, starting 4 days after treatment. GA treatment of mature tubers also resulted in an increase of reducing sugars (Clegg and Rappaport, 1970; Bailey *et al.*, 1978). The effect of GA treatments will be discussed in more detail in the next section on enzyme activities.

Furthermore, it is known that reducing sugars increase at the end of dormancy, at least prior to visible sprouting (Bailey *et al.*, 1978). Also Dimalla and van Staden (1977) found an increase in reducing sugars before visible sprouting and after this initial rise, the glucose and fructose levels decreased as the potatoes aged and sprouts developed. Note that the latter authors determined the sugars in (mainly cortical) tissue around the apical eye, lateral eye or in internodal tissue. Ross and Davies (1992) also found an increase of reducing sugars in mature tubers after harvest.

Starch

One of the main biochemical changes occurring during tuberization is the accumulation of starch. Starch is synthesized in the amyloplasts of potato parenchyma cells. The first important step in starch synthesis is the conversion of glucose-1-phosphate to the precursor for starch synthesis, ADPglucose. This conversion is catalyzed by the enzyme ADPglucose pyrophosphorylase (AGPase), which works in both directions. Starch can be broken down by amylases or starch phosphorylase. These enzymes will be discussed and compared to starch synthesizing enzymes in the next section.

Concomitantly with the visible appearance of tubers in an *in vitro* tuberization system, the fresh weight of the axillary buds increases as well as their starch content (Visser *et al.*, 1994). Also Vreugdenhil *et al.* (1998) found an increase in starch content from the day they were placed on tuber-inducing medium (no starch could be detected in axillary buds). Ross *et al.* (1994) also found an increase in starch content *in vivo*, although the rise seemed to begin at swelling of the stolon.

When GA was applied in the medium directly, starch content increased until day 4 and thereafter remained more or less constant (Vreugdenhil *et al.*, 1998), whereas the level of starch continued to increase under tuber-inducing conditions (without GA). Mares *et al.* (1981) applied GA_3 when all tubers had diameters between 1 and 3 cm (*vivo* system) and they found that the starch content was not significantly affected by the GA-treatment.

At the end of dormancy, one would expect mobilization of reserve materials from the tuber for the growth and development of a new plant. Davies and Viola (1988) indeed observed a decrease in starch content in sprouting tubers. Davies and Ross (1987) also found a sharp decrease during sprouting. Dimalla and van Staden (1977) however, found a constant level of starch during dormancy and subsequent sprouting.

Enzyme activities

The enzymes discussed here are enzymes which are related to metabolism of sucrose, generally accepted to be the transport sugar in most plants (Dimalla and van Staden, 1977) or to starch synthesis or breakdown.

Invertase and sucrose synthase

Appeldoorn *et al.* (1997) described several enzymes of carbohydrate metabolism in developing tubers, with particular emphasis on the breakdown of sucrose and its regulation. Sucrose can be hydrolyzed by invertase, resulting in glucose and fructose or converted into UDPglucose and fructose by sucrose synthase (susy). Invertase exists in different forms: acid invertases (cell wall-bound or soluble vacuolar) and a neutral cytosolic invertase. The reaction is irreversible and not subject to a high degree of fine metabolic regulation. In contrast, the reaction catalyzed by susy is reversible and under control of fine metabolic regulation.

Using an *in vitro* system, Appeldoorn *et al.* (1997) found a rapid decrease of cell wall-bound invertase before visible swelling and also a decrease of soluble acid invertase from the moment of swelling and thereafter. Susy on the other hand showed a steep increase from the moment of swelling onwards. Palmer and Barker (1972) also found a decrease in invertase activity *in vitro* and Ross *et al.* (1994) described a decrease in invertase activity and an increase in susy activity of *in vivo* growing tubers.

The decrease in invertases and the increase in susy means a shift in regulation. After tuber initiation, the predominant route for sucrose breakdown is now ruled by susy. Because susy catalyzes a reversible reaction and is highly regulated, the cells have now a mechanism of sucrose cycling and furthermore, susy will only degrade sucrose if required, which means that there can be sucrose movement into the growing tuber (Appeldoorn *et al.*, 1997).

Ross and Davies (1992) determined invertase and susy activity at harvest and during storage of *in vivo* grown tubers. They found an increase in invertase activity and a decrease in susy activity. This means that the shift from invertase to susy

during development is reversed and now invertase regulates hexose accumulation in mature, stored tubers. During sprouting, the invertase activity decreased again (Davies and Ross, 1987), which can be explained by the fact that the major source of carbohydrates (hexoses) is now starch (Renz *et al.*, 1993).

Hexokinase, fructokinase and UDPglucose pyrophosphorylase

The activity of these enzymes is of importance because of the involvement in the conversion of the sucrose degradation products (described above). Hexokinase (HK) converts glucose into glucose-6-phosphate, fructokinase (FK) converts fructose into fructose-6-phosphate and UDPglucose pyrophosphorylase (UGPase) converts UDPglucose into glucose-1-phosphate.

Initially HK activity is low, decreases during the first five days until the moment of swelling and thereafter it increases again. FK is constant until swelling and at the moment of swelling it starts to increase. UGPase showed a gradual increase over the whole period of measuring (Appeldoorn *et al.*, 1997). Ross *et al.* (1994) found a rather constant (low) level of HK activity and an increasing FK activity in *in vivo* growing tubers.

The decrease of cell wall-bound invertase and hexokinase follow parallel trends. Appeldoorn *et al.* (1997) found a positive correlation (r^2 = 0.928-0.961, day 1-5) and mentioned the fact that such a correlation between hexokinase an invertase activity has also been observed during storage of tubers.

Hexokinase and fructokinase activity during the whole life cycle of the potato tuber is described by Renz *et al.* (1993). They found a high amount of FK in growing tubers, which correlates with the need for fructokinase during sucrose mobilization via susy. During storage the HK activity increased and FK activity decreased, although FK was always higher or in the same order of magnitude as HK. The increase of HK correlates with the decrease of susy and the increase of invertase during storage. At the time of sprouting HK activity increased even more, correlating with the fact that starch is broken down, releasing only glucose.

ADPglucose pyrophosphorylase, starch phosphorylase and amylase

ADPglucose pyrophosphorylase (AGPase) catalyzes the reversible conversion of glucose-1-phosphate and ATP into ADPglucose and pyrophosphate (PPi). ADPglucose is the substrate for the synthesis of starch. Appeldoorn *et al.* (1999) determined AGPase activity of *in vitro* tubers and found an increase as soon as the tubers start to swell. Concomitantly with this increase in AGPase, they also found a temporary increase in ADPglucose and moreover the starch content is also increasing (Visser *et al.*, 1994).

Mares *et al.* (1981) determined AGPase in developing tubers after a GA-treatment (in which new stolons appeared at the tuber eyes). AGPase decreased rapidly in the first 4 days after treatment. The starch content was not significantly affected by GA_3 treatment, but did show a tendency to decline with time.

Starch phosphorylase (STP) catalyzes a reversible reaction; the conversion from $starch_n$ to $starch_{n-1}$ and glucose-1-phosphate or the other way around. The enzyme is supposed to be involved mainly in starch degradation. Appeldoorn *et al.* (1999) determined STP activity during tuber development. The activity increased as soon as the swelling of the tuber started, indicating that this activity is related to tuberization. It is clear from these measurements that the increased activity of STP parallels the accumulation of starch in the growing tuber, as also found for AGPase. They conclude that it is unknown whether STP catalyzes a net degradation or synthesis flux during tuber development. Also Japanese workers (reviewed by Bruinsma, 1962) observed an increase in starch phosphorylase activity in developing tubers (proportional to their starch content). In the subsequent period of dormancy they found a gradual decline and at the end of the dormant period a temporary rise in the cortex and the apical bud. Furthermore, both Davies and Ross (1987) and Davies and Viola (1988) found a decrease in STP activity during sprouting.

Porter (1953, in Bailey *et al.*, 1978) reported that during dormancy starch is mainly degraded by STP and to some extent also by amylases. The intact and disbudded tubers investigated by Bailey *et al.* (1978) were also assayed for activities of STP and α-amylase, which both show a general increase in activity at about the time of sprouting, followed by a decrease, while an increase in reducing sugars was found before visible sprouting. Also, Japanese workers (Bruinsma, 1962) found a striking increase in amylase activity at the beginning of sprouting. Davies and Viola (1988) found a decrease in total amylase activity during sprouting, but an initial decrease, followed by an increase of α-amylase activity.

Gibberellins are known to break dormancy and according to Clegg and Rappaport (1970) they also stimulated the formation of reducing sugars. The question arises whether gibberellins regulate hydrolytic enzyme activity in the storage tissue of the potato tuber. Bailey *et al.* (1978) tried to answer this question and in their system of intact and disbudded tubers, they found the following: when disbudded tubers were treated with GA_3 the reducing sugars increased, in accordance with the findings of Clegg and Rappaport (1970). However, the activities of starch phosphorylase and amylase did not change upon GA treatment.

Is dormancy breaking the reverse of tuber initiation?

Hormones

In figure 2.2 we try to summarize hormonal activities in potato tubers during tuber development and during dormancy and sprouting. It has to be emphasized that all hormonal activities shown in these figures are derived from data presented by different authors who all work with different systems (main differences: different phases or time-points, *vivo vs. vitro*, different varieties). Moreover, the methods used for determining these hormonal trends are also different; some are measured with chemical/physical methods, others are determined with bioassays. It is not possible to draw a clear line for ABA levels during tuber formation, based on literature data. Therefore, two contrasting options are given (ABA-1 and ABA-2). Also, the level of ethylene in dormancy is not measured, but concluded from the results of experiments in which ethylene is applied.

It is clear that not all changes occurring at dormancy breakage are the reverse of the changes in hormonal activity at tuber formation. Only the level of GA clearly shows opposite trends during the two developmental processes. For ABA no conclusion can yet be drawn because of conflicting literature data. Taking into account that GA is likely to be a major regulator of tuber formation (Vreugdenhil and Sergeeva, 2000 and references therein), it might be concluded that the question asked in the title of this paper can only partly be answered by 'yes', as far as hormones are concerned.

However, there is ample evidence that hormones other than GA are also involved in the regulation of tuber formation, indicating that dormancy breaking is more than only the reversal of tuber initiation.

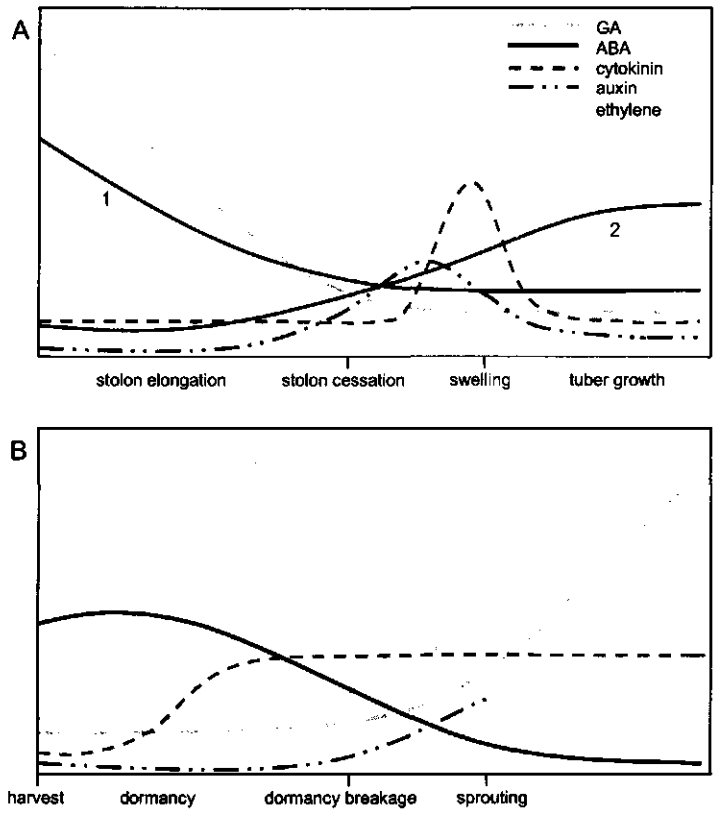

Figure 2.2 Summaries of hormonal activities during tuber development (A) and during tuber dormancy and sprouting (B). Levels of hormones are arbitrary.
A. Hormonal activities during tuber development. GA derived from Xu *et al.* (1998b); ABA-1 derived from Xu *et al.* (1998b); ABA-2 derived from Koda (1988c); Cytokinins derived from Koda (1982a) and Turnbull and Hanke (1985b); Auxin derived from Obata-Sasamoto and Suzuki (1979) and Koda and Okazawa (1983b); Ethylene derived from Suttle (1998a).
B. Hormonal activities during dormancy and sprouting. GA derived from Smith and Rappaport (1961) and Kato and Ito (1961); ABA derived from Suttle (1995); Cytokinins derived from Suttle and Banowetz (2000); Auxin derived from (Hemberg, 1949); Ethylene concluded from the data of Rylski (1974).

Carbohydrates and enzyme activities

Sucrose levels show an increase during tuber development and a decrease during dormancy and sprouting. This seems to be the reverse, although it has to be mentioned that the sucrose level of *in vitro* growing tubers decreased before visible swelling. For reducing sugars a clear reverse trend is found: both glucose and fructose decrease after visible swelling (both *in vivo* and *in vitro*) and an

increase is found at the end of dormancy, before visible sprouting. Also starch content is, not surprisingly, reversed; during tuber formation the tuber is filled with starch and during sprouting starch is mobilized for growth and development.

The enzymes fit in nicely. Sucrose breakdown is catalyzed by invertase and sucrose synthase. During tuber development invertases decrease and susy increases. A high susy activity means fine regulation of carbohydrate metabolism. In more detail: susy has a low affinity for sucrose, which offers the possibility for higher contents of sucrose and a higher sucrose content (found *in vivo*, see above) is more suitable for regulating cell growth and turgor (Appeldoorn *et al.*, 1997). Also FK and UGPase are high in developing tubers to convert the products of susy as fast as possible; moreover UGPase converts UDPglucose into glucose-1-phosphate, which can be converted into ADPglucose by AGPase to form starch. Then, during dormancy, the invertases increase and susy decreases. This shift means that sucrose is quickly mobilized via invertases, resulting in glucose and fructose. Both reducing sugars are rapidly converted via hexokinase and fructokinase (HK is increasing during dormancy, FK was already high in developing tubers and decreased somewhat during storage). At the end of dormancy, invertases are decreasing again, starch phosphorylase and amylases are increasing and will break down the starch, releasing a high amount of reducing sugars. The reducing sugars are increasing and at sprouting the HK activity increased even more.

The shift from invertase to susy and back again could be seen as a reverse trend. HK follows the invertase activity (low at development and increasing during dormancy and sprouting) and FK is high during development and decreases during storage and sprouting. The ratio HK / FK increase from 0.053 to 0.67 to 2.2 (stages: young growing tuber, stored tuber, sprouting tuber, respectively) according to Renz *et al.* (1993).

STP is present during the whole life cycle of the tuber. STP increases at tuber development, decreases during dormancy and increases again at sprouting. Let us assume that STP catalyzes a net synthetic flux during tuber development. After that, during dormancy, STP might be involved in starch cycling. At the end of dormancy STP catalyzes a net degrading flux. Then this enzyme could be seen as both high during development and sprouting, but catalyzing the reaction in opposite directions.

Conclusion

In conclusion, when comparing tuber induction and the breaking of dormancy, hormonal activities are only partly the reverse, while for carbohydrates and enzyme activities some clear reversed trends are found. For more definite conclusions, measurements should be done in the same way using the same material. Moreover, the time point of dormancy breakage should be defined more precisely to make sure that measured parameters are dormancy breaking parameters rather than sprouting parameters. Molecular genetic analysis of dormant and sprouting tubers might provide well-defined markers for the timing of dormancy breaking.

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3

Ethanol and its putative mode of action in the breaking of dormancy of potato tubers (*Solanum tuberosum* L.)

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Abstract

Ethanol was found to break dormancy of potato microtubers. The possible role of alcohol dehydrogenase (ADH) in ethanol-induced dormancy breaking of potato tubers was studied. ADH activity is necessary for ethanol-induced sprouting as appeared from 4-methyl-pyrazole inhibition of the ethanol effect. Only alcohols that can be a substrate for ADH (*e.g.* 1-propanol, but not 2-propanol) have the same effect as ethanol on dormancy breaking. As the products of the ethanol conversion (*e.g.* acetaldehyde) did not stimulate sprouting, the ADH activity itself or the NAD^+ to NADH conversion is suggested to be the causal factor in the dormancy breaking effect.

The type of secondary structure that is formed after ethanol-induced dormancy breaking depended on the sucrose concentration: a sprout developed on 1% sucrose and a secondary tuber on 8% sucrose. The theory that sucrose regulates the type of secondary structure by affecting the endogenous GA level (low sucrose leading to high GA and sprout formation, high sucrose leading to low GA and tuber formation) was tested by measuring GA levels in the tuber after transfer to ethanol containing media. It appeared that GA levels of ethanol-treated tubers always decreased to about 50% of their respective controls and that 1% sucrose in the medium resulted in a dramatic drop of GA_1, both indicating that the theory did not hold for ethanol-induced sprouting. Because this theory was originally derived from studies on tuber formation, it was concluded that regulation of sprouting is different from regulation of tuber formation. Furthermore, changes in ABA level and GA/ABA ratio also could not explain the type of secondary structure formed after ethanol treatment.

Introduction

Dormancy in general is described as 'a temporary suspension of visible growth of any plant structure containing a meristem' (Lang *et al.*, 1987). A dormancy period, which probably evolved as a survival strategy, has advantages and disadvantages: it is advantageous for the plant to survive a period unfavourable for growth (survival) or for man to store dormant plant material (*e.g.* seeds or vegetative storage/propagation organs like potato tubers) for a certain period of time, but disadvantageous when growth is necessary soon after harvest of these seeds or tubers. In case of planting dormant potato tubers, gibberellins, known as hormones favouring sprouting, germination, cell division and cell enlargement, can be used to break dormancy (see review by Claassens and Vreugdenhil, 2000). Gibberellins also break dormancy in various seeds, like in tomato seeds (Groot and Karssen, 1987) or in seeds of Arabidopsis as described by Koornneef and Van der Veen (1980). A large number of non-hormonal organic and inorganic chemicals also have an effect on (seed) dormancy. Alcohols (like ethanol) are an example of such dormancy breaking chemicals in seeds as described for red rice (Cohn *et al.*, 1989) and oat (Corbineau *et al.*, 1991). Ethanol also breaks dormancy in tubers of Jerusalem artichoke (Petel *et al.*, 1993). Indirect evidence of ethanol breaking dormancy in potato tubers was already given by Rakitin and Suvorov (1935). These authors kept young potato tubers under anaerobiosis and measured the formation of ethanol. They observed not only accumulation of ethanol, but they also observed sprouting. The control (aerobic conditions) contained only a small amount of endogenous ethanol and did not sprout.

Preliminary experiments indeed showed that ethanol is able to break dormancy in *in vitro* grown potato tubers. As a hypothesis for the working mechanism of ethanol in dormancy breaking phenomena in true seeds, it has been suggested that alcohol metabolism plays a role (Cohn *et al.*, 1989; Corbineau *et al.*, 1991). The latter authors suggest that the stimulatory effect of ethanol acts specifically through alcohol dehydrogenase (ADH) activity.

During the formation of potato tubers, unfavourable conditions, *e.g.* heat, can cause so called second growth. Different forms of secondary structures can be distinguished: deformation of tubers, formation of secondary tubers, formation of aerial sprouts from tubers and formation of stolons (Van den Berg *et al.*, 1990). Assuming that dormancy gradually develops in the tuber (Claassens and Vreugdenhil, 2000), this second growth could also be seen as breaking of (early) dormancy. Preliminary experiments on effects of ethanol on dormant *in vitro* potato tubers showed that either a shoot (sprout) is formed or a secondary tuber,

depending on the sucrose concentration in the medium. This led to the hypothesis that ethanol breaks dormancy and causes the apical bud to become active, and that other factors (sucrose) are responsible for the kind of secondary structure which develops on the tuber. That is, a low sucrose concentration and ethanol treatment leads to real sprouts, while a high sucrose concentration and ethanol treatment leads to the formation of secondary tubers (Van den Berg *et al.*, 1990). Xu *et al.* (1998) presented data indicating that sucrose affects endogenous GA levels in a negative way. Taken together, this might mean that low sucrose during ethanol treatment leads to a high GA-level resulting in sprouts and, the other way around, high sucrose causes a low GA level, which would result in a secondary tuber (see also Vreugdenhil and Sergeeva, 1999).

As described by Claassens and Vreugdenhil (2000), not only GA is supposed to have an effect on tuberization, tuber dormancy and breakage of tuber dormancy. ABA also seems to play a major role in initiation and maintenance of potato tuber dormancy (Suttle and Hultstrand, 1994). Moreover, the GA/ABA ratio might be more important in regulating developmental processes than the absolute levels of either of the two.

In this report we study in more detail the possible working mechanism of ethanol in breaking potato tuber dormancy through ethanol conversion by ADH (indicated as 'ADH theory'). Furthermore, we will investigate whether endogenous hormone levels regulate the nature of the secondary structure formed, especially whether endogenous GA levels might be regulated through exogenous sucrose (Xu *et al.*, 1998).

Materials and methods

In vitro tuberization system

In the different experiments two types of *in vitro* grown tubers were used. They will be referred to as type I and type II (micro)tubers (see also figure 1.3). The protocol to produce both type of tubers is based on the method of Hendriks *et al.* (1991).

In vitro tubers, type I: *In vitro* plants (*Solanum tuberosum*, cv. Bintje) were grown with a 16 hrs light period (50 W · m^{-2}, 20°C) and after 4 weeks these plantlets were transferred to soil. They were grown in a growth chamber for 4 weeks with a 16 hrs light period (80 W · m^{-2}, 25°C). To obtain tuber induction in these plants, the light regime was changed to short days (8 hrs light period) for three weeks more. Single node cuttings were taken and after a surface sterilization

placed on a tuber-inducing medium consisting of modified Murashige and Skoog 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. The explants were incubated in the dark at 20°C and tuber formation started on the 5th day. After 4 weeks the tubers were considered mature and dormant. The diameter of these microtubers was around 8 mm and their fresh weight approximately 500 mg. When stored in the dark at room temperature, the tubers stayed dormant for approximately 4 to 5 months.

In vitro tubers, type II: From the same *in vitro* plants as described above, single node cuttings containing an axillary bud were taken after 4 to 5 weeks. These explants were put directly on the tuber-inducing medium and incubated in the dark. Tuber formation started on the 6th day and after 4 weeks the tubers were considered mature and dormant. The diameter of these microtubers was around 3 mm and their fresh weight approximately 20 mg. When stored in the dark at room temperature, the tubers stayed dormant for approximately 5 to 6 months.

Rate of sprouting

To determine the sprouting behaviour of tubers when giving different treatments, type II microtubers were used. Each treatment was done with 45 tubers, of which 30 tubers were 8 weeks old and 15 tubers were 5 weeks old. Approximately 2 mm of the stem, bearing the tuber, was cut off to ensure uptake of test components through the stem cutting. The explants + sessile tubers were then transferred to fresh tuber-inducing medium containing the compounds to be tested and stored at 20°C in darkness. The different treatments are summarized in table 3.1. Some chemicals were applied as vapour; in these cases the compound was added as a liquid to a piece of filterpaper lying in a 3 cm petridish, placed on top of the medium in a 9 cm petridish. The explants were placed around the small petridish. The air volume in the petridish above the medium was 60 cm^3.

ADH activity

For the measurements of ADH activity, tubers of type I were used. Each sample consisted of 3 tubers of different age (2, 3 and 4 months old). Each sample was split into two, one consisting of the apical part + secondary structure (if any) and the other one consisting of the rest of the tuber tissue. The total number of tubers tested for sprouting in this experiment was about 25 per treatment. Tubers received an ethanol treatment (0.5% v/v), a GA treatment (10 μM) or no treatment (control), either with 1% or 8% sucrose in the medium.

The extraction procedure was modified after Appeldoorn *et al.* (1997). In short, 1.2 ml of extraction buffer (50 mM Hepes/KOH (pH 7.4), 5 mM $MgCl_2$, 1mM EGTA, 1 mM EDTA, 10% (v/v) glycerol, 0.1% BSA (bovine serum albumin), 5 mM DTT (dithiothreitol) and 2% (w/v) insoluble PVP (polyvinylpyrrolidone)) were added to 10 mg of powdered freeze-dried tissue. After centrifugation (5 min., 15,000 g), the supernatant was removed, frozen in liquid nitrogen and stored at -75°C until required. The extraction procedure was carried out at 4°C.

The activity of ADH was determined in 250 µl assay medium (in a 96 well plate) at 25°C, for 10 min. The medium, in which ADH was assayed, contained 100 mM Tris-HCl (pH 9.0) and 0.5 mM NAD^+, and 1% (v/v) ethanol was added to start the assay. The determination is based on the NAD^+ to NADH conversion and was measured spectrophotometrically at 340 nm, by means of an ELISA reader (SLT labinstruments 340 ATTC). Blanks had the same reaction mixture, but without substrate (ethanol).

GA determinations

Microtubers of type I, grown on tuber-inducing medium as described above, were transferred to medium containing 1% or 8% sucrose, plus or minus 0.5% (v/v) ethanol. When transferred, approximately 2 mm of the explants was cut off to promote uptake of nutrients from the medium. At the time of transfer, these tubers were 7 to 11 weeks old and assumed to be mature and dormant. On the day of transfer, the 4^{th}, and 11^{th} day of the ethanol treatment, at least 10 tubers were sampled and immediately frozen in liquid nitrogen. The total number of tubers tested for sprouting in this experiment was about 40 per treatment. When tubers showed sprouting, the secondary structure was also sampled.

Extraction for GAs was performed according to the method described by Xu *et al.* (1998) with a few minor modifications: All the glass material was pre-silanised with surfasil before use to reduce the loss of GAs caused by aspecific absorption. Instead of adding 200 ml 80% methanol (+ 0.1% ascorbic acid), 100 ml was added and stirred for 4 hrs. Then, this was centrifuged, the supernatant decanted and kept cold. Another 100 ml was added to the pellet and left stirring overnight. After centrifugation, the supernatants were pooled. Furthermore, we added deuterated gibberellins (10 ng per sample) to act as an internal standard (no tritium labelled gibberellins were added). The used QAE-Sephadex A-25 (Pharmacia) column was 10 cm long (inner diameter of 1 cm). The collected fractions of the preparative HPLC-step were not fractionated for the second time after methylation. Putative methyl-GA-fractions were dried and trimethylsilylated by dissolving in a fresh mixture of BSTFA:TMCS:pyridine (20:1:79, v/v) and

heated for 20 min at 70°C. The actual determination of GAs using a GC-MS system was performed as described by Kappers *et al.* (1997).

ABA determinations

For the ABA determinations, tubers of type I were used. Each sample consisted of 3 tubers of different age (2, 3 and 4 months old). Each sample was split into two, one consisting of the apical part + secondary structure (if any) and the other one consisting of the rest of the tuber tissue. The total number of tubers tested for sprouting in this experiment was about 25 per treatment.

The extraction procedure was modified after Berry and Bewley (1992) and Raikhel *et al.* (1987). The samples were freeze-dried and ground in liquid nitrogen. Approximately 10 mg of apical bud tissue and 50 mg of the rest of the tissue was mixed with respectively 400 and 800 µl of 80% (v/v) methanol, in which 0.1 % (w/v) BHT (2,6-Di-*tert*-butyl-4-methylphenol) was dissolved. After centrifugation (15 min., 14,000 g), the supernatant was stored at 4°C and again the same amount of this extraction buffer was mixed with the pellet. After centrifugation, the supernatants were pooled, lyophilized and resuspended in 200 µl of TBS buffer (25 mM Tris-HCl, 100 mM NaCl, 1mM $MgCl_2$, pH 8.5). ABA was then quantified using the Phytodek monoclonal antibody ELISA method (Agdia Inc.-USA).

Results

The effect of ethanol on dormancy of type I tubers is shown in figure 3.1. Ethanol treatment (0.5%), in combination with a low level of sucrose, resulted in the growth of (in this case multiple) sprouts (figure 3.1A). The same amount of ethanol in a medium with high levels of sucrose resulted in development of secondary tubers (figure 3.1B). Thus, in both cases ethanol breaks dormancy. Control tubers, not treated with ethanol, remained dormant, both on medium with 1% sucrose and with 8% sucrose. Type II potato tubers showed a comparable reaction. Furthermore, when *in vitro* tubers of type II were kept under anaerobiosis, sprouting occurred after a few weeks, while control tubers, kept in air, did not sprout, in agreement with the data found by Rakitin and Suvorov (1935). Ethanol measurements at the end of this experiment indeed showed a higher ethanol content in tubers kept under anaerobiosis (data not shown).

