# Sucrose breakdown in the potato tuber

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# Abstract

In this work different approaches are undertaken to improve the understanding of the sucrose-to-starch pathway in developing potato tubers. At first an inducible gene expression system from fungal origin is optimised for the use of studying metabolism in the potato tuber. It is found that the *alc* system from *Aspergillus nidulans* responds more rapidly to acetaldehyde than ethanol, and that acetaldehyde has less side-effects on metabolism. The optimal induction conditions then are used to study the effects of temporally controlled cytosolic expression of a yeast invertase on metabolism of potato tubers. The observed differences between induced and constitutive expression of the invertase lead to the conclusion that glycolysis is induced after an ATP demand has been created by an increase in sucrose cycling. Furthermore, the data suggest that in the potato tuber maltose is a product of glucose condensation rather than starch degradation. In the second part of the work it is shown that the expression of a yeast invertase in the vacuole of potato tubers has similar effects on metabolism than the expression of the same enzyme in the apoplast. These observations give further evidence to the presence of a mechanism by which sucrose is taken up via endocytosis to the vacuole rather than via transporters directly to the cytosol. Finally, a kinetic *in silico* model of sucrose breakdown is presented that is able to simulate this part of potato tuber metabolism on a quantitative level. Furthermore, it can predict the metabolic effects of the introduction of a yeast invertase in the cytosol of potato tubers with an astonishing precision. In summary, these data prove that inducible gene expression and kinetic computer models of metabolic pathways are useful tools to greatly improve the understanding of plant metabolism.

# Deutsche Zusammenfassung

In dieser Arbeit wurden verschiedene Ansätze verfolgt, um das Verständnis des Saccharose-zu-Stärke Stoffwechselweges in sich entwickelnden Kartoffelknollen zu untersuchen. Zunächst wurde ein induzierbares Genexpressions-System aus dem Schimmelpilz *Aspergillus nidulans* für die Untersuchung des Metabolismus von Kartoffelknollen optimiert. Es wurde herausgefunden, dass dieses sogenannte *alc* system schneller auf Acetaldehyd reagiert als auf Ethanol, und dass Acetaldehyd weniger Seiteneffekte auf den Metabolismus hat. Die optimalen Induktionsbedingungen wurden dann benutzt um die Effekte einer zeitlich kontrollierten zytosolischen Expression einer Hefe-Invertase auf den Metabolismus der Kartoffelknolle zu untersuchen. Die beobachteten Unterschiede zwischen induzierter und konstitutiver Expression der Invertase führten zu der Feststellung, dass die Glycolyse erst induziert wird nachdem ein ATP-Mangel durch erhöhtes Saccharose-Cycling kreiert wurde. Weiterhin lassen die Ergebnisse darauf schließen, dass Maltose in der Kartoffelknolle eher ein Produkt der Kondensation zweier Glucose-Einheiten ist statt ein Produkt des Stärke-Abbaus zu sein. Im zweiten Teil dieser Arbeit wurde gezeigt, dass die Expression einer Hefe-Invertase in der Vakuole von Kartoffelknollen ähnliche Effekte auf deren Metabolismus hat wie die Expression des gleichen Enzymes im Apoplasten. Diese Beobachtung ist ein weiterer Beleg für die Präsenz eines Mechanismus, bei dem Saccharose mittels Endozytose in die Vakuole aufgenommen wird anstatt über Transporter direkt ins Zytosol aufgenommen zu werden. Zum Schluß wird ein kinetisches Modell des Saccharose-Abbaus vorgestellt, das in der Lage ist diesen Teil des Stoffwechsels der Kartoffelknolle quantitativ zu simulieren. Weiterhin kann dieses Modell die metabolischen Effekte der Einführung einer Hefe-Invertase in das Zytosol von Kartoffelknollen mit erstaunlicher Präzision vorhersagen. Zusammengefasst zeigen die Ergebnisse dieser Arbeit, dass induzierbare Genexpression sowie Computermodelle von Stoffwechselwegen nützliche Hilfsmittel für eine Verbesserung des Verständnisses des Pflanzenmetabolismus sind.

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# Abbreviations

| | |
|---|---|
| 35S | 35S promoter from cauliflower mosaic virus |
| 4-MeU | 4-methylumbelliferone |
| ADP | adenosine diphosphate |
| ADPglc | adenosine diphosphate glucose |
| AGPase | adenosine diphosphate glucose pyrophosphorylase |
| AlcI | potato plants inducibly expressing a yeast invertase in their tubers |
| ATP | adenosine triphosphate |
| B33 | tuber-specific patatin promoter |
| Bq | Becquerel |
| CaMV | cauliflower mosaic virus |
| CF | carboxyfluorescein |
| $C^J$ | flux control coefficient |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid |
| EMK | ethyl methyl ketone |
| FBPase | fructose-1,6-bisphosphatase |
| Fru | fructose |
| Fru6P | fructose-6-phosphate |
| FW | fresh weight |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GC-MS | gas chromatography – mass spectrometry |
| Glc | glucose |
| Glc1P | glucose-1-phosphate |
| Glc6P | glucose-6-phosphate |
| GUS | β-glucuronidase |
| HEPES | 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid |
| HexP | hexose phosphates |
| MCA | Metabolic Control Analysis |

| | |
|---|---|
| MES | 2-morpholinoethanesulphonic acid |
| MOPS | 3-(N-morpholino)propanesulphonic acid |
| MS | mass spectrometry |
| MUG | 4-methylumbelliferyl-β-D-glucuronide |
| $NAD^+$ | oxidised nicotinamid adenine dinucleotide |
| NADH | reduced nicotinamid adenine dinucleotide |
| $NADP^+$ | oxidised nicotinamid adenine dinucleotide phosphate |
| NADPH | reduced nicotinamid adenine dinucleotide phosphate |
| PCA | Principle Component Analysis |
| PFK | phosphofructokinase |
| PGI | phosphoglucose isomerase |
| PGM | phosphoglucomutase |
| $P_i$ | inorganic phosphate |
| PK | pyruvate kinase |
| PMSF | phenylmethanesulphonyl fluoride |
| PPase | pyrophosphatase |
| $PP_i$ | pyrophosphate |
| SE | standard error |
| SPP | sucrose phosphate phosphatase |
| SPS | suscrose phosphate synthase |
| Suc | sucrose |
| Suc6P | sucrose-$6^F$-phosphate |
| SuSy | sucrose synthase |
| TCA cycle | tricarboxylic acid cycle (=Krebs cycle) |
| TPI | triose phosphate isomerase |
| Tris | tris(hydroxymethyl)aminomethane |
| UDP | uridine diphosphate |
| UDPglc | uridine diphosphate glucose |
| UGPase | uridine diphosphate glucose pyrophosphorylase |
| UTP | uridine triphosphate |
| vacI | potato plants expressing a yeast invertase in the vacuole of their tubers |

# Chapter 1. General Introduction

The aim of this work was to investigate sucrose breakdown in the potato tuber. To increase the understanding of the regulation of this pathway, different strategies were followed: (I) generation and biochemical analysis of potato plants expressing a yeast invertase in their tubers (a) in the cytosol in an inducible manner and (b) in the vacuole; (II) the simulation of this pathway *in silico* using a quantitative kinetic model. It was hoped that these strategies would provide further insight into regulation of carbohydrate metabolism, and a more direct view of the primary changes evoked upon expression of the yeast invertase. The four results chapters each contain a short introduction and discussion, making them manuscripts that can be read independently, whilst the general discussion (Chapter 7) at the end of this thesis summarises the results in a more general context.

## *1.1. The potato tuber as a model system for storage sinks*

Potato (*Solanum tuberosum* L.) is one of the most agronomically important crops. The sink organs of potato, the tubers, provide a suitable system to study the regulation of storage compound metabolism. Potato tubers are relatively homogeneous and accessible organs, in which growth and storage occur simultaneously (Reeve *et al.*, 1973). Furthermore, the development of genetic manipulation technology has led to a considerable increase in knowledge concerning the regulation of metabolic pathways of the potato. This plant is almost unique in that it is amenable for both *Agrobacterium*-mediated gene transfer protocols (Rocha-Sosa *et al.*, 1989) and a broad range of biochemical analyses (for example see (Geigenberger and Stitt, 1993; Burrell *et al.*, 1994; Tjaden *et al.*, 1998a; Trethewey *et al.*, 1998; Trethewey *et al.*, 2001; Urbanczyk-Wochniak *et al.*, 2003a).

In the source tissue of the potato plant photoassimilates are produced and the resultant reduced carbon is transported to the sink organs in the form of sucrose (Hirose and Ohsugi, 1998; Komor, 2000). This sucrose is subsequently transported via the phloem along a concentration gradient, maintained by loading of sucrose into the phloem from the source leaf mediated by an energy dependent proton/sucrose symporter (Riesmeier *et al.*, 1993; Kühn *et al.*, 1996). In developing tubers, unloading of sucrose from the phloem into the tuber tissue most probably occurs mainly via a symplastic process through the plasmodesmata directly from the sieve tubes into the parenchyma cells of the tuber (Oparka and Prior, 1988). The storage cells additionally possess a minor yet effective

mechanism for the uptake of sucrose from the apoplast (Oparka and Wright, 1988) which most probably is the predominant mechanism of sucrose unloading in very young tubers (Fernie and Willmitzer, 2001; Viola *et al.*, 2001; Kühn, 2003). Irrespective of its route of unloading the sucrose is transported via a proton/sucrose symporter to the apoplast where it is rapidly cleaved to hexoses which are then transported into the cells, most probably by the action of a hexose transporter (Smith, 1999).

## *1.2. Metabolism of growing potato tubers*

### 1.2.1. Carbohydrate Metabolism

After sucrose is taken up by the potato tuber parenchyma cells it represents the primary compound for further metabolism. The pathways whereby sucrose is converted into hexoses and starch are well understood (see for example ap Rees and Morrell, 1990; Fernie *et al.*, 2002b; Geigenberger 2003). There are two alternative possibilities for cleavage of the imported sucrose. In growing tubers the predominant pathway of sucrose catabolism is that catalysed by sucrose synthase (SuSy), the contribution of invertases within the cytosol being negligible (ap Rees, 1992; Appeldoorn *et al.*, 1997).

Sucrose synthase catalyses the interconversion of sucrose and UDP to UDPglucose and fructose. The reaction is reversible and therefore the net rate of sucrose degradation is dependent on the concentrations of fructose and UDPglucose. However, the irreversible phosphorylation of fructose to fructose-6-phosphate by fructokinase (Renz and Stitt, 1993) and the conversion of UDPglucose to glucose-1-phosphate and UTP by UDPglucose-pyrophosphorylase (UGPase) tend to drive the SuSy reaction in the direction of sucrose breakdown within the potato tuber (Oparka *et al.*, 1992). The reversible reaction catalysed by UGPase requires pyrophosphate and releases UTP. It could be demonstrated that in spinach leaves and pea embryos, the reaction is close to equilibrium (Weiner *et al.*, 1987; ap Rees and Morrell, 1990; Geigenberger and Stitt, 1993). The produced hexose monophosphates are interconverted: glucose-6-phosphate, glucose-1-phosphate and fructose-6-phosphate being equilibrated by the concerted actions of phosphoglucomutase (PGM) and phosphoglucoseisomerase (PGI), respectively (Figure 1.1). In contrast, the invertase reaction releases glucose and fructose which are rapidly converted to hexose monophosphates through phosphorylation by hexokinases or fructokinases (Renz *et al.*, 1993). These reactions are ATP dependent and the hexose monophosphates provided are utilised both in glycolytic and starch biosynthetic pathways (Figure 1.1).

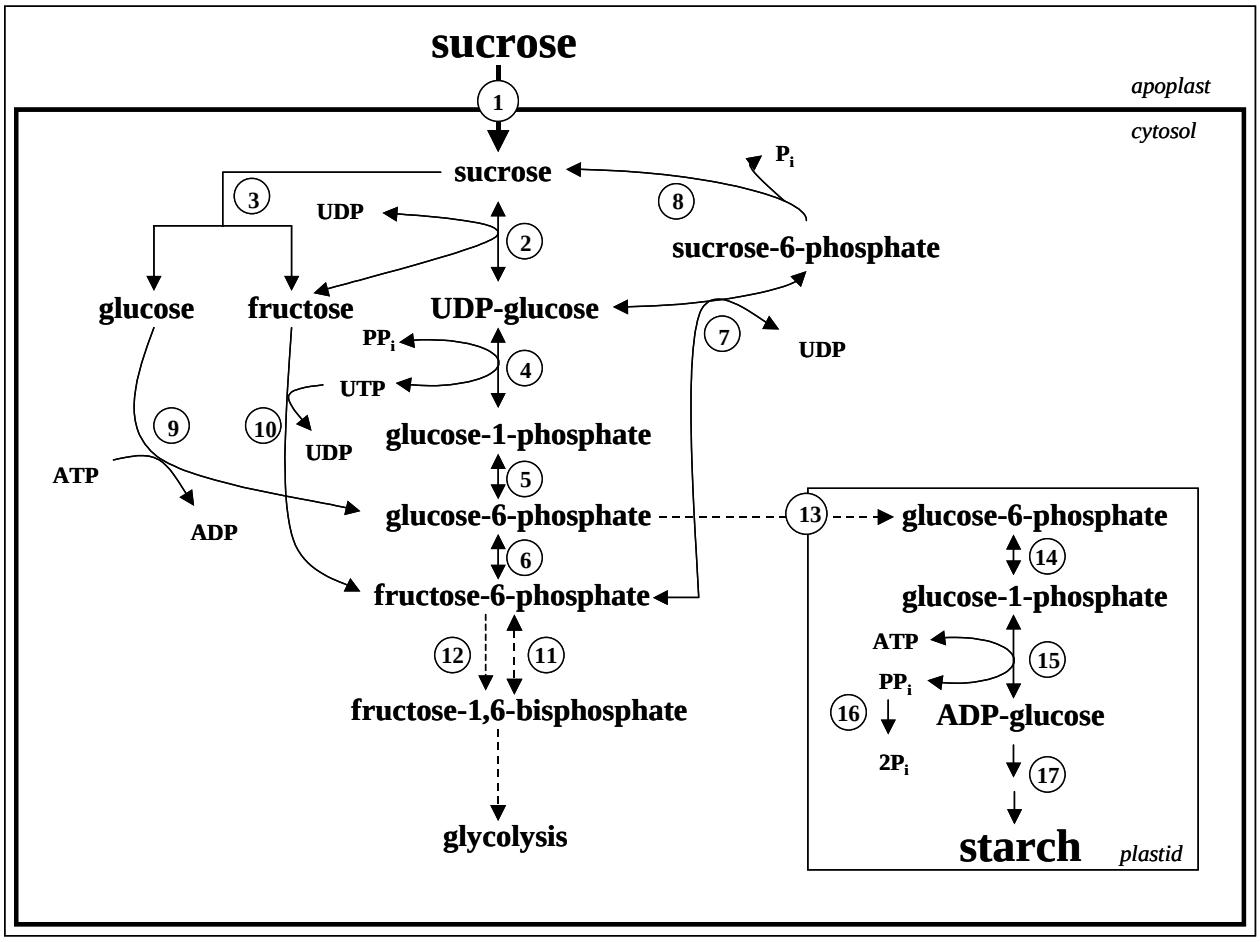



**Figure 1.1:** Sucrose/starch metabolism in heterotrophic plant cells. The numbers denote the following enzymes: (1) sucrose transporter; (2) sucrose synthase; (3) invertase; (4) UDPglucose pyrophosphorylase; (5) cytosolic phosphoglucomutase; (6) phospho-glucoseisomerase; (7) sucrose phosphate sythase; (8) sucrose phosphatase; (9) hexokinase; (10) fructokinase; (11) pyrophosphate:fructose-6-phosphate phosphotransferase; (12) phosphofructokinase; (13) plastidial glucose-6-phosphate transporter; (14) plastidial phosphoglucomutase; (15) ADPglucose pyrophosphorylase; (16) pyrophosphatase; (17) starch synthetic enzymes.

Categorical evidence that the carbon destined for starch synthesis enters the potato amyloplast at the level of hexose monophosphates, rather than triose phosphates, was provided by determinations of the degree of randomisation of radiolabel in glucose units isolated from starch following incubation of potato tuber discs with glucose labelled at the C1 or C6 positions (Hatzfeld and Stitt, 1990; Viola *et al.*, 1991). These data are in agreement with the observation that potato tubers lack plastidial fructose-1,6-bisphosphatase (FBPase) activity (Entwistle and ap Rees, 1990) and the failure to find expression of plastidic FBPase in tubers (Kossmann *et al.*, 1992). Historically there was a debate as to whether it is glucose-1-phosphate (Kosegarten and Mengel, 1994; Naeem *et al.*, 1997) or glucose-6-phosphate

(Schott *et al.*, 1995; Kammerer *et al.*, 1998; Wischmann *et al.*, 1999) that is transported into the tuber amyloplast. However, antisense inhibition of the plastidial PGM activity of potato resulted in significantly reduced starch content (Tauberger *et al.*, 2000). These data coupled with the identification of a plastidial glucose-6-phosphate transporter (Kammerer *et al.*, 1998) allows the conclusion that glucose-6-phosphate is the major precursor taken up by amyloplasts in order to support starch synthesis. Subsequently ADPglucose pyrophosphorylase (AGPase) produces ADPglucose and pyrophosphate at the cost of ATP. The pyrophosphate is rapidly removed by an inorganic alkaline pyrophosphatase located in the plastid. This reaction effectively renders the AGPase reaction irreversible in the amyloplast (Weiner *et al.*, 1987). AGPase is an important regulatory step in starch biosynthesis (Müller-Röber *et al.*, 1992; Sweetlove *et al.*, 1999; Tiessen *et al.*, 2002) and furthermore is allosterically regulated. The reaction is activated by its effector 3-phosphoglycerate and inhibited by inorganic phosphate in a range of tissues (Preiss and Sivak, 1996), most probably including potato tuber (Geigenberger *et al.*, 1998). However, starch synthesis also requires ATP to energise the production of ADPglucose. This fact is supported by observations that transgenic plants with altered activities of the plastidial ATP/ADP transporter exhibited changes in starch content (Tjaden *et al.*, 1998a). In the next steps, starch synthases catalyse the addition of glucosyl units to α-(1→4)-glucosidic chains (amylose) and branching enzymes form α-(1→6)-branch points between linear chains (amylopectin) (*for review* see Kossmann and Lloyd, 2000).

In contrast to the well characterised starch biosynthetic pathway, relatively little is known about starch degradation either of transitory starch in leaves or of the stored starch of heterotrophic tissues. Mobilisation of stored starch occurs by hydrolytic and phosphorolytic enzymes (*for review* see Beck and Ziegler, 1989; Steup, 1990; Kossmann and Lloyd, 2000). The hydrolytic enzymes include amylases, disproportionation enzyme (D-enzyme), maltase and debranching enzymes, whilst α-glucan phosphorylase and maltose phosphorylase are phosphorolytic. These release soluble sugars which are subsequently used for growth and respiration.