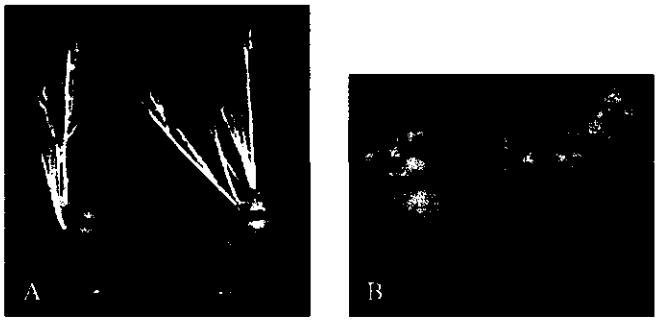

Figure 3.1 Effect of ethanol treatment on dormant microtubers (type I). Tubers are approximately 0.7-0.8 cm in diameter. Single-node cuttings were grown on tuber-inducing medium (Hendriks *et al.*, 1991), formed a tuber and were then transferred to the same medium supplemented with:
A: 1% sucrose and 0.5% ethanol, resulting in sprouting of the tubers;
B: 8% sucrose and 0.5% ethanol, resulting in secondary (and some tertiary) tubers.

Rate of sprouting

The effects of alcohols, hormones, and related compounds on the rate of sprouting are summarized in figures 3.2 and 3.3. Different approaches to investigate the validity of the 'ADH theory' (Corbineau *et al.*, 1991; Cohn and Hilhorst, 2000) were applied and the results of these experiments are shown in figure 3.2. The upper panel (figure 3.2A) shows the sprouting curves of tubers treated with the products of the ADH conversion, viz., acetaldehyde and, via conversion through aldehyde dehydrogenase, acetic acid. Within 5 days after transfer to medium containing 0.5% ethanol, more than 90% of the tubers had sprouted, whereas the control only reached about 13% in that same time period. After 30 days 100% of the ethanol-treated tubers sprouted, while the control reached 64%. Other (preliminary) control experiments never showed a sprouting percentage higher than about 20% (data not shown). A treatment with ethanol as a vapour showed approximately 64% of sprouting after 5 days, while after 30 days almost 100% sprouting was obtained. Tubers, treated with acetaldehyde or acetic acid, showed after 5 days only 11 and 9% sprouting, respectively, and the percentage of sprouting did not increase much afterwards. For this experiment, the concentration was set to 0.02% for acetaldehyde and to 0.004% for acetic acid, based on data from a preliminary experiment, showing that higher concentrations were even less active. Both treatments, even with these low concentrations, resulted in lower percentages of sprouting as compared to the control, suggesting that both compounds inhibit sprouting.

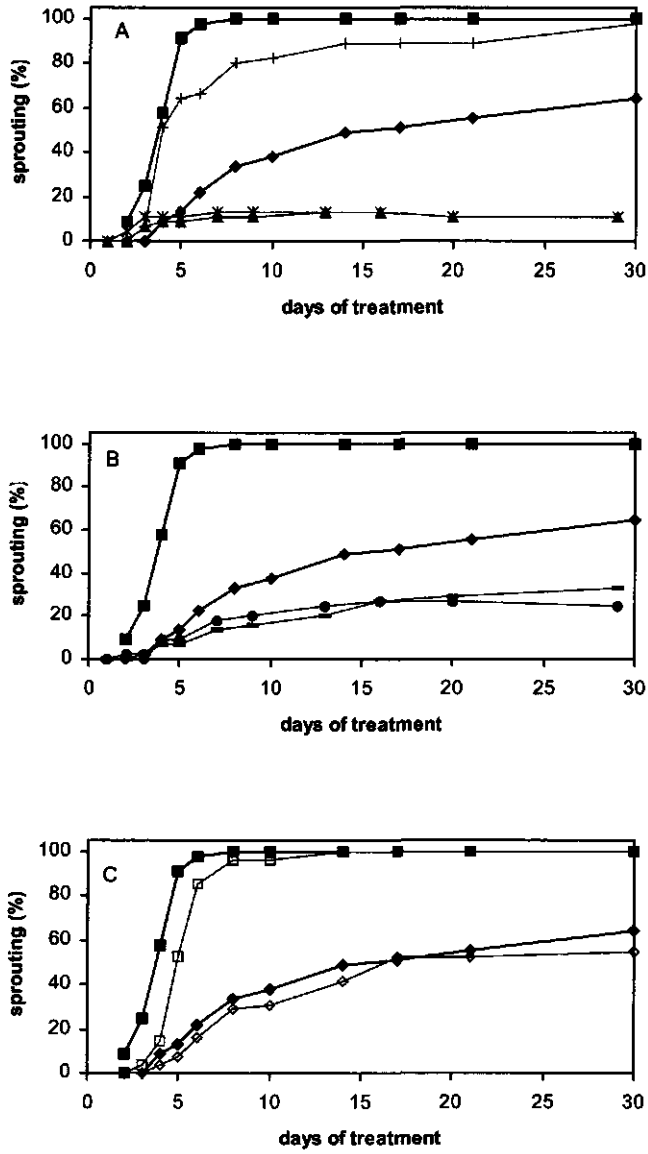

Figure 3.2 Rates of sprouting of *in vitro* grown tubers (type II) treated with different compounds, known to be involved in ADH-mediated alcohol conversion. All media contained 1% sucrose. In all panels the control (♦) and the 0.5% ethanol (■) treatment are depicted. For concentrations of tested compounds, see table 1.
A: effects of ethanol vapour (+), dissolved acetaldehyde (*) or dissolved acetic acid (▲);
B: effects of 4MP (●) or 4MP + ethanol (-);
C: effects of 1-propanol (□) or 2-propanol (◊).

Figure 3.2B shows the effect of 4MP, which inhibits the action of ADH by forming an inactive ternary complex with ADH and NAD^+ (Blomstrand *et al.*, 1979). For comparison, the control and the ethanol treatment are also depicted in this figure. The treatment with both 4MP and ethanol added to the medium reached a sprouting percentage of 7% after 5 days and 33% after 30 days, which might be explained by an inhibition of ADH by 4MP, leading to lower sprouting rates than the controls. However, the treatment with only 4MP showed approximately the same sprouting curve as the treatment with both 4MP and ethanol.

Figure 3.2C shows a comparison of effects of primary and secondary alcohols on sprouting. These types of alcohol were tested since ADH is known to convert primary alcohols to their aldehyde, whereas secondary alcohols are not converted. This graph shows that the sprouting curve of the treatment with 1-propanol follows the ethanol induced sprouting curve, although a 1 day shift is noticeable, and that the 2-propanol treatment coincided with the control.

Outgrowth of the apical bud of the tuber resulted either in a real sprout (figure 3.1A) or a secondary tuber (figure 3.1B) depending on the treatment. We define both types of secondary growth as sprouting in figures 3.2 and 3.3. The rate of sprouting of different treatments, which are expected to have an effect on the nature of the second growth structure, is shown in figure 3.3. Osmotic potential is the subject of figure 3.3A. It is clear that when ethanol is used, the concentration of sucrose has no effect on the rate of sprouting. However, the control with only 8% sucrose showed a different profile when compared to the 1% sucrose control. After 5 - 9 days of treatment the 8% sucrose control shows only 4% of sprouting and at day 29 a sprouting percentage of 42 was reached, clearly lower than the 1% sucrose control curve. When 4% sorbitol was added to tuber-inducing medium with 1% of sucrose, together equalling the osmotic potential of an 8% sucrose medium, no sprouting was observed, suggesting that this concentration of sorbitol had a negative effect on dormancy breaking or growth. The treatment with 1% sucrose + 4% sorbitol + ethanol shows no sprouting during the first week of treatment and thereafter a gradual increase is visible, reaching a sprouting percentage of 83% after 27 days of treatment. This suggests that the ethanol in the medium is capable of counteracting the negative effect of sorbitol.

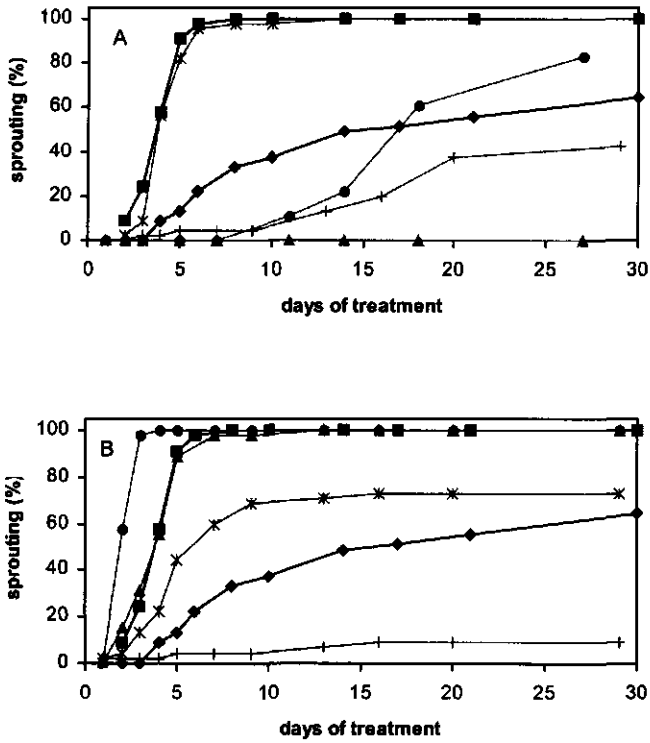

Figure 3.3 Rates of sprouting of *in vitro* grown tubers (type II) treated with different compounds, supposed to affect the nature of the secondary structure. When the apical bud shows outgrowth, it is counted as sprouting, irrespective of the nature of the newly formed structure. In both panels the control (♦) and the ethanol (■) treatment, both in the presence of 1% sucrose, are depicted. For concentrations of tested compounds, see table 1.

A: effects of 8% sucrose (+), 8% sucrose + ethanol (*), 1% sucrose + 4% sorbitol (▲) or 1% sucrose + 4% sorbitol + ethanol (●);

B: effects of ABA (+), ABA + ethanol (*), CCC + ethanol (▲) or CCC + ethanol + GA (●), all in the presence of 1 % sucrose.

The effects of hormones (GA and ABA) on the rate of sprouting are shown in figure 3.3B. ABA is believed to keep tubers dormant (Suttle and Hultstrand, 1994; Claassens and Vreugdenhil, 2000), which is clear also in this experiment. Only 9% of the ABA-treated tuber lot is sprouting after 29 days. When adding ethanol together with ABA, ethanol is able to counteract the effect of ABA on the dormant state, although sprouting does not reach 100%. After 5 days of

treatment 44% of the tubers were sprouting and after 29 days this percentage had increased to 73%.

CCC was used because it inhibits GA synthesis (Moore, 1989). When using CCC together with ethanol, the CCC does not have any effect on the sprouting curve as shown by the coinciding lines of this treatment and the ethanol treatment. When tubers were treated with GA + ethanol + CCC, it is clearly visible that this treatment results in an even faster sprouting: within 3 days almost 100% of the tubers have sprouted. Tubers treated with only GA show the same sprouting pattern (data not shown).

Type of secondary structure

Not only the rate of sprouting is affected by the treatments depicted in figure 3.2 and 3.3, also the structures that are formed differ. It was already noticed in the preliminary experiments that when ethanol was given to the tubers, the sugar percentage of the medium was of importance for the kind of secondary structure that is formed (see figure 3.1). Some examples of different secondary structures are shown in figure 3.4, viz., sprouts (A), thickened sprouts (B), and secondary tubers (C). The term 'thickened sprouts' is added, because these secondary structures are not morphologically the same as fully grown secondary tubers, but on the other hand are not like normal sprouts either.

Figure 3.4 Examples of secondary structures formed during various treatments. Tubers in the upper row (A) have formed a sprout, the middle ones (B) formed thickened sprouts and the lower tubers (C) are examples of tubers which formed secondary (and tertiary) tubers.

Figure 3.5 shows the results of the different treatments on the nature of the secondary structures after approximately 30 days. Both controls (1% or 8% sucrose) yielded only sprouts as secondary structures, whereas treatment with ethanol resulted in different secondary structures. Treatment with ethanol in vapour or dissolved in medium with 1% sucrose resulted in about 50-60% sprouts and about 40-50% thickened sprouts and secondary tubers. The same result was obtained when 4% sorbitol extra was added. Treatment with ethanol and 8% sucrose resulted in more than 80% secondary tubers while the rest consisted of thickened sprouts. Whenever the hormone ABA was used in a treatment, only sprouts were found. It was already mentioned that CCC together with ethanol did not have an effect on the rate of sprouting when compared to tubers treated with only ethanol. But if tubers were treated with both ethanol and CCC in the presence of 1% sucrose, no normal sprouts could be detected; about 50% secondary tubers were formed while the other 50% were thickened sprouts. If on top of this treatment (CCC + ethanol, 1% sucrose) GA was added, then all tubers formed sprouts; the same was observed for a treatment with only GA (data not shown).

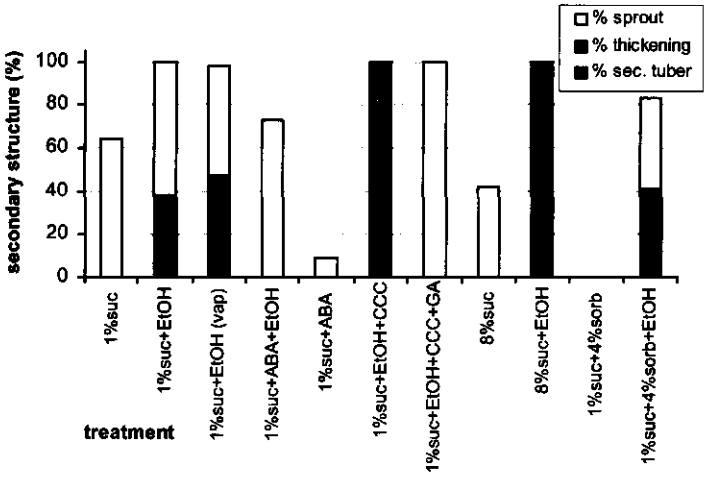

Figure 3.5 Percentages of different secondary structures formed after 30 days of various treatments of type II *in vitro* tubers. The abbreviation suc stands for sucrose, sorb for sorbitol. For further details of treatment see table 3.1.

Viability of tubers

Because not all treatments showed 100% sprouting at the time the observations of figure 3.5 were made (after approximately 1 month), the viability of the tubers

was checked. The results are shown in table 3.1. When after three months of treatment the tubers did not sprout for more than 80%, they were transferred to fresh tuber-inducing medium, containing 1% sucrose and 10 μM GA_{4+7}. After 4 months tubers from three treatments were transferred for the second time to fresh medium, also containing 1% sucrose and GA_{4+7}. Then, after almost 6 months all treatments resulted in close to 100% of sprouting, except for the acetic acid (vapour) treatment (89% of sprouting).

Table 3.1 Effect of various treatments on sprouting of dormant potato tubers (type II). Treatment conditions refer to concentrations of test compounds in the nutrient medium. Sprouting percentage was determined after 1 – 6 months of treatment. Tubers, which showed after 3 months of treatment a sprouting percentage < 80%, were transferred to fresh tuber-inducing medium containing 1% of sucrose and 10 μM GA_{4+7} to assess the viability of the explants. When these tubers did not reach a sprouting percentage > 90% after 4 months, they were again transferred to the same fresh medium as described above.

sucrose content (%-w/v)	treatment conditions	Sprouting % 1 month	2 months	3 months	4 months	6 months
1	- (control)	64	93	100	100	100
1	0.5 % ethanol	100	100	100	100	100
8	- (control)	42	91	93	93	98
8	0.5 % ethanol	100	100	100	100	100
1	5μl ethanol [a]	27	76	100	100	100
1	50μl ethanol [a]	98	98	100	100	100
1	0.02% acetaldehyde	11	40	89	100	100
1	10μl acetaldehyde [a]	20	24	31	80*	100**
1	0.004% acetic acid	11	18	76	100*	100
1	10μl acetic acid [a]	0	0	0	11*	89**
1	1 mM 4-methyl pyrazole	24	62	96	98	100
1	1 mM 4-methyl pyrazole + 0.5% ethanol	33	91	100	100	100
1	87 mM 1-propanol	100	100	100	100	100
1	87 mM 2-propanol	55	67	85	91	98
1	10 μM abscisic acid	7	7	9	58*	98**
1	10 μM abscisic acid + 0.5% ethanol	73	87	91	96	100
1	0.5% ethanol + 0.5 g/l CCC	100	100	100	100	100
1	0.5% ethanol + 0.5 g/l CCC + 10μM GA	100	100	100	100	100
1	4% sorbitol	0	70	75	100*	100
1	4% sorbitol + 0.5% ethanol	83	89	89	100	100

[a] vapour

* results after transfer to GA-containing medium

** results after second transfer to GA-containing medium

ADH activity

We determined ADH activity in tubers treated with ethanol or GA, and in control tubers. The results are shown in figure 3.6. The sprouting percentages of both GA treatments were relatively low in comparison to earlier GA treatments. We therefore sampled only sprouting tubers at day 11 and 20, to make sure that any difference in enzyme activity was due to the sprouting reaction caused by the GA or ethanol treatment.

Figure 3.6 shows the total ADH activity in the tubers during the different treatments. Both ethanol treatments showed an elevated level of activity in comparison to the other treatments. This suggests that ethanol induced ADH activity and that this is not directly related to sprouting, since the sprouting GA-treated tubers did not show such an enhanced ADH activity. ADH activity of the secondary structures was determined separately at day 20; their activity did not exceed 0.026 nmol · min^{-1} · mg FW^{-1}.

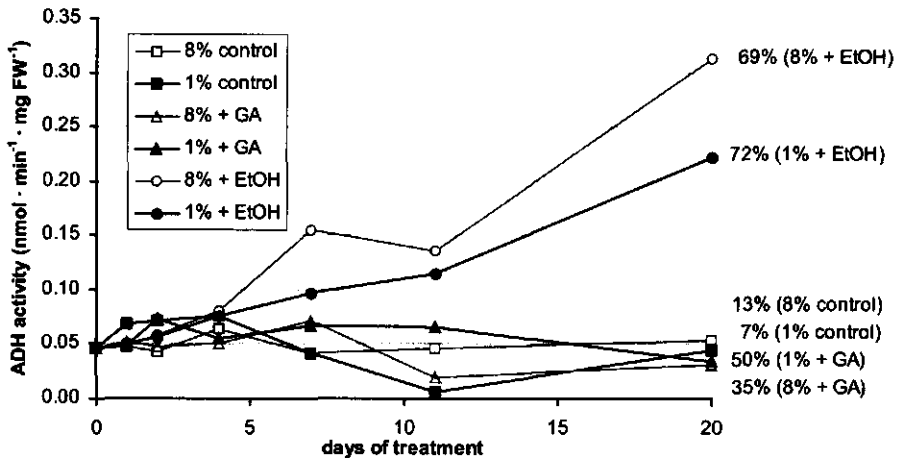

Figure 3.6 Effects of GA (10 μM), ethanol (0.5%) and sucrose (1 or 8%) on ADH activity in type I microtubers. Determinations were carried out separately for the apical parts (approximately 10% of the total tuber tissue) and the rest of the tuber (~90%). A weighed mean value was calculated afterwards and is depicted in this figure. The activity is based on duplicate measurements, consisting of 3 tubers per sample. The values at the right of the figure indicate sprouting percentages after 20 days of incubation.

GA-determinations

As shown above, both GA and ethanol break dormancy. It is also known that endogenous GA can be regulated by various external factors, such as heat and drought stress, and mechanical damage (Bodlaender *et al.*, 1964). Therefore, ethanol might have an effect on endogenous GA levels. Thus, endogenous GA_1 levels were determined in tubers treated with ethanol and 1 or 8% sucrose. The tubers did not show any sprouting on the 4th day of the treatment. On the 11th day the tubers on medium with 1% and 8% sucrose (control experiments) showed 0 and 5% sprouting, respectively, while the tubers on 1 and 8% sucrose + ethanol showed about 70% sprouting. We determined GA_1 since it is the major active GA in potato (Xu *et al.*, 1998; Vreugenhil and Sergeeva, 1999).

Figure 3.7 gives a schematic overview of the experimental set-up and results. When tubers, grown on medium with 8% sucrose, were transferred to fresh, but identical, medium, the level of GA_1 did not change appreciably (upper row). However, the GA_1 level of tubers transferred to a 1% sucrose containing medium dropped approximately 10 times after 4 days of treatment and even more after 11 days of treatment. The endogenous GA_1 levels of the ethanol treated tubers decreased about 2 times when compared to the same treatments without ethanol. Apparently, ethanol did not lead to increased (overall) GA_1 levels in the treated tubers.

ABA-determinations

It has been suggested that the GA/ABA ratio controls developmental programs, rather than the level of GA alone (Bruinsma, 1962; Koda and Okazawa, 1983; White *et al.*, 2000). In figure 3.7 the amounts of ABA are depicted for the ethanol treated tubers. The tubers used for these determinations did not show any sprouting on the 4th day of the treatment. On the 11th day the control tubers (1% and 8% sucrose) showed 5 and 15% sprouting, respectively, and the tubers treated with 1 or 8% sucrose + ethanol showed about 68% of sprouting. ABA was determined separately for the apical part and the rest of the tuber tissue. Afterwards, weighed mean values were calculated and these values are displayed in figure 3.7 (bottom values).

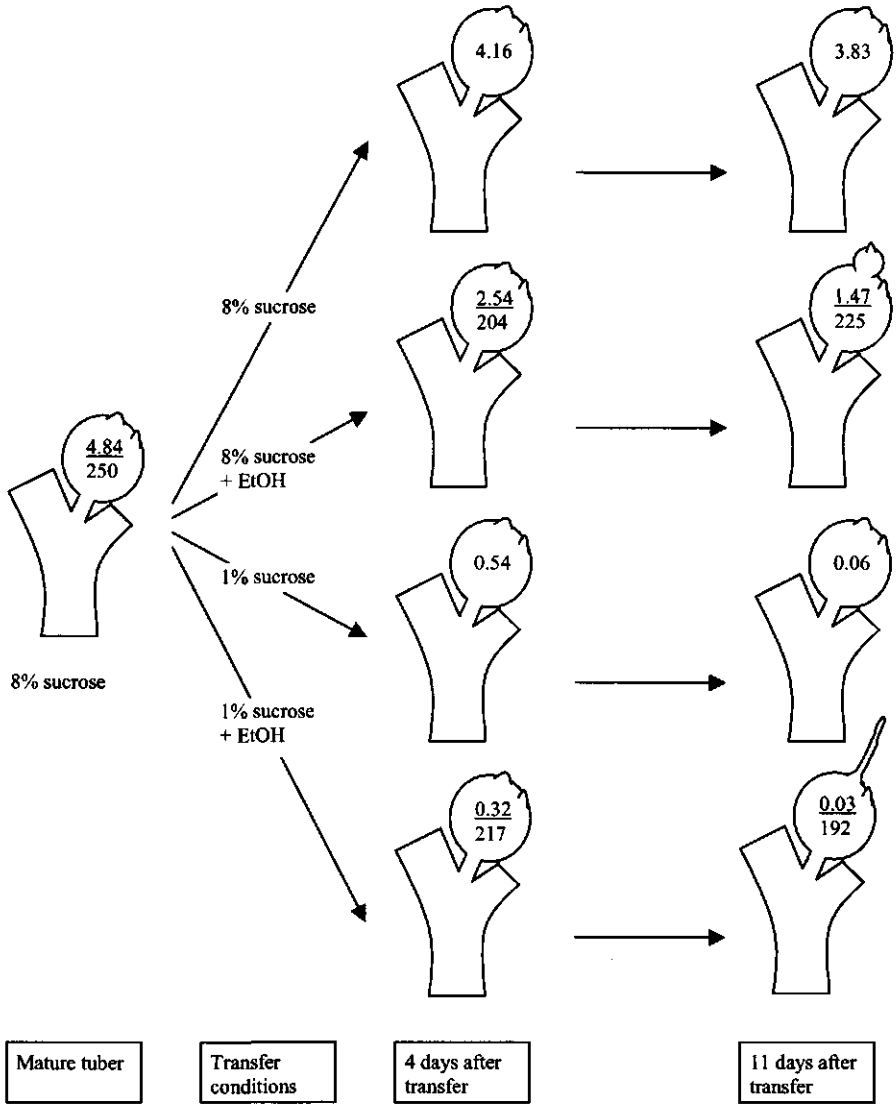

Figure 3.7 Endogenous GA_1 and ABA levels in potato microtubers (type I) during different treatments. When in the tuber drawing only one value is mentioned, this refers to the GA level. When two values are presented, the upper value refers to the GA level and the lower value to the ABA level. Both values are in ng · g FW^{-1}. Tubers were grown on medium containing 8% sucrose (mature tuber) and transferred to medium with conditions specified in the arrows. Treatment with ethanol resulted in a secondary structure, either a secondary tuber (8% sucrose) or a sprout (1% sucrose). Determinations of GA-levels were carried out on whole tubers, including the secondary structures (if present). Determinations of ABA-levels were carried out separately for the apical part + secondary structure (if any) and for the rest of the tuber tissue; the calculated weighed mean values are depicted in the figure.

The ABA levels did not change much as a reaction on different treatments (figure 3.7). ABA tended to decrease a bit when tubers were treated with ethanol. The tendency to decrease seemed to continue only for the treatment with 1% sucrose + ethanol. This could indicate that a lower ABA level is related to the formation of a sprout instead of a secondary tuber, although the measured differences were marginal. The ABA levels measured separately in the apical parts showed slightly larger differences. For tubers treated with 1% sucrose + ethanol the ABA level decreased dramatically to 49 ng · g FW^{-1}, which seems to fit in a relation between sprout formation and low ABA levels. However, after 11 days, the ABA level in the apical part increased again to a level of 425 ng · g FW^{-1}. After 4 days of treatment the ABA level in the apical part of tubers treated with 8% sucrose + ethanol almost doubled when compared to the untreated tubers (364 and 216 ng · g FW^{-1}, respectively), which could fit in a theory that high ABA levels result in a secondary tuber.

Since the mean values of the ABA levels did not change much, the changes in GA/ABA ratios showed the same trend as the changes in GA levels (figure 3.7), suggesting that the ratio GA/ABA has no specific role in the determination of the nature of the secondary structure.

Discussion

As shown in figure 3.1, ethanol indeed breaks dormancy of potato tubers. Ethanol, dissolved in the medium as well as applied in vapour, results in sprouting (figure 3.2A).

Role of ADH in ethanol-induced sprouting

Alcoholic fermentation is a process that normally takes place under anaerobic conditions. It includes the combined action of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH), in which pyruvate is converted to acetaldehyde and subsequently to ethanol (figure 3.8). This last step regenerates NAD^+, necessary for maintenance of glycolysis (Kreuzwieser *et al.*, 1999; Tadege *et al.*, 1999). This is presumably what happens in plant parts kept under anaerobiosis. In nature, this could happen for example when roots are exposed to flooding.

The fate of the ethanol, when the environment is re-oxygenated, is not certain. There are different possibilities for the degradation of ethanol: oxidation by ADH in the cytoplasm, by catalase in the peroxisomes (Kreuzwieser *et al.*, 1999) or maybe by cytochrome P450 at the endoplasmatic reticulum (Cohn and Hilhorst, 2000; Lieber, 1997). In figure 3.8 the oxidation by ADH, which is the

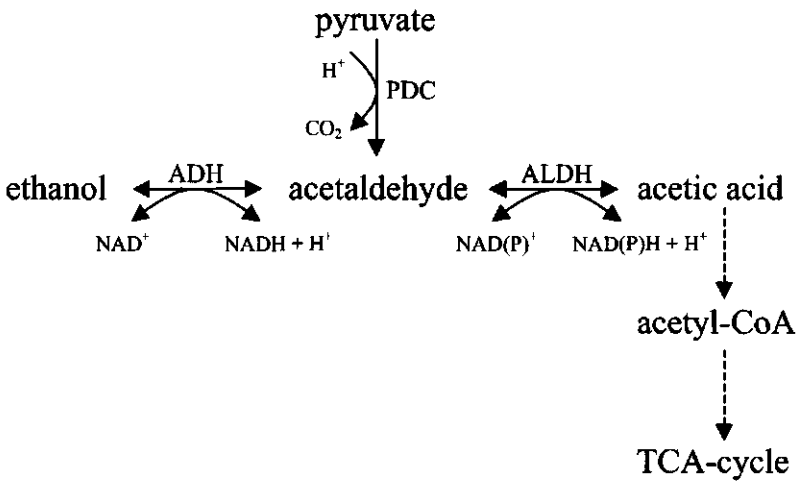

Figure 3.8 Metabolic pathway of ethanol formation and breakdown via ADH. Further details are given in the text.

most obvious route, is depicted. Ethanol, acetaldehyde and acetic acid can be emitted (through leaves; Kreuzwieser *et al.*, 1999) or ethanol may be subject to removal by diffusion (flooded roots; Liao and Lin, 1995).