### 1.2.2. Regulation of the sucrose-to-starch transition

As in many other tissues, such as seedlings of *Ricinus communis* L. (Geigenberger and Stitt, 1991), kiwi fruit (MacRae *et al.*, 1992), ripening banana (Hill and ap Rees, 1995) or heterotrophic tobacco callus (Fernie *et al.*, 2001b), there is a cycle of sucrose degradation and resynthesis in growing potato tubers (Geigenberger and Stitt, 1991). Furthermore, an increase of SuSy activity during starch

accumulation is found in many tissues including potato, barley and wheat (*for review* see Avigad, 1982; Doehlert, 1990). The activity of the enzyme changes within the tuber under different developmental and/or environmental conditions (Ross and Davies, 1992). Regulation of the reaction catalysed by SuSy occurs at many levels including regulation by changes in gene expression (Ross and Davies, 1992), post-translational modifications (Doehlert and Choury, 1991) and also allosteric regulation. SuSy is inhibited by both its substrate fructose and by glucose (Godt *et al.*, 1995; Winter and Huber, 2000). Most of the UDPglucose released following sucrose cleavage by SuSy is converted to hexose monophosphates (ap Rees, 1988). Some of these hexose monophosphates are subsequently utilised in sucrose (re)synthesis. This process is catalysed in part by SuSy operating in the reverse direction but mainly by sucrose phosphate synthase (SPS) (Geigenberger and Stitt, 1993; Geigenberger *et al.*, 1997; Tauberger *et al.*, 2000). Sucrose resynthesis by the SPS pathway proceeds from UDPglucose and fructose-6-phopshate to sucrose-6-phosphate. Subsequently sucrose-6-phosphate is dephosphorylated by a specific sucrose-6-phosphate phosphatase thereby yielding sucrose (Figure 1.1). Since sucrose is simultaneously degraded and synthesised, a substrate cycle is established. SPS is tightly controlled, it is activated by glucose-6-phosphate and inhibited by inorganic phosphate (Doehlert and Huber, 1983; Reimholz *et al.*, 1994). Furthermore, the activity of the enzyme is also controlled by phosphorylation and dephosphorylation by a sucrose-6-phosphate synthase kinase and phosphatase (Doehlert and Huber, 1983; Siegl *et al.*, 1990; Huber and Huber, 1991; Reimholz *et al.*, 1994; Geigenberger *et al.*, 1999). Several roles of substrate cycling in plants have been proposed (Geigenberger and Stitt, 1993; Geigenberger *et al.*, 1995; Geigenberger *et al.*, 1999). Such cycles involve „wastage“ of energy but may allow the net flux of a pathway to respond very sensitively to factors that modulate the rate of synthesis and degradation (Hatzfeld and Stitt, 1990). Carbohydrate metabolism in a wide range of non-photosynthetic plant tissues, including potato tuber, faba bean seeds, maize endosperm, pea root and heterotrophic cell suspension cultures of *Chenopodium rubrum* and *Saccharum* sp, are characterised by two rapid substrate cycles in the cytosol. These cycles occur between hexose- and triose phosphates (Keeling *et al.*, 1988; Hatzfeld and Stitt, 1990; Viola *et al.*, 1991; Dieuaide-Noubhani *et al.*, 1995) and between sucrose and hexose phosphates (Hargreaves and ap Rees, 1988; Dancer *et al.*, 1990; Geigenberger and Stitt, 1991; Wendler *et al.*, 1991; Dieuaide-Noubhani *et al.*, 1995). Whilst the precise metabolic role of these substrate cycles is currently unknown, the fact that the turnover of sucrose responds very sensitively to changes in the levels of metabolic intermediates and sucrose suggests that sucrose metabolism can adjust to both changes in

sucrose supply and to the demand for carbon for respiration and biosynthesis in the cell (Dancer *et al.*, 1990).

The activity of sucrose synthase is often reported to be correlated with the rate of starch synthesis. For example a progressive inhibition of sucrose mobilisation and starch synthesis could be observed in potato tuber discs following water deficiency (Geigenberger *et al.*, 1997). In conditions of water stress sucrose synthesis via SPS was stimulated whilst sucrose degradation via SuSy was inhibited. This resulted in a decreased net flux from sucrose to hexose monophosphates and consequently a decreased rate of starch synthesis. It is possible that the decreased rate in starch synthesis could be a consequence of a decreased production of 3-phosphoglycerate, the positive regulator of AGPase (see Geigenberger *et al.*, 1998). Thus interrelation of the sucrose breakdown pathways, sucrose resynthesis and starch synthesis illustrates the complexities involved in the regulation of carbon metabolism.

The level of gene expression is another important regulatory point in carbohydrate metabolism. In many studies it has been demonstrated that a variety of different genes which are involved in different metabolic pathways are regulated by the availability of soluble sugars in higher plants (*for review* see Graham, 1996; Koch, 1996; Smeekens, 2000). In comparison to yeast and bacteria, mechanisms of metabolic regulation of gene expression in plant systems are poorly understood. Driven by compelling evidence from the yeast system (*for review* see Johnston, 1999), much attention has focussed on the signalling capacity of glucose via a sensing mechanism involving hexokinase (Jang *et al.*, 1997; Jang and Sheen, 1997; Pego *et al.*, 1999). Certain data obtained using transgenic *Arabidopsis* plants with an increased or decreased level of hexokinase activity seem to be in agreement with a pivotal role for hexokinase in plants (Jang and Sheen, 1997). Whilst most of the previous studies have been performed on photosynthetic tissue, there is also considerable correlative evidence suggesting that free glucose plays a major role in regulation of metabolism in heterotrophic tissues (Borisjuk *et al.*, 1998; Weber *et al.*, 1998). Furthermore cytosolic expression of yeast invertase resulted in plants that exhibited dramatic increase in the glucose levels and a significant change in the total metabolic activity by an increased rate of glycolysis and respiration rather than starch synthesis (Trethewey *et al.*, 1998). When taken together it indicates a role for glucose in regulation of carbon partitioning in potato tubers and in analogy to the yeast system, hexokinase is a prime candidate for the mediation of glucose related changes in metabolism. Upon expression of a bacterial sucrose

phosphorylase in potato tubers, glycolysis increased without changes in glucose, suggesting that the induction of glycolysis occurs via a glucose-independent mechanism (Trethewey *et al.*, 2001).

Studies of Halford *et al.* (1999) showed that within plant sink tissue cells not only hexokinase but also other factors play a role in sugar sensing via the phosphorylation of hexoses to hexose monophosphates. As sucrose is the predominant sugar imported into the cell it may also be sensed *per se*. The presence of an active sucrose nonfermenting-1-related protein kinase complex in plants was illustrated by Halford *et al.* (1999) who showed that this complex is required for sucrose induced expression of the sucrose synthase gene in potato tuber. A further model suggested that there are not only internal sugar sensors within the cells. Lalonde *et al*. (1999) proposed that membrane-bound receptors exist. Recent studies including the use of non-transportable sucrose analogues provided further evidence for the existence of such factors (Fernie *et al.*, 2000; Fernie *et al.*, 2001c). When taken together, these data suggest that there are multiple sugar-sensing pathways in plants and that sucrose and hexoses can initiate different signals involved in gene regulation.

## *1.3. Inducible transgene expression in plants*

Genetic modifications in plants can greatly contribute to enhance the understanding of metabolic processes. In the early days of transgenic plants, the majority of them contained genes under the control of the 35S promoter from cauliflower mosaic virus (35S CaMV), resulting in the transgene being expressed in all tissue types throughout plant development. However, using this approach the observed effects were not easy to interpret in many instances since the expression of the transgene in one tissue could lead to an effect in another tissue. To overcome this problem, tissue specific promoters were used, such as the tuber-specific patatin B33 promoter (Rocha-Sosa *et al.*, 1989). Such approaches allow the controlled spatial expression of transgenes. Inducible promoters allow to express the transgene at a certain desired timepoint. Such promoters are especially interesting tools to study (i) the function of genes that would be lethal if expressed constitutively, and (ii) the short-term changes in metabolism after expression of a metabolic enzyme. There are many different types of inducible promoters that are either endogenous or have been adapted for transgene expression in plants. Generally, they can be divided into three classes. The first class comprises systems that are induced by environmental stress, such as heat-shock inducible promoters (Nagao and Gurley, 1999). The second class are endogenous chemically inducible promoters, which are responsive to growth regulators, metabolic signals, nutrients, elicitors, wound signals, herbicide safeners, and chemicals that induce the

genes for systemic acquired resistance (*for review* see Gatz, 1997). However, these two classes are not useful for metabolic studies because environmental stresses and endogenous signals itself alter metabolism.

The third class are heterologous chemically inducible systems that have been adapted for the use in plants (*for review* see Gatz, 1997; Jepson *et al.*, 1998; Zuo and Chua, 2000; Padidam, 2003; Tang *et al.*, 2004). These chemical-inducible gene expression systems are widely used and can be classified in several groups on the basis of their mechanism. This class of promoters can be subdivided into derepression or activation systems. Derepression systems such as the bacterial tetracycline repressor (TetR) (Gatz *et al.*, 1992) work by relieving the repression of transgene expression. In this system, the regulator protein TetR is released from the *tet* operator upon association with tetracycline. Using this system, the tetracycline-inactivatable system (tTA) was developed (Weinmann *et al.*, 1994). In the tTA system, the expression of the target gene is mediated by binding of tTA to the *tet* operator, which only occurs in the absence of tetracycline. The most frequently used group of inducible promoters are activation systems, which can be further divided into the two subgroups of lower and higher eukaryotic transcriptional activation systems (Jepson *et al.*, 1998). The lower systems are of fungal origin and consist of two components. One component is a regulator protein (=inducer) that changes its conformation in the presence of a chemical (=co-inducer), and only than can bind to a response element and mediate target gene transcription. The other component is a chimeric promoter that usually consists of a plant minimal 35S promoter that is not able to initiate transcription by itself, which is fused to the fungal response element. Examples of this system are the copper-inducible promoter from yeast (Mett *et al.*, 1993; Mett *et al.*, 1996) and the ethanol inducible promoter from the fungus *Aspergillus nidulans* (Caddick *et al.*, 1998). The higher eukaryotic transcriptional activation systems consist of an inactive chimeric transcription activator containing a heterologous DNA-binding domain (DBD), an activation domain, a nuclear localisation signal (NLS) and the regulatory domain of a nuclear receptor, and of one or more copies of a recognition site combined with a minimal plant promoter. Examples for this class are promoters from mammals inducible by the glucocorticoid dexamethasone (Schena *et al.*, 1991; Aoyama and Chua, 1997) and an estradiol-inducible promoter (Bruce *et al.*, 2000). Further, an even more sophisticated system was developed which exhibits dual-control of the transgene. For this system, a chimeric transcription activator TGV was made by fusing the TetR DBD (T) to the regulatory region of the rat glucocorticoid receptor (G) and the VP16 transactivating sequence (V), and the resulting promoter system is therefore dexamethasone-inducible

and tetracycline-inactivatable (Böhner *et al.*, 1999). A system that can not be assigned to any of the previous groups is the Cre-*lox* system (*for review* see Gilbertson, 2003). In this system, which is usually used in mammals and was adopted for plants, the Cre-recombinase cuts out a DNA fragment (usually between promoter and gene of interest) flanked by *lox* sites upon induction. Once activated, expression remains permanently active.

## *1.4. Analytics*

The development of tools to characterise plants has made enormous progress over the last few years. Instead of focussing on single genes, proteins or metabolites, scientists now more and more take multiparallel high-throughput approaches to characterise plant systems as a whole. On the genome level (genomics), the first fully sequenced genomes were recently published for *Arabidopsis thaliana* (Initiative, 2000) and rice (Yu *et al.*, 2002), opening unlimited possibilities for plant research. The transcript level (transcriptomics) has been assessed by mRNA transcript profiling (Lockhart *et al.*, 1996). Protein profiles (proteomics) have been established by a combination of two-dimensional gel electrophoresis and mass-spectrometry (Shevchenko *et al.*, 1996). Rapid progress has also been made in metabolomics by gas chromatography combined with mass spectrometry (GC-MS) (Fiehn *et al.*, 2000; Roessner *et al.*, 2001b). As this technique is one of the major analytical methods used in this thesis, I will explain it more thoroughly.

At first, polar and non-polar metabolites are extracted from plants with methanol or chloroform, respectively. The extracts then are derivatised by methoxymation of the carbonyl-groups of reducing sugars, and by trimethylsilylation of polar functional groups. In the GC-MS machine, the derivatised plant extracts are at first vaporised and chromatographically separated by gas-chromatography, then the sample vapour is ionised, the ions are separated on the bases of their mass, and at the end the detected signal is amplified and recorded. Several sophisticated MS systems are available, including high-resolution sector instruments, time-of-flight (TOF) separators, Fourier-transformed ion cyclotron resonance and ion trap machines, each comprising a variety of inlet and ionisation modes. Each of these systems have their specific advantages and disadvantages, so that each system has a specific biochemical application (*for review* see Hübschmann, 1996). For analysis of metabolites, quadrupole or time-of-flight spectrometers are frequently used. The first separates ions using two perpendicular magnetic fields by their deflection, the latter separates the ions by their flight time from the ion source to the detector. The advantage of a GC-MS is the two dimensional separation, in that each metabolite

is characterised by a specific retention time and a specific mass spectrum. That means two diastereomers (like e.g. glucose and galactose) having the same mass spectrum can be separated by their retention time, and two metabolites eluting at the same time from the GC column can be separated by their mass spectra. By this technique it is currently possible to quantitatively determine approximately 60-140 known and up to several hundred unknown metabolites from a twenty minute to one hour machine run (U. Roessner, O. Fiehn, J. Kopka, pers. communication).

## 1.5. Modelling of Metabolism

The large amount of data generated by the analytical procedures mentioned above need tools for handling and interpretation. These tools are generally provided by bioinformatics, which is commonly defined as the application of methods and concepts of computer science in the field of biology. Bioinformatics can be divided into three main topics. The first major topic is the sequence analysis or genome informatics, its tasks being assembling sequence fragments, automatic annotation, pattern matching and implementation of database systems, like EMBL (http://www.ebi.ac.uk/embl/), KEGG (Kanehisa and Goto, 2000; http://www.genome.ad.jp/kegg/), etc. The second major topic of bioinformatics is protein design, in which the goal is to develop useful models that allow the automatic calculation of 3D structures, including the prediction of the molecular behaviour of this protein. The third major topic is Metabolic Engineering. Its goal is the analysis and synthesis of metabolic processes. The basic molecular information of metabolic pathways is stored in database systems. There are general databases like for example KEGG, which comprises genes, pathways and metabolites for a large number of organisms, and Brenda (http://www.brenda.uni-koeln.de/), which contains enzyme data. Further on, there are organism-specific databases like for example AraCyc (Müller *et al.*, 2003; http://aracyc.stanford.edu/), which contains pathway data for the model plant *Arabidopsis thaliana*. Based on the molecular and physiological knowledge represented by these database and information systems, pathway models can be established that allow the implementation of tools to analyse these pathways.

Pathway modelling can be done either in qualitative or in quantitative ways. One qualitative approach is the analysis of the elementary (flux) modes of a pathway, which are defined as “a minimal set of enzymes that can operate at steady state, with all the irreversible reactions operating in the appropriate direction” (Schuster and Hilgetag, 1994). Basically, elementary mode analysis elucidates the main routes that can be taken in a pathway, which is a very useful information for pathway

analysis (*for review* see Schuster *et al.*, 1999). Another qualitative approach, which is using Petri nets, can provide much information about the general properties of a pathway of interest, such as the presence of traps (accumulating metabolites), dead ends and *t-invariants* (possible paths that can be taken from the substrate to the product of a pathway). Interestingly, the *t-invariants* from Petri nets are calculated with an algorithm that is very similar to the algorithm to calculate *elementary flux modes* (I. Koch and M. Heiner, pers. communication). Petri nets are mainly used outside of biology, e.g. for analysis of technical systems, financial systems, operating systems, communication systems and software dependabilities (Heiner *et al.*, 1994). For pathway analysis, Petri nets are used in cases when there is not enough data to produce a kinetic model, or when the structure of a pathway is more important than the kinetic, e.g. in signalling cascades. A Petri net model of sucrose breakdown in the potato tuber is described elsewhere (Koch *et al.*, 2004).

The quantitative approach is usually a kinetic model of a pathway including rate laws with all their kinetic constants for velocity, as well as binding of substrates and inhibitors. The pathway is here described by a set of differential equations that can be mathematically solved. A steady state can be calculated, and by changing the maximal catalytic activities for an enzyme in the model it is possible to predict the effect of an overexpression or reduced expression of a gene on metabolism. There are different platforms on which these kinetic models are established, for example the Windows-based simulation tool Gepasi (http://www.gepasi.org; Mendes, 1993), Unix-based systems like Scamp or ScrumPy (http://mudshark.brookes.ac.uk/sware.html), or the multiple-platform virtual cell project E-Cell (http://www.e-cell.org). Metabolic Control Analysis (MCA), a theory established in the seventies by two seminal works (Kacser and Burns, 1973; Heinrich and Rapoport, 1974), is implemented in some of these programs. In MCA, the concept of a 'rate-limiting enzyme' was replaced by a coefficient expressing the degree of control over flux exerted by every enzyme (flux control coefficient $C^J$), thereby pointing out targets for Metabolic Engineering. Another quantitative approach to analyse metabolic pathways is to determine mass action ratios from flux measurements (for example Geigenberger *et al.*, 2004).

## *1.6. Research strategies*

From the preceding sections it is clear that within the potato tuber the pathway of sucrose breakdown is well characterised, but not fully understood. It was also pointed out that there are many tools like inducible promoters, metabolite profiling and kinetic pathway modelling that could help to

improve this understanding. At first, the establishment of an inducible gene expression system to study metabolism is described. Then, this system was used to study the short-term effects of the expression of a yeast invertase in a late developmental phase of the potato tuber. Further, the effects of a vacuolar yeast invertase on potato tuber metabolism are outlined. Finally, a kinetic model of the sucrose breakdown pathway is described. Chapter 2 is listing the Materials and Methods used for the lab-based results chapters 3-5. Chapter 6 contains a separate Methods section describing the model itself. This method section was not incorporated into Chapter 2 (Materials and Methods) because it is essential for understanding of the model.

# Chapter 2. Materials and Methods

## 2.1. Chemicals

General chemicals were purchased from Roche (Mannheim), Sigma-Aldrich (St. Louis, MO, USA) or Merck (Darmstadt). Acetaldehyde was obtained from Fluka Chemie GmbH (Buchs, Switzerland). Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Roche (Mannheim). Restriction enzymes and buffers were obtained either from Roche (Mannheim) or from New England Biolabs (Beverly, Massachusetts, USA). Except where noted otherwise, all biochemical enzymes were purchased from Roche (Mannheim). [U-$^{14}$C]-α-D-glucose was obtained from Amersham Buchler (Braunschweig). ADP, ATP, $NAD^+$, NADH, $NADP^+$, fructose-6-phosphate, fructose-1,6-bisphosphate, glucose-6-phosphate, glucose-1,6-bisphosphate, glucose-1-phosphate, glyceraldehyde-3-phosphate, phospho*enol*pyruvate, 2-phosphoglycerate, 3-phosphoglycerate and sucrose-6$^F$-phosphate were obtained from Roche (Mannheim).

## 2.2. Plasmids and Cloning

Preparation and restriction of plasmids, cloning, and gel electrophoresis were performed using standard procedures according to Sambrook *et al.* (1989). Ligations were performed using the T4 DNA Ligase (Roche, Mannheim) according to the manufacturer's protocol. DNA fragments were eluted from the gel and purified using the QIAEX II Gel Extraction Kit (Qiagen, Hilden) according to the manufacturer's protocol. Aqueous plasmid stocks were kept at –20°C prior to use.

The plasmid pB33-Alc-GUS was cloned by exchanging the 35S-promoter from the vector p35S:*alcR,alcA*:GUS-in-pBin19 (Sweetman *et al.*, 2002) with the tuber-specific B33 patatin promoter (Rocha-Sosa *et al.*, 1989). This cloning procedure was done by Chengcai Chu at the IPK Gatersleben.

The plasmid pB33-Alc-Inv (Figure 2.1) was produced in a two-step cloning procedure. First, the vector pUC18-suc2 (Andrea Leisse, MPI Golm) was cut with *Bam*HI in order to isolate the *suc2* gene from *Saccharomyces cerevisiae*. The fragment was then ligated into the *Bam*HI-site of the vector pUC19-AlcA (Uwe Sonnewald, IPK Gatersleben). In a second step, the resulting plasmid was cut with *Hin*dIII, and the resulting fragment was ligated into the *Hin*dIII-cut vector pB33-Alc-GUS (Uwe Sonnewald, IPK Gatersleben; *Hin*dIII cuts out the *alcA*:GUS cassette from this vector).

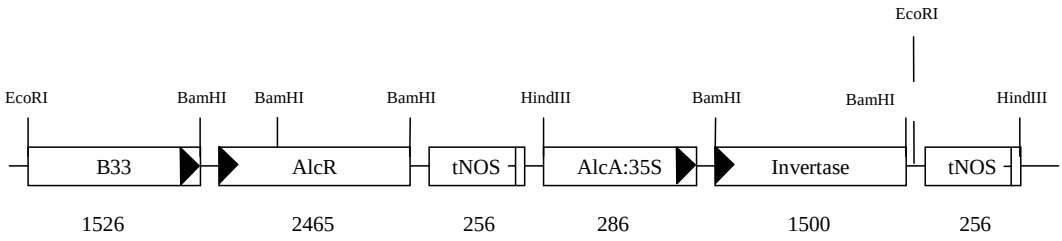



**Figure 2.1:** Map of the plasmid pB33-Alc-Inv including gene lengths (in base-pairs) and restriction sites. Invertase = *suc2* from *Saccharomyces cerevisiae*.

To generate the plasmid pB33-vacInv, the *suc2* gene from *S. cerevisiae* was excised with *Bam*HI from the vector pUC18-suc2 (Andrea Leisse, MPI Golm) and cloned into the *Bam*HI-site of pBluescriptSK$^-$ (Stratagene, La Jolla, California, USA). The resulting vector then was PCR-amplified using primers introducing a *Sal*I- (5') and a *Pst*I-site (3'), and ligated into pTA (Invitrogen, Karlsruhe). The 1.5kb *Sal*I/*Pst*I-fragment of this vector then was cloned into the vector IGF#34 (Arnd Heyer, MPI Golm) that had been previously linearised by *Sal*I and *Pst*I restriction. This vector then was cut with *Bsp*120I and *Sma*I to obtain a 1.8kb chimeric fragment (in which *suc2* is cloned in frame behind the vacuolar target sequence) that was filled in with the Klenow fragment (Roche, Mannheim) and ligated into the *Sma*I-site of pBinB33Hyg (Liu *et al.*, 1990).

## *2.3. Bacterial Strains*

Bacterial glycerol stocks were generated as described by Sambrook *et al.* (1989) and stored at -80°C. The strain *Escherichia coli* XL1 Blue MRF' (Stratagene, La Jolla, California, USA) was used for all cloning steps. The strain *Agrobacterium tumefaciens* C58C1 pGV2260 (Deblaere *et al.*, 1985) was used to transform potato plants.

## *2.4. Transformation and cultivation of bacteria*

Competent *E. coli* XL1 Blue cells were prepared and transformed by electroporation according to Miller *et al.* (1988). The cells were grown at 37°C on YT-medium plus appropriate selective antibiotic as described by Sambrook *et al.* (1989). Competent *A. tumefaciens* cells were prepared according to Hoefgen and Willmitzer (1990) and transformed by electroporation according to Miller *et*

*al.* (1988). The cells were grown at 28°C on YEB-medium plus appropriate selective antibiotic according to Vervliet *et al.* (1975).

## *2.5. Plant material*

*Solanum tuberosum* L. cv. Desirée (tetraploid) was supplied by Saatzucht Lange AG (Bad Schwartau). The line U-IN2-30, expressing a yeast-derived invertase under the control of the tuber specific patatin promoter B33 has been described earlier (Sonnewald *et al.*, 1997).

## *2.6. Transformation and cultivation of* Solanum tuberosum

Transformation of potato plants was carried out by *A. tumefaciens* mediated gene transfer following the method of Rocha-Sosa *et al.* (1989). The selection of transgenic plants was performed on medium containing hygromycin (Dietze *et al.*, 1995). Regenerants were screened for expression of the transgene by determining enzyme activity. Positive transformants were vegetatively propagated in tissue culture.

Plants were maintained in tissue culture on MS-medium (Murashige and Skoog, 1962) containing 2% (w/v) sucrose, 0.8% (w/v) Select Agar and 125 µg/ml Claforan under the following conditions: 22°C, 50% relative humidity, 3000 Lux and a 16h light, 8h dark regime. In the greenhouse, plants were grown on soil (Einheitserde P, Tantau, Uetersen) in 2.5L pots under the same light regime with a minimum daytime illumination of 250 µmol photons $m^{-2}$ $s^{-1}$ at 22°C and a relative humidity of 60%. The plants were watered by an automatic watering system that was increased according to the plant age from 50 to 250 ml per day. The water was permanently supplied with 0.7g/L of the fertiliser Hakaphos Rot (Compo, Münster).

## *2.8. Determination of enzyme maximum catalytic activities*

### 2.8.1. Extraction procedures and assay conditions

Extracts of tuber slices or powdered tuber slices (80 to 120 mg tissue) were prepared and desalted according to Trethewey *et al.* (1998). The desalted extracts were kept in aliquots at –80°C until assayed. All enzymes (except GUS) were extracted in 50 mM HEPES-KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 0.5 mM PMSF, 0.1% (v/v) Triton X-100, 10% (v/v)

glycerol, 2 mM benzamidine, 2 mM ε-aminocaproic acid. β-glucuronidase (GUS) was extracted in 500 µl GUS extraction buffer (Rao and Flynn, 1992) using a RZR 2040 grinder (Heidolph Instruments, Schwabach, Germany). After thawing, extracts were kept at 4°C prior to assaying. Unless otherwise noted, enzyme assays were carried out at 25°C in a final reaction volume of 300 µl as stated below. The change in absorbance at 340 nm was continuously followed using an Anthos ht II microtiter-plate reader (Anthos Labtec Instruments, Hanau). Activities of invertases, GUS, PPase and SPP were determined in stopped assays. All coupling enzymes provided as ammonium sulphate suspension were desalted by centrifuging at 4°C and 20,000 × *g* for 10 min, the supernatant being discarded and the sediment dissolved in the corresponding reaction buffer. If needed for evaluation, the protein content of the desalted extracts was determined according to the method of Bradford (1976).

### 2.8.2. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)

Glyceraldehyde-3-phosphate dehydrogenase was assayed in the direction of glyceraldehyde-3-phosphate production in a reaction mixture modified from Plaxton (1990). The assay contained 10 µl desalted extract in 100 mM HEPES-KOH (pH 8.0), 8 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA, 0.3 mM NADH, 2 mM ATP, 3.33 U $ml^{-1}$ phosphoglycerate kinase. The reaction was started by the addition of 3-phosphoglycerate to a final concentration of 6 mM.

### 2.8.3. Hexokinase (EC 2.7.1.1)

Hexokinase was assayed in the direction of glucose-6-phosphate production as described by Renz *et al.* (1993). The assay contained 10µl desalted extract in 30 mM HEPES-NaOH (pH 7.5), 2 mM $MgCl_2$, 0.6 mM EDTA, 9 mM KCl, 1 mM $NAD^+$, 1 mM ATP, 1 U $ml^{-1}$ $NAD^+$-linked glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*). The reaction was started by the addition of glucose to a final concentration of 1 mM.

### 2.8.4. Fructokinase (EC 2.7.1.4)

Fructokinase was assayed in the direction of fructose-6-phosphate production as described by Renz *et al.* (1993). The assay contained 10µl desalted extract in 30 mM HEPES-NaOH (pH 7.5), 2 mM $MgCl_2$, 0.6 mM EDTA, 9 mM KCl, 1 mM $NAD^+$, 1 mM ATP, 1 U $ml^{-1}$ $NAD^+$-linked glucose-6-

phosphate dehydrogenase (*Leuconostoc mesenteroides*), 1 U $ml^{-1}$ phosphoglucose isomerase. The reaction was started by the addition of fructose to a final concentration of 1 mM.

## 2.8.5. Phosphofructokinase (EC 2.7.1.11)

Phosphofructokinase was assayed in the direction of fructose-1,6-bisphosphate production as described by Burrell *et al.* (1994). The assay contained 50 µl desalted extract in 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.1 mM NADH, 1 mM ATP, 0.83 U $ml^{-1}$ Aldolase, 1.13 U $ml^{-1}$ glycerol-3-phosphate dehydrogenase, 2.17 U $ml^{-1}$ triose-phosphate isomerase. The reaction was started by the addition of fructose-6-phosphate to a final concentration of 5 mM.

## 2.8.6. Pyruvate kinase (EC 2.7.1.40)

Pyruvate kinase was assayed in the direction of pyruvate formation as described by Burrell *et al.* (1994). The assay contained 10 µl desalted extract in 50 mM MOPS (pH 7.0), 100 mM KCl, 15 mM $MgCl_2$, 0.15 mM NADH, 1 mM ADP, 5 U $ml^{-1}$ lactate dehydrogenase. The reaction was started by the addition of phospho*enol*pyruvate to a final concentration of 5 mM.

## 2.8.7. Sucrose phosphate phosphatase (EC 3.1.3.24)

Sucrose phosphate phosphatase was assayed in the direction of sucrose formation in a stopped reaction (John Lunn, MPI Golm, pers. communication). The reaction mixture (total volume 100 µl) contained 50 mM HEPES-KOH (pH 7.1), 5 mM $MgCl_2$, 2 mM sucrose-$6^F$-phosphate. The reaction was started by the addition of 25 µl desalted enzyme extract. After incubation at 25°C for 60 min, the reaction was stopped by the addition of 10 µl 2M TCA. The samples were then incubated for 10 min on ice, with a subsequent centrifugation step of 1 min. The produced phosphate was assayed by adding 20 µl of the above reaction mix to 280 µl 0.3% (w/v) ammonium molybdate and 3% (w/v) ascorbic acid in 0.35 M sulphuric acid. After 40 min incubation at 30°C, the developed colour was measured at 820 nm using an Anthos ht II microtiter-plate reader (Anthos Labtec Instruments, Hanau).

## 2.8.8. Acid and Alkaline Invertase (=β-fructosidase) (EC 3.2.1.26)

Invertases were assayed in the direction of sucrose cleavage in a stopped reaction by determining the amount of hexoses released (Stitt *et al.*, 1989; Zrenner *et al.*, 1995). The reaction

mixture contained 10 µl extract and 90 µl buffer and was incubated at 30°C for 90 min (wild type) or 10 min (transgenics). The reaction was stopped at 95°C for 5 min. Control samples were directly heat-inactivated without prior incubation. For acid invertase the buffer contained 20 mM sodium acetate (pH 4.7) and 100 mM sucrose (high purity). Prior to stopping the reaction mixture was neutralised with 10 µl 1M sodium phosphate buffer (pH 8.0). For alkaline invertase the buffer contained 20 mM HEPES-KOH (pH 7.5) and 100 mM sucrose (high purity). The produced glucose was then determined as described in Chapter 2.9 with 100 µl reaction mixture in a total volume of 300 µl.

## 2.8.9. β-glucuronidase (=GUS, EC 3.2.1.31)

β-glucuronidase was assayed by fluorometry as described by Rao and Flynn (1992). The assay contained 5 µl extract in 95 µl buffer (50 mM sodium phosphate, pH 7.5, 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (v/v) Sarkosyl, 10 mM β-mercaptoethanol). The reaction was started by the addition of 4-methylumbelliferyl-β-D-glucuronide (MUG, Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 0.5 mM. After 30 min incubation at 37°C, the reaction was stopped by the addition of 300 µl 0.2M sodium carbonate (pH 11.2). The whole assay volume was measured for fluorescence in a Fluoroscan Ascent platereader fluorometer (Labsystems, Franklin, MA, USA). 4-methylumbelliferone (4-MeU, Sigma-Aldrich, St. Louis, MO, USA) was used as a standard.

## 2.8.10. Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13)

Fructose-1,6-bisphosphate aldolase was assayed in the direction of glyceraldehyde-3-phosphate and dihydroxoacetone phosphate production as described by Burrell *et al.* (1994). The assay contained 20 µl desalted extract in 40 mM HEPES-KOH (pH 7.8), 0.2 mM NADH, 1.43 U $ml^{-1}$ glycerol-3-phosphate dehydrogenase, 4.17 U $ml^{-1}$ triose-phosphate isomerase. The reaction was started by the addition of fructose-1,6-bisphosphate to a final concentration of 5 mM.

## 2.8.11. Enolase (EC 4.2.1.11)

Enolase was assayed in the direction of phospho*enol*pyruvate production as described by Burrell *et al.* (1994). The assay contained 10 µl desalted extract in 100 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 0.2 mM NADH, 2.7 mM ADP, 4.17 U $ml^{-1}$ pyruvate kinase, 5 U $ml^{-1}$ lactate dehydrogenase. The reaction was started by the addition of 2-phosphoglycerate to a final concentration of 0.5 mM.

### 2.8.12. Triose-phosphate isomerase (EC 5.3.1.1)

Triose-phosphate isomerase was assayed in the direction of dihydroxyacetone phosphate production as described by Burrell *et al.* (1994). The assay contained 5 µl desalted extract in 100 mM HEPES-NaOH (pH 8.0), 5 mM EDTA, 0.2 mM NADH, 0.83 U $ml^{-1}$ glycerol-3-phosphate dehydrogenase. The reaction was started by the addition of glyceraldehyde-3-phosphate to a final concentration of 1.5 mM.

### 2.8.13. Phosphoglucose isomerase (EC 5.3.1.9)

Phosphoglucose isomerase was assayed in the direction of glucose-6-phosphate formation as described by Burrell *et al.* (1994). The assay contained 5 µl desalted extract in 100 mM HEPES-KOH (pH 7.4), 5 mM $MgCl_2$, 0.33 mM $NAD^+$, 0.42 U $ml^{-1}$ $NAD^+$-linked glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*). The reaction was started by the addition of fructose-6-phosphate to a final concentration of 1 mM.

### 2.8.14. Phosphoglucomutase (EC 5.4.2.2)

Phosphoglucomutase was assayed in the direction of glucose-6-phosphate formation as described by Sweetlove *et al.* (1996). The assay contained 3 µl desalted extract in 50 mM HEPES-KOH (pH 7.5), 5 mM $MgCl_2$, 100 µM glucose-1,6-bisphosphate, 2 mM $NAD^+$, 0.42 U $ml^{-1}$ $NAD^+$-linked glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*). The reaction was started by the addition of glucose-1-phosphate to a final concentration of 2 mM.

## *2.9. Determination of sugars, starch and hexose phosphates*

Starch and the soluble sugars glucose and sucrose were extracted as described by Trethewey *et al.* (1998) and determined photometrically. The change in absorbance was continuously followed at 340 nm using an Anthos ht II microtiter-plate reader (Anthos Labtec Instruments, Hanau). Soluble sugars were determined with a method modified from Stitt *et al.* (1989). The reaction mixture consisted of 20 µl ethanolic extract and 275 µl of 100 mM imidazol (pH 6.9), 5 mM $MgCl_2$, 2 mM $NADP^+$, 1 mM ATP and 2 U $ml^{-1}$ $NADP^+$-linked glucose-6-phosphate dehydrogenase (yeast). To start the reactions, the respective enzymes were sequentially added to the following final concentrations:

for glucose 1 U $ml^{-1}$ hexokinase (yeast overproducer), for fructose 0.5 U $ml^{-1}$ phosphoglucose isomerase (yeast), and for sucrose 11.5 U $ml^{-1}$ invertase (β-fructosidase from yeast). Starch content was measured using a commercially available starch determination kit (UV method; Cat.No. 207 748, Roche, Mannheim). The assay is based on the enzymatic hydrolysis of starch by α-amyloglucosidase and the determination of glucose in a coupled assay with hexokinase and glucose-6-phosphate dehydrogenase.

Hexose phosphates were determined in a platereader-based substrate-cycling assay exactly as described by Gibon *et al.* (2002).

## *2.10. Histochemical staining*

Tuber material was stained for β-glucuronidase activity in 6-well microtiter plates containing GUS staining buffer (Jefferson *et al.*, 1987) over night at 37°C. Staining of invertase activity was performed with two different methods for whole slices and micrographs, respectively. Whole slices (thickness: 1mm) were stained exactly as described by Hajirezaei *et al.* (2003).

For micrographs, tuber sections were stained for invertase activity with a method modified from Doehlert and Felker (1987). The sections were cut with a razor-blade, fixed with paraformaldehyde as described by Wittich and Vreughdenhil (1998) and washed 5 times over a period of 20h to remove all endogenous sugars. Subsequently, sections were incubated for 45 min at 37°C in a buffer containing 0.38 M sodium phosphate (pH 6.0), 0.24 mg $ml^{-1}$ nitro blue tetrazolium, 0.14 mg $ml^{-1}$ phenazine methosulphate, 5 mg $ml^{-1}$ sucrose (high purity) and 25 units $ml^{-1}$ glucose oxidase. Control sections were incubated with the reaction mixture without sucrose. After rinsing in water, the sections were stored in 15% (v/v) ethanol and later photographed through a microscope.

## *2.11. Metabolite profiling by gas chromatography - mass spectrometry (GC-MS)*

The relative levels of a wide range of metabolites were determined and quantified following the protocol from Roessner *et al.* (2001b).

## *2.12. Flux measurements*

For tuber disc labelling experiments tuber discs (diameter 10mm) were cut from the cap region of growing tubers attached to the fully photosynthesising mother plant following a 24h induction with acetaldehyde. These discs were then washed three times with 10mM 2-morpholinoethanesulphonic acid (MES; pH 6.5, KOH) and incubated (using conditions defined in Fernie *et al.*, 2002a) for 2h in 10 mM MES-KOH (pH 6.5) containing 2 mM glucose including 7.4 GBq/ml [$U^{14}C$]glucose (Amersham-Buchler, Freiburg, Germany). The discs were then harvested, washed three times in buffer and frozen in liquid nitrogen. Tissue was subsequently fractionated exactly as described by Fernie *et al.* (2001b).

*In planta* labelling experiments with intact tubers were performed as described by Bologa *et al.* (2003). Tubers were evacuated, taking care not to bend the stolons, a fine channel (1-2mm in diameter) was bored through the middle of the tuber and filled with 0.2% (v/v) acetaldehyde and after 24h 0.74 GBq/ml of [$U^{14}C$]glucose (specific activity 11.5 GBq/mmol), equivalent to approximately 37MBq per tuber. After 2 h of incubation in radiolabel, a concentric ring of tissue of (10mm diameter) was removed for analysis. During the entire experiment tubers remained attached to the mother plants via their stolons. Tissue was fractionated exactly as described by Fernie *et al.* (2001b).

## *2.13. Density measurements*

The term ‘tuber density’ refers to the specific tuber density, *p*, which was derived from the formula:

$$p = \text{mass of tubers in air}/(\text{mass of tubers in air} - \text{mass of tubers in water})$$

## *2.14. Statistical analysis of data*

T-tests were performed as described by Motulsky (1995). The expression “significant” is used only when an alteration has been confirmed to be statistically significant ($P < 0.05$) with Student’s t-test. For Principle Component Analysis (PCA) of metabolite profiles, data from each set of measurements was first normalised to the average of the corresponding wild type samples. The resulting data were then log10-transformed to get approximately a Gaussian distribution and to minimise the influence of outliers. PCA analysis was performed using the “prcomp” function of the library “mva” of the statistical software package “R” (http://www.r-project.org). Components were exported as text files and visualised with the program Sigma Plot (SPSS, Chicago, IL, USA).

# Chapter 3. Induction conditions

## 3.1. Introduction

A variety of gene expression systems that allow temporal control of transgene expression by environmental or chemical means have been recently characterised (Gatz, 1997; Jepson *et al.*, 1998; Zuo and Chua, 2000). It was envisaged that such systems would both avoid lethality problems that can be associated with constitutive overexpression and furthermore allow a more direct analysis of the primary effects of introducing a foreign protein into a cell. Promoters such as the dexamethasone inducible promoter (Aoyama and Chua, 1997) have been employed to great effect in the delineation of the immediate targets of the transcription factors CONSTANS and APETALA3/PISTILLATA in *Arabidopsis* (Sablowski and Meyerowitz, 1998; Samach *et al.*, 2000) and in dissecting the roles of the UNUSUAL FLOWER ORGANS (UFO) gene at various stages of floral development in *Arabidopsis* (Laufs *et al.*, 2003). To date, however little use of these promoters has as yet been made in the understanding of metabolism.