The ADH reaction is reversible; the K_m value of ADH of different species varies from 9.6 to 19 mM for ethanol (Cohn and Hilhorst, 2000; Corbineau *et al.*, 1991; Leblovà and Perglerova, 1976; Liao and Lin, 1995) and ranges from 0.69 to 1 mM for acetaldehyde (Leblovà and Perglerova, 1976; Liao and Lin, 1995). These values imply that this reaction proceeds in favour of the reduction of acetaldehyde.

Leblová *et al.* (1976) described seed germination as a period of natural anaerobiosis, during which ethanol is formed and a preceding increase in ADH activity is noticed. Does the increase in ADH activity precede seed germination and, moreover, is this increase (or the subsequent formation of ethanol) the cause of germination? When ethanol is applied, seeds generally germinate (Corbineau *et al.*, 1991; Cohn and Hilhorst, 2000). Moreover, ethanol might induce ADH; also in ethanol-treated potato tubers the levels of ADH activity were increased (figure 3.6). For *Aspergillus nidulans*, three isozymes of ADH are described of which ADH I is induced by ethanol, while ADH II is repressed by ethanol and ADH III is specifically induced by anaerobic stress (Sealy-Lewis and Fairhurst, 1995).

Cohn *et al.* (1989) stated that 'the relative activity of many dormancy-breaking chemicals is generally a function of their lipophilicity (the ease of penetration) and further modulated by the nature of the functional groups present

(subsequent metabolic conversion)'. Footitt *et al.* (1995) suggested that propanol is metabolised to an acid form, after which this compound causes embryo acidification, which, in its turn, is presumably associated with the dormancy breaking process. According to these findings it is important that the alcohol is metabolised to its aldehyde or acid, which in its turn can break dormancy.

Therefore, it was expected that acetaldehyde or acetic acid would also break dormancy in potato microtubers. Treatment with acetaldehyde or acetic acid did not lead to more than 11% of sprouting after approximately 1 month, much less than the sprouting percentage of the control; in this way these 'products' of the ethanol conversion seem to inhibit sprouting rather than promoting it. Because neither acetaldehyde nor acetic acid promotes sprouting, conversion of ethanol to these products does not seem to be required for breaking of dormancy in potato tubers.

Treatment with 4MP or 4MP + ethanol inhibited sprouting. As an inhibitor of ADH, 4MP should indeed counteract the effect of ethanol, presuming that conversion of ethanol is necessary for breaking dormancy. The fact that 4MP alone also inhibits sprouting suggest that the compound itself inhibits dormancy breaking, or that ADH activity is necessary for breaking dormancy, also in the absence of exogenous ethanol.

Corbineau *et al.* (1991) mention that secondary alcohols (like 2-propanol) are non-oxidizable for (plant/oat) ADH. Also Cohn and Hilhorst (2000) state the fact that methanol, isopropanol and isobutanol are not ADH1 substrates. The results of the experiment, in which the effect of a primary and a secondary alcohol on sprouting was tested, supported the 'ADH theory'. The 1-propanol had a similar effect on sprouting as ethanol, while 2-propanol showed the same sprouting curve as the control. This indicates that an alcohol should be a substrate for ADH, to be effective in breaking dormancy.

Taken together, it is concluded that the products of ADH conversion are not important in breaking dormancy in potato tubers, but the conversion by ADH itself might be. The conversion of alcohols to their aldehydes by ADH is accompanied by the reduction of NAD^+ to NADH (see figure 3.8). Cohn (1987) discusses data on changes in $NADP^+$/NADPH and NAD^+/NADH ratios during germination. He mentions that levels as well as ratios did not change with imbibition time in dormant vs. non dormant seeds, nor did they change when gibberellin or nitrite were applied as triggering agents to dormant seeds, suggesting that NADH formation plays no role in breaking dormancy. On the other hand, Gallais *et al.* (1998) studied pyridine nucleotides levels and redox charges of non-dormant and dormant caryopses of oat (*Avena sativa* L.) and

noticed an increase in NADH content and CRC (catabolic redox charge: NADH/(NADH + NAD^+)) during early germination of non-dormant caryopses, which differed significantly from the NADH content and CRC of non-germinating dormant caryopses. They also found that ethanol provokes increases in NADH and consequently in CRC, which suggests that germination is enhanced or accelerated when the NADH content increases (Gallais *et al.*, 1998). They discussed that the rise in NADH could be due to glycolysis. However, when ethanol is applied to seeds or potato tubers, ADH-mediated ethanol conversion accompanied by NAD^+ to NADH conversion will also lead to an increase in NADH. Also 4MP can interfere with these NAD^+ and NADH levels or CRC. By forming a complex with ADH and NAD^+ (an effect of 4MP; Blomstrand *et al.*, 1979), the level of NAD^+ will decrease and the CRC will concomitantly increase. This will lead to a new (changed) NAD^+/NADH balance, which might affect germination.

Type of secondary structures

Ethanol breaks dormancy in potato tubers and, depending on the sucrose concentration in the medium, different secondary structures are formed (figure 3.1). Ethanol apparently caused the activation of cell divisions and elongation while the sucrose concentration in the medium somehow affected the nature of the secondary structure formed. High sucrose is known to induce tuber formation (Vreugdenhil *et al.*, 1998), whereas low sucrose is known as a non-tuber-inducing treatment (Xu *et al.*, 1998). Sucrose in the medium may regulate (local) sucrose concentrations in the tuber and in this way affect the nature of the secondary structure formed. Measurements of sucrose concentration in whole tubers showed no significant difference after ethanol treatment (8% sucrose, data not shown). On the other hand, Vreugdenhil *et al.* (1998) showed that local differences in sucrose concentration may exist, which were not measured in these experiments.

If osmotic potential would affect the type of secondary structure, one would expect that the treatment with 1% sucrose + 4% sorbitol + ethanol would have the same effect as the 8% sucrose + ethanol treatment. However, treatment with only 1% sucrose + 4% sorbitol inhibited sprouting. When ethanol is added during this treatment, the effect is partly counteracted. When treated with 1% sucrose + 4% sorbitol + ethanol, the nature of the secondary structure was the same as without sorbitol; still sprouts and not secondary tubers were formed. It can be concluded from these results that osmotic potential does not have an effect on the type of secondary structure.

CCC, an inhibitor of GA synthesis, was added to a treatment with 1% sucrose + ethanol. The sprouting rate of this treatment showed no difference with the treatment with 1% sucrose and ethanol, thus CCC itself had no effect on the sprouting rate. However, this particular treatment gave more secondary tubers and thickened sprouts than the treatment without CCC (figure 3.5). This might suggest that a high level of GA caused by the low level of sucrose, is prevented by CCC and consequently no sprouts but secondary tubers are formed.

The possible effect of sucrose on hormone levels has been described before. Xu *et al.* (1998) suggested that endogenous GA levels were regulated by sucrose: single node cuttings formed stolons or tubers depending on the concentration of sucrose used. Low sucrose leads to high GA levels and stolon formation, while high sucrose caused a low GA level and the formation of a tuber. If this theory would hold also for dormant tubers, then tubers treated with ethanol and a low percentage of sucrose should have a high endogenous GA level, leading to sprouts, while a treatment with high sucrose is expected to lead to low GA levels and formation of secondary tubers.

However, the presumed role of GA on the nature of the secondary structure, could not be confirmed by direct measurements of GA_1 levels. Tubers transferred to fresh medium (also containing 8% sucrose), showed only a small decrease in GA levels: after 11 days 20% reduction was observed. The changes in GA level after transfer to medium containing only 1% of sucrose were enormous: the GA level decreased with almost 90% after 4 days, while after 11 days only 1% of the GA was left. The levels in the presence of ethanol were 40 to 60% of the levels in their respective controls, both with 1 and 8% sucrose in the medium. Evidently ethanol does not activate second growth via increased levels of GA.

Clearly, sucrose has an effect on hormone levels (GA), but not the expected effect as described by Xu *et al.* (1998) for developing tubers. Their theory assumed that a low exogenous sucrose level would result in a high endogenous GA level and vice versa. In our experiments a low exogenous sucrose level resulted in a low GA level, a high exogenous sucrose level did not change the level of GA. Moreover, when single-node cuttings were put on a tuber-inducing medium with ethanol, no growth of the axillary bud was observed (data not shown), while this same experiment with GA instead of ethanol gave stolon or shoot growth (Xu *et al.*, 1998). When mature tubers were transferred to medium containing GA or ethanol, (secondary) growth was always observed. From all data, it can be concluded that the process of tuber formation from an axillary bud and the process of tuber sprouting are differently regulated (see also Claassens

and Vreugdenhil, 2000). The sucrose/GA theory proposed by Xu *et al.* (1998) for tuber formation does not hold for tuber sprouting. However, it must be noted that no data are available on GA sensitivity or localisation in this set-up, while it is known that environmental factors can alter the responsiveness to GA (Ross and Reid, 1992 and references therein).

Treatments with ABA, known as a dormancy hormone with a negative effect on seed germination, cell division and elongation, showed indeed that ABA keeps the tubers dormant, with only 9% of them sprouting after 1 month (figure 3.3B). Adding ethanol together with ABA shows that ABA under these conditions is not able to keep the tubers totally dormant; 73% of the tubers is now sprouting after 1 month. ABA promotes tuberization on normal stem cuttings according to Xu *et al.* (1998). Also, the GA/ABA ratio will decrease by adding ABA. ABA alone as well as the GA/ABA ratio would theoretically favour the formation of secondary tubers. Figure 3.5 shows the opposite: only sprouts were formed in the treatment with ABA + ethanol. This suggests that ABA during second growth does not affect the nature of the secondary structure formed.

ABA levels were measured separately around the apical bud and in the 'rest' of the tuber tissue, the mean values are depicted in figure 3.7. When tubers were transferred to 8% sucrose + ethanol the level in the bud-part was almost twice as high as in the bud-part of the untreated tuber, which might explain the formation of a secondary tuber at this site (see Xu *et al.*, 1998). The difference is not so much visible anymore after 11 days. When tubers are transferred to medium containing 1% sucrose + ethanol, the ABA level in and around the bud is very low, which could account for the formation of a sprout. However, after 11 days the amount of ABA is very high in and around the apical bud, which does not fit to the assumption that a low level of ABA is related to sprout growth. The GA/ABA ratio could have an effect on the type of secondary structure (Bruinsma, 1962, Koda and Okazawa, 1983). However, the calculated GA/ABA ratios in figure 3.7 are not different from the trends visible in GA data; low sucrose means a low GA and a low GA/ABA ratio. Hence, these ratios do not explain the observed developmental patterns either.

Although Macnicol and Jacobsen (2001) show that ADH gene expression in the aleurone layer of barley seeds is regulated by an ABA/GA interaction, GA does not seem to regulate ADH enzyme activity in potato tubers (figure 3.6). However, our data indeed suggest that ADH plays an important part in the ethanol-induced breaking of dormancy. Furthermore, Koch *et al.*, (2000) discusses the fact that in maize root tips, alcohol dehydrogenase-1 responds to sugars and that other stresses can also affect *Adh1* expression.

In summary, it was found that ethanol breaks dormancy in potato microtubers. ADH seems to be involved in the breaking of dormancy, but a role for the (acidic) product as described for seeds could not be shown. We suggest a role for ADH in the NAD^+ to NADH conversion, which will presumably result in an increase in NADH and CRC. The reasons for the different types of secondary structure remain uncertain. The sucrose/GA theory is not valid in this situation, which confirms that the mechanism of the regulation of sprouting differs from the one of the regulation of tuber formation, although the endogenous sucrose concentrations and localization should be determined more precisely. It is clearly shown that ethanol by no means increase the level of GA_1.

Controlled expression of transgenes in plants is often used for the characterization of gene function and to regulate growth and development. An alcohol-inducible promoter is already used in tobacco, potato and Arabidopsis (Roslan *et al.*, 2001). However, such an alcohol-inducible promoter should be handled with care, at least in potato (and maybe other plants), because the ethanol concentration used to switch on this promoter, can affect other processes which may interfere with the processes under investigation. Furthermore, research upon storage of potato tubers under controlled atmosphere (CA) conditions with low oxygen (De Graaf, 1991) can cause alcoholic fermentation, which in its turn induces sprouting, which is not always desirable. Especially in the case of seed potatoes a very accurate CA-storage regime should therefore be applied.

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Starch-synthesizing and -degrading enzymes during potato tuber dormancy and sprouting

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Abstract

The possibility of starch cycling at different phases of the tuber life cycle was investigated by studying different enzymes involved in starch synthesis and breakdown throughout the dormancy period. Enzyme activities of ADP-glucose pyrophosphorylase (AGPase), starch phosphorylase (STP) and amylases were determined and all showed a decrease during the first stage of dormancy. However, no clear changes were detected at the time of dormancy breaking and sprouting. AGPase was also determined by *in situ* staining during the whole dormancy period; with this method a clear decrease at the initiation of dormancy and a large increase before visible sprouting could be observed. This increase was especially clear near the vascular tissue and at the apical bud, which showed a very intensive staining. Different isozymes or differences in extraction efficiency for developing and sprouting tubers could be an explanation of the differences observed with both methods. *In situ* staining of STP activity in a sprouting tuber showed that the tissue distribution of STP was the same as observed for AGPase. As a possible explanation direct cycling of starch was suggested: STP produces glucose-1-phosphate during starch breakdown, which can be directly used as a substrate by AGPase for starch synthesis.

Also gene expression studies with the AGPase S promoter coupled to the luciferase reporter gene clearly showed a higher activity in sprouting tubers as compared to dormant tubers. It was not clear, however, whether this activity already increased before visible sprouting.

The presence of amylase activity already during dormancy initiation (quantitative measurements) and AGPase activity still present at the sprouting stage (*in situ* studies), suggests cycling of starch throughout the whole period of dormancy. According to the *in situ* studies, the AGPase activity increases well before visible sprout growth and could therefore be one of the first physiological determinants of dormancy breakage.

Introduction

Potato tubers are formed underground at so called stolons, which develop as lateral shoots at the base of the stems of the potato plant. In order to form tubers, the plants have to become induced to tuberize, *e.g.* by length of photoperiod, temperature or nitrogen limitation. Tuber induction leads to tuber initiation, which is defined as a swelling of the stolon to twice its original diameter (Ewing and Struik, 1992). Tuber initiation is followed by tuber growth and maturation. Then the tuber enters a period of dormancy, which presumably is already initiated during tuber development (Burton, 1978; Claassens and Vreugdenhil, 2000). The length and depth of this dormancy period is dependent on cultivar and environmental factors occurring during tuber development and storage. At the end of the dormancy period the tuber starts sprouting and a new plant is formed, thus completing the potato plant's life cycle.

Potato tubers can also be formed *in vitro* and they mainly differ in size from soil-grown tubers (Xu *et al.*, 1998). In many other aspects, *e.g.*, ultrastructure and the relative activities of enzymes involved in carbohydrate metabolism, they are similar to soil-grown tubers (Fernie and Willmitzer, 2001).

The developing potato tuber, a storage/sink organ by definition, accumulates starch during its development. Starch accumulation occurs already at tuber initiation and is accompanied by an increase in enzymes involved in its biosynthesis (Visser *et al.*, 1994). At maturity, approximately 15 – 25 % of the tuber fresh weight consists of starch. This reserve carbohydrate can be used to provide energy for (future) plant metabolism, as occurring during sprouting. The former sink has then turned into a source and starch is broken down by different enzymes and used to support growth of the new plant.

Starch exists of linear amylose and branched amylopectin. The rate-limiting step in starch biosynthesis is the conversion of glucose-1-phosphate to the precursor for starch synthesis, ADP-glucose (Ball *et al.*, 1998). This reversible conversion is catalysed by the enzyme ADP-glucose pyrophosphorylase (AGPase). ADP-glucose is the substrate for starch synthases, which catalyze the formation of new linear α-1,4 bonds between pre-existing primers and glucose units. The α-1,6 branch point linkages in amylopectin are formed by branching enzymes. All enzymes involved in starch synthesis in potato are supposed to be confined to the amyloplast (Prat *et al.*, 1990), although a cytosolic isoform of AGPase is found in cereal seeds (Denyer *et al.*, 1996; Thorbjørnsen *et al.*, 1996).

Degradation of starch coincides with increases in the activity and products of α-amylase, β-amylase, starch phosphorylase (STP), maltase and debranching enzymes (Preiss and Levi, 1980). Both amylose and amylopectin are hydrolyzed by α-amylase, which hydrolyzes internal α-1,4 linkages yielding glucose, maltose, maltotriose and dextrins. β-Amylase hydrolyzes starch into maltose by sequential removal of disaccharide units from non-reducing ends. Maltase hydrolyzes maltose and maltotriose resulting from the amylase conversions, yielding glucose units. Starch debranching enzymes are capable of hydrolyzing the α-1,6 branch-points, although they are also supposed to play a role in starch synthesis (Van de Wal, 2000). STP catalyzes a reversible reaction: the conversion from $starch_n$ to $starch_{n-1}$ and glucose-1-phosphate (using free phosphate) or the other way around, implying that this enzyme could be involved in both synthesis and breakdown (Brisson *et al.*, 1989).

The last few years evidence is accumulating that futile cycles, *i.e.*, concomitant synthesis and breakdown of metabolites, are part of many metabolic pathways (Krook, 1999; Stitt and Heldt, 1981). It is thought that such cycles offer rapid, flexible and sensitive regulation of fluxes through these pathways. Also for starch metabolism, futile cycles have been suggested (Pozueta-Romero and Akazawa, 1993; Geigenberger *et al.*, 1994; Sweetlove *et al.*, 1996). If present, amylases, STP and AGPase are likely to be part of these cycles and in this report, we will thus focus on these enzymes.

Literature presents conflicting data on the activity of amylases during tuber development, dormancy and sprouting. Davies and Viola (1988) reported a decrease in total amylase activity during sprouting, although the decrease in α-amylase was only transient. Bailey *et al.* (1978) reports a transient increase for α-amylase at the time of sprouting and Biemelt *et al.* (2000) showed an increase of both α- and β-amylase in the sub-eye tissue after the onset of sprouting. The last authors also showed a decrease of β-amylase during the first phase of dormancy, but did not discuss this further.

Also for STP, conflicting data are presented. Literature agrees on an increase in STP activity during dormancy initiation (Bruinsma, 1962; Appeldoorn *et al.*, 1999). However, the latter authors conclude that it is unclear whether STP catalyzes a net degradation or synthesis of starch in this case. During dormancy breaking / sprouting, a temporary rise of STP activity is mentioned by Bruinsma (1962). Davies and Ross (1987) and Davies and Viola (1988) found a decrease in STP activity during that same period, which was also described by Biemelt *et al.* (2000). Bailey *et al.* (1978) reported a transient increase of STP activity at the time of sprouting.

AGPase activity increases during tuberization (Sowokinos, 1976; Visser *et al.*, 1994; Appeldoorn *et al.*, 1999), which parallels the accumulation of starch. However, no data are available on the possible role of AGPase during potato tuber dormancy and sprouting. Therefore, we decided to focus mainly on AGPase activity around the time of tuber sprouting. For comparison, we also determined amylase and STP activities in the same samples.

The discrepancies mentioned above for STP activities might partly be explained by tissue specific changes in activity of this enzyme. Therefore, we also include histochemical analyses of STP and AGPase activities, in order to detect local changes, possibly overlooked when whole tubers are extracted for enzyme assays. Additionally, preliminary AGPase gene expression studies are shown to support the histochemical analyses of AGPase.

Materials and methods

In vitro tuberization system

The protocol to produce microtubers is based on the method of Hendriks *et al.* (1991). *In vitro* plants (*Solanum tuberosum*, cv. Bintje for data on enzymes, cv. Desiree for data on gene activity) were grown with a 16 hrs light period (50 W · m^{-2}, 20°C) and after 4 weeks these plantlets were transferred to soil. They were grown in a growth chamber for 4 weeks with a 16 hrs light period (80 W · m^{-2}, 20°C). To obtain tuber induction in these plants, the light regime was changed to short days (8 hrs light period) for three weeks more. Single node cuttings were taken and after surface sterilization placed on a tuber-inducing medium consisting of modified Murashige and Skoog 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. The explants were incubated in the dark at 20°C and tuber formation started on the 5th day. After 4 weeks the tubers are considered mature and dormant. The diameter of these microtubers is around 8 mm and their fresh weight approximately 500 mg. When stored in the dark at room temperature, the tubers stayed dormant for approximately 4 to 5 months. Tubers used for analysis of enzymes were stored still attached to the stem cutting and on the medium (cv. Bintje). Tubers used for analysis of gene activity were air-dried for 4 to 5 days at 20°C, in darkness at a RH of approximately 80%. In this way, the stem cuttings (and stolons if present) dried out and could be cut off, leaving a minimal wound area (cv. Desiree).

Quantitative measurements of enzyme activities

For the measurements of AGPase, STP and α- and β-amylase activities, samples were taken from various batches differing in physiological state, ranging from the end of tuber development to dormancy, dormancy breakage and the subsequent sprouting. The first sample consisted of tubers of 23 days old, which were considered as still developing. The next samples were of tubers of 86, 149 and 184 days old, when tubers were considered dormant. Approximately half of the batch of tubers of 212 days old was sprouting, and both sprouting and non-sprouting tubers were sampled and analysed separately. At day 240 all tubers were sprouting. In the non-sprouting samples, the apical part was sampled separately from the rest of the tuber. In the sprouting samples, also the sprouts were sampled separately. All data are based on 3 to 6 samples, each consisting of 2 to 4 (parts of) tubers or sprouts.

AGPase and STP: The extraction procedure for AGPase and STP was modified after Appeldoorn *et al.* (1997). In short, 1.2 ml of extraction buffer (50 mM Hepes/KOH (pH 7.4), 5 mM $MgCl_2$, 1mM EGTA, 1 mM EDTA, 10% (v/v) glycerol, 0.1% BSA (bovine serum albumin), 5 mM DTT (dithiothreitol) and 2% (w/v) insoluble PVP (polyvinylpyrrolidone)) was added to 10 mg of powdered freeze-dried tissue. After centrifugation (5 min, 15,000 g), the supernatant was removed and frozen in liquid nitrogen and stored at -75°C until required. The extraction procedure was carried out at 4°C.

Activities of AGPase and STP were determined in 250 µl assay medium (in a 96 well plate) at 30°C, for 10 min. The reactions were started by adding extract or substrate and were measured spectrophotometrically at 340 nm (based on the NAD to NADH conversion), by means of an ELISA reader (SLT labinstruments 340 ATTC).

AGPase was assayed after Appeldoorn *et al.* (1999). The reaction mixture contained 75 mM Hepes/KOH (pH 8.0), 0.44 mM EDTA, 5 mM $MgCl_2$, 3 mM dithiothreitol (DTT), 0.1% bovine serum albumine (BSA), 2 mM ADP-glucose, 1.0 mM NAD, 2.5 U PGM (rabbit muscle; Boehringer, Mannheim), 7.5 U G6PDH (*Leuconostoc mesenteroides*; Boehringer, Mannheim), 20 µM glucose-1,6-bisphosphate, 2 mM 3-phosphoglycerate (3PGA) and 10 mM NaF. This reaction mixture was added to 30 µl of extract and the reaction was started adding 2 mM pyrophosphate (PPi). Blanks had the same reaction mixture, but without substrate (PPi).

STP was also assayed after Appeldoorn *et al.* (1999). The reaction mixture contained 50 mM Hepes/KOH (pH 7.0), 0.4% soluble starch, 0.4 mM NAD, 1.0 U PGM, 1.0 U G6PDH, 15 µM glucose-1,6-bisphosphate and 10 mM Na_3PO_4.

The reaction was started by adding the total reaction mixture to 30 µl of extract. Blanks were carried out with heat-denaturated extracts.

α-amylase and β-amylase: The extraction procedure for α-amylase and β-amylase was modified after Cochrane *et al.* (1991). In short, 0.5 ml of extraction buffer (30 mM ethylenediamine dihydrochloride (pH 7.0 with KOH), 3 mM $CaCl_2$, 20% (v/v) glycerol, 10 mM DTT and 0.5% (w/v) nonidet P-40) was added together with 10 mg of insoluble PVP (polyvinylpyrrolidone) to 10 mg of powdered freeze-dried tissue. After centrifugation (20 min, 6,000 g), the supernatant was removed and frozen in liquid nitrogen and stored at -75°C until required. The extraction procedure was carried out at 4°C.

For measuring α- and β-amylase activity, the 'Ceralpha' and the 'Betamyl' kit (Megazyme, Ireland) were used. All solutions were prepared according to the instruction of the kits and both assays were performed in a 96 well plate. For α-amylase, 20 µl of substrate solution (blocked *p*-nitrophenyl maltoheptaoside, glucoamylase and α-glucosidase) was added to 15 µl of extract and this mixture was incubated at 30°C for 90 min. For β-amylase, 20 µl of substrate solution (*p*-nitrophenyl maltopentaoside, α-glucosidase and stabilisers) was added to 10 µl of extract and this mixture was incubated at 30°C for 60 min. After incubation, both reactions were stopped by adding 200 µl of stopping reagent (1% Trizma base) and the absorbance was read at 405 nm by means of an ELISA reader (SLT labinstruments 340 ATTC). Blanks were carried out without substrate solution.

In situ staining of enzyme activities

For staining of AGPase, tubers of the same batches and thus the same age (with an additional sample of 58 days old) as described for the quantitative measurements have been used. For STP, only sprouting tubers have been stained. Staining is based on the coupling of the reduction of NAD to the reduction of NBT (nitroblue tetrazolium), which results in precipitation of the blue tetrazolium salt. The whole procedure was carried out as described by Sergeeva and Vreugdenhil (2002). In short, sections of 200 µm thickness were cut with a sledge microtome. Sections were immediately fixed in a mixture of 2% paraformaldehyde, 2% PVP 40 and 0.001 M DTT, pH 7.0 at 4°C for 1 h. After fixation, sections were rinsed with water at least five times to remove soluble carbohydrates and stored overnight in water.

AGPase staining was performed by incubating the tissue sections at 30°C for 30 min in 1 ml of reaction medium. This medium is similar to the reaction mixture for the quantitative measurements, with addition of NBT. The medium consists of 75 mM Hepes/NaOH (pH 8.0), 0.44 mM EDTA, 5 mM $MgCl_2$, 0.1% BSA, 1

mM NAD, 2 U PGM, 6 U G6PDH, 20 µM glucose-1,6-bisphosphate, 2 mM 3PGA, 10 mM NaF, 1.4 mM PPi, and 0.03% NBT. The reaction was started by adding 2 mM ADP-glucose. The control reaction was carried out by omitting the substrate ADP-glucose (Sergeeva and Vreugdenhil, 2002).

STP staining was performed by incubating the sections at 30°C for 30 min in 1 ml of reaction medium, consisting of 50 mM Hepes/KOH (pH 7.0), 0.4% soluble starch, 0.4 mM NAD, 1.0 U PGM, 1.0 U G6PDH, 15 µM glucose-1,6-bisphosphate, 10 mM Na_3PO_4 and 0.03% NBT. This medium is based on the quantitative measurement as described above and derived from Appeldoorn *et al.* (1999). Controls were carried out without soluble starch or Na_3PO_4, or both.

Stained sections were studied with a Leica binocular or a Nikon Optiphot microscope in bright field mode. A digital Panasonic Colour Video Camera or a Sony CCD camera DKR 700 was used to take photographs.

AGPase gene expression

For monitoring AGPase gene expression throughout the dormancy period, transgenic plants were used, containing the luciferase reporter gene (from the American firefly, *Photinus pyralis*) fused to the AGPaseS promoter (Verhees *et al.*, 2002).

The AGPaseS-luc transgenic tubers were sprayed with the substrate solution (1 mM firefly D-luciferin, sodium salt, Duchefa; 0,01% Tween 80) 24 h and 3 h before measurement. For monitoring the light emitting reaction of luciferase, tubers were placed under an intensified CCD camera (C2400-77, Hamamatsu Photonics, Japan). Images were generated by integrating emission of photons for 15 min. The pixel intensity of these images is a direct measurement for the activity of the luciferase enzyme present in the tubers and is shown by a false colour scale. These measurements were done at different time points during dormancy and sprouting of one batch of tubers.

Results

Quantitative measurements of enzyme activities

Activities of AGPase and STP during tuber development, dormancy and sprouting are depicted in figure 4.1. Activities were determined separately in the apical part, comprising approximately 10% of the tuber tissue around and including the apical bud, and in the rest of the tuber tissue. When tubers were sprouting, the sprouts were sampled separately, viz., at days 212 and 240. At day

212 50% of the tubers had sprouted and the enzyme activities are shown separately for sprouting and non-sprouting tubers.

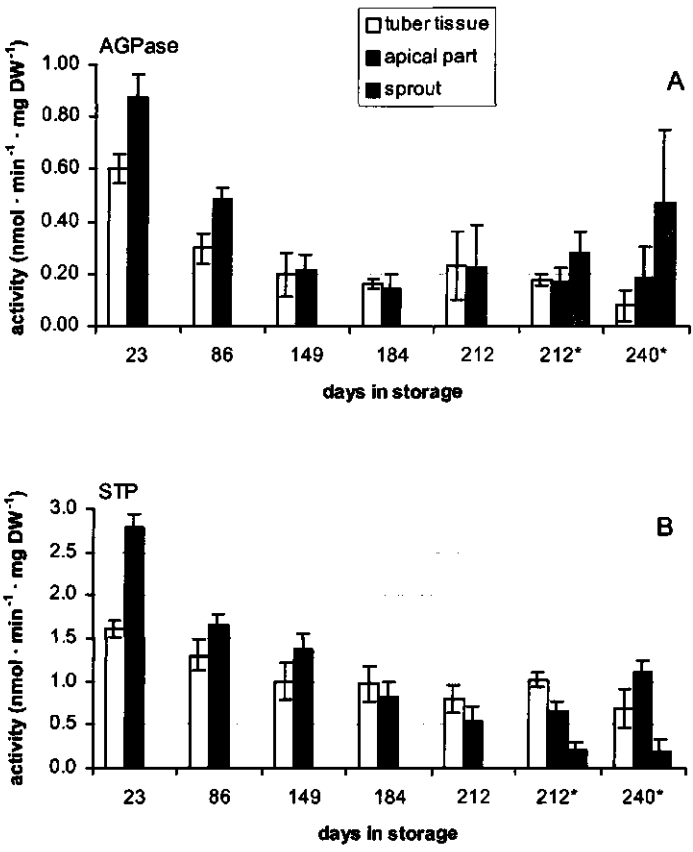

Figure 4.1 AGPase (A) and STP (B) activities in different parts of potato microtubers during dormancy and sprouting. Activities were determined in the apical part of the tuber (approximately 1/10th of total tuber mass), the rest of the tuber tissue and in the sprouts (if present). Asterisk (*) indicates that tubers are sprouting. Tubers of 212 days old are split into sprouting (212*) and non-sprouting tubers (212). Activity is presented as nmol · min^{-1} · mg DW^{-1} ± SD. All data are based on 3 to 6 samples, each consisting of 2 to 4 (parts of) tubers or sprouts.

It is clear that the AGPase activity of both the apical eye and the rest of the tuber tissue declined during the first phase of dormancy. At the first two days of sampling (days 23 and 86) the AGPase activity in the apical part was higher than in the rest of the tuber. This difference disappeared when the tubers were stored for a longer time. No significant differences in AGPase activity were observed between sprouting tubers and non-sprouting tubers at the end of the dormancy

period (*i.e.* non-sprouting tubers of 184 and 212 days old and sprouting tubers of 212 and 240 days old). In tubers that are just sprouting (day 212) the AGPase activity in the sprout does not differ significantly from the activity in the rest of the tuber. However, in older tubers (day 240) the activity in the sprout seemed to increase.

The STP activity, depicted in figure 4.1B, also showed a decline in activity during the first phase of dormancy, most noticeably in the apical part of the tuber. At the end of the dormancy period, not much difference in STP activity was found between dormant and sprouting tubers, although a slight tendency to increase was observed for the apical parts of sprouting tubers. The STP activity in the sprouts is remarkably low, significantly lower than in the apical part and the rest of the tuber tissue.

Also for the α-amylase, depicted in figure 4.2A, a decrease in activity during the first phase of dormancy was found. In developing tubers (day 23), a higher activity in the apical part can be seen when compared to the rest of the tuber tissue. During dormancy, the activity of α-amylase gradually declined until the end of the dormant period (day 212, non-sprouting). During sprouting an increase was observed, but only in the apical parts of the tubers.

The decrease in activity in the first phase of dormancy was also observed for β-amylase, both for the apical part as well as for the rest of the tuber tissue. During later phases of dormancy and the initial phase of sprouting, the levels of β-amylase activity were constant. In sprouting tubers (day 240) β-amylase activity increased in the apical part (figure 4.2B).

In situ staining of enzyme activities

The same batches of tubers as used for the quantitative measurements of enzyme activities were also used for *in situ* staining of AGPase enzyme activity. For localization of STP activity, only sprouting tubers were stained. Figures 4.3A-1 to A-7 represent tubers through a complete dormancy period, including sprouting and the sprout itself. At the end of tuber development (23 days old tuber, figure 4.3A-1), the overall AGPase activity appeared to be very low, although some staining is visible in the apical bud, mainly in vascular tissue. During the first phase of dormancy (58 days old), some activity remained in the vascular tissue in the tuber, towards the apical bud. The 86 days old tubers, *i.e.*, dormant ones, did not show any activity (figure 4.3A-3). Similarly, at day 149, no activity was observed (data not shown). When the tubers were 184 days old, and did not show visibly sprouting yet, high AGPase activity was visible in the vascular tissue

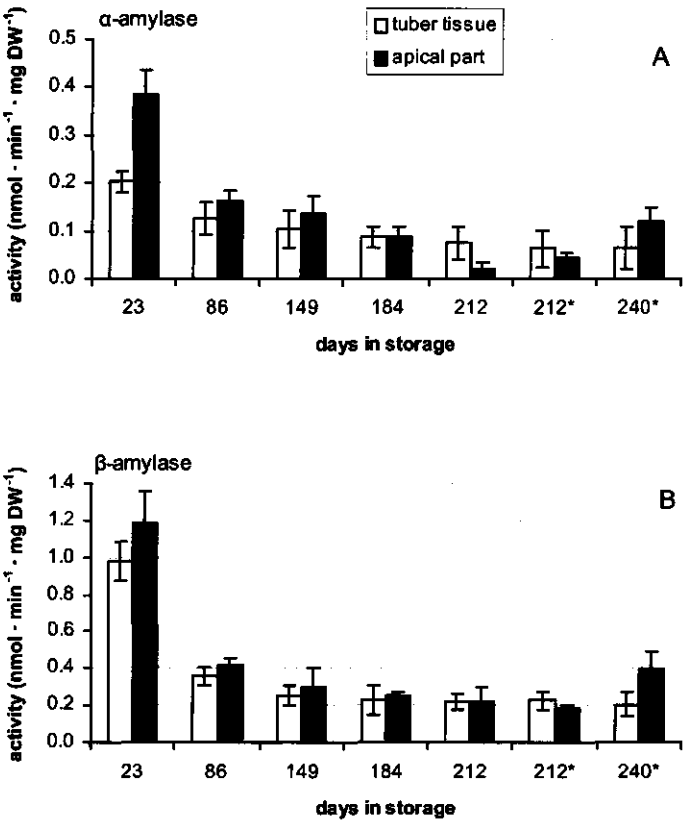

Figure 4.2 α-amylase (A) and β-amylase (B) activities in different parts of potato microtubers during dormancy and sprouting. Activities were determined in the apical part of the tuber (approximately 1/10th of total tuber mass) and in the rest of the tuber tissue. Asterisk (*) indicates that tubers are sprouting. Tubers of 212 days old are split into sprouting (212*) and non-sprouting tubers (212). Activity is presented as nmol · min^{-1} · mg DW^{-1} ± SD. All data are based on 3 to 6 samples, each consisting of 2 to 4 (parts of) tubers.

towards and in the apical part of the tuber, and also in the vascular tissue in the rest of the tuber (figure 4.3A-4). The same pattern was observed for tubers of 212 days old and 225 days old, *i.e.*, a non-sprouting and a sprouting tuber, respectively (figure 4.3A-5,6). Also the vascular tissue of the sprout was stained, as shown in figure 4.3A-6 and A-7, and in an enlarged view in figure 4.3A-11.

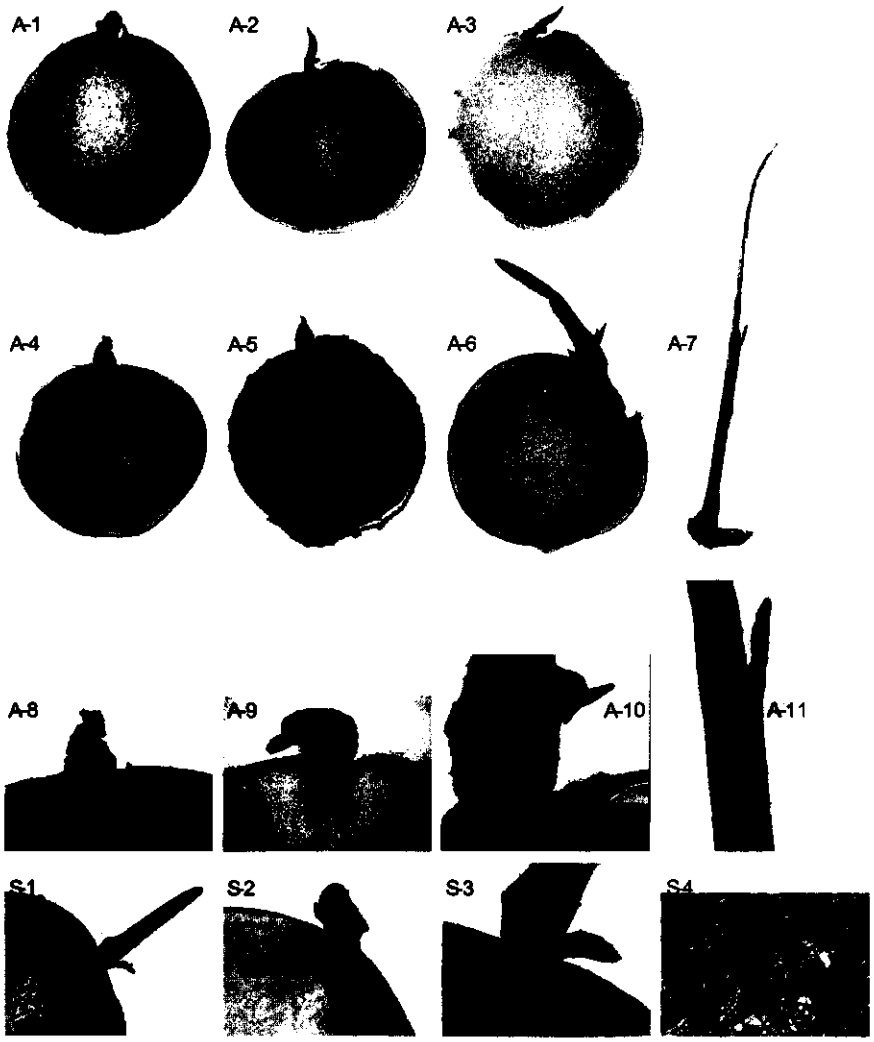

Figure 4.3 Localization of AGPase (A) and STP (S) activity in potato microtubers at different stages of their life cycle.
A-1: AGPase staining in developing tuber, almost mature, 23 days old; A-2: dormant tuber, 58 days old; A-3: dormant tuber, 86 days old; A-4: dormant tuber, presumably around dormancy breaking, 184 days old; A-5: non-sprouting tuber from a sprouting batch, 212 days old; A-6: sprouting tuber, 225 days old; A-7: sprout, 240 days old.
A-8: enlargement of A-4, very high staining in apical part (eye) and vascular tissue; A-9: control staining of sprouting tuber (without ADPglucose), no staining visible; A-10: enlargement of base of sprout: staining in vascular tissue and meristems of the buds; A-11 enlargement of sprout (A-7), showing high staining in vascular tissue.
S-1: STP staining in sprouting tuber, 240 days old; S-2: control staining of sprouting tuber (without soluble starch and Na_3PO_4), staining visible in vascular tissue, parenchyma cells and in phelloderm cells; S-3: enlargement of S-1, sprouting tuber, staining in vascular tissue; S-4: enlargement of S1, staining in first layer of phelloderm.

Figure 4.3A-8 is an enlargement of A-4, revealing the very dark staining in the apical bud. For comparison, a control (without ADP-glucose), completely lacking blue staining is shown in A-9. Figure 4.3A-10 shows an enlargement of the base of a sprout, in which staining in vascular tissue and meristems is visible.

Figure 4.3S-1 shows results on STP staining in a sprouting tuber. Like AGPase activity, it was present in the vascular tissue in tuber and sprout. Also, a band of activity appeared to be present in or around the phellogen layer. A control staining (without soluble starch and phosphate) is shown in S-2, in which some staining is still visible in the apical bud and around the phellogen layer, although much lower than in the presence of the STP substrates. Figure 4.3S-3 is an enlargement of S-1, clearly showing high activity in vascular tissue, storage parenchyma and meristems. S-4 is an enlargement of the band of staining around the phellogen layer, revealing that the first layer of the phelloderm is stained rather than the phellogen layer itself.

AGPase gene expression

For measuring AGPase gene expression, microtubers were obtained from an AGPase-*luc* transformant, carrying the firefly luciferase gene under control of the AGPase S promoter. The microtubers were stored in a 24 well plate. Just before measurement with the highly sensitive camera, the apical buds were turned upwards facing the camera. The same tubers were measured every few weeks and figure 4.4 shows a luciferase picture of dormant tubers (A, 128 days old) and the same tubers but sprouting (B, 191 days old). The batch of tubers measured here sprouted for 87%. The light emitted by the luciferase enzyme is visualized in a pseudo colour scale (blue: low activity, red: high activity).

There was a large difference in luciferase activity, represented by pseudo colours in figure 4.4, between dormant and sprouting tubers. Almost none of the 48 dormant tubers (figure 4.4A) showed luciferase activity, except three of the tubers in the upper plate and maybe 1 or 2 tubers in the lower plate. Of the 23 tubers, still present in the wells after 191 days, at least 20 showed high luciferase activity, the remaining three also showed activity but lower. Some of the tubers showed luciferase activity at two spots, which either represents sprouting of a lateral bud or the presence of an axillary bud on the main sprout.

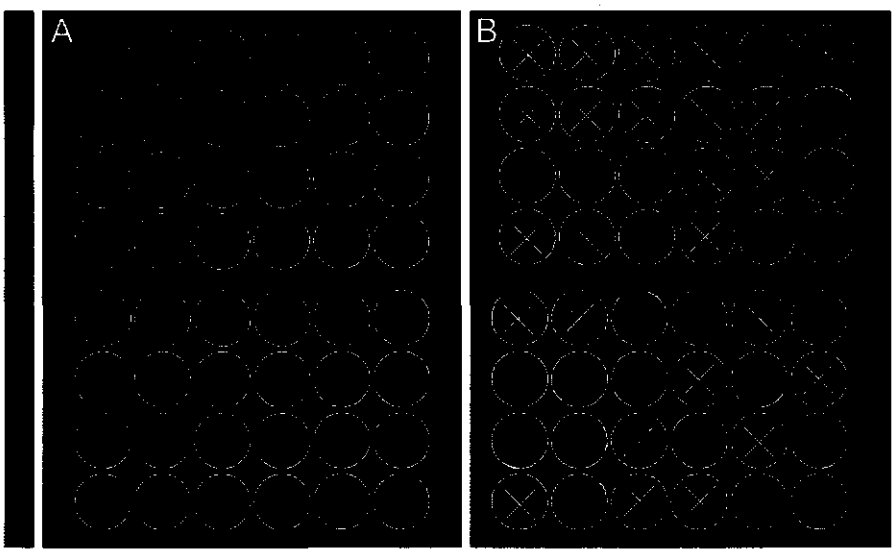

Figure 4.4 AGPase gene expression in transgenic potato microtubers. AGPase-*luc* expression pattern of the same batch of tubers was measured after 128 days (A; dormant tubers) and after 191 days (B; sprouting tubers). The luciferase activity is displayed in pseudo colours, ranging from blue (low activity) via green and yellow to red (high activity). In each well, 1 microtuber is present and the apical bud is turned to the camera. Wells marked with a cross did not contain a tuber anymore.

Discussion

Timing of sprouting

Although an *in vitro* system is very synchronous in tuber formation (start of visible tuber formation takes place within 2-3 days for all explants), sprouting occurs over a period of approximately 4 weeks (figure 1.4), which is similar to the sprouting period for field grown tubers (Claassens and Vreugdenhil, 2000). Moreover, the start of the sprouting period of a batch of microtubers can differ up to 4 weeks for various microtuber batches. In theory, this could mean that two successive independent batches of tubers, for example the batches investigated after 184 and 212 days in storage in figure 4.1 and 4.2, are in the same physiological state or, that the difference in physiological age is not 4 weeks but 8 weeks, something to keep in mind when interpreting the results.

In general, dormancy is described as 'a temporary suspension of visible growth of any plant structure containing a meristem' (Lang *et al.*, 1987). This means that non-sprouting tubers of 212 days old are, strictly speaking, dormant tubers. However, the term breaking of dormancy, which is only defined as something

happening at a time-point before visible sprouting, could also refer to these tubers, supposing they are about to sprout in the coming two weeks. For changes occurring during the breaking of dormancy, both samples of 212 days old and the tubers of 184 and 149 days old are of importance. Visibly sprouting tubers (212 and 240 days) are not dormant anymore and have broken dormancy already. Non-sprouting tubers of 212 days old can have broken dormancy. Tubers of 184 days could start sprouting at that very moment and so part of this batch maybe also have broken dormancy; on the other hand, the total batch could also be still dormant. Tubers of 149 days old are dormant; the term deep dormant may be used to distinguish them from tubers just entering the dormant period (23 days old) and tubers at the end of dormancy (184 days old).

Quantitative measurements of enzyme activities

The activity of AGPase in the first phase of dormancy decreased, as can be seen in figure 4.1A. It is quite logical that the enzyme activity is very high during tuber development (Appeldoorn *et al.*, 1999), as the tuber is a sink organ which accumulates a lot of starch in this period (Visser *et al.*, 1994). In the experiment shown in figure 4.1A, we also measured 9 days old microtubers (not separated in tuber tissue and apical part), which showed for AGPase an activity of approximately 3 nmol substrate · min^{-1} · mg DW^{-1}, *i.e.*, approximately 4 times higher than at day 23 (results not shown).

Also STP and amylase activities (figure 4.1B and 4.2) show a clear decline in the beginning of dormancy. Compared to AGPase which decreases from 3 nmol · min^{-1} · mg DW^{-1} during tuber development to 0.2 during dormancy, the decrease of STP activity is not as steep, *i.e.* from 2.5 nmol · min^{-1} · mg DW^{-1} during tuber development (data not shown) to approximately 1.0 during tuber dormancy. Although it is clear that STP activity is present, it is not known whether it is involved in starch synthesis or breakdown (Brisson *et al.*, 1989), but during tuber development breakdown is likely to occur through amylases, which also show a high activity.

In general, none of the enzymes discussed above showed a clear trend in activity during the sprouting period. Only, some marginal effects can be seen. For AGPase, the activity of the sprout was a bit elevated, although the standard deviation was very high in these measurements. We indeed found some starch in the (base of the) sprout, a lot less though than in the tuber (data not shown). For STP, an increase in the apical part was observed when comparing activity in sub-eye tissue of 240 days old tubers to both sprouting and non-sprouting tubers of 212 days old (figure 4.1B). Because the comparison is made to 212 days old tubers, these changes may be related to sprouting and not to breakage of

dormancy. The same can be concluded for α-amylase (figure 4.2A). For β-amylase, there seems to be a small increase in activity in sub-eye tissue of 240 days old tubers compared to tubers of 184 and 212 days old. As the sprouting tubers of 212 days old did not show this increase, this increase in β-amylase might be a (late) result of sprouting, as already mentioned by Biemelt *et al.* (2000).

In situ staining of enzyme activities

The results on AGPase activity *in situ* (figure 4.3) are in contrast with the quantitative measurements discussed above. Sergeeva and Vreugdenhil (2002) show high AGPase staining in a developing tuber of 12 days old, resembling the tubers in figures 4.3A-4 and A-5, with clear staining connected with the vascular bundles. The activity still visible at 58 and 86 days (figure 4.3A-1 and A-2) completely disappeared at 149 and 186 days (figure 4.3A-3). So, the initial decrease of AGPase activity during and after tuber development (figure 4.1B) is also visible *in situ*.

However, in contrast to the activity measurements, already before and during the sprouting period an elevated level of AGPase activity, especially at the apical eye, is clearly shown (figure 4.3A-4, A-5, A-6). Also the sprout itself shows a blue precipitate, which indicates AGPase activity. The fact that AGPase staining is clearly visible before visible sprouting, indicates that AGPase is someway connected to dormancy breaking or is at least one of the first signals of the breaking of dormancy in potato tubers.

The possibility that nitroblue tetrazolium is precipitated by another enzyme than AGPase is very low. Figure 4.3A-9 shows a control staining without the substrate ADP-glucose, in which no blue precipitate could be observed, indicating that the enzyme giving the blue precipitate uses ADP-glucose as a substrate. Recently, another enzyme breaking down ADP-glucose has been described, viz. ADP-glucose pyrophosphatase (AGPPase), which breaks down ADP-glucose to glucose-1-P and AMP, and in doing so, it may limit starch synthesis *in vivo* (Kleczkowski, 2001). However, the ADP-glucose breakdown catalyzed by AGPPase is inhibited in a competitive manner by PPi and 3PGA and its optimal activity occurs at pH 6.0 (Rodríguez-López *et al.*, 2000), whereas the AGPase in our experiments was assayed at pH 8.0 in presence of PPi and 3PGA. Moreover, a control for AGPase was carried out without both PPi and 3PGA, which gave no staining at all (data not shown). Thus, the precipitate shown in figure 4.3A-1 to A-11 is very likely to be the result of AGPase activity only.

The remarkable increase of AGPase activity at the apical eye, shown well before visible sprouting, did not appear from the quantitative measurements, even not in

extracts of the apical part. The difference between the quantitative and *in situ* measurements could be caused by the presence of different isoforms of AGPase (La Cognata *et al.*, 1995; Thorbjørnsen *et al.*, 1996); during the extraction procedure for the quantitative activity measurements the isoform responsible for the *in situ* staining might not be extracted. This would also mean that different isoenzymes of AGPase are active during tuber development and dormancy breaking / sprouting. On the other hand, the extraction of AGPase enzyme may be simply more difficult from amyloplasts in older tubers, or there could be a component present in the older tubers, which interferes with the AGPase activity measurements.

STP activity (figure 4.3S-1 to S4) shows approximately the same staining pattern as AGPase activity, except for the first phelloderm layer. In this layer no AGPase activity is present, while STP activity clearly is. Moreover, starch granula disappeared from this layer. The presence of the STP activity here indicates that the enzyme is involved in starch breakdown.

The experiments described above, show the importance of accurate localization studies (Sergeeva and Vreugdenhil, 2002) and the necessity for critical evaluation of the current AGPase extraction methods.

AGPase gene expression

The AGPase enzyme is a heterotetramer composed of two large and two small subunits, AGPase S and AGPase B, respectively. There are three genes in potato encoding the large subunit and one gene encoding the small subunit (Smith *et al.*, 1997) and all four are expressed in the tuber. Furthermore, the expression of these genes can be differentially regulated, *e.g.*, AGPase S2 is expressed stronger in sink leaves than in source leaves and its expression is induced by exogenous sucrose (La Cognata *et al.*, 1995). The AGPase S-*luc* transformant used in this study showed an elevated expression in sprouting tubers (figure 4.4). Whether this expression increased before visible sprouting remains unclear, mostly due to the asynchronicity of the sprouting period and the low number of tubers in this experiment. The increased level of the expression of this subunit would indicate that this subunit could be part of the AGPase isozyme, which showed an elevated activity in the enzyme staining studies, mentioned earlier. If this is the case, then another AGPase isozyme has to be active during tuber development and not during dormancy breaking. As the promoter of the subunit studied here also shows high activity during tuber development (Verhees *et al.*, 2002), the presence of different isozymes during tuber development and dormancy breaking becomes less likely. This gene expression study indicates regulation of AGPase on the transcriptional level rather than at the translational level.

Starch cycling?

Active amylases could be extracted from developing tubers, suggesting starch breakdown during this period. The increase in AGPase enzyme activity *in situ*, well before visible sprouting, suggests starch synthesis before and during the sprouting period. These findings suggest starch cycling during all periods of potato tuber life cycle.

The staining patterns in sprouting tubers for AGPase and STP are similar, which might suggest a direct starch cycling, with STP degrading starch, forming glucose-1-phosphate, being the substrate for AGPase. On the other hand, STP itself may also be involved in starch synthesis (Müller-Röber *et al.*, 1992), and in this case, both enzymes would form starch in the direct neighbourhood of the vascular tissue. One might hypothesize then that amylases degrade starch in storage parenchyma cells, the resulting sugars are transported towards the vascular tissue, and starch is synthesized again around the vascular tissue. The high activity of AGPase and STP found in the bud could be well explained by the fact that vascular bundles converge towards the bud.

Local increases in sucrose might upregulate AGPase expression (La Cognata *et al.*, 1995) and subsequent enzyme activity here. Geiger *et al.* (1998) showed that exogenous supply of sucrose indeed stimulated starch synthesis. In that case, the AGPase activity observed in this report would be an early result of the dormancy breaking process, and not a trigger. On the other hand, the increase in AGPase activity as seen in figure 4.3A-4 is relatively early and worthwhile to study in more detail. Activities and localization of other enzymes involved in sugar metabolism, *e.g.*, sucrose synthase, invertase, hexokinase and phosphoglucomutase in the same *in vitro* system will be further investigated.

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5

Sugar metabolism in dormant and sprouting potato tubers (*Solanum tuberosum* L.)

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Abstract

Sucrose metabolism and its relation to starch cycling was investigated throughout the period of dormancy and sprouting of potato tubers. Neutral sugars decreased in the tuber during dormancy, reaching a constant level before visible sprouting occurs. Activities of susy as well as invertases were very low in the tuber during dormancy and sprouting. In histochemical analysis, invertase could not be detected in the tuber, whereas susy showed only a low level of staining. These results suggest that sucrose breakdown is very limited in the tuber and that the observed decrease of sucrose during the dormancy period is probably the result of susy activity.

The sprout showed a very high activity of invertases, while susy activity was very low again. Sucrose content was high in very young sprouts, indicating that breakdown via invertase started somewhat later. In older sprouts, sucrose and fructose decreased again, while glucose accumulated. This accumulation is explained by the fact that there was almost no hexokinase activity in the sprout. Although not so high in activity as well, there seemed to be enough fructokinase for phosphorylating a large part of the fructose present in the sprout.

In apical parts of sprouting tubers, a high level of glc-1-P was found and the ratio between glc-6-P and glc-1-P was altered, in favour of glc-1-P, presumably due to a modified PGM activity. In this way PGM can channel carbohydrates in the direction of starch synthesis. However, the low level of 3PGA (which is a positive regulator of AGPase and so of starch synthesis) found in the tuber during early sprouting might indicate that the elevated level of glc-1-P is not used for starch synthesis but for sucrose synthesis, necessary to supply carbon to the growing sprout.

In young sprouts, especially glc-1-P and 3PGA increased next to frc-6-P, glc-6-P and UDPglc. The latter increases could indicate a higher level of glycolysis, OPPP and cell wall synthesis, respectively. The high levels of glc-1-P (presumably supplied via PGM) and 3PGA suggest a stimulated starch synthesis. In this way the sprout is able to store carbohydrates (temporarily) and adjust the carbon fluxes to the requirements of the cell. Sucrose cycling is unlikely to occur in the sprout, due to the high invertase activities.

The results presented here suggest an important role for starch cycling during dormancy breaking and sprouting. PGM is supposed to perform a regulating role in this cycling.

Introduction

Freshly harvested potato tubers do not sprout, since they are dormant. This dormancy state is presumably already initiated during tuber development (Burton, 1978; Claassens and Vreugdenhil, 2000). After a certain period of time (dependent on cultivar and environmental factors occurring during tuber development and storage), dormancy ends, leading to sprouting and a new plant is formed.