One exception to this is the expression of a yeast invertase in tobacco under the control of the two-component ethanol inducible system: the *alc*R encoded transcription factor (ALCR) and a promoter derived from the *alc*A promoter of *Aspergillus nidulans* (Caddick *et al.*, 1998; Salter *et al.*, 1998). The authors demonstrated that using this promoter high levels of invertase expression were recorded following induction (but not in the absence of inducer). They demonstrated the advantages that such an approach has over constitutive manipulation of enzyme activities as it allows the consequences of a manipulation at a specific point in time. Since this initial publication several further characterisations of the utility of the *alc* gene expression system in plants have been carried out. These studies demonstrated the efficiency of this inducible system in a wide range of plant hosts including *Arabidopsis thaliana, Brassica napus, Nicotiana tabacum* and in detached tubers of potato (Salter *et al.*, 1998; Roslan *et al.*, 2001; Sweetman *et al.*, 2002). In all instances the induction of the *alc* gene system has been documented following application of ethanol supplied to the plants by drenching the roots in ethanol solution (Caddick *et al.*, 1998; Salter *et al.*, 1998; Roslan *et al.*, 2001) or exposing the plants or plant parts to ethanol vapour (Sweetman *et al.*, 2002). Recently, a functional inducible RNA interference constructs that was confined to designated cell types has been developed using this system

(Chen *et al.*, 2003). However, since it has recently been demonstrated by elegant, yet indirect experiments in *A. nidulans* that acetaldehyde is the sole physiological inducer of the *alc* gene system (Flipphi *et al.*, 2001), it is conceivable that this is also a better inducer of plant transgene expression under the control of this system.

For this reason, in this section of work the inducing capability of acetaldehyde on the *alc* gene expression system in the developing potato tuber was tested. It was found out that the *alc* gene switch is induced more rapidly with low concentrations of acetaldehyde than with the corresponding concentrations of ethanol. Furthermore it is illustrated that the application of the inducers to wild type tissues has minimal effects on the metabolic complements of this tissue suggesting the great potential of the use of this system in studying temporal aspects of metabolism. However, observations of the pattern of expression following induction of B33-Alc-GUS transgenic potato lines indicate that expression of the transgene is not uniform across this bulky tissue suggesting the need for great caution in planning and interpreting the results following the use of this promoter.

## *3.2. Results*

### 3.2.1. Preparation and selection of transgenic lines

The aim of this section of work was to find a suitable and rapid mechanism of controlled gene expression in a bulky, hypoxic system such as the potato tuber. For this purpose I chose to express the two component *alc* gene system from *A. nidulans* under the control of the class I patatin promoter (Rocha-Sosa *et al.*, 1989) in order to additionally confer tissue-specific expression. A total of 74 independently transformed potato lines were generated following an *Agrobacterium*-mediated gene transfer protocol (Dietze *et al.*, 1995) that contained the construct pB33-Alc-GUS (kindly provided by Chengcai Chu, IPK Gatersleben; Figure 3.1). These lines were initially selected on kanamycin and subsequently transferred to the greenhouse. Tubers were formed in 2.5 L pots, and after ten weeks of growth 150 ml 0.2% (v/v) ethanol was applied twice daily to the soil for 4 days. 7% of the lines showed observable staining for β-glucuronidase (GUS). Surprisingly, the induction of GUS expression was not uniform across the tissue in these transformants. At the developmental stage used in these experiments no tubers yielded detectable GUS expression in the absence of the inducer, however mature tubers (> 100g in weight) yielded very strong expression of GUS in the absence of inducer (Figure 3.2). Interestingly the pattern of staining was the opposite of that observed following chemical

induction being strongest in the centre of the tuber, a fact that is probably best explained by the fact that hypoxic conditions develop in larger tubers (Geigenberger *et al.*, 2000). From the initial screening three B33-Alc-GUS lines were selected, amplified in tissue culture, and the resultant clones grown in the greenhouse for further study.

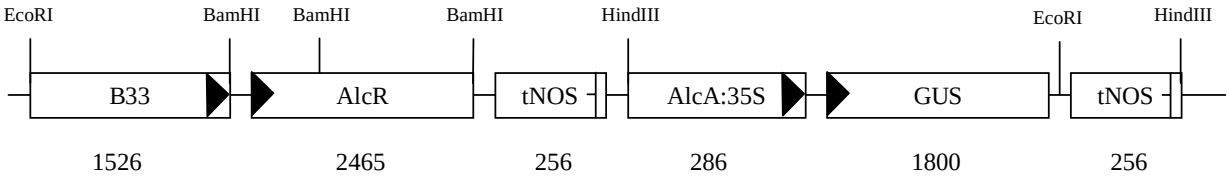



**Figure 3.1:** Map of the plasmid pB33-Alc-GUS. The antibiotic resistance parts from the binary vector pBinAR are not shown. The vector was constructed by Chengcai Chu, IPK Gatersleben.

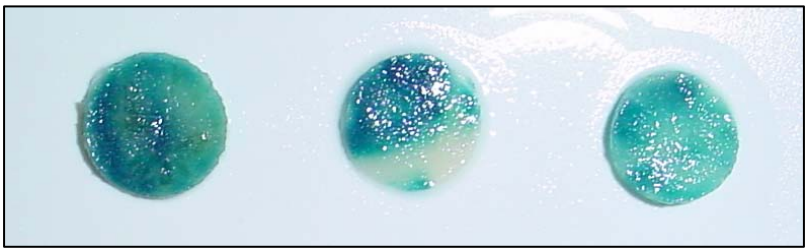


**Figure 3.2:** Auto-induced GUS expression in B33-Alc-GUS plants. Histochemical staining of 1 mm thick representative discs (1 cm diameter) from the centre of large tubers (>100 g) was carried out on tubers isolated from 16-week old senescing plants from the transgenic line B33-Alc-GUS 10 without application of any chemical inducer.

## 3.2.2. Pattern of gene expression following induction of the *alc* gene system

To further investigate the non-uniform expression observed in the preliminary characterisation of these lines we performed a larger experiment in which 10-week-old potato plants were induced with 0.2 % (v/v) ethanol for 24h or 96h. In addition, driven by the recent finding that acetaldehyde is the physiological inducer of the *alc* gene system *in situ* (Flipphi *et al.*, 2001) I evaluated the patterns of expression following induction under identical conditions. Figure 3.3 shows representative photographs of the patterns of expression observed under the above-mentioned induction conditions. In all cases the initial observation that the pattern of expression following induction was non-uniform was confirmed with no expression observable in the centre of the tubers. Interestingly in all cases the highest staining was observed in the stolon and apex regions of the tuber. In addition, as best illustrated by the acetaldehyde-induced samples, expression of GUS is prominent in patches close to

the tuber skin. Close scrutiny revealed that these patches were adjacent to the tuber lenticles. Despite the lack of uniformity in expression it is very clear from this qualitative data that induction of the *alc* gene system by acetaldehyde is effective in plant systems.

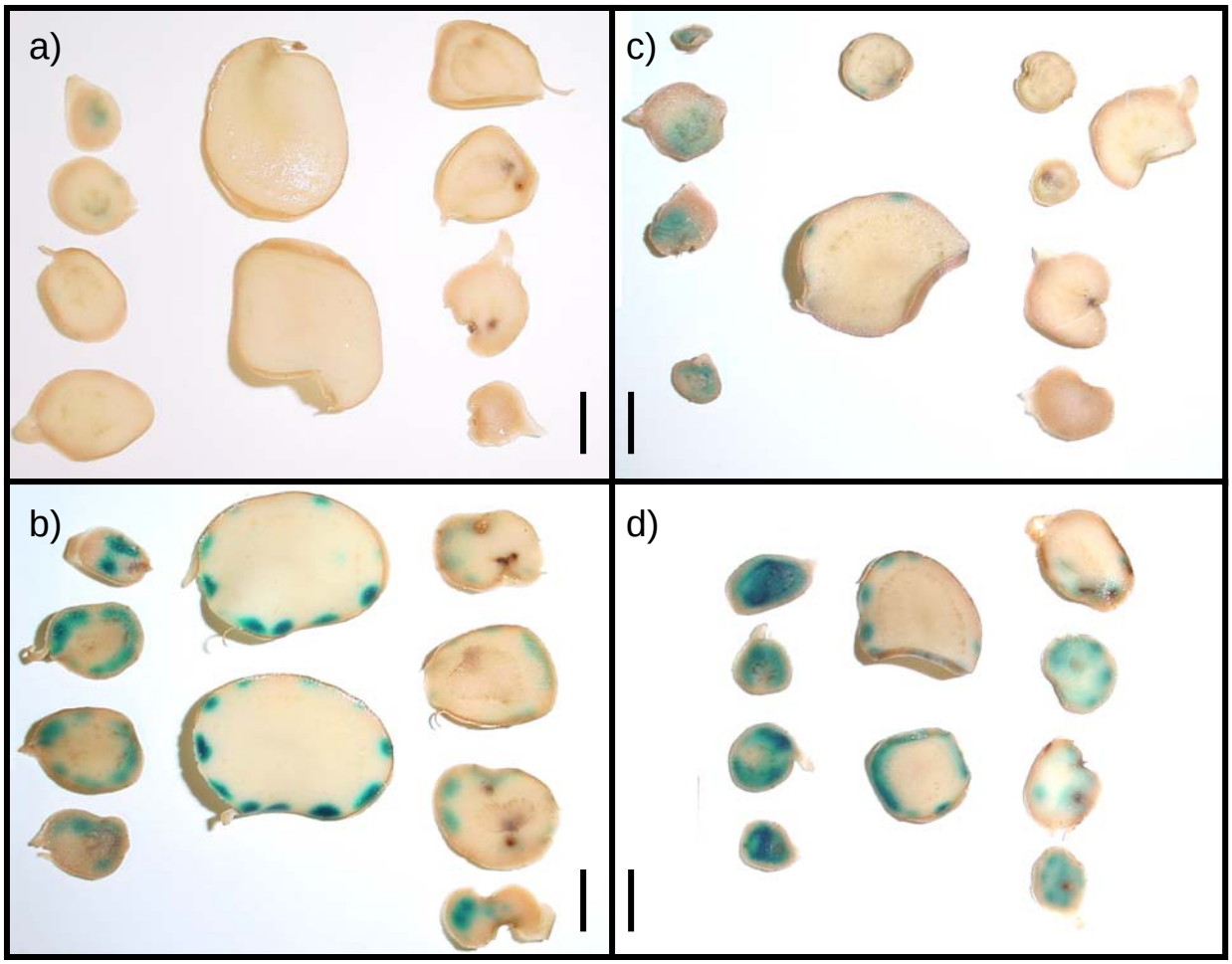



**Figure 3.3:** Inducible GUS expression in B33-Alc-GUS plants. Histochemical staining of 1 mm thick tuber slices was carried out on tubers isolated from 10-week old soil grown plants from the transgenic line B33-Alc-GUS 10. Staining of wild type and non-induced tubers from lines B33-Alc-GUS 7, 10 and 13 did not reveal any GUS expression. Induction was achieved by soil drenching with 150 ml 0.2 % (v/v) solvent for the stated time interval (twice daily) before analysis. Representative staining patterns are presented for the following treatments: ethanol, 24h (a); acetaldehyde, 24h (b); ethanol, 96h (c); acetaldehyde, 96h (d). The photographs show the expression pattern down the tuber following the stolon to apex axis (every 2$^{nd}$ slice is shown). Scale bars = 1 cm.

### 3.2.3. Comparison of induction following the application of different chemical inducers

Having established the effectiveness of acetaldehyde for the induction of the *alc* system in potato tubers I next decided to evaluate the possibility of inducing the system with 2-butanone (= ethyl methyl ketone, EMK), which has been demonstrated to be the best (non-lethal) inducer of the system in *A. nidulans*, and with acetone which showed close to 80% of inducing activity in *A. nidulans* compared to EMK (Creaser *et al.*, 1985). Whilst histochemical staining of tissue from B33-Alc-GUS line 10 allowed visualisation of GUS expression, the pattern of expression with EMK as inducer was similar to that observed with ethanol and acetaldehyde and the level of expression was less intense (Figure 3.4). The situation observed following induction with acetone was even less promising with very little expression visible even 96h following the initial induction. Given these results I decided to concentrate subsequent experiments on the quantitative comparison of the relative efficacy of induction by ethanol and acetaldehyde.

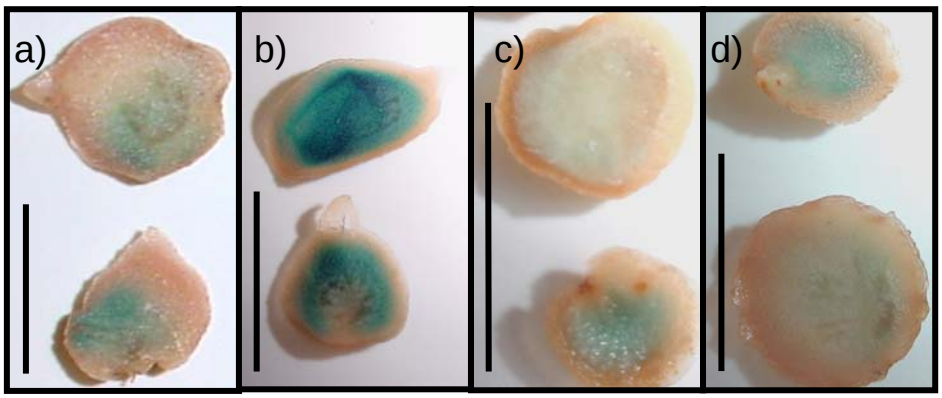



**Figure 3.4:** Detailed comparison of GUS expression following induction with a range of solvents. Histochemical staining of 1mm thick tuber slices was carried out on tubers isolated from 10-week old soil grown plants from the transgenic line B33-Alc-GUS 10. Staining of wild type and non-induced tubers from lines B33-Alc-GUS 7, 10 and 13 did not reveal any GUS expression. Induction was achieved by soil drenching with 150 ml 0.2 % solvent for 96h (application twice daily) before analysis. Representative staining of slices from the cap of the tuber are presented for the following solvents: ethanol (a); acetaldehyde (b); 2-butanone (c); acetone (d). Scale bars = 1cm.

### 3.2.4. *In vitro* induction of whole tuber slices and induction by injection

The characteristic induction pattern described in chapter 3.2.2. could either be related to a not uniform gene expression (AlcR regulator protein or GUS) or it could be a consequence of insufficient diffusion of the inducer. To assess this question, I cut whole slices from tubers of 10 week-old, non-induced plants from line AlcI-34 and incubated them in 6-well macrotiter plates containing 0.2% acetaldehyde (control: 0%) for 6h with consecutive staining for GUS activity. The control slices showed a slight stain in some cases, which might be due to the submergence for 6h in water and later for additional 18h in GUS staining solution: the slices could have low oxygen and, as a consequence, start to ferment. Probably the produced ethanol then could induce the GUS gene. The slices incubated in 0.2% acetaldehyde are stained more or less homogenously, suggesting that the spotted pattern observed in chapter 3.2.2. is a result of limited diffusion of the inducer.

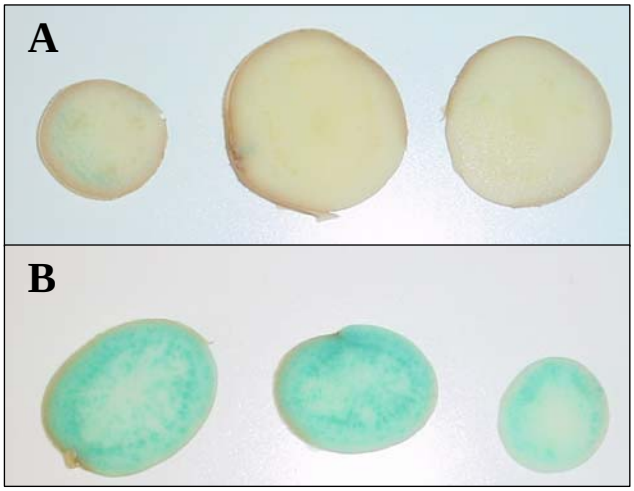



**Figure 3.5:** *In vitro* induction of whole tuber slices. Tubers from 10 week-old plants from line AlcI-34 were sliced and incubated for 6 h in water (A) and 0.2% acetaldehyde (B) with subsequent staining for GUS activity. Representative slices were photographed. The large slices are about 4 cm in diameter.

To address the same question as above, the induction potential of acetaldehyde injection into tubers was tested. Tubers were evacuated, but remained attached to the mother plant. A fine borehole was then made through the tubers and filled with 0.2% (v/v) acetaldehyde, after 24h hours a concentric cylinder was cut around the hole, sliced and stained for GUS activity (Figure 3.6). It can clearly be seen that the parenchyma of the tuber is stained some millimetres around the hole. This again suggests that the potential to be induced is present at all regions of the tuber.

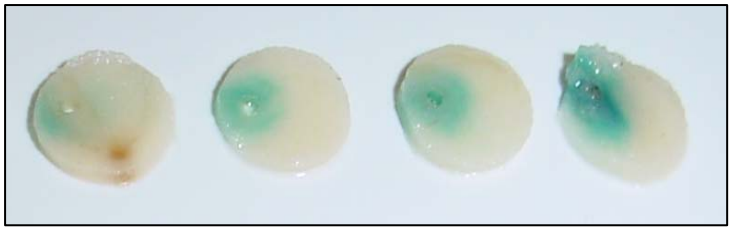


**Figure 3.6:** GUS induction by acetaldehyde injection. 0.2% (v/v) acetaldehyde was injected directly into intact tubers (*in planta*) into a fine borehole (1 mm diameter). After 24h, a concentric cylinder was cut around the borehole, sliced and stained for GUS activity. The slices are 1cm in diameter.

## 3.2.5. Time course of induction

A time course of induction with either acetaldehyde or ethanol was carried out on tubers from B33-Alc-GUS line 10 by assessing GUS enzyme activity, from tissue extracted from directly under the stolon, at regular intervals over a period of nine days (Figure 3.7). GUS activity was first detected at appreciable levels 7h after application of acetaldehyde peaking at 24h after the initial application, the enzyme then remained present at stable activity for two days before slowly decreasing. In keeping both with my earlier qualitative interpretation and with the findings described by Sweetman *et al.* (2002) the activity of GUS was not detectable in the ethanol treated samples until 24h after the initial application thereafter it remained stable for three days before decreasing. This observation was reproduced in three independent experiments. GUS activity in the untreated transformant control was negligible in all instances. Interestingly the mean coefficient of variation was lower for the samples induced with acetaldehyde than with ethanol (0.81 and 0.99, respectively) across all three experiments indicating that the reproducibility of induction may be slightly higher in response to acetaldehyde.

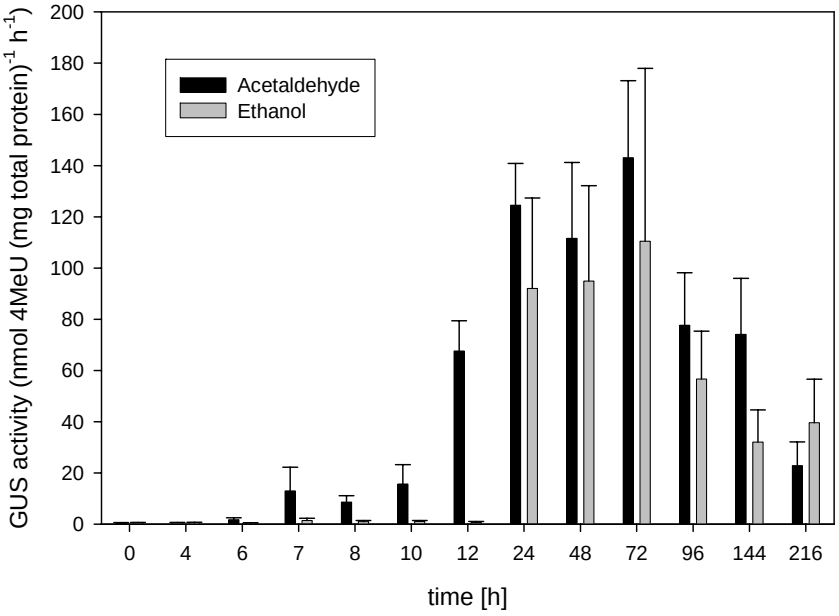



**Figure 3.7:** Ethanol and acetaldehyde induction of reporter gene expression with time. GUS activity was determined using fluorometry; the values are mean ± SE of 6 independent determinants. Plants were left untreated (control watering only) in a separate growing area or induced by soil-drenching with 150ml of 0.2% solvent at t=0 and 6h. A disk was taken from the region directly under the stolon from a total of 6 tubers from 2 plants and GUS activity was determined in protein extracts by fluorometry. GUS activity was undetectable in untreated samples. 4MeU: 4-methylumbelliferone.