Within this life cycle, the tuber represents both source and sink organs. Source organs are defined as net exporters of carbohydrates, sink organs as net importers. Two kinds of sink organs can be distinguished; utilisation sinks, which are metabolically very active, rapidly growing organs, in which the imported assimilates are mainly subjected to respiration and used for biosynthesis of cellular structures; the other type of sinks are storage sinks, which use most of the imported assimilates for synthesis and accumulation of reserve material (Müller-Röber *et al.*, 1990). In the potato tuber, reserves are mainly stored as carbohydrates (starch). Thus, a (growing) stolon is an utilisation sink and as soon as tuber initiation and growth starts, it can be defined as a storage sink. During dormancy breaking a change from storage sink to a source organ is likely to occur, although the apical meristem may be defined as an utilisation sink, while the rest of the tuber is a source organ. The emerging sprout is then a rapidly growing utilisation sink.

The assimilates, produced in source tissues such as leaves, are transported mainly as sucrose. During tuber development, sucrose is loaded into the phloem (in the leaves) and transported to the underground developing tubers (storage sink organs), where it can be unloaded and transported within the tissue in an apoplastic or symplastic way. Apoplastic uptake of sucrose by the cells can take place via sucrose or hexose transporters, the latter only after hydrolysis of sucrose by (cell wall-bound) invertase, leading to glucose and fructose. Sucrose in the cell can be degraded by sucrose synthase (susy), leading to fructose and UDP-glucose, or by invertase. The hexoses can be phosphorylated and the resulting hexose-phosphates can enter glycolysis, the oxidative pentose phosphate pathway (OPPP), cell wall synthesis or starch synthesis. In developing tubers, a major part of the hexose-phosphates will enter starch synthesis.

At dormancy breakage, sucrose and hexoses already present in the cells can be utilised. Thereafter, starch has to be mobilized, either by amylases or by starch phosphorylase, yielding glucose or glucose-1-phosphate. For transport, hexoses or hexose-phosphates are usually converted into sucrose. Glucose can be

converted into glucose-6-phosphate and subsequently to glucose-1-phoshate. The latter one can be converted into UDP-glucose and together with fructose-6-phosphate this forms sucrose-6-phosphate, which will end up as sucrose by the action of sucrose phosphate phosphatase. Susy can also synthesize sucrose from UDP-glucose and fructose, although it is generally assumed that this enzyme is mainly involved in sucrose breakdown. After transport, sucrose will be broken down again, via susy or invertases to be used for glycolysis, the oxidative pentose phosphate pathway (OPPP), cell wall synthesis or starch synthesis. As described in the previous chapter, starch cycling is likely to occur in potato tubers, as suggested by the high activity of AGPase at the end of dormancy. However, during dormancy breaking and sprouting starch synthesis is not the major process going on in these tubers.

Appeldoorn *et al.* (1999) showed an increase in AGPase during tuber development, which leads to the substrate for starch synthesis (ADPglc), from the moment the stolon starts to swell. Concomitantly, a switch occurred from a hydrolytic sucrose degradation route (invertase) to a predominantly susy-catalysed one (Appeldoorn *et al.*, 1997). As starch synthesis presumably also takes place at the end of the dormancy period, the question arises whether sucrose breakdown is related to this cycling of starch, and, if so, what kind of degradation route is used.

The molecule sucrose seems to be very important in various regulatory processes in plant cells. For example, exogenous supply of sucrose to sliced discs of potato tubers leads to stimulation of starch synthesis (Geiger *et al.*, 1998), while in premature cotyledons of *Vicia faba*, exogenous sucrose tends to stop mitosis and induces cellular differentiation and storage processes (Weber *et al.*, 1996). Sucrose in developing seeds of transgenic *Vicia narbonensis*, in which a yeast cell wall-bound invertase was introduced, is converted into glucose and fructose. As a consequence, sucrose and starch content are reduced, together with the activity of sucrose synthase and AGPase (Weber *et al.*, 1998). These and other findings lead to the suggestion that starch accumulation in storage sink tissues could be a function of sucrose concentration (Weber *et al.*, 1998). Finally, the switch from an invertase- to a susy-catalysed sucrose breakdown allows a higher cytosolic sucrose content, as susy shows a lower affinity for sucrose than invertase (Appeldoorn *et al.*, 1997 and references therein), which might affect AGPase activity. Not only the sucrose content or enzymes involved in sucrose breakdown (susy and invertases) may influence to starch synthesis, but also other metabolites and enzyme activities may have effect. One enzyme in particular may be of interest, viz. phosphoglucomutase (PGM), catalyzing the reversible reaction between glc-6-P and glc-1-P. These two components are usually supposed to be in

equilibrium. However, Hattenbach and Heineke (1999) mention that PGM can be removed from equilibrium by special conditions, such as osmotic stress, whereas PGM was demonstrated to be inhibited by accumulation of fructose-1,6-bisphosphate and /or ribulose-1,5-bisphosphate. Furthermore, Fernie *et al.* (2001; -2002) state that both the plastidial and cytosolic PGM play an important role in the regulation of starch synthesis or accumulation within the tuber.

The previous chapter suggested starch cycling during tuber development as well as during tuber sprouting. In this report, the suggested close relationship between sucrose and starch metabolism is studied throughout tuber dormancy. Therefore, the levels of various carbohydrates and the activities of enzymes, which convert these carbohydrates, are determined, as their regulation is very tightly connected (Appeldoorn *et al.*, 1999). Since these processes might be different in source and sink parts of sprouting tubers, localization studies of enzymes have also been included.

Materials & Methods

In vitro tuberization system

The protocol to produce microtubers is based on the method of Hendriks *et al.* (1991). *In vitro* plants (*Solanum tuberosum*, cv. Bintje) were grown with a 16 hrs light period (50 W · m^{-2}, 20°C) and after 4 weeks these plantlets were transferred to soil. They were grown in a growth chamber for 4 weeks with a 16 hrs light period (80 W · m^{-2}, 20°C). To obtain tuber induction in these plants, the light regime was changed to short days (8 hrs light period) for three weeks more. Single node cuttings were taken and after surface sterilization placed on a tuber-inducing medium consisting of modified Murashige and Skoog 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. The explants were incubated in the dark at 20°C and tuber formation started on the 5th day. After 4 weeks the tubers are considered mature and dormant. The diameter of these microtubers is around 8 mm and their fresh weight approximately 500 mg. When stored in the dark at room temperature, the tubers stayed dormant for approximately 4 to 5 months. Tubers used for analysis of neutral and phosphorylated sugars were air-dried for 4 to 5 days at 20°C, in darkness at a RH of approximately 80%. In this way, the stem cuttings (and stolons if present) dried out and could be cut off, leaving a minimal wound area. Tubers used for analysis of enzymes were stored in the dark at 20°C, still attached to the stem cutting and on the medium.

Neutral and phosphorylated sugars

For determinations of neutral and phosphorylated sugars, tubers were air-dried as described above and these microtubers were then stored at 10°C in the dark. During the dormancy period, the apical halves of the tubers were sampled every four weeks. As soon as the first tubers of the batch started to sprout, another sample was taken, while the last sample was taken two weeks later; in all cases apical halves of non-sprouting tubers were used. At the last time point, also sub-apical tuber tissue of sprouting tubers (cube of 2 by 2 by 2 mm) and very young sprouts (2 to 3 mm) were sampled.

Extraction was performed following the protocol earlier described by Herbers *et al.* (1997). In short, frozen tuber tissue was homogenized to powder in liquid nitrogen, in a mortar placed on dry ice (solid CO_2). The powdered material was then further homogenized in 2.0 ml 16% (w/v) trichloroacetic acid (TCA) in diethylether (4°C) and left for incubation for 15 min. After this short incubation 0.8 ml 16% TCA in water, containing 5 mM EGTA (4°C) was added to the homogenate and incubation was continued for 3 h at 4°C. The homogenate was then centrifuged for 7 min. at 15,000 g and the water phase was washed 3 times with 600 µl water-saturated ether, after which it was neutralized with 5 M KOH in 1 M triethanolamine. This extract was frozen in liquid nitrogen and stored at -75°C until required.

Glucose, fructose and sucrose were determined as described by Stitt *et al.* (1989), with an ELISA-reader at 340 nm in a 96 wells plate. These compounds were measured in a buffer containing 100 mM Imidazol-HCl, 5 mM $MgCl_2$, 2 mM NAD, 1 mM ATP and 2 U glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*) with a final pH of 6.9. Hexokinase (HK) was added to start the reaction for measuring glucose, then phosphogluco-isomerase (PGI) was added to measure fructose and finally invertase was added to measure sucrose.

UDPglucose (UDPglc), glucose-1-phosphate (glc-1-P), glucose-6-phosphate (glc-6-P), fructose-6-phospate (frc-6-P) and 3-phosphoglycerate (3PGA) were determined photometrically as in Stitt *et al.* (1989), using a dual wavelength spectrophotometer (Sigma-ZWS II, Eppendorf GmbH, Hamburg). In short, glc-6-P, frc-6-P and glc-1-P were measured using the extracts in a buffer, containing 50 mM Hepes, 5 mM $MgCl_2$ and 15 mM NADP. The reaction was started by adding G6PDH (yeast) to measure glc-6-P, then PGI was added to measure frc-6-P, and finally PGM was added to measure glc-1-P. UDPglc was measured in a glycine buffer, pH 8.7, with 5 mM $MgCl_2$ and 30 mM NAD. This reaction was started by adding UDPglucose dehydrogenase. Finally, 3PGA was measured in a buffer containing 100 mM Tris-HCl, pH 8.1, with 5 mM $MgCl_2$, 40 mM ATP and

3 mM NADH. The reaction was started using phosphoglycerate kinase and glyceraldehyde-P dehydrogenase.

The distribution of neutral sugars in sprouting tubers, stored at 10°C in the dark, was measured using Dionex HPLC. Sprouting tubers were split into 5 parts: basal half of tuber, apical half of tuber, basal part of sprout, middle part of sprout and the tip of the sprout. The extraction was performed using approximately 10 mg of powdered freeze-dried tissue in 1 ml 80% methanol, containing 0.5 mg / ml melizitose as an internal standard. This mixture was boiled for 15 min at 76°C and the remaining solvent was removed by vacuum centrifugation (Speedvac, 2 hrs.). The pellet was then resuspended in 0.5 ml water, centrifuged for 5 min at 15,000 g, and the supernatant was further diluted. The diluted extract (20 µl) was injected into a Dionex HPLC system (DX300 Chromatography System, Dionex Corp., Sunnyvale, USA) equipped with a CarboPac PA1 column (4x 250 mm) and a similar guard column (4 x 50 mm) preceding the previous one. Sugars were eluted with a flow rate of 1 ml · min^{-1} using 85 mM NaOH as eluens at room temperature. The compounds were monitored using pulsed amperometric detection and identified and quantified by comparison with elution profiles of sugar standards.

Quantitative measurements of enzyme activities

For the measurements on susy, invertases, HK, FK, PGM, PGI, UGPase, G6PDH and 6PGDH activities, samples were taken from different batches in various developmental stages, ranging from the end of tuber development to dormancy, dormancy breakage and subsequent sprouting. The first sample consisted of tubers of 23 days old, which were considered as still developing. The next samples consisted of tubers of 86, 149, and 184 days old, when tubers were considered dormant. Approximately half of the batch of tubers of 212 days old was sprouting, and both sprouting and non-sprouting tubers were sampled and analysed separately. At day 240 all tubers were sprouting. In the non-sprouting samples, the apical part was sampled separately from the rest of the tuber. In the sprouting samples, also the sprouts were sampled separately. All data are based on 3 to 6 samples, each consisting of 2 to 4 (parts of) tubers or sprouts.

The extraction procedure for susy, soluble invertase, HK, FK, PGM, PGI, UGPase, G6PDH and 6PGDH was modified after Appeldoorn *et al.* (1997) and is also described in the previous chapter. In short, 1.2 ml of extraction buffer (50 mM Hepes/KOH (pH 7.4), 5 mM $MgCl_2$, 1mM EGTA, 1 mM EDTA, 10% (v/v) glycerol, 0.1% BSA (bovine serum albumin), 5 mM DTT (dithiothreitol) and 2% (w/v) insoluble PVP (polyvinylpyrrolidone)) was added to 10 mg of powdered freeze-dried tissue. After centrifugation (5 min, 15,000 g), the

supernatant was removed and frozen in liquid nitrogen and stored at -75°C until required. The extraction procedure was carried out at 4°C. Cell wall-bound invertase was extracted from the pellets obtained after centrifugation of the homogenate of the soluble enzymes (see above), according to Appeldoorn *et al.* (1997).

Activities of susy, HK, FK, PGM, PGI, UGPase, G6PDH and 6PGDH were determined in 250 µl assay medium (in a 96 well plate) at 30°C, for 10 min. The reactions were started by adding extract or substrate and were measured spectrophotometrically at 340 nm (based on the NAD to NADH conversion), by means of an ELISA reader (SLT labinstruments 340 ATTC). Activities of soluble and cell wall-bound invertases were determined by measuring the glucose formed during a 45 min incubation as described by Appeldoorn *et al.* (1997). All buffers and chemical solutions used to perform these assays are described by Appeldoorn *et al.* (1997; -1999), except that volumes were adapted to the 96 wells plate used in this context.

Histochemical localization of enzyme activities

For staining of invertase, susy, UGPase, PGM, PGI, HK and FK activity, tubers were taken from different batches in various developmental stages, ranging from dormancy to dormancy breakage and subsequent sprouting. Staining is based on the coupling of the reduction of NAD to the reduction of NBT (nitroblue tetrazolium), which results in precipitation of the blue tetrazolium salt. The whole procedure was carried out as described by Sergeeva and Vreugdenhil (2002). In short, sections of 200 µm thickness were cut with a sledge microtome. Sections were immediately fixed in a mixture of 2% paraformaldehyde, 2% PVP 40 and 0.001 M DTT, pH 7.0 at 4°C for 1 h. After fixation, sections were rinsed with water at least five times to remove soluble carbohydrates and stored overnight in water. Staining for the different enzymes was performed by incubating the sections in 1 ml of incubation medium in a water bath at 30°C. Incubation lasted 3 hrs for invertase and susy, 1.5 hrs for UGPase, PGM, HK and FK, and 12 hrs (overnight) for PGI. HK, FK and invertase assays were also performed after 12 hrs incubation but this did not yield additional information. All incubation media were derived from Sergeeva and Vreugdenhil (2002). After the incubation, all enzyme reactions were terminated by rinsing the sections in distilled water. The stained sections were then stored at 4°C in water until photographic studies were made. Stained sections were studied with a Leica binocular or a Nikon Optiphot microscope in bright field mode. A digital Panasonic Colour Video Camera or a Sony CCD camera DKR 700 was used to take photographs.

Results

Neutral sugars

Figure 5.1A shows the levels of neutral sugars during dormancy and sprouting. Sucrose was by far the most prominent sugar in the tubers. The levels of all sugars decreased during storage, reaching a constant level, several weeks before sprouting (figure 5.1A). The results in the figure are all based on apical halves of non-sprouting tubers. At the last sampled time-point (189 days), also apical cubes of sprouting tubers were sampled. The levels of these samples are shown in table 5.1; for comparison also the levels of the different neutral sugars in the apical halves of the non-sprouting tubers are added. The levels of sugars in tuber tissue of sprouting and non-sprouting tubers were quite similar. At the same time-point (189 days), also some very young sprouts were sampled separately and neutral sugars determined (table 5.1, last column). Remarkably high amounts of all neutral sugars were found in these young sprouts, when compared to tuber tissue.

Table 5.1 Levels of sucrose, hexoses and P-sugars in parts of non-sprouting and sprouting tubers of 189 days old. The levels of the neutral sugars are in μmol · g FW^{-1} (± SD), the levels of the phosphorylated compounds are in nmol · g FW^{-1} (± SD). Data are averages of four independent replicates in non-sprouting tubers and of two independent replicates in tuber tissue and sprouts of sprouting tubers.

Intermediate	Apical halves non-sprouting tubers	Apical cubes sprouting tubers	Young sprouts (2 –3 mm)
sucrose	10.4 ± 1.9	9.10	44.2
glucose	0.94 ± 0.33	0.92	13.4
fructose	1.14 ± 0.17	1.30	9.30
3PGA	81.0 ± 11	29.8	211.5
glucose-6-P	97.3 ± 18	92.0	183.3
fructose-6-P	21.2 ± 2.8	21.8	48.1
glucose-1-P	7.9 ± 1.8	13.5	40.4
UDP-glucose	16.1 ± 2.3	19.1	52.2

Local sucrose or hexose contents could have more specific effects and therefore sprouting tubers, with sprouts of several cm, were divided into several pieces in order to analyze possible differences in localization. The results of this study are shown in figure 5.1B. It is clear that the reducing sugars were very low in the tuber compared to the sucrose level, confirming the results shown in figure 5.1A and table 5.1. The basal and apical parts of the tuber were similar in sugar contents and the sucrose level at the base of the sprout is also similar to the amount in the tuber. However, in the middle and the tip of the sprout, sucrose

levels appeared to decrease. Glucose levels showed a steep increase along the sprout, of which the tip showed a much higher level than the base of the sprout. Fructose also increased, with again the tip of the sprout showing the highest level, although the absolute level remained much lower than that of glucose.

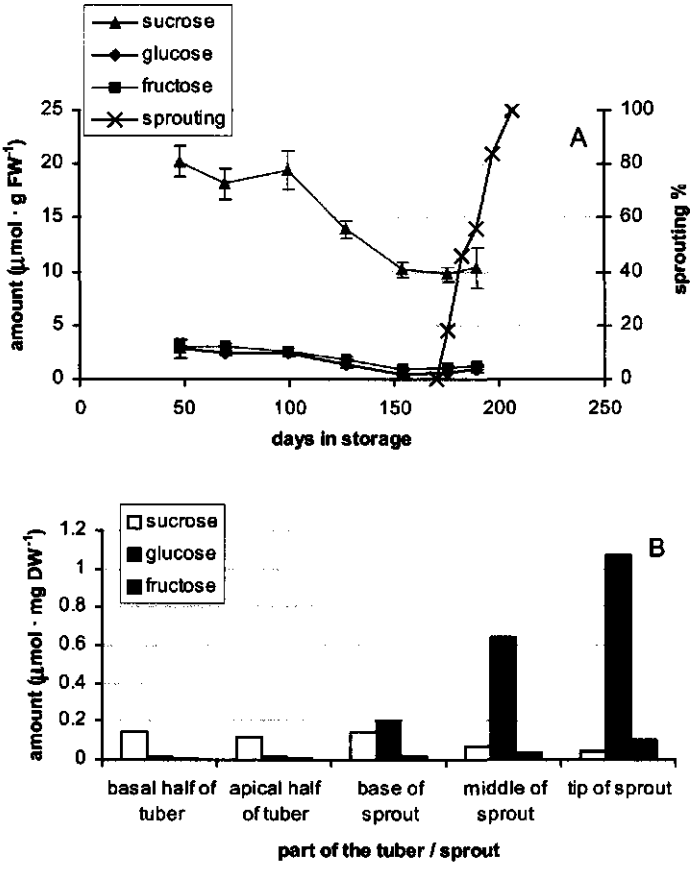

Figure 5.1 Neutral sugars in potato tubers during dormancy and sprouting (A) and in different parts of a sprouting tuber (B).
A: The amounts of sugars are measured in apical halves of non-sprouting tubers and presented as µmol · g FW^{-1} ± SD. At the end of the dormancy period (after 170 days of storage) tubers started sprouting. During this period only the data for non-sprouting tubers are depicted. The sprouting curve is also depicted in the figure (right axis) and is based on 50 microtubers. All data are based on 4 samples, each consisting of 2 parts of different tubers.
B: Single measurements of samples consisting of at least 4 (parts of) different tubers and sprouts. The tubers are 307 days old and the batch started to sprout at 163 days in storage. The sugar amounts are presented as µmol · mg DW^{-1}.

Phosphorylated compounds

As different phosphorylated sugars can affect sucrose and / or starch metabolism, either as substrates for important conversions or by being a regulatory compound, several of these compounds were measured. Results are displayed in figure 5.2. All intermediates showed a decreasing trend during dormancy, except for glucose-1-P. This decrease was the steepest early in dormancy. Glucose-1-P was very low during the whole period of dormancy and was the only compound showing a tendency to increase at the end of dormancy.

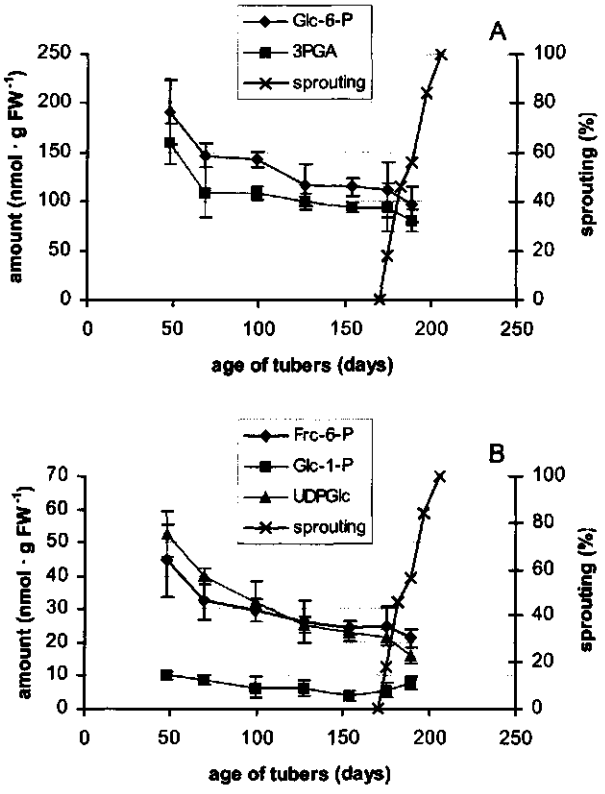

Figure 5.2 Phosphorylated compounds during dormancy and sprouting.
A: The amounts of glc-6-P and 3PGA;
B: The amounts of frc-6-P, glc-1-P and UDPglc.
All compounds are measured in apical halves of non-sprouting tubers and presented as nmol · g FW^{-1} ± SD. At the end of the dormancy period (after 170 days of storage) tubers started sprouting. During this period only the data for non-sprouting tubers are depicted. The sprouting curve is also depicted in the figure (right axis) and is based on 50 microtubers. All data are based on 4 samples, each consisting of 2 parts of different tubers.

When apical halves of non-sprouting tubers of sprouting batches (189 days old) are compared to apical tissue cubes of sprouting tubers (189 days old), the 3PGA level showed a clear decrease, while glucose-1-P increased. In the young sprouts, on the other hand, all intermediates increased to a very high level (table 5.1).

Quantitative measurements of enzyme activities

Appeldoorn *et al.* (1997) found an enzymatic switch in sucrose breakdown, coinciding with an increased AGPase activity (Appeldoorn *et al.*, 1999) in developing tubers. An increased AGPase activity was also found at the end of potato tuber dormancy (previous chapter). So, besides the above reported (P-) sugar contents, the enzymes involved in sucrose breakdown (invertases and sucrose synthase) as well as the enzymes involved in the conversion of their degradation products (UGPase, FK, HK) were investigated. Also activities of some enzymes, involved in subsequent conversions of P-sugars, were measured, viz.,

- PGI, catalyzing the reversible conversion between glc-6-P and frc-6-P;
- PGM, supposedly involved in starch synthesis through the reversible conversion of glc-6-P to glc-1-P, the latter one being the substrate for AGPase;
- G6PDH and 6PGDH, two important enzymes in the OPPP.

The activities of enzymes for sucrose breakdown are shown in figure 5.3. Both soluble (figure 5.3A) and cell wall-bound invertase (figure 5.3B) are low during the whole period of dormancy and during sprouting in the tuber (tuber tissue and apical part), in contrast to the high activity in the sprout itself. Tubers of 23 days old showed sucrose synthase activity (figure 5.3C), which was higher in the apical part than in the rest of the tuber. Afterwards, the activity dropped to a low level and did not change during dormancy breaking and sprouting. The sprouts showed a level of activity similar to the low level of activity in the tuber.

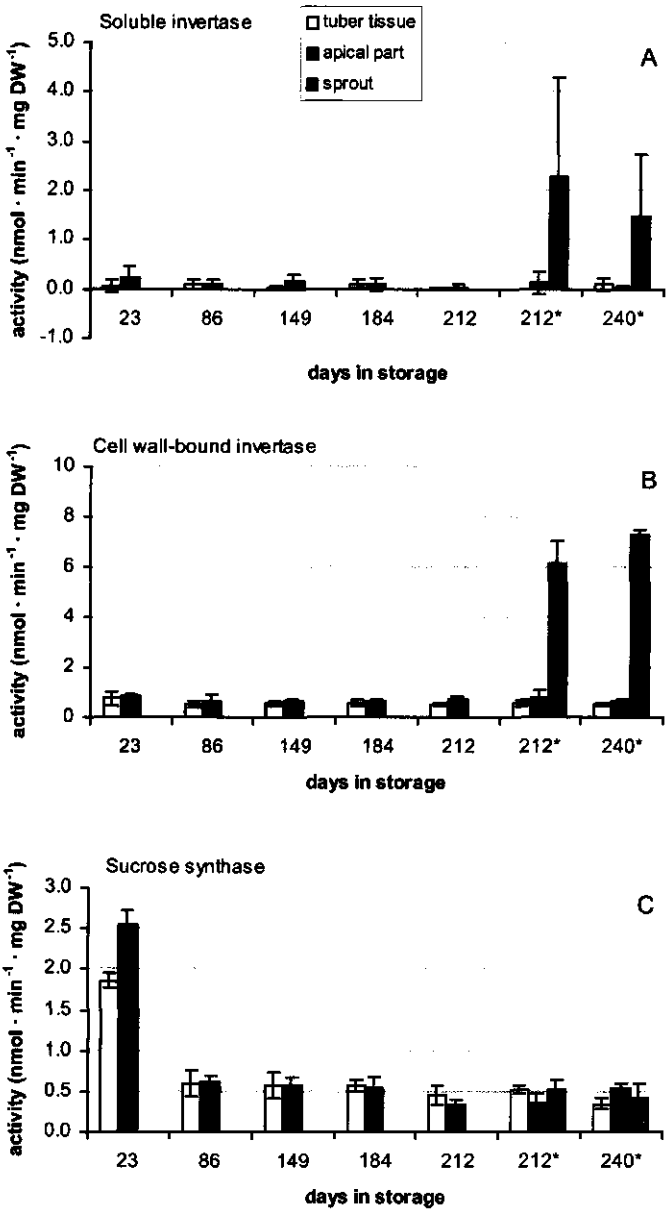

Figure 5.3 Soluble invertase (A), cell wall-bound invertase (B) and sucrose synthase (C) activities in different parts of potato microtubers during dormancy and sprouting. Activities were determined in the apical part of the tuber (approximately 1/10th of total tuber mass), the rest of the tuber tissue and in the sprouts (if present). Asterisk (*) indicates that tubers were sprouting. Tubers of 212 days old were split into sprouting (212*) and non-sprouting tubers (212). Activity is presented as nmol · min^{-1} · mg DW^{-1} ± SD. All data are based on 3 to 6 samples, each consisting of 2 to 4 (parts of) tubers or sprouts.

Enzyme activities of UGPase, FK and PGI are shown in figure 5.4. HK activity is not shown because of very low levels throughout dormancy and sprouting, viz., less than 0.15 nmol · min · mg DW^{-1}. UGPase (figure 5.4A) in tuber tissue decreased a bit during the first phase of dormancy and remained on a constant relatively high level afterwards, also at the end of dormancy and during sprouting. UGPase in the apical part seems first to decrease more, while a small increase could be observed here for sprouting tubers. UGPase in the sprouts had about the same activity as in the tubers. Fructokinase (figure 5.4B) still had a high level in 23 days old tubers in both tuber tissue and apical parts of the tuber. The FK activity was a bit higher in the apical part. After these 23 days, the FK activity dropped to a low level, which seemed to decrease even further during the dormancy period. Any changes for tubers of 184 days old and more were not significant. The sprouts showed a low level of activity similar to the activity in tuber tissue. For PGI (figure 5.4C) in tuber tissue, a fairly constant high level throughout the whole dormancy period was observed. The activity of PGI in the apical parts showed a decrease towards the end of dormancy (212 days old, non-sprouting tubers), while at 240 days the activity was higher again in this part. Sprouts had a higher activity than the tubers.

PGM (figure 5.5A) showed a decrease during the first phase of dormancy for both the apical part and the rest of the tuber tissue. The apical parts of 23 days old tubers showed a higher activity than the rest of the tuber, but this difference disappeared during the course of dormancy and even reversed in 212 days old tubers (both sprouting and non-sprouting). At the end of dormancy no major changes were observed for either tissue, while the sprouts showed a similar activity as the tuber.

G6PDH, catalyzing the first step in the OPPP, also showed a decreasing trend during the first phase of dormancy (figure 5.5B). The apical parts again showed a higher activity in 23 days old tubers, and also here this difference disappeared during the course of dormancy and reversed at 212 days old tubers. At the end of dormancy no remarkable changes were observed. The sprouts showed a similar or somewhat higher activity than the tubers. Finally, 6PGDH showed a much lower activity than G6PDH and this activity also decreased during the first phase of dormancy (figure 5.5C). For the sprouting tubers of 240 days old a higher level of 6PGDH in the apical part appeared to be present. The sprouts showed an elevated level of activity, which was more obvious in the older sprouts.

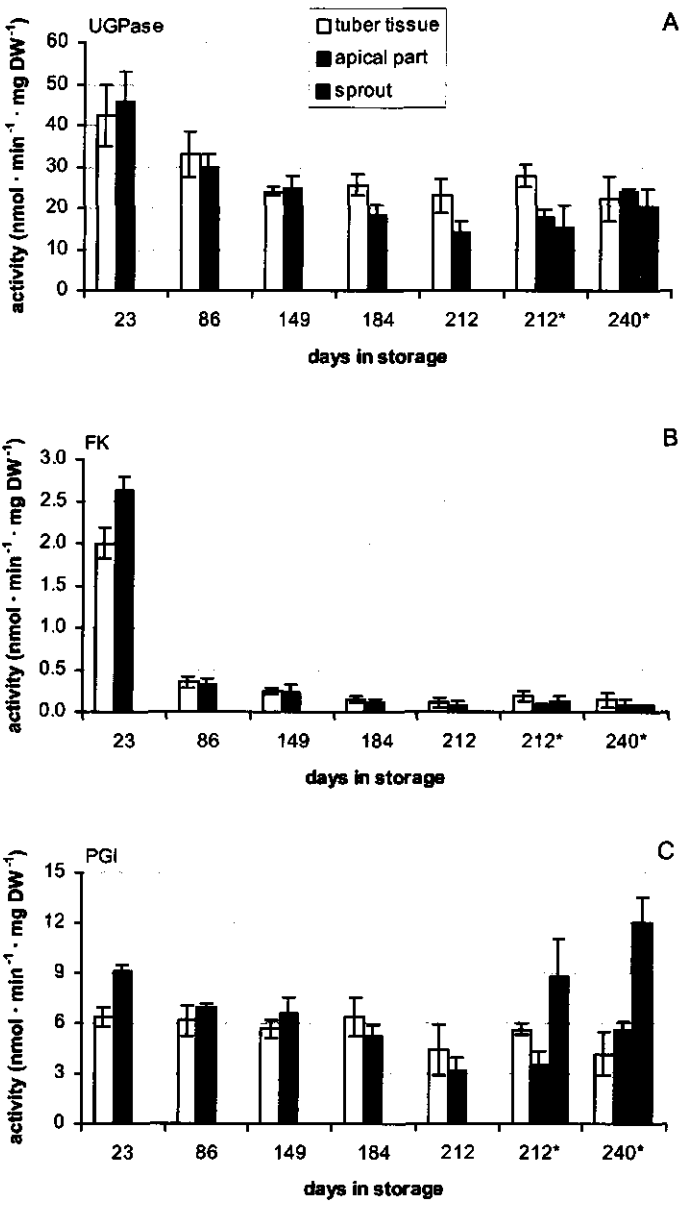

Figure 5.4 UGPase (A), fructokinase (B) and phosphoglucо-isomerase (C) activities in different parts of potato microtubers during dormancy and sprouting. Activities were determined in the apical part of the tuber (approximately 1/10th of total tuber mass), the rest of the tuber tissue and in the sprouts (if present). Asterisk (*) indicates that tubers were sprouting. Tubers of 212 days old were split into sprouting (212*) and non-sprouting tubers (212). Activity is presented as nmol · min^{-1} · mg DW^{-1} ± SD. All data are based on 3 to 6 samples, each consisting of 2 to 4 (parts of) tubers or sprouts.

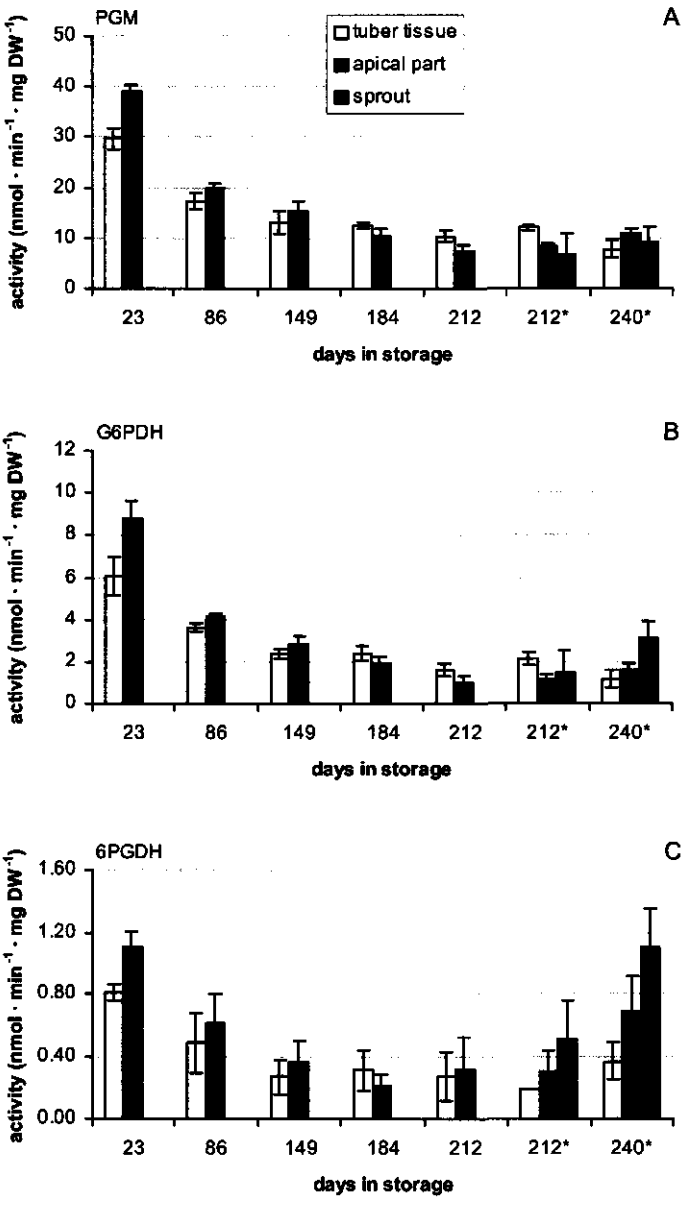

Figure 5.5 Phosphoglucomutase (A), glucose-6-phosphate dehydrogenase (B) and 6-phosphogluconate dehydrogenase (C) activities in different parts of potato microtubers during dormancy and sprouting. Activities were determined in the apical part of the tuber (approximately 1/10th of total tuber mass), the rest of the tuber tissue and in the sprouts (if present). Asterisk (*) indicates that tubers were sprouting. Tubers of 212 days old were split into sprouting (212*) and non-sprouting tubers (212). Activity is presented as nmol · min^{-1} · mg DW^{-1} ± SD. All data are based on 3 to 6 samples, each consisting of 2 to 4 (parts of) tubers or sprouts.

Histochemical localization of enzyme activities

Some of the enzyme activities discussed above were also histochemically analysed. Tubers were analysed at different time-points through dormancy, a selection of which is shown in figure 5.6. For comparison a dormant tuber and a sprouting one are placed next to each other for each enzyme.

The overall results of this histochemical stainings are very similar to the quantitative measurements. For invertase, no activity in the tuber nor in or near the apical eye could be seen during the dormancy period. In the sprouting tuber, the sprout showed a very high activity, the tuber itself showed no staining. The blue spot visible in the tuber (figure 5.6, Inv-B) is most likely an artefact caused by the presence of another sprout in the same incubation medium, and thus no real staining of the tuber itself. Sucrose breakdown can also be accomplished by susy, but this enzyme showed no activity, neither in dormant nor in sprouting tubers (figure 5.6, susy-A and susy-B). When these photographs were enlarged, however, some susy activity could be detected in phloem companion cells in the tuber (both in dormant and sprouting ones; results not shown).

The sucrose degradation products can be further metabolised by UGPase, FK and HK. Dormant tubers showed UGPase activity all over the tuber, particularly in the vascular bundles. In sprouting tubers, the vascular bundles converging to the sprout seem to be more stained than in the dormant tubers. The vascular tissue in the sprout is also intensively stained (figure 5.6, UGP-A and UGP-B). Any of these presumed differences between sprouting and dormant tubers were not confirmed by the quantitative measurements (figure 5.4A). FK showed some activity in the vascular tissue in dormant tubers (figure 5.6, FK-A). Activity in other tissues, like storage parenchyma or in / around the apical eye, was very low. The activity in the sprouting tubers (figure 5.6, FK-B) seemed to be even lower than in the dormant tuber, only some activity in vascular tissue leading towards the base of the sprout was visible. Very low activity was detected at the (vascular tissue of the) sprout. For HK, a very low amount of activity could be detected in the vascular tissue of sprouting tubers (figure 5.6, HK-B). Most of this activity was present in the vascular tissue in the apical part, leading to the sprout.

PGM and PGI both showed a high staining. Again the vascular tissue was highly stained although other tissues also showed blue precipitate. For both enzymes it was observed that the apical eye in the dormant tuber was highly stained. The sprout also showed activity for both enzymes. For none of the enzymes obvious differences between dormant and sprouting tubers could be seen in these figures. In summary, the histochemical analysis revealed relatively high overall activities for UGPase, PGI and PGM and a high activity for invertase in the sprout,

confirming the results of the quantitative measurements (figures 5.3A, 5.3B, 5.4A, 5.4C and 5.5A).

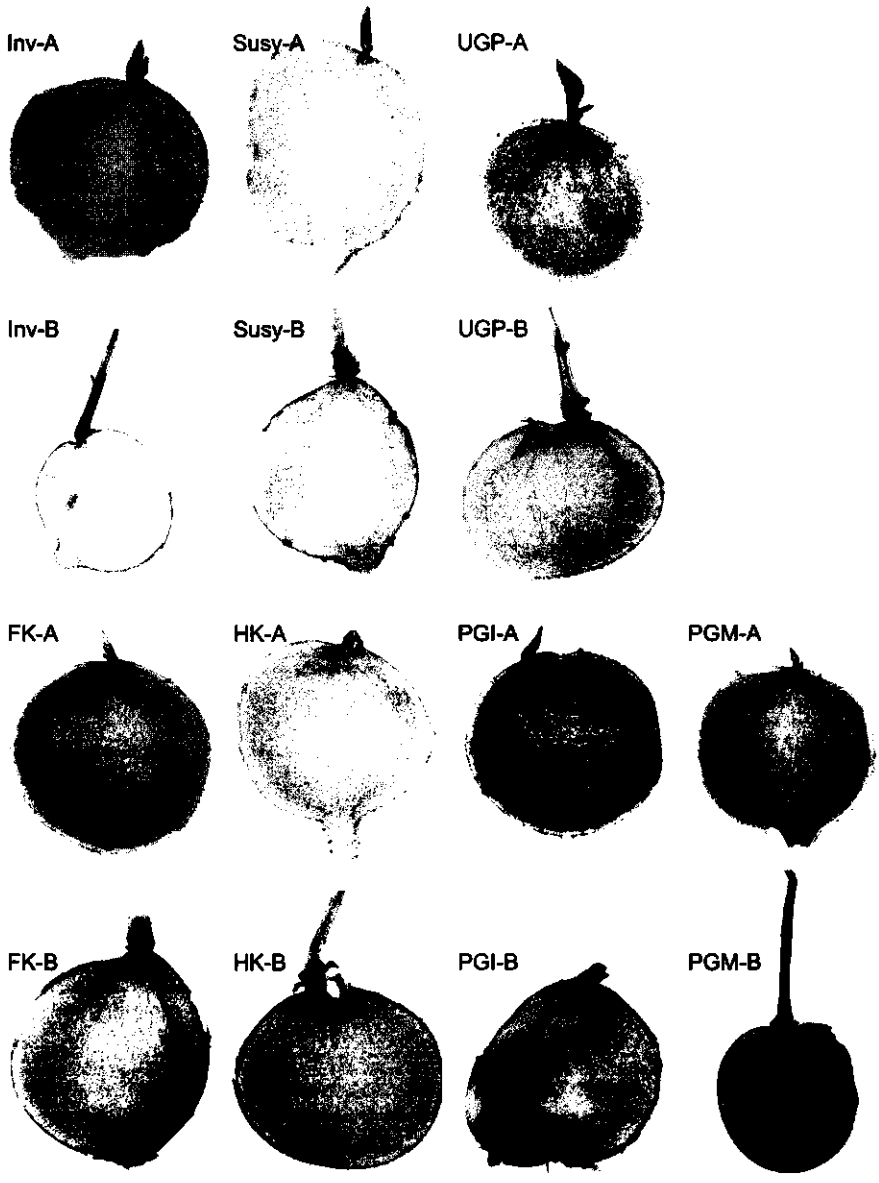

Figure 5.6 Histochemical analysis of different enzyme activities in potato microtubers during dormancy and sprouting. A represents dormant tubers, 113 days old; B represents sprouting tubers, 225 days old. Abbreviations: Inv = invertase, Susy = sucrose synthase, UGP = UGPase, FK = fructokinase, HK = hexokinase, PGI = phosphogluco-isomerase, PGM = phosphoglucomutase. For incubation times and other details of the procedure for staining: see Materials and Methods.

Discussion

Sucrose metabolism

Appeldoorn *et al.* (1997) discussed the switch from a hydrolytic sucrose breakdown, catalyzed by invertase, to a sucrose synthase driven one, occurring during the initiation of potato tubers. The reaction catalysed by invertase is highly exothermic and irreversible (Avigad, 1982) and invertase activity appeared to be high in rapidly elongating tissues (utilisation sinks) (Ross *et al.*, 1994). The reaction by sucrose synthase, on the other hand, is readily reversible and subject to a high degree of fine metabolic regulation (Geigenberger and Stitt, 1993). The switch to sucrose synthase in sucrose breakdown during tuber development offers the cell a mechanism of sucrose cycling. In this way, a cell is able to adjust the net rate of sucrose breakdown to the supply of sucrose and the requirement for carbon in the biosynthetic and respiratory pathways in the cell (Appeldoorn *et al.*, 1997 and references therein). Susy is involved in this sucrose cycling together with sucrose-phosphate synthase, which converts UDP-glucose and frc-6-P into sucrose-6-phosphate, which in its turn is broken down by a phosphatase to yield sucrose and Pi.

Some sucrose cycling was expected to occur in sprouting tubers. As described in the previous chapter, it is hypothesized that, at the moment of dormancy breaking and sprouting, starch is broken down in storage parenchyma, converted into sucrose and transported towards the phloem. Because of the high AGPase activity near the vascular tissue, it was suggested that starch might be re-synthesised (temporarily) in cells near the phloem. If so, the sucrose would be broken down and converted into glc-1-P and finally to starch. When carbon is needed, starch will be broken down, and sucrose will be again formed and transported to the phloem, and via the phloem to the place where it is needed (the apical eye / sprout). During this presumed sucrose and starch synthesis and breakdown, sucrose could also be degraded for respiratory use. During the process of dormancy breaking / sprouting, susy is thus hypothesized to be active in tuber tissue, as this enzyme is able to adjust sucrose breakdown (cycling) to the supply and requirement for carbon, although invertase could also be active when massive breakdown of sucrose is needed for growth.

Sucrose metabolism in the tuber tissue

As can be seen in figure 5.3 and 5.6, both enzymes involved in sucrose breakdown did not show high activity in the tuber and no significant changes in the levels of the enzyme activities were observed at dormancy breaking /

sprouting. Only a very low amount of cell wall-bound invertase (CWI) and susy seemed to be present as a kind of background activity (figure 5.3). The staining experiments in figure 5.6 did not shown any blue precipitate for invertase and susy in the tubers, although enlargements with very high magnification showed some susy activity in phloem companion cells in the tuber (both in dormant and sprouting ones; results not shown). Ross and Davies (1992) found an increase in invertase during storage, which decreased again during sprouting, whereas we found almost no activity.

From the enzymes involved in conversion of sucrose degradation products, UGPase, FK and HK were investigated. UGPase activity was very high, decreased somewhat during the first phase of dormancy but remained high throughout dormancy (figure 5.4A, 5.6). FK dropped to a low level and did not change afterwards (figure 5.4B, 5.6). HK was very low and sometimes not measurable at all; moreover, no changes could be deduced (data not shown). The histochemical experiments also did not show much staining for HK activity (figure 5.6), but enlargements showed some very weak staining at the vascular tissue and storage parenchyma in tuber tissue of both dormant and sprouting tubers (data not shown). Renz *et al.* (1993) found an increasing HK activity and a decreasing FK activity during storage and at the time of sprouting the HK activity increased even more, in contrast to our findings (see also chapter 2).

All neutral sugars reached a steady level at the end of dormancy and did not change thereafter in the tuber itself (figure 5.1A). Several studies showed an increase in the level of reducing sugars in mature tubers after harvest (Ross and Davies, 1992) or just before visible sprouting (Bailey *et al.*, 1978; Dimalla and Van Staden, 1977). Biemelt *et al.* (2000) could not show a clear trend for reducing sugars in different varieties during dormancy. For sucrose, however, a clear decrease from the moment that storage began was visible (Biemelt *et al.*, 2000). Other studies on sucrose were also quite similar to our results; either a decreasing trend during dormancy and sprouting was found (Dimalla and Van Staden, 1977) or a rather constant level during dormancy (Ross and Davies, 1992).

The above results do not support the proposed theory that sucrose cycling takes place during dormancy breaking and sprouting, as described in the previous section. It can be derived from the results described above that sucrose breakdown is not likely to occur within the tuber tissue at that time or only at a very low rate. This low level of sucrose breakdown then might be catalyzed by susy, as invertase could not be detected in the tuber tissue with the histochemical analysis. Because of the very low levels of net sucrose breakdown, it might be concluded that the carbon required for sprout growth is supplied by sucrose,

synthesized from starch degradation products (*e.g.* from glc-1-P via UGPase, SPS and SPP, see figure 5.7B) Sucrose did decrease during the dormancy period and it is likely that this part of sucrose is broken down (probably by susy) for tuber maintenance.

The low levels of HK and FK activity are in agreement with the very low activities of invertase and the low activity of susy. The high level of UGPase activity found in tuber tissue is supposedly involved in the reversed reaction from glucose-1-P (presumably derived from starch breakdown through STP) to UDP-glucose, which can act as a substrate for sucrose-P synthase to form sucrose or is a substrate for cell wall synthesis.

Sucrose metabolism in the sprout

In the experiment of figure 5.1A, also young sprouts were sampled (from 189 days old tubers), and the data are shown in table 5.1. All neutral sugars were very high in these very young, 2-3 mm long, sprouts. Different parts of much older sprouting tubers were analysed separately and in these sprouts sucrose decreased from base to apex (figure 5.1B). At the base of the sprout, the sucrose level was the same as in the tuber. Fructose levels increased from base to apex, as was also observed for glucose: the level in the base of the sprout was already high (higher than in the tuber) and increased even more to the tip of the sprout.

The activity of invertases was high in the sprout (figure 5.3A and 5.3B). This high level of invertase activity is also displayed in the histochemical analysis, which does not discriminate for the different invertases (figure 5.6). As sucrose content was very high in young sprouts (table 5.1), this indicates that the breakdown of sucrose by invertase started somewhat later. Susy (figure 5.3C) showed some activity in the sprout, although not much. Enlargements of the photographs on enzyme staining of the sprout tissue, however, showed some weak staining in companion cells of the phloem (data not shown), so a very low amount of susy activity was indeed present in the sprouts.

The activities of the enzymes involved in the conversion of the degradation products of either of the two enzymes responsible for sucrose breakdown are shown in figure 5.4. UGPase is also present in the sprout at a level similar to the level in the tuber tissue (figure 5.4A). Activity could also be demonstrated by histochemical analysis (figure 5.6). Fructokinase seems to be present in the sprout (figure 5.4B), but at a low level, similar to the level of the tuber tissue. Activity is also shown in the histochemical analysis, in which activity is indeed present in the base of the sprout (figure 5.6). HK activity is almost not detectable (data not

shown) and also the histochemical staining of this enzyme shows only a very low level of activity, of which most was present at the base of the sprout.

These data indicate a total different carbohydrate metabolism in the sprout than in the tuber tissue itself. First of all, sucrose is supposed to be imported from the tuber, and in the sprout this sucrose is mainly converted by invertase to form glucose and fructose. The activity of FK, mostly present at the base of the sprout, seems to be high enough for further conversion of fructose (figure 5.1B). However, the HK activity, present in very low amounts at the base of the sprout, is not enough to degrade glucose and thus high levels of glucose accumulate in the sprout. Both invertase and glucose are known to be present in rapidly dividing tissue (Ross *et al.*, 1994; Borisjuk *et al.*, 1998), which is again shown in this experimental set-up.

Starch metabolism in the tuber

In line with the conclusions of the previous chapter, we will now focus on the possibility of starch cycling during dormancy and sprouting, based on the data on sucrose metabolism in the tuber and sprout, as presented in this chapter. As discussed and concluded in the previous sections, not much sucrose breakdown, and, corresponding to this conclusion and the fact that the sucrose level is constant during the last phase of dormancy, also not much sucrose synthesis takes place in the tuber tissue. As the sucrose levels decrease during the first phases of dormancy (figure 5.1A), it was even suggested that the disappearing sucrose is used for maintenance of the tuber implying that sucrose synthesis during dormancy would be absent or only taking place at a very low rate. These conclusions are summarised in figure 5.7A (dormant tuber) and 5.7B (sprouting tuber).

It is not unlikely that also starch breakdown would participate in (part of) tuber maintenance during dormancy and certainly in respiration during sprouting. As amylase activity is low in tuber and sprout (see also chapter 4) and also hexokinase activity is very low in tuber and sprout (during dormancy as well as during sprouting), degradation via amylases is not the most obvious route for starch breakdown. Starch phosphorylase (STP) activity, on the other hand, is higher during dormancy and (early) sprouting (chapter 4). Moreover, starch degradation via STP leads to glc-1-P, which can easily be channelled to UDPglc (sucrose synthesis), or glc-6-P (OPPP, glycolysis), as both UGPase and PGM are very active during dormancy and sprouting. If necessary, glc-1-P can even be channelled back into starch synthesis, thus offering the cell a mechanism of starch cycling, which can be used to fine-regulate the requirement for carbon in

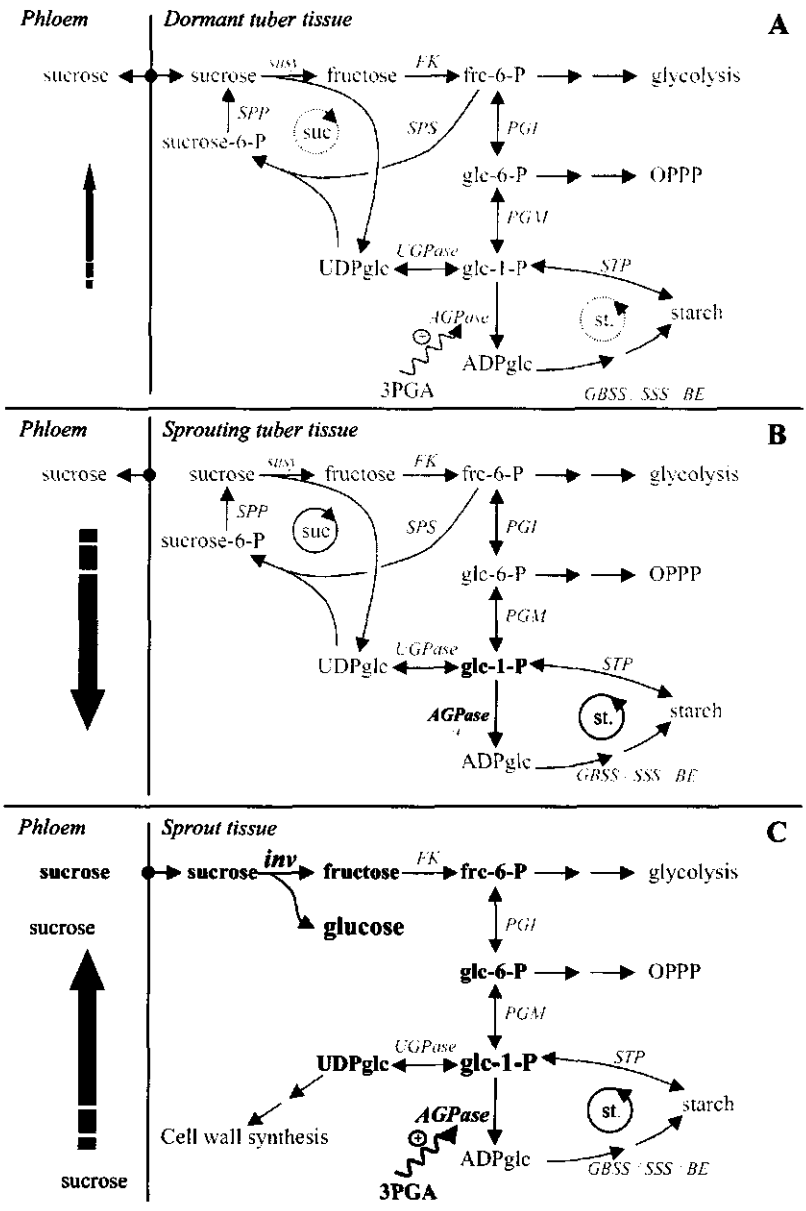

Figure 5.7 Suggested model for sucrose and starch metabolism in tuber tissue of dormant tubers (A), sprouting tubers (B) and tissue of the sprout (C). In each panel, only a phloem element and a representative part of tuber / sprout tissue is depicted, in which the tissue part represents both the apoplast and a (parenchyma) cell with all its compartments, *e.g.* vacuole and amyloplast. Sucrose cycling and starch cycling are indicated by 'suc' and 'st' inside a cycle. Dotted circles indicate that the activity of this cycle is (very) low. Elevated levels of intermediates and enzyme activities are depicted in bold; when these levels increased further or were very high a larger font was also applied. See discussion for comments on this model.

the cell. During dormancy, all measured intermediates of the above-discussed conversions are quite low and some of the involved enzyme activities are also low, so it is assumed that the suggested conversions take only place on a kind of background level (figure 5.7A).

Amylases are supposed to become active after sprouting (Biemelt *et al.*, 2000; previous chapter). HK activity is then expected to increase because it is needed to phosphorylate the released glucose. Our results do not show such an HK increase, but samples might be taken too early to show this increase. Biemelt *et al.* (2000), also did not notice a real increase in glucokinase either.

Early in the sprouting process, some changes take place in the hexose phosphates in the tuber tissue. Glc-1-P almost doubled while glc-6-P did not change much (table 5.1). Under normal conditions, the ratio between glucose-6-P, fructose-6-P and glucose-1-P is supposed to be constant. This equilibrium mostly depends on the reversible reactions between these compounds catalysed by PGM and PGI (ap Rees, 1988; Viola, 1996). Table 5.2 shows the ratios of the components of this hexose-P pool. This ratio should be around 10 : 3 : 1 in potato tubers (Hajirezaei, personal communication), although also a ratio for glc-6-P to glc-1-P of 10 : 0.5 is mentioned for other tissues (Stryer, 1988). Results in Hajirezaei and Sonnewald (1999) gave a 10 : 2.7 : 0.64 for growing and 10 : 2.4 : 0.67 for stored soil-grown tubers. These ratios are similar to what we found in stored dormant *in vitro* tubers (table 5.2). The ratio changes at 189 days of storage: the relative level of glc-1-P increases then already in the apical part of non-sprouting tubers. This increase is even more visible in the apical cubes of tuber tissue of sprouting tubers. The activities of the enzymes involved in these conversions, PGI and PGM (figure 5.7) are not changing in the tuber tissue during sprouting (figure 5.4C and 5.5A).

Table 5.2 Amounts and / or ratios of different phosphorylated compounds during dormancy and sprouting in potato tuber tissue and sprouts. Until day 175, tuber tissue of non-sprouting (non-spr.) tubers are analysed. Levels of 3PGA are displayed as nmol · g FW $^{-1}$ ± SD, all other values are ratios.