## 3.2.6. Dose-dependency of induction

In a separate experiment I quantified the levels of GUS enzyme activity following induction by soil drenching with different concentrations of inducer for a 24h period (Figure 3.8). When the results are analysed it is clear both for acetaldehyde and ethanol that induction is negligible in concentrations below 0.05% (v/v) inducer. Furthermore, in both instances the expression is significantly higher following induction with 5.0 % (v/v) inducer than 0.2 % (v/v) inducer. Surprisingly, the highest GUS enzyme activity recorded following acetaldehyde induction occurred at the intermediate concentration of 1.0 % (v/v). With the exception of this concentration GUS activities are comparable between the two treatments.

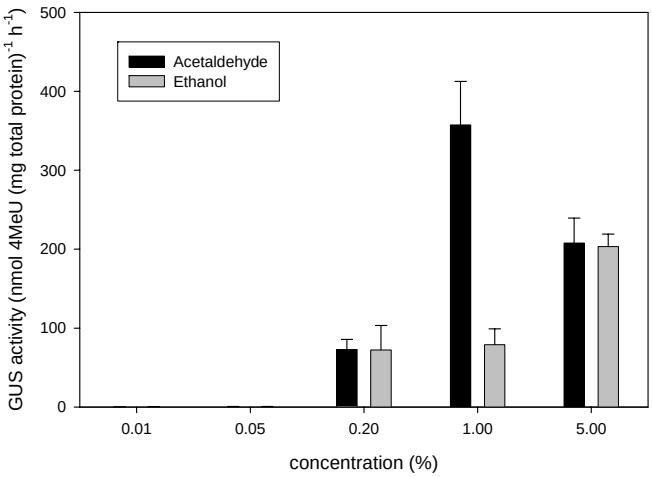



**Figure 3.8:** Ethanol and acetaldehyde induction of reporter gene expression with concentration. GUS activity was determined using fluorometry; the values are mean ± SE of 6 independent determinants. Plants were left untreated (control watering only) in a separate growing area or induced by soil-drenching with various concentrations of solvent at t = 0 and 6h. After 24h a disk was taken from the region directly under the stolon from 6 tubers from one plants and GUS activity was determined in protein extracts by fluorometry. GUS activity was undetectable in untreated samples. 4MeU: 4-methylumbelliferone.

## 3.2.8. Effect of soil-drenching on metabolism in the potato tubers

Having established that, at least in the case of the region below the stolon, high levels of expression could be achieved following induction in 0.2 % (v/v) ethanol or acetaldehyde for 24h, I next decided to investigate the effect of these treatments on potato tuber metabolism. For this purpose I soil-drenched untransformed wild type plants with 0.2 % (v/v) of each of the inducer species or with water and took sub-stolon tuber tissue samples after 24h. This tissue was rapidly extracted and frozen in liquid nitrogen prior to metabolite analysis using a recently established GC-MS protocol (Roessner *et al.*, 2001b). This analysis allowed to identify and quantify more than 60 metabolites of primary metabolism including sugars, sugar alcohols, amino acids and organic acids (Table 3.1). Surprisingly there was very little change in the metabolite complements following exposure to either of these chemicals. This was particularly noticeable in the case of acetaldehyde in which the levels of only three metabolites significantly altered with respect to the control watering (alanine decreased, whilst aspartate and inositol increased). Tuber tissue that was exposed to ethanol solutions exhibited far greater a number of significant changes (thirteen in all) including a general decrease in the levels of sugars coupled to a large increase in several organic and amino acids, notably succinate and alanine.

**Table 3.1:** Comparison of metabolite levels in tubers of wild type potato plants watered for four days twice daily with 150 ml of 0.2% ethanol, 0.2% acetaldehyde or as normal (i.e. twice daily with 150 ml water). Data are normalised to the mean response calculated for the control samples. Values presented are the mean ± SE of six independent determinants. Those that are significantly different from the control are identified in bold type.

| **Metabolite** | **Water** | **Ethanol** | **Acetaldehyde** |
|---|---|---|---|
| raffinose | 1.00 ± 0.17 | 0.55 ± 0.16 | 0.97 ± 0.18 |
| melezitose | 1.00 ± 0.24 | 0.79 ± 0.17 | 0.79 ± 0.23 |
| maltose | 1.00 ± 0.17 | **0.50 ± 0.07** | 0.86 ± 0.12 |
| sucrose | 1.00 ± 0.17 | 0.90 ± 0.08 | 0.69 ± 0.12 |
| glucose | 1.00 ± 0.30 | **0.18 ± 0.05** | 0.87 ± 0.22 |
| gluconate | 1.00 ± 0.14 | 0.98 ± 0.10 | 0.82 ± 0.11 |
| galactose | 1.00 ± 0.09 | **0.65 ± 0.11** | 1.14 ± 0.11 |
| galacturonate | 1.00 ± 0.10 | 1.12 ± 0.10 | 1.08 ± 0.08 |
| mannose | 1.00 ± 0.10 | 0.72 ± 0.13 | 1.15 ± 0.13 |
| mannitol | 1.00 ± 0.11 | 0.82 ± 0.06 | 1.27 ± 0.13 |
| fructose | 1.00 ± 0.22 | 0.92 ± 0.24 | 0.85 ± 0.19 |
| fructose-6-phosphate | 1.00 ± 0.05 | 1.01 ± 0.07 | 1.34 ± 0.18 |
| saccharate | 1.00 ± 0.08 | **1.31 ± 0.05** | 1.14 ± 0.12 |
| ribose | 1.00 ± 0.24 | 0.82 ± 0.10 | 0.51 ± 0.02 |
| glycerate | 1.00 ± 0.07 | 1.06 ± 0.05 | 1.22 ± 0.09 |
| glycerol-3-phosphate | 1.00 ± 0.04 | 0.99 ± 0.03 | 1.04 ± 0.04 |
| citrate | 1.00 ± 0.20 | 1.09 ± 0.14 | 1.25 ± 0.18 |
| aconitate | 1.00 ± 0.20 | 1.99 ± 0.95 | 1.54 ± 0.56 |
| succinate | 1.00 ± 0.09 | **3.09 ± 0.59** | 0.99 ± 0.33 |
| γ-aminobutyrate | 1.00 ± 0.20 | 1.54 ± 0.22 | 0.84 ± 0.17 |
| fumarate | 1.00 ± 0.11 | 1.17 ± 0.05 | 1.25 ± 0.17 |
| malate | 1.00 ± 0.22 | 0.77 ± 0.12 | 1.19 ± 0.13 |
| serine | 1.00 ± 0.10 | **2.59 ± 0.46** | 1.02 ± 0.03 |
| glycine | 1.00 ± 0.12 | **1.75 ± 0.29** | 0.84 ± 0.16 |
| cysteine | 1.00 ± 0.25 | 1.22 ± 0.13 | 0.89 ± 0.10 |
| alanine | 1.00 ± 0.06 | **2.12 ± 0.42** | **0.53 ± 0.07** |
| valine | 1.00 ± 0.05 | 1.03 ± 0.10 | 1.07 ± 0.14 |
| shikimate | 1.00 ± 0.08 | 1.13 ± 0.11 | 1.01 ± 0.15 |
| quinic acid | 1.00 ± 0.08 | 0.98 ± 0.07 | 0.90 ± 0.10 |
| tyrosine | 1.00 ± 0.25 | 1.24 ± 0.20 | 1.57 ± 0.52 |
| phenylalanine | 1.00 ± 0.10 | 0.82 ± 0.10 | 0.72 ± 0.07 |
| glutamate | 1.00 ± 0.05 | 1.01 ± 0.05 | 1.19 ± 0.09 |
| pyroglutamate | 1.00 ± 0.09 | 1.10 ± 0.15 | 1.29 ± 0.20 |
| glutamine | 1.00 ± 0.11 | 1.08 ± 0.32 | 1.37 ± 0.31 |
| ornithine | 1.00 ± 0.18 | 0.85 ± 0.05 | 0.82 ± 0.21 |
| aspartate | 1.00 ± 0.18 | **1.72 ± 0.23** | **1.62 ± 0.20** |
| asparagine | 1.00 ± 0.24 | 1.24 ± 0.52 | 1.21 ± 0.40 |
| lysine | 1.00 ± 0.21 | 0.67 ± 0.09 | 0.74 ± 0.19 |
| homoserine | 1.00 ± 0.14 | **1.87 ± 0.33** | 1.07 ± 0.24 |
| threonine | 1.00 ± 0.12 | 1.12 ± 0.24 | 1.37 ± 0.28 |
| isoleucine | 1.00 ± 0.11 | 0.80 ± 0.09 | 1.12 ± 0.20 |
| methionine | 1.00 ± 0.10 | 0.97 ± 0.08 | 1.02 ± 0.09 |
| β-alanıne | 1.00 ± 0.18 | **2.56 ± 0.53** | 0.86 ± 0.17 |
| ascorbate | 1.00 ± 0.26 | 2.14 ± 0.52 | 3.44 ± 1.20 |
| dehydroascorbate | 1.00 ± 0.08 | 0.87 ± 0.11 | 1.13 ± 0.04 |
| threonate | 1.00 ± 0.06 | 1.01 ± 0.10 | 0.88 ± 0.06 |
| benzoate | 1.00 ± 0.06 | 1.02 ± 0.04 | 0.94 ± 0.05 |
| inositol | 1.00 ± 0.05 | **0.66 ± 0.05** | **1.51 ± 0.07** |
| inositol-phosphate | 1.00 ± 0.06 | 1.07 ± 0.05 | 0.99 ± 0.06 |
| glutarate | 1.00 ± 0.09 | 0.94 ± 0.07 | 0.88 ± 0.08 |
| uracil | 1.00 ± 0.33 | 1.32 ± 0.32 | 1.00 ± 0.23 |
| nicotinic acid | 1.00 ± 0.23 | 1.15 ± 0.27 | 0.97 ± 0.26 |
| hydroxylamine | 1.00 ± 0.06 | 1.13 ± 0.08 | 0.93 ± 0.08 |
| putrescine | 1.00 ± 0.08 | **0.80 ± 0.04** | 0.82 ± 0.04 |
| spermidine | 1.00 ± 0.18 | 1.53 ± 0.59 | 1.90 ± 1.20 |
| tyramine | 1.00 ± 0.08 | 1.31 ± 0.22 | 1.25 ± 0.08 |
| phosphate | 1.00 ± 0.04 | 1.10 ± 0.08 | 0.94 ± 0.04 |

## *3.3. Discussion*

The strategy described in this Chapter was attempted in order to characterise the utility of the inducible *alc* gene system in a tissue-specific manner. The aims of this study were two-fold, firstly to develop an expression system that allowed modulation of potato tuber metabolism independently of leaf metabolism, and secondly to establish the optimal conditions for the induction of this system for studies of metabolism within the growing potato tuber. The expression of a GUS reporter construct under the control of the *alc* gene system and the B33 patatin promoter demonstrates that this strategy was, at least to a limited extent, successful. Expression was, however, not uniform across the tuber with high level expression limited to the stolon and apex of the tuber and to parenchyma tissue adjacent to the lenticles. Two different explanations can be postulated to explain this pattern of expression – it could be due either to the activity of the patatin promoter or to accessibility of cells to the inducing agent. It seems more likely that the later hypothesis is correct for several reasons. First, previous GUS reporter expression studies carried out with the patatin promoter expressed constitutively reveal that the promoter is active in all regions of the tuber (Rocha-Sosa *et al.*, 1989). Secondly, induction studies with ethanol vapour on detached tubers expressing the *alc* gene system under the control of the CaMV 35S promoter revealed that expression was also considerably higher on the outside of the tuber (Sweetman *et al.*, 2002). Thirdly, in the current study I observed that large tubers from 16-week-old plants auto-induce expression of GUS even in the absence of inducer. Under these growth conditions the pattern of GUS expression is completely different with expression throughout the tuber but maximal in its centre.

Despite the fact that high-level induction is confined to certain regions of the tuber providing the experiments are carried out with caution this system is clearly capable of conferring tissue-specific inducible expression. In order to optimise the induction of this system a range of potential inducers were applied to 10-week old plants. These experiments demonstrated that the *alc* gene system is sensitive to soil drenchings of low concentrations of EMK, acetaldehyde and to a lesser extent to acetone as well as the previously reported effects of ethanol (Caddick *et al.*, 1998; Salter *et al.*, 1998; Roslan *et al.*, 2001; Sweetman *et al.*, 2002). In concordance with these previous studies *alc* background activity was negligible in all tests. As was previously observed in detached tubers (Sweetman *et al.*, 2002) the response of the *alc* system to ethanol is much slower for potato tubers than for leaves from *Arabidopsis*, rapeseed or tobacco (Caddick *et al.*, 1998; Salter *et al.*, 1998; Roslan *et al.*, 2001). However, in the potato tuber the *alc* system responds to acetaldehyde within 7h. Recent

experiments in *Aspergillus nidulans* have revealed that this is the true physiological inducer in the fungus (Flipphi *et al.*, 2001). Given that it was also demonstrated that its application to wild type tubers had little effect on metabolism (in sharp contrast to ethanol which resulted in a large decrease in sugar content and an induction of anaerobic metabolism), it is proposed that this chemical should routinely be used for induction in bulky systems.

In conclusion, when used in conjuncture with the B33 patatin promoter the *alc* gene expression system is a very effective way of expressing inducible transgenes in a tissue-specific manner. The induction of this system in the potato tuber is much more rapid following the application of acetaldehyde than that of ethanol. Moreover, application of acetaldehyde to potato tubers produces far fewer changes in the metabolite complement than does that of ethanol. When taken together these data indicate that acetaldehyde is a promising method by which to induce the *alc* gene system particularly for metabolic studies.

# Chapter 4. Inducible Invertase

## 4.1. Introduction

During the last decade, a variety of inducible gene expression systems have been adapted for use in plants (Gatz, 1997; Jepson *et al.*, 1998; Zuo and Chua, 2000; Padidam, 2003). They have been used with great success in developmental studies in the model plant *Arabidopsis thaliana* (Sablowski and Meyerowitz, 1998; Samach *et al.*, 2000; Laufs *et al.*, 2003). However, the only time these promoters have been used to enhance the understanding of metabolism was the expression of a yeast invertase in tobacco under the control of the two-component ethanol-inducible system (Caddick *et al.*, 1998; Salter *et al.*, 1998). The transgenics produced were utilised as a proof of concept that the promoter allows tight and regulable control since it was previously known that expression of invertase on the leaves of tobacco yielded a severe thickening and curling of leaves alongside a leaf chlorotic phenotype (Sonnewald *et al.*, 1991) and these characteristics were only observed following induction (Caddick *et al.*, 1998). Since this initial publication several further demonstrations of the utility of the *alc* gene system have been published demonstrating the efficiency of this inducible system in a wide range of plant hosts including *Arabidopsis thaliana* (Roslan *et al.*, 2001), *Brassica napus* (Sweetman *et al.*, 2002), *Nicotiana tabacum* (Sweetman *et al.*, 2002), and in both detached (Sweetman *et al.*, 2002), and growing potato tubers (see Chapter 3). However, in all instances the examination of the metabolic changes accompanying the chemical induction of gene expression has been rather cursory. In Chapter 3 promoter activities of this tissue specific construct in response to watering with a range of solvents as chemical inducer were shown. These studies revealed that acetaldehyde was a more effective inducer of expression within the tuber system, a fact that most probably reflects its role as the physiological inducer of the *alc* gene system in *Aspergillus nidulans* (Flipphi *et al.*, 2001). Moreover, analysis of wild type tuber material following a mock induction (i.e. harvesting 24h after watering with 0.2% acetaldehyde) revealed that there is very little effect of the inducer *per se* on the metabolism of the tuber (Chapter 3). In this section of work here, transgenic plants expressing a yeast invertase cytosolically under the tuber-specific control of the *alc* gene system were generated and the expression of the chemically-induced transgene characterised. The resultant transformants were then grown for 10-weeks before the induction of transgene expression (by the application of low concentrations of

acetaldehyde) and the metabolic consequences of its expression were analysed. Particular attention was paid to metabolic characteristics that had previously been documented to change following the constitutive expression of the same gene as it was hoped that the transgenics described in this study would allow to dissect direct changes from the expression of the transgene at a single time point from more pleiotropic effects that result from the expression of a transgene throughout development of this organ.

## *4.2. Results*

### 4.2.1. Preparation and selection of transgenic lines

The aim of this work was to compare and contrast the effects of expressing a yeast invertase in the potato tuber in a controlled manner at a given developmental time point to those observed following overexpression of the same coding region under the control of a constitutively active promoter. For this purpose I created novel transgenic lines expressing the yeast invertase under the control of the two component *alc* gene system from *A. nidulans* and the class I patatin promoter (Rocha-Sosa *et al.*, 1989). A total of 88 independently transformed potato lines were generated following an *Agrobacterium*-mediated gene transfer protocol (Dietze *et al.*, 1995) that contained the construct pB33-Alc-INV. These lines were selected on kanamycin and subsequently transferred to the greenhouse. Tubers were formed in 2.5L pots and harvested 24h after induction by watering 10 week-old plants with acetaldehyde. 56 lines showed appreciably increased invertase activity in the cap region (just below the point at which the tuber attaches to the mother plant via the stolon) (Fig 4.1A). From this initial screening six AlcI lines were selected, amplified in tissue culture, and the resultant clones grown in the greenhouse for further study.

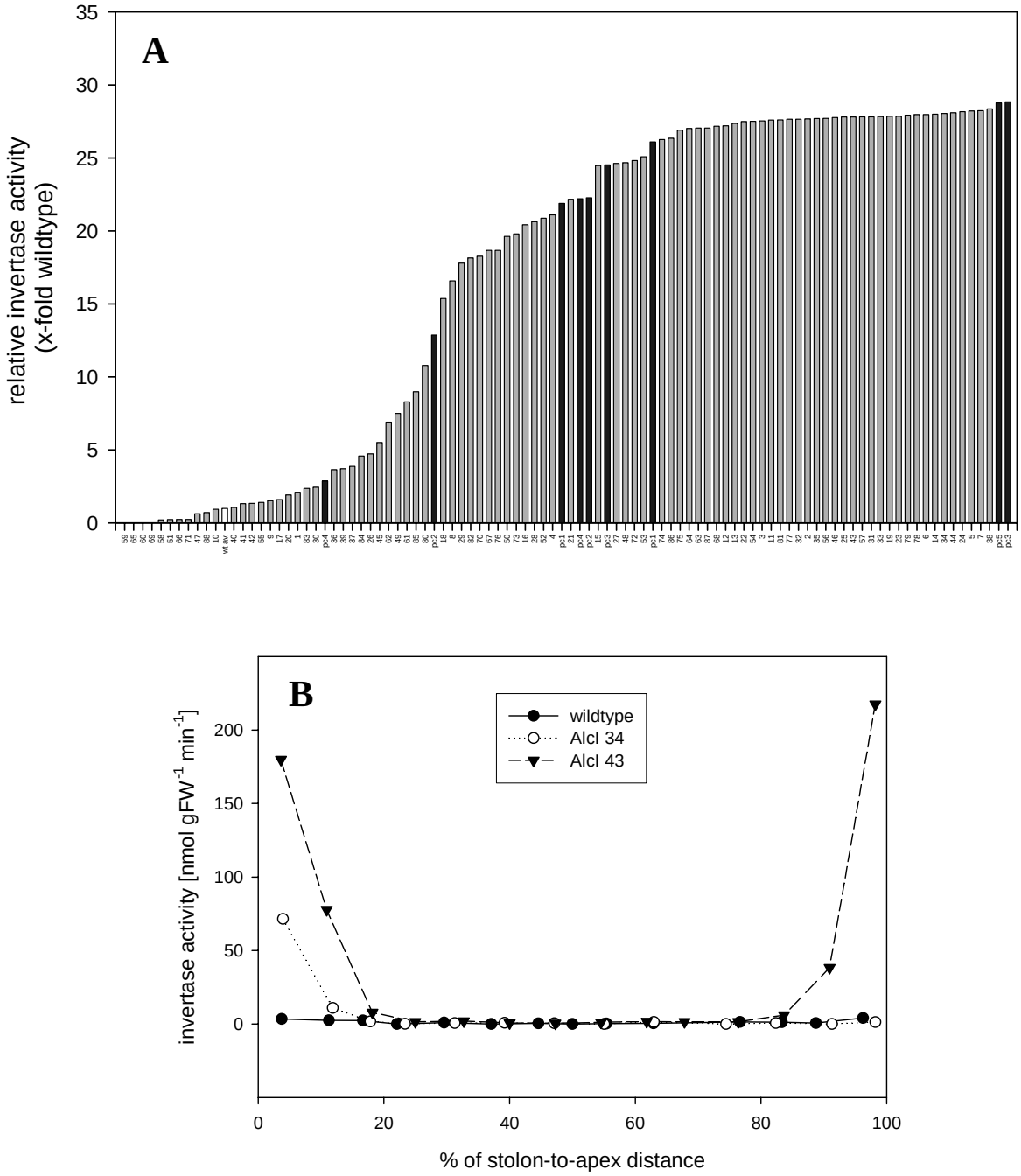



**Figure 4.1:** Overexpression of yeast invertase under the control of a B33 patatin driven alc gene system (A). Tuber samples were taken from 10-week-old AlcI plants following 24h induction by watering with 0.2% (v/v) acetaldehyde. Activities are presented as x-fold wild type activity. Wild type plants served as a negative control, and the tuber-specific, cytosolic invertase line U-IN2-30 served as a positive control (black bars, pc1-5). Gradients of invertase activity (B) along the longitudinal axis of growing potato tubers from wild type and the inducible lines AlcI-34 and AlcI-43. For each genotype a core was bored from the basal end to the apical end and sectioned into 2mm thick discs. Alternate discs were taken to determine invertase activities and glucose content (data not shown). Results are the mean of two replicate cores per genotype. Activities are presented in nmol $gFW^{-1}$ $min^{-1}$.