Days in storage	3PGA	UDPglc / glc1P	Glc6P : frc6P : glc1P
48	158.8 ± 21	5.2	10 : 2.3 : 0.53
69	109.4 ± 26	4.5	10 : 2.2 : 0.60
99	108.8 ± 6.3	5.0	10 : 2.1 : 0.45
127	100.4 ± 7.9	4.0	10 : 2.2 : 0.55
154	94.1 ± 5.0	5.9	10 : 2.1 : 0.33
175	93.7 ± 24	3.8	10 : 2.2 : 0.50
189 non-spr. tubers	81 ± 11	2.0	10 : 2.2 : 0.81
189 spr. tubers	29.8	1.4	10 : 2.4 : 1.46
189 sprouts	211.5	1.3	10 : 2.6 : 2.20

While the reaction catalyzed by PGI appears to remain close to equilibrium throughout dormancy and sprouting, the reaction catalyzed by PGM shifts away from equilibrium, in favour of glc-1-P, at the end of the dormancy period and during sprouting. As mentioned before, a similar observation was made by Hattenbach and Heineke (1999) in water stressed leaf disks of spinach. They found that accumulation of fructose-1,6-bisphosphate (next to ribulose-1,5-bisphosphate) inhibited PGM activity. This sugar bisphosphate is unfortunately not measured in our experiments.

Also the ratio of UDPglc to glc-1-P changes in favour of glc-1-P, although UGPase activity did not change much during sprouting in the tuber tissue (figure 5.4A). The decrease in ratio UDPglc / glc-1-P might suggest that this is in favour of sucrose breakdown by susy (taking away one of the degradation products). However, as can be seen from the absolute levels (see table 5.1), it is not a clear decrease of UDPglc but only the increase of glc-1-P that is responsible for the lower ratio.

All these data are summarised in figure 5.7B (sprouting tuber), in which glc-1-P and AGPase both show increasing levels (bold). Glc-1-P is supposedly formed via starch breakdown. By an as yet unknown mechanism, the glc-6-P to glc-1-P ratio is altered in favour of glc-1-P, possibly by PGM (Hattenbach and Heineke, 1999). Also the ratio UDPglc to glc-1-P decreases, but the UDPglc level stays more or less constant. AGPase activity increases (chapter 4), suggesting starch synthesis. However, AGPase is known to be allosterically activated by 3PGA (Preiss and Levy, 1980) and the low level of 3PGA in 189 days stored sprouting tubers (table 5.2) suggests therefore a reduction of starch synthesis at this moment. Starch cycling is suggested, although regulation via 3PGA is probably present. Although no direct evidence was obtained, sucrose synthesis at this stage, accompanied by a rapid export from the tuber, is very plausible as the sprout receives large amounts of sucrose (table 5.1). Thus, it was hypothesized that starch cycling and a shifted equilibrium of PGM is involved in the total regulation of carbon fluxes at the moment of sprouting (figure 5.7B).

Starch metabolism in the sprout

As discussed earlier, sucrose metabolism in the sprout is based on the flow of sucrose entering the sprout, which is degraded by invertase, as susy shows no or very low activity and invertase activity is very high in the sprout. As hexokinase activity was very low in the sprout, glucose accumulates (figure 5.1B). Also fructose seems to accumulate (table 5.1), but after a while fructokinase activity seems to be high enough for phosphorylating most of the fructose (figure 5.1B).

The proposed pathways for sucrose breakdown in the sprout are summarised in figure 5.7C.

No evidence for sucrose formation was found. Moreover, the high level of invertase would immediately break down any formed sucrose. Furthermore, the phloem probably supplies enough sucrose from the tuber to provide for the needs of all cells in the sprout.

The hexose-phosphates all showed an increased level compared to tuber tissue, just like UDPglc and 3PGA (table 5.1). Glc-1-P increased more than the other P-sugars, illustrated by the ratio of the hexose-phosphates (table 5.2). Apparently, PGM is not able to establish the 'normal' ratio of glc-1-P and glc-6-P. The higher levels of frc-6-P, glc-6P and UDPglc could be an indication of higher glycolysis, OPPP and cell wall synthesis, respectively. The increase in glc-1-P is, in first instance, a result of the flux through the hexose-phospates from sucrose breakdown, rather than from turnover of starch, since in the growing sprout, starch would first have to be synthesized before it could be degraded. The high level of 3PGA (more than 7 times as high as in the tuber tissue of sprouting tubers, table 5.2), together with the high level of AGPase, especially near the vascular bundles, appearing from histochemical staining (figure 4.3), suggests also a high level of starch synthesis. Once starch has been synthesized, part of it can be broken down again in a controlled manner to meet the carbon requirements for the different routes. The high glc-1-P and AGPase activity are emphasized in figure 5.7C. Once again, starch cycling, possibly regulated by PGM (Fernie *et al.*, 2001; -2002) seems to be involved in controlling carbon fluxes.

For the sake of simplicity, no cellular compartments have been indicated in figure 5.7. Starch biosynthesis and degradation occur in the amyloplast, and cell wall invertase is of course localized in the cell wall / apoplast. Since our measurements do not allow to discriminate between different isozymes, *e.g.* plastidial and cytosolic PGM, no attempts have been made to include subcellular localization of enzymes and metabolites in figure 5.7. Interestingly, it has been suggested that both the plastidial and the cytosolic form of PGM might be involved in the regulation of starch biosynthesis (Fernie *et al.*, 2001; -2002).

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6

General discussion

In this chapter the results presented in the preceding chapters will be combined and discussed further. At the end of chapter 5, the possible role of sucrose and starch cycling and their mutual relations were already discussed in relation to carbohydrate metabolism during dormancy and sprouting of potato tubers. A schematic overview of the changes in carbohydrate metabolism, with emphasis on sucrose and starch cycling, during the switch of dormancy to sprouting is presented at the end of the previous chapter (figure 5.7). In this chapter these results will be compared with the results of Appeldoorn *et al.* (1997, 1999) on potato tuber initiation, to broaden the discussion of chapter 2. Furthermore, spontaneous and induced (by GA and ethanol) sprouting will be compared, based on the data on enzyme activities and intermediates, to discuss the value of the different ways of induced sprouting as a model for spontaneous sprouting. As *in vitro* tubers were used in all experiments described in this thesis, the first section of this general discussion will be devoted to the question whether these microtubers are a good model system for soil-grown tubers.

In vitro grown potato microtubers as a model for soil-grown tubers?

One of the reasons for using microtubers in the present study, viz., a supposedly synchronous release of dormancy, appeared to be non-valid (figure 1.4). However, the use of *in vitro* tubers offered several other advantages for our experiments. As mentioned in chapter 1, the *in vitro* system leads to one harvest per month instead of one harvest per year, as would have been the case with field-grown tubers. Moreover, the use of field-grown tubers would mean that potatoes were growing each year under different environmental conditions, leading to unpredictable and non-controllable differences between the different batches of tubers, *e.g.* length of dormancy or occurrence of second growth. Pot-grown tubers in greenhouses are less dependent on weather conditions; pot-grown tubers in climate chambers are not dependent on weather conditions at all and more harvests per year can be obtained. However, this system is disadvantageous for practical reasons: a lot of space in climate chambers is necessary and the storage of these tubers under specified conditions also needs a lot of space. On the other hand, storage of *in vitro* grown tubers also takes a lot of space, as these tubers are sometimes stored on medium, still attached to their stem cutting. However, microtubers are sterile and more uniform in size and physiological state. Moreover, a major advantage of this tuberization method is the fact that different compounds can be easily introduced into the tuber by dissolving them in tissue culture medium and transferring cuttings, bearing tubers, to this medium. This method, in which uptake of test components takes place via the stem to the tuber, is applied in chapter 3.

There are definitely differences between field-grown tubers and *in vitro* grown microtubers, of which the size is the most obvious one. The final size of the *in vitro* grown tubers in our system is about 8 mm, whereas field-grown tubers can grow much larger. The reason that the microtubers do not reach the size of field-grown tubers is the lack of a perimedullary region (Xu *et al.*, 1998). As soon as field-grown tubers reached a diameter of 8 mm, longitudinal divisions stopped and randomly orientated cell division and enlargement occurred in the perimedullary region and continued until tubers reached their final diameter. These developmental processes do not occur in microtubers. Because of this difference in size, there is a large difference in the ratio surface / volume of the two types of tubers, which may result in large consequences for uptake and removal of gaseous compounds via the epidermis, and for the distribution of a particular compound within the tuber.

Apart from the differences in size, the *in vitro* grown tubers seemed to be a good model for field-grown tubers, because the physiological processes occurring, are in general quite comparable. Xu *et al.* (1998) concluded that the morphology and the processes of cell division and cell enlargement of *in vitro* tubers are similar to those observed during early stages of 'normal' tuber formation in soil. Furthermore, Veramendi *et al.* (1999 and references therein) summarized that enzymes, important during the tuberization process, *e.g.* susy, invertase, FK, HK and UGPase, show similar patterns in *in vitro* microtubers and in soil-grown tubers. The same authors compared enzyme activities and metabolite levels at key steps in primary carbohydrate metabolism for both types of tubers and discussed the applicability of this system as a model. They conclude that there is a reasonable similarity with respect to starch metabolism and metabolite levels and find the system suitable for use as a model, although they warn for some putative differences in the activities of the enzymes of sucrose degradation and glycolysis. One year later, Roessner *et al.* (2000) reported on a new method for simultaneous analysis of metabolites by gas chromatography-mass spectrometry. This method was applied in comparing *in vitro* grown microtubers to soil-grown tubers and the conclusion was drawn that major differences were found between the two types of tubers, especially with respect to the levels of amino acids, which were found to be higher in *in vitro* grown tubers. This report urged us to re-evaluate our own *in vitro* system, which differs from the one used by Veramendi *et al.* (1999) and Roessner *et al.* (2000). The main differences between their *in vitro* system and ours are the medium and the photoperiod used for growing the microtubers. Veramendi *et al.* (1999) used normal MS medium, while ours is modified and contains only 1/10 part of the standard amount of KNO_3 and NH_4NO_3. Moreover, Veramendi *et al.* (1999) incubated the nodal cuttings for 3 weeks under

long day conditions, followed by treatment under short day conditions, whereas the microtubers in our laboratory were growing in the dark. As a consequence, tuberization in their system occurred only in the 6th week and tuberization only increased from 5 to 85% during this week.

To compare the *in vitro* tubers obtained with our system with soil-grown tubers, the results on soil-grown tubers obtained by Hajirezaei and Sonnewald (1999), Biemelt *et al.* (2000) and Roessner *et al.* (2000) or summarised by Veramendi *et al.* (1999) were used. Table 6.1 gives an overview of different metabolites and enzyme activities found in the above-mentioned reports and results from our own experiments.

As can be derived from the table, the data obtained for many of the compounds or enzyme activities did not differ much for the *in vitro* system used in this research and for soil-grown tubers. Moreover, the differences were often similar as those obtained when various experiments with soil-grown tubers are compared. The data for soil-grown tubers are derived from different cultivars (Desirée, Adretta, Solara, Monalisa), whereas the data for *in vitro* grown tubers are all derived from experiments with cultivar Bintje.

Striking differences for the two types of tubers were found for several parameters:

- both glucose and fructose in developing tubers seem to be quite high in *in vitro* grown tubers, although data obtained during this research (see table 6.1) were similar to data from soil-grown tubers;
- Glc-1-P in developing microtubers also seems to be high, but is should be mentioned that measurements are carried out with HPLC and are probably measured around the detection level;
- Hexokinase in developing *in vitro* tubers is much lower than in soil-grown tubers while susy is relatively high in both developing and stored *in vitro* tubers.

The conclusion drawn by Roessner *et al.* (2000) was particularly based on the observations on the high levels of amino acids in their *in vitro* grown tubers in comparison with soil-grown tubers. In our microtubers, on the other hand, levels were of the same orders of magnitude (table 6.1, asparagine, glutamine and proline) or a lot lower (glycine and phenylalanine), compared to the soil-grown tubers of Roessner *et al.* (2000). Like these authors also suggest themselves, the differences found for their microtubers could be a consequence of the high amount of nitrate supplied to the microtubers used in their system. However, in

Table 6.1 Levels of intermediates and enzyme activities in soil-grown and *in vitro* grown tubers. Neutral sugars, starch and anions are in μmol · g FW^{-1}, phosphorylated compounds in nmol · g FW^{-1}, enzyme activities in nmol · min^{-1} · mg FW^{-1} (results of Biemelt *et al.*, 2000 were recalculated assuming 3.8 mg protein per gram FW, Hajirezaei and Sonnewald, 1999).

metabolite / enzyme activity	**soil-grown tubers, growing**	***in vitro* tubers, growing**	**soil-grown tubers, stored / sprouting**	***in vitro* tubers, stored / sprouting**
Sucrose	6.1[1] 22.4 –33.8[2] 25.9[3]	± 15[6] ± 27[7] ± 40[8b]	2.5 – 14.8[1] 1.9 – 7.9[2]	± 10[8a]
Glucose	1.8[1] 6.9 – 33.5[2] 23.8[3]	± 56[6] ± 62[7] ± 29[8b]	0.72 – 1.2[1] 4.1 - 30.3[2]	± 0.7[8a]
Fructose	0.5[1] 0.2 – 1.9[2] 0.01[3]	± 8[6] ± 5.5[7] ± 1.9[8b]	0.36 – 1.1[1] 0.4 – 7.6[2]	± 1.0[8a]
Starch[9]	505[1] 664 – 968[2]	± 670[6] ± 280[7]	322 -380[1] 510 – 935[2]	± 990[8b]
UDPglc	60.2[1] 104[4]	± 70[5]	43[1]	21.4[8a]
3PGA	54.1[1] 60[4]		69.3[1]	93.7[8a]
Glc-6-P	96.9[1] 210[3] 143[4]	± 160[5]	126[1]	112[8a]
Frc-6-P	26.1[1] 37[4]	± 60[5]	30.2[1]	24.8[8a]
Glc-1-P	6.2[1] 14[4]	± 40[5]	8.5[1]	5.6[8a]
Citric acid	18.86[3] 2.3 – 11.4[4]	12.6 – 15.9[8b]		
Malic acid	5.39[3] 2.2 – 2.5[4]	10.8 – 14.0[8b]		
Asparagine	5.62[3]			2.25[8b]
Glutamine	1.08[3]			0.99[8b]
Glycine	0.23[3]			0.014[8b]
Phenylalanine	0.59[3]			0.013[8b]
Proline	0.18[3]			0.28[8b]
Hexokinase	0.132[4]	± 0.06[5]		< 0.04[8b]
Fructokinase	0.24 – 1.2[4]	± 2.0[5]	0.029 – 0.072[2]	± 0.040[8a]
PGM	4.54[4]	± 17[5]		
Susy	1.01[4]	± 6.0[5]	0.003 – 0.012[2]	± 0.150[8a]
UGPase	21.3[4]	± 24[5]		
AGPase	0.99[4]	± 2.0[5]		
α-amylase			0.024 – 0.035[2]	± 0.025[8a]
β-amylase			0.022 – 0.061[2]	± 0.070[8a]

[1] results derived from Hajirezaei and Sonnewald, 1999; [2] results derived from Biemelt *et al.*, 2000; [3] results derived from Roessner *et al.*, 2000; [4] results derived from Veramendi *et al.*, 1999; [5] results derived from Appeldoorn *et al.*, 1997, -1999; [6] results derived from Vreugdenhil *et al.*, 1998; [7] results derived from Visser *et al.*, 1994; [8a] data obtained during the research, described in this thesis; [8b] unpublished data; [9] starch levels are represented as μmol glucose units · g FW^{-1}.

our system a tuber-inducing medium consisting of modified Murashige and Skoog medium (containing only 1/10 part of the standard amount of KNO_3 and NH_4NO_3) was used together with a high amount of sucrose to induce tuberization of the nodal cuttings. As a consequence, the levels of amino acids of our microtubers were also al lot lower, confirming the suggestion made by Roessner *et al.* (2000).

In conclusion, the *in vitro* tubers used in our experiments are in general similar to soil-grown tubers and therefore thought to be a good model-system. Also Fernie and Willmitzer (2001) agree that, at the levels of ultrastructure and the relative activities of enzymes of carbohydrate metabolism, *in vitro* tubers have been shown to be similar to soil-grown tubers.

In chapter 3, a special type of *in vitro* tubers is used (microtuber, type II). As mentioned before, the ratio surface / volume of these microtubers is totally out of proportions and therefore it is possible that the same concentrations of compounds as used in microtubers of type I, lead to much stronger responses. This may for instance be the case in treatments with ethanol, as this treatment gave 100% sprouting within 8 days with microtubers of type II (figure 3.2A) and approximately 70% sprouting after 20 days when microtubers of type I are used (figure 3.6). Moreover, some attempts were made to enforce second growth in developing, larger soil-grown tubers with a 1% ethanol solution, but no second growth could be observed under these conditions with this concentration of alcohol (unpublished data).

Induced sprouting as a model for spontaneous sprouting?

Studies aimed at unravelling physiological processes occurring during the breaking of dormancy in potato tubers, are hampered by the long period of dormancy, viz., 3 to 6 months under normal storage conditions. Several methods are available to break dormancy of tubers prematurely. Such methods are used in agricultural practice and might also be useful in physiological studies, provided that the sequence of events, evoked by these methods, is similar to that occurring during spontaneous sprouting. Gibberellins are well known as dormancy breaking compounds. However, gibberellins might affect all kinds of processes within the tuber, some of which are not related to sprouting. To circumvent this problem, the application of another component to induce sprouting was investigated. As already mentioned in the introduction of this thesis and again in chapter 3, alcohols are known to break dormancy in seeds of various plant species. We investigated the effects of ethanol on induction of sprouting in potato tubers and the supposed mode of action of inducing sprouting (or breaking dormancy, which results in sprouting) via alcohol dehydrogenase, as

discussed in chapter 3. It was shown in that chapter that ADH increased in activity. This increase was suggested to be specifically induced by ethanol, as the GA-induced sprouting did not show this ADH increase, indicating that it was not related to the sprouting process itself. Spontaneously sprouting tubers neither showed an increase in ADH activity (data not shown).

Levels of intermediates and enzyme activities related to carbohydrate metabolism were measured, concentrating on sucrose and starch metabolism and their inter-relationship. Their significance during the spontaneous sprouting process was discussed in chapter 4 and 5. The same factors were investigated in induced sprouting to obtain information on whether the induced sprouting is regulated in a similar way as spontaneous sprouting. Data on changes in tuber tissue and in the (secondary) structure resulting after sprouting will be discussed separately.

Tuber. For the enzyme activities, no dramatic changes were observed in the tuber during dormancy breakage of spontaneously sprouting tubers, except for AGPase, for which we observed an increase in the histochemical analysis. Unfortunately, histochemical analysis was not carried out in the experiments with induced sprouting. For the intermediates, only 3PGA and glucose-1-P showed significant changes during spontaneous sprouting. Intermediates and enzyme activities were also investigated for ethanol- and GA-induced sprouting. The data are summarised in table 6.2; results on PGI, PGM, STP, G6PDH, 6PGDH, sol. invertase, FK, HK, UGPase and AGPase are not included, since for these enzymes under none of the investigated conditions, significant changes in the activity measurements were found. The assays for enzyme activity were carried out using the same experimental set-up as described in chapter 3. The control treatments were included to check for possible changes due to transfer of tubers to fresh media.

It is clear from table 6.2 that the changes in enzyme activities and metabolite levels during GA- and ethanol-induced sprouting are not always identical and that both ways of induced sprouting gave sometimes different results as compared to spontaneous sprouting. GA-induced sprouting leads to an increase of the level of cell wall-bound invertase and possibly related to this, of the levels of reducing sugars. The increased level of sucrose suggest a higher rate of sucrose synthesis. Measurements of the levels of SPS or SPP activity might confirm these data. The increased levels of CWI and reducing sugars are suggested to be a direct result of the GA-treatment, since during spontaneous sprouting these increased levels did not occur.

Table 6.2 Changes in levels of intermediates and enzyme activities in tuber tissue of spontaneously sprouting tubers and in tubers forced to sprout by treatment with GA or ethanol. Tubers were 2 - 4 months old, when the treatment started. Explanation of signs: c = constant level; ++ = major increase; + = minor increase; -- = major decrease; - = minor decrease, nd = not determined.

intermediate / enzyme	spontaneous sprouting	GA 1% suc	GA 8% suc	ethanol 1% suc	ethanol 8% suc	control 1% suc	control 8% suc
ADH	c	c	c	++	++	c	c
α-amylase	c	c	c	+	++	c	c
β-amylase	c	c	--	+	+	--	--
CWI	c	+	++	-	--	-	--
susy	c	c	c	c	+	c	c
Amino acids	++	c	nd	++	nd	nd	nd
3PGA	--	c	nd	-	nd	nd	nd
Glc-1-P	+	-	nd	-	nd	nd	nd
Sucrose	c	+	nd	c	nd	nd	nd
Reducing sugars	c	+	nd	c	nd	nd	nd

Ethanol-induced sprouting was accompanied by an increased level of ADH activity, as already discussed in chapter 3. Moreover, ethanol appeared to increase the levels of amylases, although no upregulation of hexokinases could be detected and no increase in reducing sugars (glucose) occurred either. The increase of the amylase activities in both treatments with ethanol needs further investigation. Furthermore, an increase could be observed in amino acid synthesis, just as found and described for spontaneous sprouting (Brierley *et al.*, 1996). Such an increase of amino acids was not observed during GA-induced sprouting. Also a decrease in 3PGA was observed, suggesting a lower level of starch synthesis. In the ethanol treatment, combined with 8% sucrose, susy activity increased, which is in agreement with the type of secondary structure formed during this treatment (a tuber, see next section on secondary structures).

The control transfer treatments showed decreasing trends for β-amylase and CWI activity, so the decreasing levels of CWI activity during ethanol induced-sprouting and of β-amylase in GA-induced sprouting with 8% sucrose are most probably due to transfer of the tubers to fresh media. The constant level of β-amylase after a GA-treatment with 1% sucrose, instead of the expected decrease as observed for the controls, may be a positive effect of the GA-treatment.

Sprout / secondary tuber. Sprouts originating from spontaneously sprouting tubers showed elevated levels of all neutral sugars and phosphorylated compounds, as described in chapter 5. Also in the sprouts, which originated from treatments with GA or ethanol with 1% sucrose, the neutral sugars and phosphorylated compounds increased (results not shown). Of the measured enzyme activities

during spontaneous sprouting, the invertases clearly increased, while also PGI showed an increase compared to the tuber tissue (figure 5.3A, 5.3B and 5.4C). Also in the sprouts formed during dormancy breaking with GA and ethanol (on 1% sucrose) and in the secondary tubers, formed with ethanol and 8% sucrose, PGI activity increased (table 6.3). PGM, UGPase and G6PDH also showed increasing levels in activity, after dormancy breakage with GA or ethanol, which were not observed in spontaneously formed sprouts.

For 6PGDH, such an increase was not found for ethanol and 1% sucrose. The reasons for these differences are unclear. Both types of invertases, however, showed only an increase when a sprout was formed, but not in the secondary tuber. This is consistent with differences in sucrose breakdown in tubers and shoots, as described by Appeldoorn *et al.* (1997) and Ross *et al.* (1994); the preferred way of sucrolysis in tubers is via susy, whereas in shoots, sucrose is mainly hydrolyzed by invertases. Whether the increase in activity of invertases is causing the difference in secondary structures, or whether the formation of a sprout leads to an increased invertase activity, can not be concluded from our data.

Table 6.3 Enzyme activities in sprouts or secondary tubers, induced by a GA- or ethanol-treatment, compared to the activities in sprouts arising during spontaneous sprouting. Enzyme activities are based on single measurements of samples, existing of at least 4 different structures (sprouts or secondary tubers) per sample. Enzyme activities are expressed in nmol · min^{-1} · mg DW^{-1}. The last column represents the mean enzyme activity in the tubers undergoing the treatments.

treatment	spontaneous sprouting	GA 1% sucrose	ethanol 1% sucrose	ethanol 8% sucrose	mean activity in tuber
structure	sprout	sprout	sprout	sec. tuber	
PGI	11	13	18	11	5
PGM	8	21	31	24	10
UGPase	18	36	42	29	15
G6PDH	2.1	4.5	3.8	7.6	2.2
6PGDH	0.8	2.0	0.53	1.6	0.4
CWI	6.8	6.7	5.1	0.45	0.4
Sol. inv	1.9	2.9	1.4	0.62	0.5

In conclusion, both GA- and ethanol-induced sprouting show too many differences compared to spontaneous sprouting to be used as a model for investigating spontaneous dormancy breaking and sprouting in general. Also the comparison between GA- and ethanol-induced sprouting reveals that both compounds regulate dormancy breaking and subsequent sprouting in different ways.

Is dormancy breaking the reverse of tuber initiation in terms of carbohydrate metabolism?

In the conclusion of chapter 2 the remark is made that for more definite conclusions on an answer to the title 'Is dormancy breaking of potato tubers the reverse of tuber initiation?', measurements should be done with the same methods using the same material. The research of Appeldoorn *et al.* (1997, 1999) on tuber initiation is done in the same laboratory as the research presented in this thesis. Moreover, the *in vitro* tuberization method and most of the methods for measuring intermediates and enzyme activities are identical for the research described in this thesis, and in their papers on tuber initiation. Thus, a more powerful tool is now available to compare carbohydrate metabolism during tuber initiation and dormancy breaking / sprouting.

Central in the results of Appeldoorn *et al.* (1997) was the shift from a hydrolytic breakdown of sucrose in the stolon via invertases, to a sucrose synthase driven one in the tuber. This shift is accompanied by an increase in fructokinase activity in the tuber and a high overall activity of UGPase, which support the switch to the susy catalysed breakdown by converting the sucrose degradation products. Concomitantly with the decrease in invertases, a decrease in hexokinases (glucokinases) was found, as glucose is not a product anymore of sucrose degradation. Unfortunately, no sucrose phosphate synthase (SPS) or sucrose phosphate phosphatase (SPP) were measured in this context. Appeldoorn *et al.* (1999) found, not surprisingly, an increase in AGPase during tuber formation. Also an increase in STP was found, for which it is still unclear whether it catalyses a degradation or a synthesis step, or whether it does both depending on the developmental stage in which different regulatory components may play a role. Furthermore, a few enzymes were found to increase temporarily at the onset of tuberization, but remaining rather constant or decreased slightly afterwards. PGM seemed to increase during tuberization, just like G6PDH and the ratio G6PDH / 6PGDH. Measurements of hexose-phosphates showed a temporary increase in frc-6-P just before the onset of tuberization, whereas glc-6-P showed a general decline and glc-1-P a general increase. After the onset of tuberization, the ratios of these hexose-phosphates is totally out of equilibrium (see also table 5.2). ADPglc temporarily rises during the onset of tuberization, while UDPglc showed a general decrease during tuberization.

Part of the findings of Appeldoorn *et al.* (1997, 1999) as described above are schematically depicted in figure 6.1. The changes in carbohydrate metabolism during tuberization, with special emphasis on sucrose and starch cycling, are depicted separately for stolon tissue (figure 6.1A; before tuberization) and tuber tissue (figure 6.1B, after tuberization). Also the carbohydrate metabolism of

sprouting tubers is once again depicted (figure 6.2), as derived from figure 5.7. Figure 6.2A represents the tuber tissue of a sprouting tuber and figure 6.2B is the sprout tissue. Some differences between figure 6.2A and figure 5.7B can be noticed. While figure 5.7 only shows changes in measured components, figure 6.2A also shows deducted changes, *e.g.* sucrose synthesis via SPS and SPP, which are presented in bold as if they were found to show a higher activity, while they were not measured during our research. As sucrose is massively available in the young sprout for growth and sucrose has to come from the tuber, in which it has to be synthesised, high activities of SPS and SPP are supposed to be present here.

Comparing figures 6.1 and 6.2, many similarities, but also some differences can be noticed. First of all, there is a structural difference, which is not as such visible in the figures. During tuber formation, there is first growth of the stolon and then the stolon tissue is converted into tuber tissue as the stolon starts to swell and forms a tuber. During sprouting, the tuber supplies carbohydrates for the new sprout, which is, in contrast to a newly formed tuber that exists partly of stolon tissue, all newly formed tissue.

Furthermore, sucrose unloading from the phloem into the tissue is depicted in figures 6.1A, 6.1B and 6.2B and sucrose loading in figure 6.2A (indicated by the direction of the arrows between the phloem and tissue and in the phloem). Finally, the presence of HK is indicated in figure 6.1A, and although both the substrate and the product are present, HK is not indicated in figure 6.2B (see below).