## 4.2.2. Pattern of gene expression following induction of the *alc* gene system

Whilst the above results confirm the results of Chapter 3 that the *alc* gene system is expressed in the cap region of the tuber they do not exclude the possibility that appreciable expression occurs in other parts of the tuber. To further investigate this possibility, and thus establish which is the most suitable tissue for further experimentation I carried out a number of preliminary experiments on whole tubers from 10-week old plants of lines AlcI-34 and AlcI-43 that had been induced with 0.2% (v/v) acetaldehyde for 24h. It has previously been demonstrated that in wild type tubers both invertase activity and glucose content follow a steep negative gradient from stolon to apex (Merlo *et al.*, 1993). For this reason a 10 mm stolon-apex core through large (20-30g) developing tubers was taken and both the activity of invertase and the level of glucose (which is presumably an indicator of the *in vivo* invertase activity) along this gradient were analysed. It was found that invertase activity was higher than that found in the wild type in the cap region but fell dramatically towards the centre of the tuber before rising again toward the tuber apex in line AlcI-43 but not AlcI-34 (Figure 4.1B). Surprisingly, the content of glucose was not markedly altered lines across the entire core in either of the transgenic lines. As a second experiment the invertase expression by the performance of *in vivo* histochemical staining was localised. Cross-sections of potato tubers were collected and washed three times with bi-distilled water to remove endogenous soluble sugars. Subsequently, slices were incubated in a sucrose-containing buffer and invertase activity was visualized by the formation of a brown colour (see Chapter 2). Although slight staining of the endogenous invertase was detected throughout control tubers (data not shown) invertase activity of transgenic plants was strongly enhanced in the cap and base of the tuber and also in regions adjacent to the lenticels (Figure 4.2). This expression pattern was exactly in accordance with that observed following expression analysis experiments in which the *alc* gene system was used to drive the expression of a GUS-reporter gene (Chapter 3).

**Figure 4.2:** Histochemical staining of invertase. Tubers of line AlcI-34 were cross sectioned from stolon to apex and slices were washed with water and incubated in enzymatic buffer mixture to allow the localisation of areas of high enzyme activity. These areas correspond to the same areas in which GUS-reporter gene activity was observed in Chapter 3.

### 4.2.3. Effect of induction of yeast invertase expression on sugar levels

Having established where, and under what conditions, the transgene can be induced I next wanted to evaluate the effect inducing the transgene had on metabolism. For this reason the newly created transgenic lines were then grown alongside the thoroughly characterised invertase overexpressing line U-IN2-30 (Sonnewald *et al.*, 1997; Trethewey *et al.*, 1998; Roessner *et al.*, 2001b) prior to induction. Following 10 weeks of growth the expression of invertase was induced by application of 0.2% (v/v) acetaldehyde to the soil. Harvesting of AlcI lines, the constitutive invertase overexpressor U-IN2-30 and the wild type controls was then carried out by sampling only material from the region directly under the stolon. Since it was shown in Chapter 3 that transgene induction is not uniform across experiments, the cap region of all the tubers of a given plant were pooled to provide enough material for determinations of enzymes, carbohydrates and metabolites within a single sample. As a first experiment I confirmed that expression of the transgene had been induced by measuring the invertase activity in these pooled samples (Table 4.1). Since it has previously been shown that the constitutive cytosolic expression of a yeast invertase in potato tubers resulted in a large decrease in sucrose coupled to an increased glucose content (Sonnewald *et al.*, 1997; Trethewey *et al.*, 1998), next these parameters were evaluated. The level of sucrose fell dramatically in the AlcI lines, to below 25% in the lines 23 and 34, which is a similar level of reduction to that observed in the constitutive line U-IN2-30 (despite displaying the same levels as wild type prior to induction; data not shown). Interestingly, the level of sucrose in the inducible lines roughly correlated with the level of invertase expression (Figure 4.3 A). In contrast, the level of glucose in these samples was largely

unaltered in the lines expressing the yeast invertase only for a period of hours, despite the fact that the material from the constitutive control (grown alongside these plants under identical greenhouse conditions) was characterised by a six fold increase in glucose content (Figure 4.3 B). Despite the lack of change in glucose levels in the AlcI lines, there is a clear change in the sucrose to glucose ratio (Figure 4.3 C) – which gives evidence for the *in vivo* activity of the expressed gene. In all cases the results obtained from the plants expressing the invertase in a constitutive manner resemble those recorded previously (Trethewey *et al.*, 1998; Roessner *et al.*, 2001b).

The levels of starch in the AlcI lines were, with the exception of line AlcI-18 (which is the weakest expressing line), unaltered with respect to wild type. In contrast the level of starch in the constitutive control line was markedly reduced in this study (Figure 4.3 D), in accordance with previous studies (Trethewey *et al.*, 1998; Hajirezaei *et al.*, 2000).

**Table 4.1:** Acid invertase activities in developing wild type and transgenic lines expressing a yeast invertase under the control of the *alc* system (AlcI) or the tuber-specific constitutively active B33 patatin promoter (line U-IN2-30). Activities are given as nmol $min^{-1}$ $gFW^{-1}$, values presented are means ± SE (n = 6; using 6 independently pooled samples each from a separate plant).

| Line | Acid invertase activity | | |
|---|---|---|---|
| Wild-type | 2.2 | ± | 0.3 |
| AlcI-18 | 29.7 | ± | 3.8 |
| AlcI-38 | 80.9 | ± | 15.3 |
| AlcI-3 | 122.6 | ± | 17.7 |
| AlcI-23 | 141.9 | ± | 5.6 |
| AlcI-43 | 206.9 | ± | 32.0 |
| AlcI-34 | 260.9 | ± | 33.9 |
| U-IN2-30 | 407.1 | ± | 32.4 |

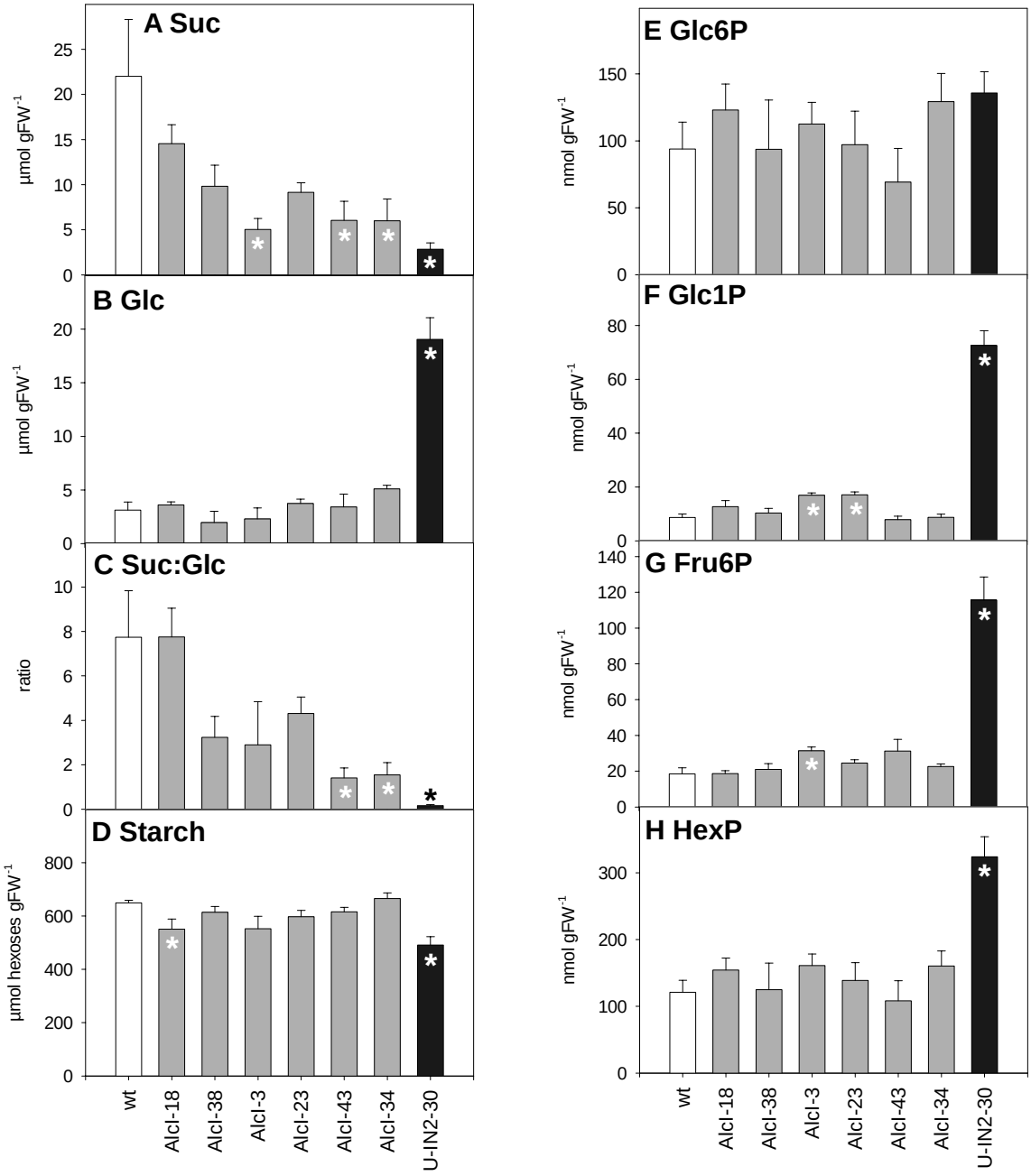



**Figure 4.3:** Carbohydrates and hexose phosphates in wild type (white bar), constitutively-expressed transgenic control (black bar) and AlcI plants (grey bar). Extracts were prepared from the exact samples as those used for the determination of enzyme activities presented in Table I. Sucrose (a) and glucose (b) were determined in extracts from six individual plants per line following these determinations the sucrose to glucose ratio (c) was deduced. Starch (d) was determined after hydrolysis to hexoses. Glc6P (e), Glc1P (f), Fru6P (g) and total hexose phosphates (h) were determined from four individual plants per line. Carbohydrate data and hexose phosphate data are presented in µmol $gFW^{-1}$ and nmol $gFW^{-1}$, respectively, as mean ± SE. Significant changes from the wild type ($P < 0.05$ using the Student's t test) are marked with an asterisk.

### 4.2.4. Effect of induction of yeast invertase expression on other primary metabolites

Given that the constitutive cytosolic expression of a yeast invertase in potato tubers resulted in dramatic increases in the levels of the majority of other metabolites of primary metabolism (Roessner *et al.*, 2001b) I next decided to evaluate the effect inducing the expression of invertase late in tuber development had on the level of these metabolites (Table 4.2). These determinations largely confirmed the results of the enzyme based tests presented above in showing that the sucrose content was reduced in the AlcI lines whilst the glucose levels are largely unchanged. Interestingly, they also revealed a large increase in the levels of maltose in the AlcI lines which was found to be present in excess of 20-fold higher than that found in wild type (but still 10-fold lower than that found in the constitutively-expressed transgenic control line). Further on, the levels of glucose 6-phosphate and fructose 6-phosphate only increased marginally in the AlcI lines, despite of an over tenfold increase in the constitutive control line (Table 4.2 and Trethewey *et al.*, 1998). Looking at metabolites further removed from the sucrose cleavage reaction it becomes apparent that although the levels of metabolites were also dramatically altered in the AlcI lines the changes in metabolite content were generally in the opposite direction to those found in the constitutively-expressed transgenic control line. For example, the levels of the majority of amino acids were enhanced in the constitutively-expressed transgenic control by as much as six-fold, however, in the AlcI lines the majority of the amino acid levels were depressed to approximately 50% of the wild type level. One obvious exception to this was proline which was found to increase in several of the AlcI lines despite decreasing in the constitutively-expressed transgenic control. Similarly, changes in the levels of ascorbate, shikimate, inositol and erythritol changed contrapuntally in the constitutively expressing transgenic control and the AlcI lines. The data set presented here also revealed that levels of the fatty acids 18:0 and 16:0 were significantly increased in the constitutively expressing transgenic control (a fact that has not been documented previously) but also in the AlcI lines. The situation is less clear regarding intermediates of the TCA cycle since although fumarate decreases in the AlcI lines, in contrast to the observed increase in the constitutively expressing transgenic control, the level of succinate is elevated in both the AlcI lines and the constitutively expressing transgenic control.

**Table 4.2:** Metabolite levels in developing wild type, constitutively-expressed transgenic control and AlcI plants. Metabolites were determined using the same samples from developing tubers (chemically-induced where appropriate) as those used to measure enzyme activities and carbohydrates presented in Table 4.1, Figure 4.3 and Figure 4.4. Data are normalised to the mean response calculated for the wild type. Values presented are the means ± SE of six independent determinants. Those that are significantly different from the wild type are identified in bold face. GABA, 4-amino butyric acid.

| | WT | AlcI-18 | AlcI-38 | AlcI-3 | AlcI-23 | AlcI-43 | AlcI-34 | U-IN2-30 |
|---|---|---|---|---|---|---|---|---|
| 1 arginine | 1.00 ± 0.54 | 0.93 ± 0.42 | 0.53 ± 0.19 | 0.81 ± 0.21 | 0.38 ± 0.18 | 0.40 ± 0.18 | 0.43 ± 0.15 | 2.07 ± 1.01 |
| 2 asparagine | 1.00 ± 0.03 | 0.96 ± 0.03 | 0.91 ± 0.04 | 0.90 ± 0.04 | 0.99 ± 0.18 | **0.66 ± 0.06** | **0.64 ± 0.14** | 1.04 ± 0.09 |
| 3 β–alanine | 1.00 ± 0.20 | 0.68 ± 0.06 | 0.65 ± 0.09 | 0.82 ± 0.11 | 0.63 ± 0.04 | 0.60 ± 0.04 | 0.73 ± 0.04 | 0.87 ± 0.05 |
| 4 cystein | 1.00 ± 0.20 | 1.41 ± 0.16 | 1.05 ± 0.17 | 0.80 ± 0.19 | 1.27 ± 0.31 | 0.71 ± 0.09 | **0.44 ± 0.07** | 0.96 ± 0.10 |
| 5 GABA | 1.00 ± 0.07 | 0.93 ± 0.06 | **0.77 ± 0.06** | 1.01 ± 0.05 | 0.86 ± 0.04 | 0.86 ± 0.06 | 0.99 ± 0.04 | 1.09 ± 0.08 |
| 6 glutamate | 1.00 ± 0.18 | 0.78 ± 0.21 | 0.62 ± 0.13 | 0.75 ± 0.07 | 0.54 ± 0.12 | **0.42 ± 0.08** | **0.41 ± 0.07** | 0.71 ± 0.17 |
| 7 glycin | 1.00 ± 0.13 | 0.82 ± 0.08 | 0.70 ± 0.07 | 0.86 ± 0.11 | 0.79 ± 0.07 | 0.71 ± 0.06 | 0.78 ± 0.03 | **1.39 ± 0.06** |
| 8 isoleucine | 1.00 ± 0.19 | 0.99 ± 0.13 | 0.87 ± 0.06 | 0.69 ± 0.14 | 0.82 ± 0.14 | 0.74 ± 0.08 | 0.79 ± 0.02 | 0.86 ± 0.25 |
| 9 leucine | 1.00 ± 0.04 | 0.86 ± 0.11 | **0.61 ± 0.07** | **0.76 ± 0.08** | 0.78 ± 0.20 | **0.56 ± 0.07** | **0.58 ± 0.05** | **1.65 ± 0.23** |
| 10 lysine | 1.00 ± 0.39 | 0.67 ± 0.17 | 0.49 ± 0.09 | 0.51 ± 0.06 | 0.27 ± 0.07 | 0.32 ± 0.13 | 0.26 ± 0.09 | 6.17 ± 3.33 |
| 11 methionine | 1.00 ± 0.09 | 0.76 ± 0.09 | 0.85 ± 0.09 | 0.89 ± 0.09 | 0.74 ± 0.14 | **0.51 ± 0.06** | **0.65 ± 0.04** | 1.02 ± 0.11 |
| 12 norvaline | 1.00 ± 0.04 | 0.97 ± 0.05 | 1.01 ± 0.03 | 0.83 ± 0.07 | 0.94 ± 0.11 | **0.78 ± 0.07** | 0.99 ± 0.06 | 0.87 ± 0.07 |
| 13 ornithine | 1.00 ± 0.63 | 1.14 ± 0.49 | 0.70 ± 0.17 | 1.03 ± 0.28 | 0.52 ± 0.20 | 0.52 ± 0.21 | 0.65 ± 0.17 | 2.58 ± 1.14 |
| 14 phenylalanine | 1.00 ± 0.08 | 0.76 ± 0.13 | **0.60 ± 0.06** | **0.64 ± 0.06** | **0.53 ± 0.10** | **0.48 ± 0.08** | **0.54 ± 0.05** | **2.51 ± 0.33** |
| 15 proline | 1.00 ± 0.14 | 1.46 ± 0.22 | 0.97 ± 0.14 | 1.83 ± 0.85 | 3.05 ± 0.92 | 1.82 ± 0.58 | 1.59 ± 0.29 | 0.69 ± 0.08 |
| 16 pyroglutamate | 1.00 ± 0.22 | 1.79 ± 0.44 | 1.71 ± 0.33 | 1.11 ± 0.24 | 0.90 ± 0.13 | 0.47 ± 0.16 | 0.70 ± 0.16 | **1.78 ± 0.20** |
| 17 serine | 1.00 ± 0.12 | 0.84 ± 0.07 | 0.78 ± 0.08 | 0.86 ± 0.08 | 0.79 ± 0.11 | 0.68 ± 0.08 | 0.74 ± 0.06 | **1.55 ± 0.06** |
| 18 4-OH-proline | 1.00 ± 0.27 | 1.96 ± 0.52 | 1.99 ± 0.39 | 1.18 ± 0.31 | 0.98 ± 0.19 | 0.69 ± 0.17 | 0.77 ± 0.21 | **2.07 ± 0.28** |
| 19 threonine | 1.00 ± 0.07 | 1.20 ± 0.09 | 1.03 ± 0.07 | 1.00 ± 0.06 | 1.06 ± 0.11 | 0.90 ± 0.12 | 0.97 ± 0.05 | 1.04 ± 0.03 |
| 20 valine | 1.00 ± 0.10 | 1.00 ± 0.11 | 1.02 ± 0.08 | 0.92 ± 0.11 | 0.85 ± 0.05 | 0.76 ± 0.05 | 0.90 ± 0.02 | 1.00 ± 0.07 |
| 1 18:0 | 1.00 ± 0.03 | **1.18 ± 0.01** | **1.18 ± 0.07** | **1.17 ± 0.05** | **1.26 ± 0.09** | **1.18 ± 0.05** | **1.19 ± 0.04** | **1.43 ± 0.07** |
| 2 16:0 | 1.00 ± 0.06 | 1.04 ± 0.02 | 1.02 ± 0.04 | 1.03 ± 0.04 | 1.14 ± 0.07 | 1.02 ± 0.03 | 1.07 ± 0.03 | **1.27 ± 0.06** |
| 3 9,12(Z,Z)-18:2 | 1.00 ± 0.09 | 0.94 ± 0.08 | 0.94 ± 0.05 | 0.89 ± 0.07 | 0.90 ± 0.04 | 0.95 ± 0.14 | 0.97 ± 0.08 | 1.29 ± 0.13 |
| 4 ascorbate | 1.00 ± 0.26 | 0.75 ± 0.11 | 1.03 ± 0.24 | 0.79 ± 0.13 | 0.66 ± 0.07 | 0.64 ± 0.07 | 0.59 ± 0.05 | 2.44 ± 1.17 |
| 5 benzoate | 1.00 ± 0.09 | 0.92 ± 0.08 | 0.84 ± 0.05 | 1.04 ± 0.10 | 0.96 ± 0.08 | 0.99 ± 0.04 | 1.13 ± 0.05 | 1.44 ± 0.34 |
| 6 citramalate | 1.00 ± 0.10 | 0.98 ± 0.09 | **1.29 ± 0.08** | 1.15 ± 0.11 | 0.99 ± 0.19 | 1.20 ± 0.07 | **1.55 ± 0.21** | **2.00 ± 0.24** |
| 7 citrate | 1.00 ± 0.15 | 0.66 ± 0.06 | 0.98 ± 0.15 | 0.65 ± 0.13 | 0.55 ± 0.16 | 0.59 ± 0.12 | 0.53 ± 0.17 | 1.27 ± 0.04 |
| 8 dehydroascorbate | 1.00 ± 0.08 | 0.81 ± 0.06 | 0.79 ± 0.07 | **0.72 ± 0.06** | **0.75 ± 0.04** | **0.67 ± 0.06** | **0.67 ± 0.06** | **0.49 ± 0.03** |
| 9 fumarate | 1.00 ± 0.08 | **1.84 ± 0.25** | **1.62 ± 0.10** | 1.38 ± 0.15 | **1.78 ± 0.13** | **1.62 ± 0.21** | **1.89 ± 0.22** | **0.58 ± 0.05** |
| 10 gluconate | 1.00 ± 0.08 | 0.98 ± 0.05 | 1.01 ± 0.07 | 1.02 ± 0.11 | 0.99 ± 0.10 | 1.01 ± 0.10 | 0.79 ± 0.08 | 1.02 ± 0.10 |
| 11 glycerate | 1.00 ± 0.04 | 1.00 ± 0.03 | 0.97 ± 0.04 | 0.91 ± 0.06 | 0.92 ± 0.07 | **0.84 ± 0.04** | 0.90 ± 0.05 | **4.38 ± 0.22** |
| 12 malate | 1.00 ± 0.08 | 0.96 ± 0.04 | 1.15 ± 0.07 | 0.96 ± 0.07 | 1.01 ± 0.05 | 1.12 ± 0.04 | 0.81 ± 0.17 | **1.36 ± 0.07** |
| 13 quinate | 1.00 ± 0.09 | 0.91 ± 0.04 | 0.83 ± 0.05 | 0.90 ± 0.05 | 1.01 ± 0.05 | 0.83 ± 0.11 | 0.89 ± 0.05 | 0.76 ± 0.10 |
| 14 shikimate | 1.00 ± 0.18 | 1.07 ± 0.12 | 1.31 ± 0.05 | 0.88 ± 0.08 | 0.91 ± 0.04 | 0.75 ± 0.06 | 0.72 ± 0.07 | 1.49 ± 0.22 |
| 15 succinate | 1.00 ± 0.25 | 0.77 ± 0.03 | 1.37 ± 0.11 | 1.49 ± 0.21 | 1.41 ± 0.05 | **1.96 ± 0.14** | **2.09 ± 0.29** | **2.90 ± 0.12** |
| 16 threonate | 1.00 ± 0.19 | 0.70 ± 0.05 | 0.76 ± 0.04 | 0.77 ± 0.05 | 0.82 ± 0.03 | 0.92 ± 0.10 | 0.99 ± 0.06 | 1.18 ± 0.10 |
| 1 fructose | 1.00 ± 0.49 | 1.02 ± 0.24 | 1.05 ± 0.22 | 1.09 ± 0.47 | 1.39 ± 0.25 | 1.33 ± 0.09 | 1.17 ± 0.33 | 1.15 ± 0.22 |
| 2 fucose | 1.00 ± 0.09 | 0.87 ± 0.05 | 0.81 ± 0.02 | **0.73 ± 0.06** | **0.78 ± 0.02** | 0.95 ± 0.13 | 0.79 ± 0.06 | **0.55 ± 0.04** |
| 3 galactose | 1.00 ± 0.18 | 1.20 ± 0.15 | 1.00 ± 0.11 | 0.87 ± 0.16 | 1.11 ± 0.15 | 1.29 ± 0.15 | 0.75 ± 0.05 | **0.40 ± 0.08** |
| 4 glucose | 1.00 ± 0.28 | 0.54 ± 0.09 | 0.51 ± 0.14 | 0.78 ± 0.35 | 0.55 ± 0.12 | 1.43 ± 0.36 | 0.81 ± 0.16 | **7.46 ± 0.71** |
| 5 inositol | 1.00 ± 0.15 | 0.95 ± 0.07 | 0.88 ± 0.06 | 0.88 ± 0.09 | 1.07 ± 0.06 | 1.18 ± 0.10 | 1.08 ± 0.04 | **0.51 ± 0.03** |
| 6 maltose | 1.00 ± 0.16 | 0.91 ± 0.23 | 1.87 ± 0.64 | 8.02 ± 6.35 | **1.74 ± 0.18** | **22.26 ± 4.81** | **23.32 ± 7.47** | **240.45 ± 30.96** |
| 7 mannose | 1.00 ± 0.19 | 0.94 ± 0.08 | 0.95 ± 0.14 | 0.75 ± 0.10 | 0.97 ± 0.12 | 1.16 ± 0.13 | 0.68 ± 0.05 | **4.64 ± 0.58** |
| 8 mannitol | 1.00 ± 0.08 | 1.01 ± 0.06 | **1.28 ± 0.07** | 1.02 ± 0.09 | 1.09 ± 0.03 | 0.80 ± 0.06 | **0.79 ± 0.03** | **3.52 ± 0.10** |
| 9 ribose | 1.00 ± 0.13 | 0.93 ± 0.05 | 0.92 ± 0.05 | 0.88 ± 0.10 | 0.84 ± 0.03 | 0.85 ± 0.06 | 0.84 ± 0.12 | **3.24 ± 0.09** |
| 10 sorbitol/galactitol | 1.00 ± 0.10 | 1.39 ± 0.22 | 1.49 ± 0.24 | 1.41 ± 0.16 | **1.41 ± 0.09** | **1.39 ± 0.11** | **1.93 ± 0.19** | **14.71 ± 1.99** |
| 11 sucrose | 1.00 ± 0.24 | 0.97 ± 0.16 | 0.84 ± 0.16 | 1.02 ± 0.56 | **0.33 ± 0.08** | 0.44 ± 0.17 | 0.37 ± 0.19 | **0.05 ± 0.00** |
| 12 xylose | 1.00 ± 0.16 | 0.62 ± 0.08 | **0.55 ± 0.11** | **0.42 ± 0.05** | **0.56 ± 0.10** | 0.79 ± 0.19 | **0.39 ± 0.04** | **0.11 ± 0.00** |
| 1 fructose-6-P | 1.00 ± 0.20 | 1.08 ± 0.18 | 1.80 ± 0.32 | 1.14 ± 0.20 | 1.00 ± 0.23 | 1.53 ± 0.22 | 1.35 ± 0.18 | **12.29 ± 0.86** |
| 2 glucose-6-P | 1.00 ± 0.22 | 1.04 ± 0.24 | 1.73 ± 0.33 | 1.12 ± 0.19 | 0.96 ± 0.20 | 1.60 ± 0.22 | 1.35 ± 0.15 | **10.89 ± 0.60** |
| 3 glycerol-1-P | 1.00 ± 0.06 | **0.80 ± 0.05** | 0.93 ± 0.04 | 0.87 ± 0.07 | **0.77 ± 0.08** | 0.92 ± 0.14 | 0.90 ± 0.09 | 1.15 ± 0.06 |
| 4 inositol-1-P | 1.00 ± 0.22 | 0.78 ± 0.28 | 0.67 ± 0.22 | 0.69 ± 0.18 | 0.52 ± 0.10 | 0.95 ± 0.18 | 0.79 ± 0.11 | **4.43 ± 0.58** |
| 5 phosphate | 1.00 ± 0.05 | 1.01 ± 0.04 | 0.96 ± 0.02 | 1.01 ± 0.07 | 0.95 ± 0.03 | 1.00 ± 0.03 | 1.04 ± 0.05 | 1.04 ± 0.03 |
| 1 α-tocopherol | 1.00 ± 0.09 | 1.02 ± 0.20 | 0.89 ± 0.12 | 1.11 ± 0.07 | 1.03 ± 0.05 | 1.05 ± 0.11 | 1.23 ± 0.06 | **1.68 ± 0.12** |
| 2 erythritol | 1.00 ± 0.04 | 1.04 ± 0.19 | 1.21 ± 0.17 | **0.69 ± 0.07** | **0.70 ± 0.05** | **0.73 ± 0.05** | **0.83 ± 0.05** | **10.21 ± 2.28** |
| 3 glycerol | 1.00 ± 0.12 | 0.89 ± 0.04 | 1.03 ± 0.15 | 0.83 ± 0.09 | 0.85 ± 0.06 | 0.91 ± 0.06 | 0.81 ± 0.05 | 0.89 ± 0.05 |
| 4 putrescine | 1.00 ± 0.10 | 1.20 ± 0.05 | 1.15 ± 0.05 | 1.09 ± 0.04 | 1.08 ± 0.04 | 1.08 ± 0.08 | 0.89 ± 0.05 | **0.49 ± 0.02** |
| 5 tyramine | 1.00 ± 0.14 | 0.90 ± 0.16 | 1.39 ± 0.25 | 0.89 ± 0.04 | 1.33 ± 0.08 | **1.50 ± 0.17** | 1.44 ± 0.15 | 1.31 ± 0.04 |

## 4.2.5. Effect of controlled expression of invertase on glycolytic activities

The constitutive cytosolic expression of a yeast invertase results in an induction of glycolysis both at the level of enzyme activity and of flux (Trethewey *et al.*, 1998; Trethewey *et al.*, 1999). The exact mechanism behind this induction has thus far proven elusive, however, given that a similar induction is also observed following the expression of a bacterial sucrose phosphorylase it was previously postulated that the induction is linked to a signal coming either from the low sucrose content or from the additional energy demands placed on the cell by an induction of a cycle of sucrose degradation and (re)synthesis (Trethewey *et al.*, 1998; Fernie *et al.*, 2002a). For this reason I was interested to see the effect that the inducible expression of invertase had on the activities of several key enzymes of the glycolytic pathway (Figure 4.4). Using tissue extracted from the same samples as those taken for the sugar analysis defined above, the activities of ten enzymes of glycolysis were determined in desalted extracts using assays that had previously been optimised for potato tuber extracts (Trethewey *et al.*, 1998; Veramendi *et al.*, 2002). The transgenic lines created for this study exhibited altered activities of several glycolytic enzymes, with a few notable exceptions activities of the AlcI lines increased, however these changes were generally less dramatic than those observed in the constitutively-expressed transgenic control and were only significant in a handful of cases. The activities of hexokinase of the AlcI lines were marginally (but not significantly) elevated above the wild type level, whereas that observed in line U-IN2-30 was decreased to approximately 60% of the activity found in wild type (Figure 4.4 A). Similarly, the activity of phosphoglucomutase was elevated by up to 40% above the wild type in both the AlcI lines and the constitutively expressed control (significantly so in lines AlcI-23 and AlcI-38; Figure 4.4 C). Fructokinase activity was reduced in both the AlcI lines and constitutively expressed transgenic control with respect to the wild type (significantly so in line AlcI-43; Figure 4.4 B) and phosphoglucose isomerase was significantly reduced with respect to the wild type in all invertase expressing lines tested (Figure 4.4 D). Phosphofructokinase and enolase activities were significantly enhanced in the constitutively expressed transgenic control but were unaltered in the AlcI lines (Figures 4.4 E and I) whereas aldolase was unaltered in the constitutively expressed transgenic control but was significantly decreased in two of the three strongest AlcI lines (Figure 4.4 F). Similarly triose phosphate isomerase was only significantly different from the wild type in one line AlcI-3 – in which it was elevated (Figure 4.4 G). Glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase were significantly increased in three

of the six AlcI lines, but to a lesser extent than that observed in the constitutively expressed transgenic control (Figures 4.4 H and J).

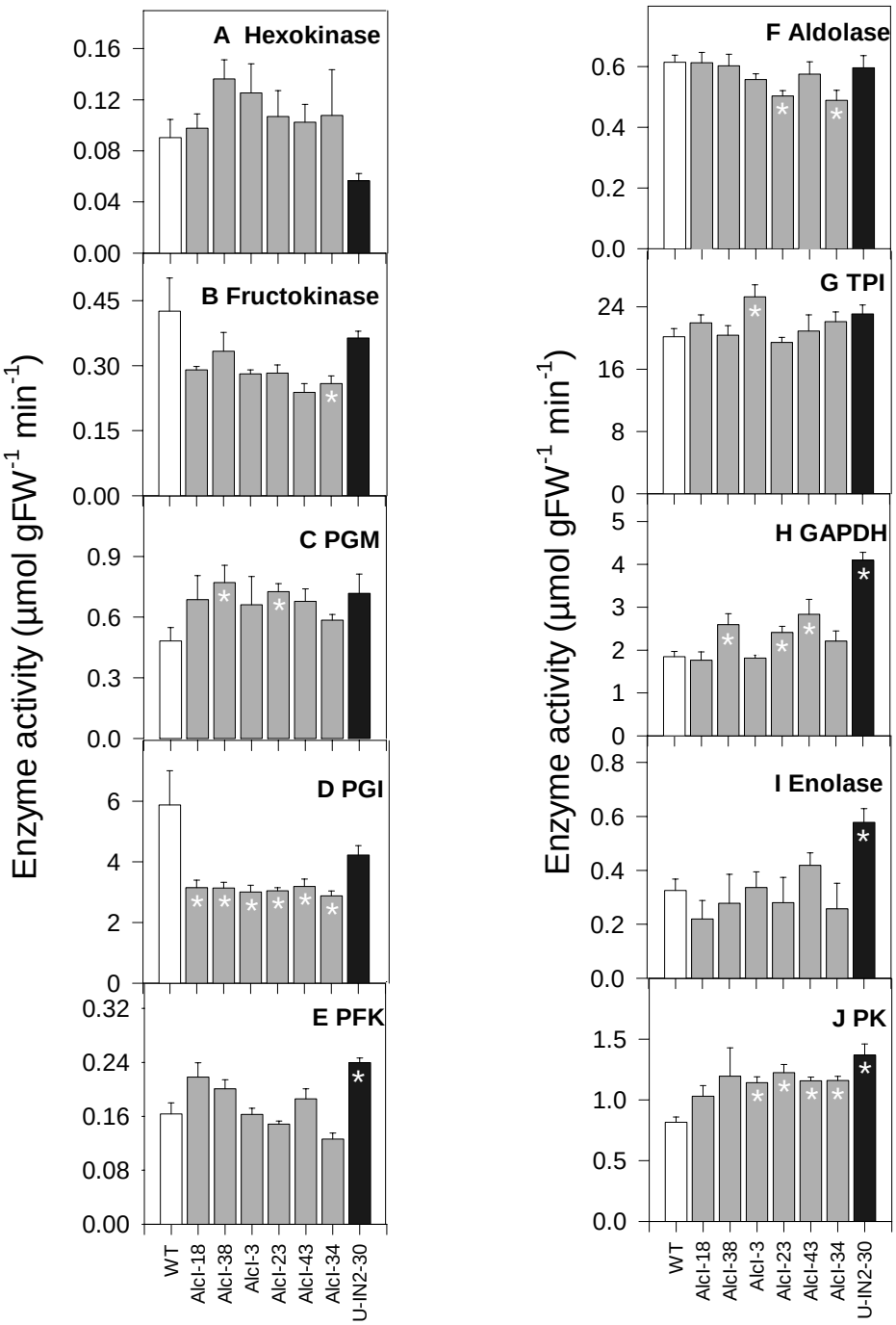



**Figure 4.4:** Maximal catalytic activities of enzymes of glycolysis in wild type (white bar), constitutively-expressed transgenic control (black bar) and AlcI plants (grey bar). PGM, phosphoglucomutase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; TPI, triose phosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PK, pyruvate kinase. Values re presented in µmol $gFW^{-1}$ $min^{-1}$ as means ± SE (n = 6). Significant changes from the wild type ($P < 0.05$ using the Student's t test) are marked with an asterisk.

## 4.2.6. Metabolism of labelled glucose in tuber discs of the transgenic lines

In order to analyse the dominant fluxes of carbohydrate metabolism in the transgenic lines, freshly cut slices of growing potato tubers were incubated with 2 mM [U$^{14}$C]glucose (Figure 4.5). Labelled glucose was preferred to sucrose due to the large changes in sucrose content in the transformants. Furthermore, this choice of radiolabelled substrate also provided the opportunity to measure the rate of sucrose resynthesis and allowed comparison of the results obtained from the wild type and constitutive transgenic control line with those documented previously (Trethewey *et al.*, 1999). The results obtained for the constitutive transgenic control line were in very close agreement in comparison to the wild type control between this and the previous study so attention was focussed on the AlcI lines. There was no difference in the rate of [U$^{14}$C]glucose taken up by discs of the transgenics in comparison with the wild type (Figure 4.5 A). The proportion of radiolabel that was subsequently metabolised was however significantly different between the transformants with a minor decrease in metabolism observed in lines AlcI-3, AlcI-23 and AlcI-43 and a dramatic decrease in the constitutive transgenic control line (Figure 4.5 B). The pattern of label incorporation was generally similar between the AlcI lines and the constitutive transgenic control with dramatic decreases in label incorporation in starch (Figure 4.5 C), and increases in that found in sucrose (these increases were even greater in the AlcI lines; Figure 4.5 D). Similarly, with the exception of line AlcI-18 which displayed a decreased label retention, the AlcI lines were also characterised by elevated maltose contents (although in this instance to a far lesser extent than observed in the constitutive transgenic control; Figure 4.5 E). Furthermore both the label released as $^{14}CO_2$ during the experiment (Figure 4.5 G), and the label retained in organic acids (Figure 4.5 H) followed a similar trend in the AlcI lines to that observed for the constitutive transgenic control with decreased label in $CO_2$ and decreased label in organic acids observed in the strongest AlcI expressors (AlcI-23, -43, -34 in the case of $CO_2$ and AlcI-43, -34 in the case of organic acids). Although most of the patterns of label incorporation are similar between the inducible lines and the constitutive transgenic control there was a notable differences in the label retained in the phosphoester pool (Figure 4.5 F). This was dramatically reduced in the constitutive transgenic control but was unchanged or even increased (lines AlcI-38 and AlcI-23) in the AlcI lines.