Figure 6.1A shows the carbohydrate metabolism in the stolon. In general, sucrose is unloaded into the apoplast and degraded by invertases, followed by phosphorylation of the products. As the stolon is a fast growing tissue, the carbon flow is then channelled into glycolysis, OPPP and cell wall synthesis. Starch synthesis was not mentioned in Appeldoorn *et al.* (1997), but some AGPase is present (Appeldoorn *et al.*, 1997) and it is known that starch deposition also takes place in a stolon (Vreugdenhil *et al.*, 1998), when sucrose concentrations are high, although at a much lower rate than in the tuber. STP is also present (Appeldoorn *et al.*, 1997) in stolon tissue and therefore some starch cycling might occur in growing stolons. Starch cycling would be a way to temporarily store carbohydrates and regulate the flow in the different directions, via UGPase, PGM and PGI.

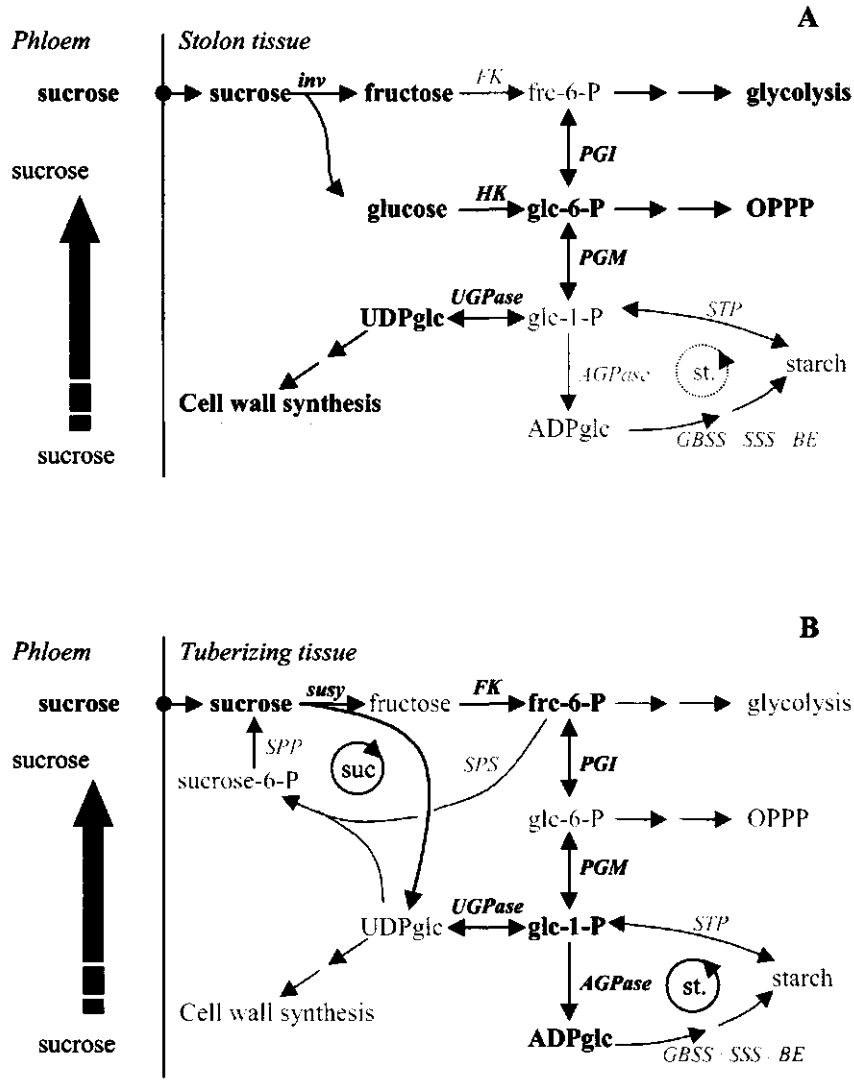

Figure 6.1 Suggested model for the changes in sucrose and starch metabolism before (A, stolon) and after (B, tuber tissue) tuberization of potato. For each panel, only a phloem element and a representative part of the tissue is depicted, in which the tissue part represents both the apoplast as well as a (parenchyma) cell with all its compartments, *e.g.* vacuole and amyloplast. Sucrose cycling and starch cycling are indicated by 'suc' and 'st' inside a cycle. Dotted circles indicate that the activity of this cycle is (very) low. High levels of intermediates and enzyme activities are depicted in bold. Enzyme activity patterns and levels of intermediates are derived from Appeldoorn *et al.* (1997, 1999).

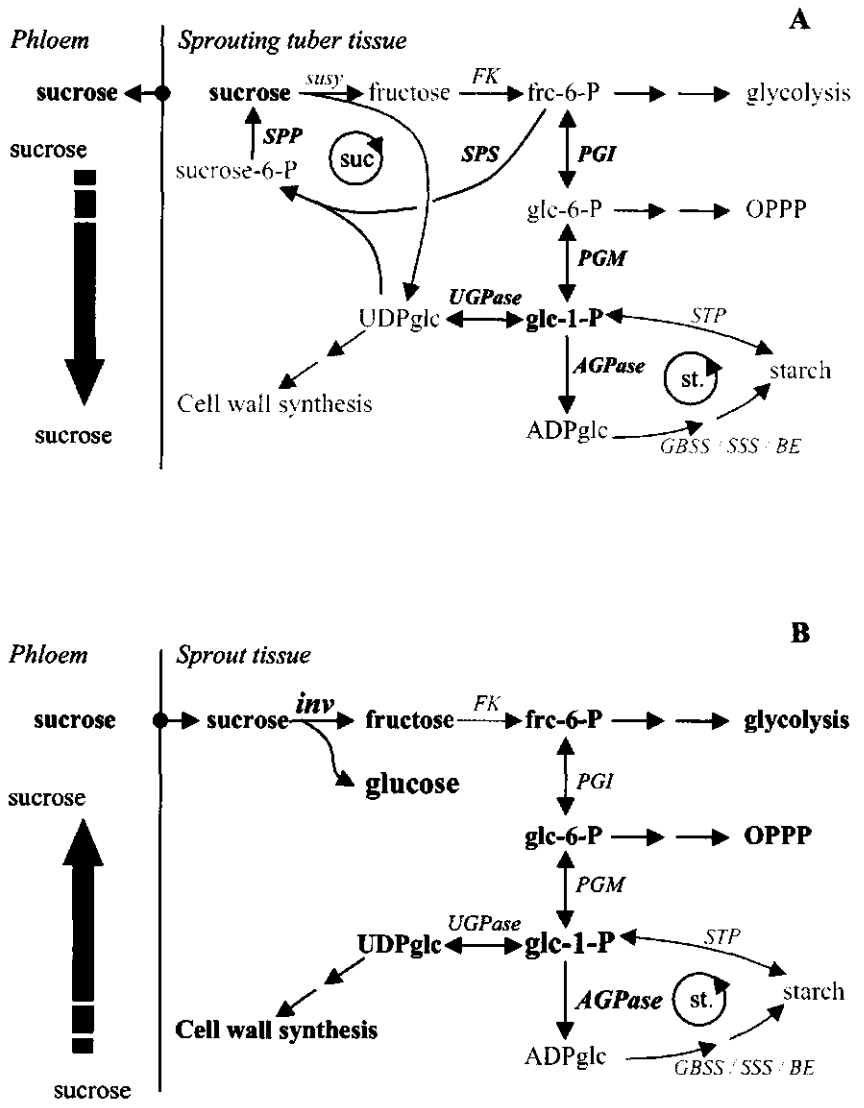

Figure 6.2 Suggested model for sucrose and starch metabolism during dormancy breaking and sprouting in tuber (A) and sprout tissue (B); see also figure 5.7. For each panel, only a phloem element and a representative part of the tissue is depicted, in which the tissue part represents both the apoplast as well as a (parenchyma) cell with all its compartments, *e.g.* vacuole and amyloplast. Sucrose cycling and starch cycling are indicated by 'suc' and 'st' inside a cycle. The levels of intermediates and enzyme activities, which increased or are thought to increase (see text) are depicted in bold.

When tuber formation starts, sucrose is thought to be unloaded symplastically (Viola *et al.*, 2001); it enters the cell and is subsequently converted by susy (figure 6.1B). The degradation products are mostly channelled into starch synthesis, and only a small part enters glycolysis, OPPP and cell wall synthesis. As also STP increases, starch degradation can take place and starch cycling might occur. Carbon flow in figure 6.1B is centred across a line going from top left to bottom right in the figure. This can be compared to figure 6.2A, in which carbon flows from starch degradation (bottom right) to sucrose synthesis (top left), after which the sucrose can be loaded into the phloem. Thus, carbohydrate metabolism in both figures uses the same pathways for sucrose and starch metabolism, including cycling, but net fluxes are in opposite directions.

The sprout tissue (figure 6.2B) is quite similar to the stolon tissue (figure 6.1A), although the starch synthesis is more prominent in the sprout, as both the results of the measurements of glc-1-P and 3PGA content (chapter 5) as well as the AGPase activity (chapter 4) point to that direction. It has to be mentioned that the phosphorylated components are measured using different methods: Appeldoorn *et al.* (1999) uses an HPLC method, whereas in this thesis the components are measured enzymatically (chapter 5). The latter method is believed to be more sensitive and was needed for measuring the very low amounts during potato tuber dormancy. The other difference between figure 6.1A and 6.2B is the already mentioned hexokinase. In figure 6.2B, the HK is not depicted as almost no activity was found in both quantitative measurements and histochemical analysis during dormancy and sprouting in both tuber and (young) sprout (chapter 5), in contrast to earlier findings by Renz *et al.* (1993) as already discussed in chapter 2. Moreover, it was suggested in chapter 2 that this increased HK activity found by Renz *et al.* (1993) was in agreement with the increase of invertase activity during storage (Ross and Davies, 1992). However, invertases were also low in our experiments in the tuber during storage and only increased in the sprout, both in the quantitative measurements and in histochemical analysis. The HK activity of the sprout remained also low in our experiments, in agreement with the accumulation of glucose found in sprouts (figure 5.1B). Appeldoorn *et al.* (1997) found a relatively higher HK activity in stolons, which is therefore depicted in figure 6.1A. This HK activity in the stolon is also in agreement with the glucose levels in the stolon (Visser *et al.*, 1994), which are similar to the fructose levels. Glucose increased when swelling occurred, corresponding with decreasing HK acitvity (Appeldoorn *et al.*, 1997).

In conclusion, when comparing enzyme activities and levels of intermediates in tuberizing and sprouting tuber tissue, it is quite clear from the schematic models (figure 6.1B and 6.2A), that indeed the processes studied here use the same

pathways (sucrose and starch cycling), with net fluxes in opposite directions. Carbohydrate metabolism in stolon and sprout tissue (figure 6.1A and 6.2B), on the other hand, are largely similar, also with respect to the flux direction. We assume that the carbohydrate metabolism in stolon and sprout shows the typical characteristics for rapidly growing tissue, which overshadows any minor changes in carbohydrate metabolism related to initiation or breakage of tuber dormancy. Therefore, research on carbohydrate metabolism involved in initiation and breakage of dormancy has to focus on the processes taking place in the tuber tissue.

In spite of the difficulties in establishing the exact time-point of dormancy breaking, the measurements done in tubers before visible sprouting and during early sprouting resulted in a schematic model, which can be compared to earlier studies on tuber initiation (and dormancy initiation) by Appeldoorn *et al.* (1997, 1999). This comparison lead to the conclusion that the same pathways are active in these two different developmental stages of the potato tuber, yet with opposite net carbon fluxes. In chapter 2 of this thesis, it was already concluded that for levels of carbohydrates and enzyme activities involved in carbohydrate metabolism, some clear reverse trends were found when tuber initiation and dormancy breakage are compared. These 'reverse trends' are now described more precisely as changes in carbon fluxes in sucrose and starch cycling. Further research on these carbon fluxes, especially during dormancy breaking, can further support this hypothesis.

It was also concluded in chapter 2 that hormonal activities were only partly the reverse, during tuber initiation and dormancy breakage. Within the scope of this thesis, it is suggested that it is necessary to use the same material and the same methods to draw more definite conclusions on the role of hormonal activities, which was already suggested in chapter 2.

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Summary

Potato tuber dormancy is part of the vegetative life cycle of potato. It refers to a period, in which no growth occurs, although the tuber is stored under conditions that are favourable for growth. Factors in the tuber are responsible for this growth arrest; soon after dormancy is broken, sprouting occurs. Dormancy is already initiated during tuber formation.

Potato tubers are often placed in short- to long-term storage for a stable year round supply. The occurrence of sprouting can be detrimental for further (industrial) processing of the tubers. On the other hand, sometimes sprouting of the tubers is already necessary soon after harvest, *e.g.* when tubers need to be planted for the next growing season. Therefore, it is of major interest to investigate the physiological regulation of potato tuber dormancy, dormancy breaking and sprouting. In this research, microtubers obtained with an *in vitro* system were used; it was concluded (chapter 6) that microtubers were a good model for soil-grown tubers.

Some physiological processes during tuber dormancy initiation (tuber formation) and dormancy breakage (just before visible sprouting), seemed to be reversed, *e.g.* increasing levels of GA were necessary to induce sprouting during dormancy initiation, while at the end of dormancy lower doses were sufficient. Also the endogenous levels of ABA, known as a dormancy hormone, are reported to increase during the initiation of dormancy and to decrease again at the end of dormancy, thus initiating sprouting. Starch, which is the major carbohydrate reserve in potato, is synthesized during tuber formation and degraded again during sprouting to supply carbohydrates for growth of the new plant. The hypothesis that the developmental processes of initiation leading to dormancy (tuber formation) and dormancy breakage / sprouting were more or less the opposite of each other in terms of levels of endogenous hormones, carbohydrates and intermediates and with respect to the involved enzymatic conversions, is reviewed in chapter 2. Especially the endogenous hormone levels during tuber formation and dormancy breakage / sprouting are extensively discussed (summarised in figure 2.2) and it was concluded that these hormone levels were partly the reverse in the two different developmental stages.

Carbohydrates and enzymes involved in sugar and starch metabolism are also discussed in chapter 2, although literature about data during dormancy breaking was relatively scarce. It was concluded that indeed carbohydrate metabolism related processes might be reversed when dormancy initiation and dormancy breaking are compared.

The effect of GA application on dormancy breaking is undisputed; however, the observed changes in carbohydrate metabolism after GA-application could be the result of the developmental process of dormancy breaking, or a direct result of the hormone and not related to sprout induction. Therefore, another way of inducing sprouting, by the use of ethanol, is investigated and discussed in chapter 3. In this chapter, the role of ADH in ethanol-induced dormancy breaking was investigated. It was concluded that ADH seemed to be involved in dormancy breaking, but a role for the (acidic) products could not be shown. Therefore, a role for ADH in the NAD^+ to NADH conversion was suggested. Applying ethanol with 1% sucrose in the medium resulted in a sprout while ethanol with 8% sucrose in the medium resulted in a secondary tuber. We hypothesized, based on a theory developed for tuber formation, that sucrose could affect endogenous GA levels in a negative way. This would mean that high sucrose would result in low endogenous GA, which in its turn would result in a secondary tuber and vice versa. Determinations of endogenous GA levels did not confirm this hypothesis, low sucrose levels even lead to a decrease of GA_1 levels. Apparently, the role of sucrose and GA in the regulation of sprouting differed from their role in the regulation of tuber formation. Also ethanol, by no means, increased the level of GA_1.

As already mentioned, data on sugar and starch metabolism during dormancy and sprouting were rare. In chapter 4, starch cycling during tuber formation, dormancy and sprouting was investigated by determining enzyme activities involved in starch synthesis and degradation. AGPase activity appeared to increase before visible sprouting, but this was only shown in histochemical staining and not in activity measurements. Also AGPase S-*luc* tranformants showed an elevated expression in sprouting tubers. Amylase activity was also high during tuber formation and decreased during dormancy. It was only after sprouting that the activity of β-amylase increased. STP was high during tuber formation, decreased during dormancy and remained constant during sprouting. STP was suggested to be involved in starch degradation and starch cycling was suggested to be active during all developmental stages of the tuber.

The fact that a switch in sucrose breakdown from an invertase to a susy-regulated one occurs during tuber formation made us investigate sucrose breakdown during dormancy and sprouting. Also enzymes involved in the conversions of the sucrose degradation products were investigated, as well as the levels of sugars and intermediates. The results are discussed in chapter 5. Both invertase and susy activities were very low in the tuber both during dormancy and sprouting. As only susy showed some activity in histochemical staining, sucrose degradation in the tuber was suggested to be supported by susy. At the end of dormancy,

glucose-1-phosphate, derived from starch breakdown by STP, is supposed to be converted by UGPase, SPS and SPP into sucrose, which is being exported from the sink tuber to the growing sprout. Invertase showed a huge activity in the sprout and hexokinase showed almost no activity. As a result, high levels of glucose were found in the sprout. The results of chapter 4 on starch cycling are implemented in the results on sucrose metabolism in chapter 5. A schematic model shows the roles of starch and sucrose cycling in both the tuber and the sprout, during dormancy breakage.

In the general discussion (chapter 6), starch and sucrose metabolism during tuber formation and sprouting are compared and it could be concluded that both developmental processes use the same pathways (sucrose and starch cycling), with net fluxes in opposite directions, in the tuber. The conclusion of chapter 2, could be extended with the above conclusion. Carbohydrate metabolism in stolon and sprout are largely similar, also with respect to the flux direction.

Furthermore, carbohydrate metabolism during induced sprouting (by GA or ethanol) is compared to the one occurring during spontaneous sprouting (chapter 4 and 5). It could be concluded that both methods for induced sprouting showed too many differences compared to spontaneous sprouting to use them as a model for the processes occurring during spontaneous sprouting (chapter 6).

Samenvatting

Aardappelknollen maken deel uit van de vegetatieve levenscyclus van de aardappel en ondergaan na de ontwikkeling een periode van kiemrust. Ondanks het feit dat de knollen bewaard worden onder condities welke gunstig zijn voor groei, treedt in de periode van kiemrust geen groei op. Factoren in de knol zelf zijn verantwoordelijk voor het tegenhouden van de groei: als de kiemrust gebroken is, treedt spruitgroei wel op. De initiatie van de kiemrust vindt al plaats tijdens de knolontwikkeling.

Omdat een stabiele aanvoer van aardappelknollen het hele jaar door noodzakelijk is, worden de knollen vaak gedurende kortere of langere tijd opgeslagen. Tijdens deze bewaring kan het breken van de kiemrust zorgen voor de groei van spruiten, wat veelal nadelig is voor de (industriële) verwerking van deze knollen. Daarentegen kan het soms ook noodzakelijk zijn dat de aardappelen al uitlopen kort nadat ze geoogst zijn, bijvoorbeeld als de knollen gepoot moeten worden voor het nieuwe groeiseizoen. Kortom, twee belangrijke redenen waarom onderzoek naar de fysiologische regulatie van de kiemrust van aardappelen van groot belang is. In het hier beschreven onderzoek werd gebruik gemaakt van microknollen, die werden verkregen met een *in vitro* systeem; in hoofdstuk 6 zijn deze microknollen vergeleken met grond-gegroeide knollen, waarbij geconcludeerd werd dat de microknollen een goed model vormden voor het uitvoeren van dit onderzoek.

Sommige fysiologische processen tijdens de initiatie van de kiemrust (de fase van knolontwikkeling) en tijdens het breken van de kiemrust (de fase voor zichtbare spruitgroei), lijken elkaars tegengestelde. Dit geldt bijvoorbeeld voor de gibberelline (GA)-niveau's die nodig zijn om de kiemrust te breken: steeds hogere niveau's zijn nodig tijdens de initiatie van de kiemrust terwijl aan het einde van deze periode lagere hoeveelheden GA volstaan. Daarnaast is beschreven dat de endogene gehaltes van het hormoon abscisinezuur (ABA) toenemen tijdens de initiatie van de kiemrust en afnemen aan het einde van deze periode en zodoende het spruiten initiëren. Zetmeel, dat de belangrijkste opslagvorm van koolhydraten is in aardappel, wordt gesynthetiseerd tijdens knolvorming en weer afgebroken tijdens het spruiten van de knol om bouwstenen en energie te leveren voor de groei van de nieuwe plant. Dit leidde tot de hypothese dat de ontwikkelingsprocessen die ten grondslag liggen aan de initiatie van kiemrust en aan het breken van kiemrust min of meer tegengesteld zijn aan elkaar in termen van endogene hormoon-, koolhydraat- en intermediairniveau's. Ook de betrokken enzymatische conversies zouden een tegengesteld karakter vertonen.

Een overzicht van de bestaande literatuur hierover is gegeven in hoofdstuk 2. Het zijn voornamelijk de endogene hormoonniveau's tijdens de initiatie en het breken van de kiemrust die uitgebreid aan de orde komen (samengevat in figuur 2.2) en hieruit is geconcludeerd dat deze hormoonniveau's gedeeltelijk een omgekeerde trend lieten zien in de twee besproken ontwikkelingsfasen.

Literatuur betreffende koolhydraten en enzymen betrokken bij het suiker- en zetmeelmetabolisme tijdens de kiemrustbreking bleek schaars te zijn. Geconcludeerd werd dat inderdaad enkele processen gerelateerd aan koolhydraatmetabolisme in tegengestelde richting verliepen bij vergelijking van de initiatie en het breken van de kiemrust, maar informatie over de regulatie hiervan bleek grotendeels te ontbreken.

Een behandeling met GA kan de kiemrust breken. Veranderingen in koolhydraatmetabolisme na een behandeling met GA kunnen echter zowel het resultaat zijn van deze kiemrustbreking, als een direct gevolg van de hormoonbehandeling en niet gerelateerd aan het uitlopen van de knollen. Een andere manier om kiemrust te breken, namelijk via het gebruik van ethanol, werd daarom onderzocht (hoofdstuk 3). Met name de rol van alcoholdehydrogenase (ADH) in deze door ethanol geïnduceerde spruitgroei bleek van belang en werd vanuit verschillende invalshoeken onderzocht. ADH scheen betrokken te zijn bij de kiemrustbreking, maar een effect van de producten van deze omzetting kon niet aangetoond worden. Aan het eind van hoofdstuk 3 wordt dan ook een rol van ADH in de regulatie van de NAD^+ / NADH omzetting en verhouding gesuggereerd.

Verder leverden ethanolbehandelingen gecombineerd met verschillende concentraties suiker in het medium verschillende resultaten op wat betreft het type secundaire structuur dat werd gevormd. Ethanol in combinatie met 1% suiker in het medium resulteerde in een spruit, terwijl ethanol met 8% suiker resulteerde in een secundaire knol. De hypothese dat sucrose (saccharose) een effect had op de endogene GA gehaltes en daardoor op het type secundaire structuur, werd onderzocht. Deze hypothese was gebaseerd op een theorie die ontwikkeld was voor de knolontwikkeling, waarbij hoge concentraties suiker een laag endogeen GA gehalte tot gevolg hadden en vice versa. Lage concentraties suiker zouden dan leiden tot een spruit (hoog GA) en hoge concentraties suiker tot een secundaire knol (laag GA). Echter, de bepalingen van de endogene GA gehaltes konden deze hypothese niet onderbouwen; laag sucrose leidde zelfs tot een afname in de GA_1 concentratie. Blijkbaar verschilt de rol van sucrose en GA in de regulatie van de kiemrustbreking en de regulatie van de knolontwikkeling. Ook ethanol zelf leidde niet tot een verhoging van de GA_1-concentratie.

Zoals boven reeds aangegeven, waren gegevens over suiker- en zetmeelmetabolisme tijdens kiemrustbreking en spruitgroei schaars. In hoofdstuk 4 wordt dan ook dieper ingegaan op de synthese en afbraak van zetmeel tijdens knolvorming, de kiemrustperiode en de periode waarin de knollen spruiten. De enzymen ADPglucose-pyrofosforylase (AGPase, betrokken bij synthese), zetmeelfosforylase (STP, kan zowel betrokken zijn bij synthese als afbraak) en amylase (afbraak) staan centraal in dit hoofdstuk. AGPase vertoonde een hoge activiteit tijdens de knolvorming en nam daarna af. Voordat zichtbare spruitgroei optrad werd een verrassende toename in activiteit waargenomen; deze kon echter alleen aangetoond worden met behulp van histochemische kleuring en niet met behulp van metingen in extracten. Ook AGPase S-*luc* transformanten vertoonden een verhoogd expressieniveau in spruitende knollen. Verder bleek er ook verhoogde amylase-activiteit op te treden tijdens knolvorming, welke weer afnam tijdens de kiemrustperiode en pas weer omhoog ging nadat spruitgroei was waargenomen. STP was hoog in activiteit tijdens knolvorming, nam af tijdens de kiemrustperiode en bleef daarna min of meer constant. STP lijkt voornamelijk betrokken te zijn bij de afbraak van zetmeel. Zowel zetmeelsynthese als -afbraak lijken plaats te vinden gedurende de verschillende ontwikkelingsstadia van de knol en er kan dus gesproken worden van een constant actieve zetmeelcyclus.

Het was al bekend dat tijdens knolvorming een verandering plaatsvindt in de afbraak van sucrose, waarbij het enzym invertase plaats moet maken voor sucrose-synthase (susy). Daarom werden ook de verschillen sucrose-afbraakroutes tijdens de kiemrustperiode en de daaropvolgende spruitgroei nader onderzocht. Hierbij werden ook de enzymen betrokken bij de verdere omzetting van de sucrose-afbraakproducten en de veranderingen in suiker- en intermediairniveau's bepaald. De resultaten zijn besproken in hoofdstuk 5. Zowel de activiteit van invertase als die van susy zijn erg laag in de knol tijdens de periode van kiemrust en gedurende de daaropvolgende spruitgroei. Met behulp van histochemische kleuring kon alleen susy activiteit worden aangetoond in de knol en dus is de geringe sucroseafbraak die plaatsvindt in de knol hoogstwaarschijnlijk het resultaat van susy-activiteit. Aan het eind van de kiemrustperiode wordt glucose-1-fosfaat, afkomstig van zetmeelafbraak via STP, waarschijnlijk omgezet via UDPglucose-pyrofosforylase, sucrose-fosfaat-synthase en sucrose-fosfaat-fosfatase in sucrose, dat vervolgens uit de knol geëxporteerd wordt naar de groeiende spruit. In de spruit is een hoge invertase activiteit aanwezig en bijna geen hexokinase activiteit. Dit leidt tot hoge glucose hoeveelheden in de spruit. De resultaten van hoofdstuk 4 en hoofdstuk 5 zijn verwerkt in een schematisch model, dat de rol laat zien van een zetmeel- en een sucrosecyclus in knol en spruit tijdens kiemrustbreking.

In de algemene discussie (hoofdstuk 6) worden zetmeel- en sucrosemetabolisme tijdens knolvorming en tijdens het uitlopen van de knol met elkaar vergeleken. Er werd geconcludeerd dat dezelfde routes (sucrose- en zetmeelcyclus) gebruikt worden gedurende beide ontwikkelingsstadia, echter de netto fluxen verlopen in de knol in tegengestelde richting. Het koolhydraatmetabolisme in de stolon en de spruit zijn grotendeels hetzelfde, ook met betrekking tot de richting van de flux.

Ook is in hoofdstuk 6 het koolhydraatmetabolisme tijdens kunstmatig opgewekte spruitgroei (met GA of ethanol) vergeleken met spontaan optredende spruitgroei. De conclusie werd getrokken dat beide methoden om spruitgroei te induceren te veel verschillen vertoonden in vergelijking met spontaan optredende spruitgroei om ze te kunnen gebruiken als een model om processen te bestuderen die samenhangen met natuurlijke spruitgroei (hoofdstuk 6).

Curriculum Vitae

Margo Maria Joseph Claassens werd geboren op 25 april 1973 in het Zeeuwsche Sint Jansteen (gemeente Hulst). In 1991 behaalde zij haar VWO-B diploma aan de toenmalige RK Jansenius Scholengemeenschap te Hulst. In datzelfde jaar begon zij aan haar studie Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen. Binnen deze studie heeft zij twee afstudeervakken gedaan. Het eerste afstudeervak heeft zij bij de vakgroep Organische Chemie gedaan; dit betrof een onderzoek aan de zuivering van een enzym betrokken bij terpeensynthese in de witlofwortel. Voor haar tweede afstudeervak verrichtte zij onderzoek bij de vakgroep Biochemie, waarbij het metabolisme en de toxiciteit van een aantal verbindingen bestudeerd werd in de rat. Zij behaalde haar diploma Moleculaire Wetenschappen, chemisch-biologische en biotechnologische oriëntatie, in augustus 1996. Vervolgens is zij behulpzaam geweest bij promotie-onderzoek van anderen bij de vakgroep Biochemie. In juni 1997 is zij als Assistent in Opleiding haar eigen promotie-onderzoek bij de vakgroep Plantenfysiologie aan de Landbouwuniversiteit Wageningen begonnen. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Sinds 15 juli 2002 is zij werkzaam bij de Stichting HIV Monitoring in Amsterdam als Data Monitor.

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