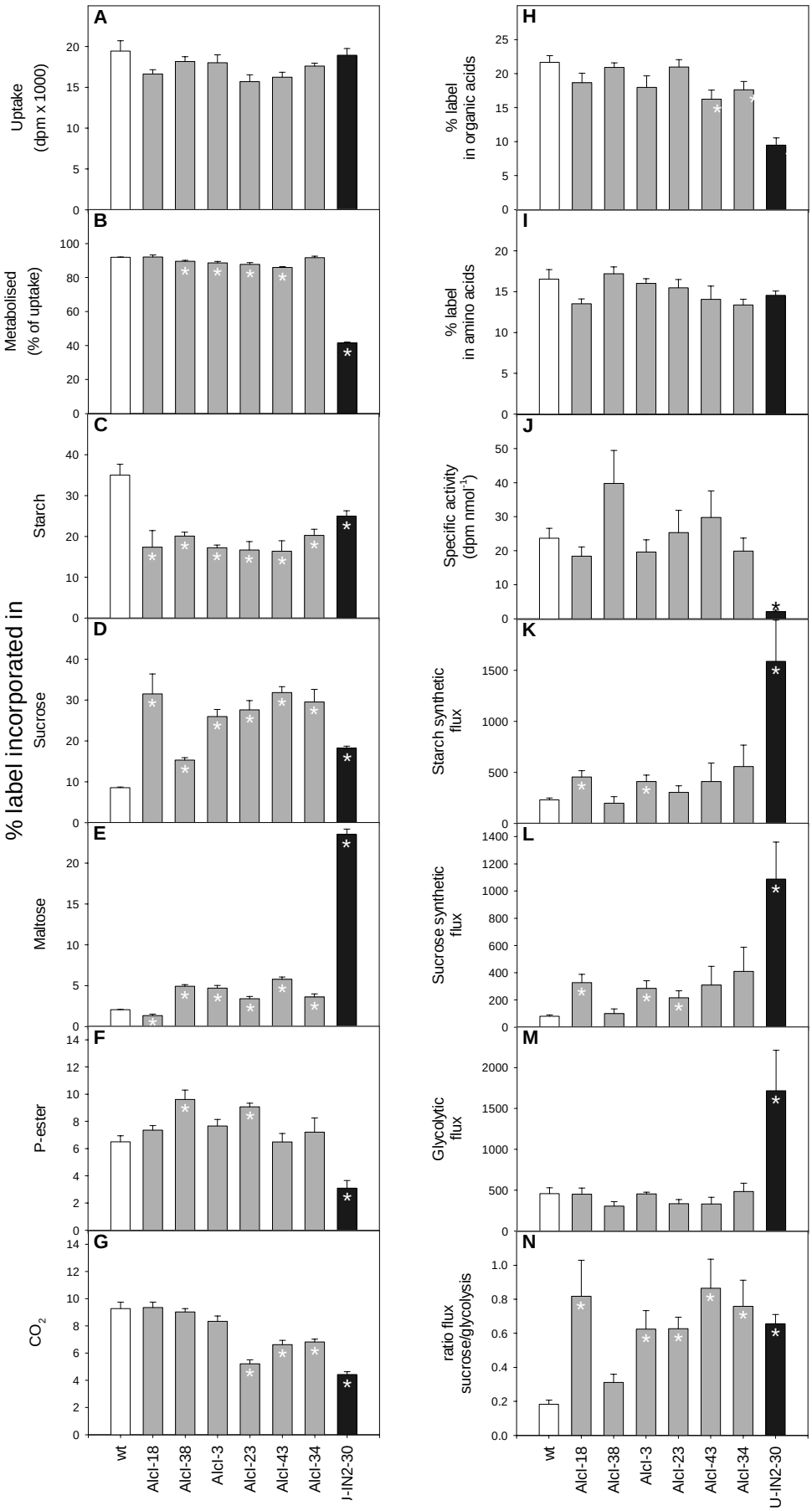



Figure 4.5: legend see next page

**Figure 4.5 (previous page):** Metabolism of [U$^{14}$C]glucose in wild type, constitutively-expressed transgenic control and AlcI tuber discs. 2mM Glc including 7.4GBq/ml [U$^{14}$C]glucose was supplied to discs taken from the cap region of the tuber following 24h induction with 2% (v/v) acetaldehyde. [U$^{14}$C]glucose absorbed by the tissue (a); % of label that is metabolised to other compounds (b). Incorporation of $^{14}$C into starch (c), sucrose (d), maltose (e), phosphoesters (f), carbon dioxide (g), organic acids (h) and amino acids (i). The specific activity of the hexose-P pool (j) was estimated by dividing the label retained in the phosphate ester pool by the summed carbon of the hexose phosphates, and was used to calculate absolute fluxes to starch (k), sucrose (l), glycolysis (m) and the ratio between sucrose synthesis and glycolysis (n). Values represent the mean ± SE ($n$ = 4). Significant changes from the wild type ($P < 0.05$ using the Student's t test) are marked with an asterisk.

## 4.2.7. Estimation of the rates of sucrose synthesis, starch synthesis and glycolysis

Since interpretation of labelling experiments is especially complicated when treatments are compared that modify label uptake or turnover (Geigenberger *et al.*, 1997; Fernie *et al.*, 2001a) I next determined the specific activity of the hexose phosphate pool (Figure 4.5 J) in order to estimate absolute rates of sucrose synthesis, starch synthesis and glycolysis. This was achieved by division of the label retained in the hexose phosphate pool by the summed carbon in the hexose phosphate pool. The assumptions implicit in this estimation are discussed in detail in (Geigenberger *et al.*, 1997). In contrast to the constitutive transgenic control which displayed dramatically decreased specific activities the specific activities of the hexose phosphate pools of the AlcI lines were largely unaltered with respect to wild type**.** These specific activities were then used to calculate the absolute fluxes to sucrose, starch and glycolytic products (organic and amino acids, protein and carbon dioxide). The flux to starch (Figure 4.5 K) was moderately increased in lines AlcI-18 and AlcI-3 but unaltered in the other AlcI lines, furthermore these changes were very mild in comparison to the large increase observed in the constitutive transgenic control. The sucrose biosynthetic flux was increased in all AlcI lines by up to fivefold (significantly so in lines AlcI-18, -3 and -23), however, this was considerably less than the 13-fold increased seen in the constitutive transgenic control (Figure 4.5 L). In contrast the rate of glycolysis was unchanged in the AlcI lines although the constitutive transgenic control displayed a threefold increase in the flux through this pathway (Figure 4.5 M). Consequently, the deduced ratio of sucrose synthetic flux to glycolytic flux was dramatically increased in the AlcI lines

with respect to wild type and (with the exception of line AlcI-38) approximate that seen in the constitutive transgenic control (Figure 4.5 N).

### 4.2.8. *In vivo* feeding experiments

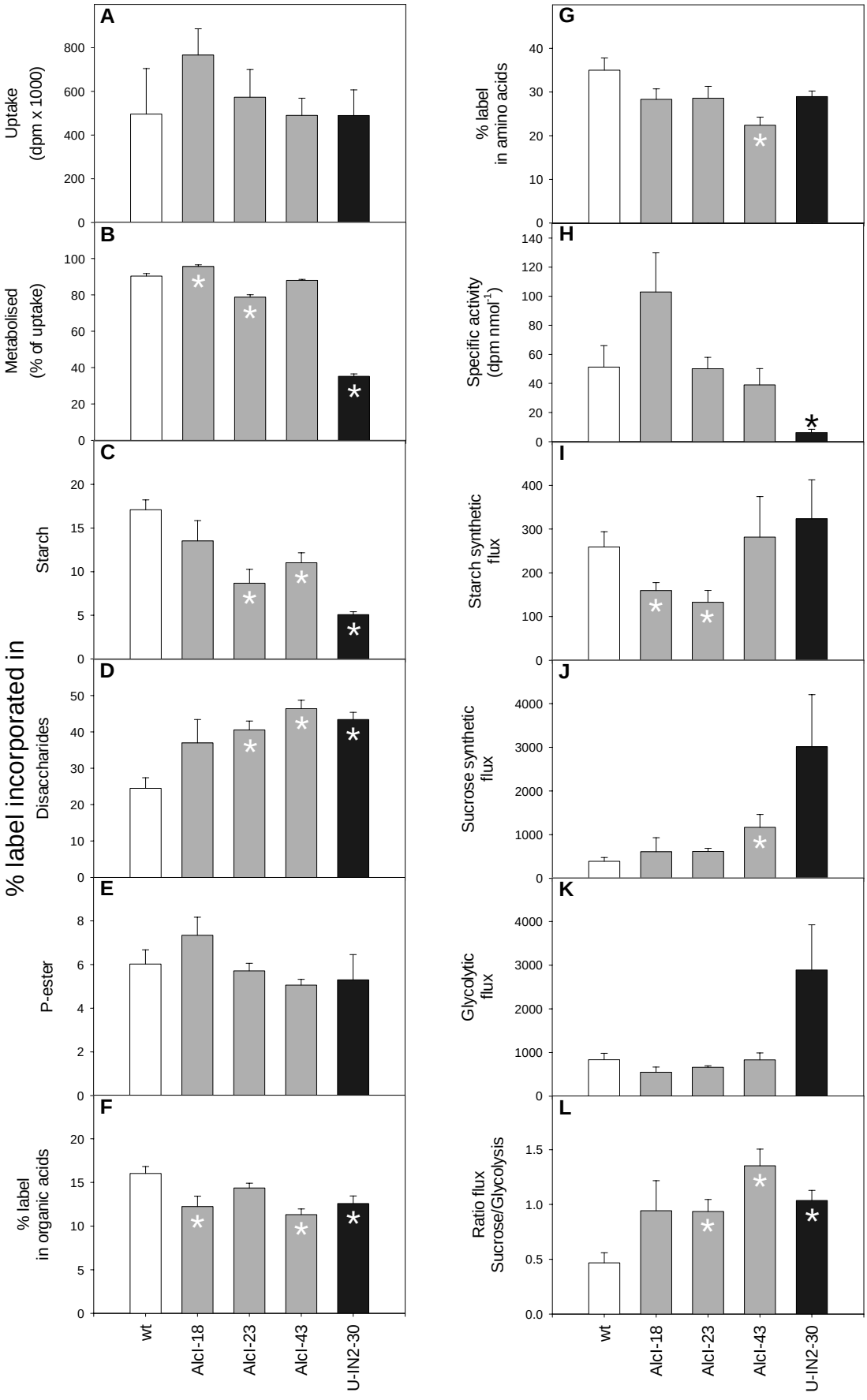
Given that it has recently been demonstrated that growing potato tubers are characterised as having low internal oxygen (Geigenberger *et al.*, 2000), and that this is exacerbated by the introduction of an alternate pathway of sucrose degradation to the endogenous sucrose synthase pathway that predominates in later stages of tuber development (Bologa *et al.*, 2003), I decided to repeat the above experiments under *in vivo* conditions. For this purpose some modifications to the induction procedure were made so as to also induce regions of parenchyma in the centre of the tuber. In Chapter 3 it was shown that if a fine borehole is made through a tuber expressing GUS under the control of the tuber specific *alc* gene system and 0.2% (v/v) acetaldehyde is injected into it then after 24 h GUS expression can be visualised along the transect. This approach was taken to induce individual tubers from the AlcI plants for a period of 24 h after which the acetaldehyde was washed out and replaced with 65 mM [$U^{14}C$]glucose for a period of 2h. This experiment was restricted to three inducible transgenic lines (AlcI-18, -23 and -43) and both control lines.

Following the incubations cores of 10 mm diameter of tuber tissue, concentric to the borehole, were taken and frozen in liquid nitrogen prior to extraction. In keeping with the experiment described above the total radiolabel in the sample at the end of the incubation was the same in the AlcI lines as it was in the wild type and the constitutive transgenic control (Figure 4.6 A). Moreover the relative rate of metabolism was also similar to that described above with a dramatic reduction in label metabolism in the constitutive transgenic control and a mild reduction in the line AlcI-43, however, the proportion of radiolabel metabolised was marginally higher in line AlcI-18 than in the wild type in this experiment (Figure 4.6 B). The redistribution of radiolabel was also qualitatively similar between experiments with a general decrease in label incorporation in starch (Figure 4.6 C) and an increase in disaccharides (sucrose + maltose; Figure 4.6 D) in both the AlcI lines and the constitutive transgenic control. Similarly, there was little change in phosphoesters (Figure 4.6 E) or amino acids (Figure 4.6 G) and a reduction in organic acids (Figure 4.6 F) in all transgenic lines. Estimation of the specific activities of the hexose phosphate pool was carried out as described in Chapter 4.2.7 and revealed similar patterns in the *in vivo* experiment (Figure 4.6 G). The estimated specific activities of the hexose phosphate pools of the AlcI lines were the same as those observed in wild type whereas that of

the constitutive transgenic control was dramatically reduced. The *in vivo* starch fluxes were somewhat different from those recorded in the discs with no change in the rate of starch synthesis in the constitutive transgenic control and the highest expressing AlcI line and a reduced starch synthesis in the two other AlcI lines studied in this experiment (Figure 4.6 I). However, the (lack of) changes observed above in the absolute rates of sucrose synthesis and glycolysis were also seen *in vivo* (Figures 4.6 J and 4.6 K, respectively). Consequently, the changes in the deduced sucrose synthesis to glycolysis ratio were also conserved between experiments (Figure 4.6 L).

Following page:

**Figure 4.6:** Metabolism of [U$^{14}$C]glucose in wild type, constitutively-expressed transgenic control and AlcI tubers. [U$^{14}$C]glucose (specific activity: 11.5 GBq/ mmol) was injected directly into intact tubers (*in planta*) into a fine borehole that had for the previous 24h been filled with 0.2% (v/v) acetaldehyde. [U$^{14}$C]glucose absorbed by the tissue (a); % of label that is metabolised to other compounds (b); incorporation of $^{14}$C into starch (c), disaccharides (d), phosphoesters (e), organic acids (f) and amino acids (g). The specific activity of the hexose-P pool (h) was estimated by dividing the label retained in the phosphate ester pool by the summed carbon of the hexose phosphates, and was used to calculate absolute fluxes to starch (i), sucrose (j), glycolysis (k) and the ratio between sucrose synthesis and glycolysis (l). Values represent the mean ± SE (n = 4). Significant changes from the wild type ($P < 0.05$ using the Student's t test) are marked with an asterisk.


% label incorporated in
A
Uptake (dpm x 1000)
B
Metabolised (% of uptake)
C
Starch
D
Disaccharides
E
P-ester
F
% label in organic acids
G
% label in amino acids
H
Specific activity (dpm nmol-1)
I
Starch synthetic flux
J
Sucrose synthetic flux
K
Glycolytic flux
L
Ratio flux Sucrose/Glycolysis
wt
AlcI-18
AlcI-23
AlcI-43
U-IN2-30

## *4.3. Discussion*

**The expression of yeast invertase in the tuber cytosol under the control of the two component Alc gene system**

This study represents the first comprehensive biochemical study of the consequences of the expression of a metabolic enzyme under the control of an inducible promoter. Transgenic lines expressing a yeast invertase in the potato tuber cytosol under the control of a tissue specific inducible promoter were generated for two reasons. Firstly, the constitutive expression of the same enzyme in the cytosol has previously been shown to have dramatic consequences on metabolism and lines harbouring this transgene have been very well characterised at a broad range of levels (Sonnewald *et al.*, 1997; Trethewey *et al.*, 1998; Hajirezaei *et al.*, 2000; Roessner *et al.*, 2001b; Urbanczyk-Wochniak *et al.*, 2003a). Secondly, recent studies on lines constitutively expressing invertase in the tuber cytosol have indicated that this bypass of the tubers endogenous sucrose synthase path of degradation leads to low internal oxygen and impaired metabolic performance (Bologa *et al.*, 2003). Given that some of the metabolic effects observed following the constitutive expression of the yeast invertase may be secondary changes resulting from the long term perturbation of the sucrose catabolism of the tuber it can be rationalised that using an inducible promoter may allow the dissection of direct from secondary or pleiotropic effects of the transgene expression. For this purpose it was chosen to use induction of the Alc gene system from *Aspergillus nidulans* by application of acetaldehyde. This systems offered the advantages that its expression is very tightly controlled, that its expression in the tuber was previously characterised and that it was confirmed that the application of acetaldehyde itself has little effect on tuber metabolism (Chapter 3). A further advantage of the Alc gene system is that it has previously been demonstrated to be capable of driving appreciable expression of the same gene that was used here in early proof of concept studies that tested the utility of this promoter in plants (Caddick *et al.*, 1998). In this study it was confirmed that the non-uniform pattern of expression of the two component system was due to accessibility of the chemical inducer. Lines of evidence supporting this conclusion include the similarity of the *in situ* staining of invertase activity in the transformants to that previously observed in GUS potato lines, the fact that elevated invertase activities were only routinely observed at the tip of the tuber and the fact that following acetaldehyde induction of whole tuber slices of Alc-GUS lines staining was uniform (Chapter 3).

Since the induction was very much confined to local areas within the tuber parenchyma I decided to carry out all biochemical experiments in this region. For this purpose six AlcI lines were chosen that showed activities ranging from 29 to 266 nmol $gFW^{-1}$ $min^{-1}$ following 24h induction with 0.2 % (v/v) acetaldehyde – for comparison the maximal invertase activity achieved on constitutive expression was 407 nmol $gFW^{-1}$ $min^{-1}$. All further experiments on these lines were performed using both wild type and the constitutive transgenic line U-IN2-30 (Sonnewald *et al.*, 1997) as controls.

**Metabolic consequences of induced expression of invertase in the developing tuber**

As stated in the Introduction the main reason for carrying out these studies was to determine the effect of expressing a yeast invertase cytosolically in potato tubers at a given time point as opposed to its constitutive expression throughout development. I chose to induce gene expression under the control of the alc gene system by the application of acetaldehyde after 10 weeks of plant growth since this time point is representative for growing potato tubers and the majority of previous measurements on tubers with altered sucrose metabolism have been carried out at this point. Induction of gene expression at this time point had the further advantage that at this stage of tuber development photoassimilate delivery to the tuber has been demonstrated to be by symplastic mechanisms with sucrose subsequently being metabolised by sucrose synthase (Viola *et al.*, 2001), discussed in (Fernie and Willmitzer, 2001). This is in contrast to early stages of tuber development in which apoplast unloading and degradation of sucrose by invertase predominate (Appeldoorn *et al.*, 1997; Viola *et al.*, 2001).

Intriguingly, when invertase was expressed at this specific timepoint several similarities but also some dramatic differences were observed in the resultant metabolic phenotype when compared to that obtained following constitutive expression. The level of sucrose in both the AlcI transformants and the constitutive transgenic control were dramatically reduced. There were, however, clear differences in the pattern of change in the levels of the products of this cleavage: glucose accumulating considerably on constitutive expression but not in the AlcI lines following induction over a 24h period (fructose was unaltered with respect to wild type in all transgenic lines tested here). Similarly, there was no accumulation of hexose phosphates in the AlcI lines whereas these were more than 10-fold higher in the constitutive transgenic control (all data presented here for this line is in close agreement to that recorded previously (Sonnewald *et al.*, 1997; Trethewey *et al.*, 1998; Trethewey *et al.*, 1999)). The exact reason for these differences is not apparent, however, two hypotheses can be put forward to

explain the lack of accumulation of hexose and hexose phosphates in the AlcI lines. Firstly, it was previously speculated that the accumulation of hexose in the constitutive lines was due to the fact that in the potato tuber hexokinase is present at low activities and as such is a relatively inefficient step (Trethewey *et al.*, 2001; Veramendi *et al.*, 2002), that is unable to cope with the additional substrate available on the constitutive expression of invertase. The studies by Viola *et al.* (2001), described above, suggest that the hexokinase is not normally highly involved in primary metabolism in the potato tuber: a fact supported by reverse genetic studies on the importance of this enzyme in the tuber (Veramendi *et al.*, 1999; Veramendi *et al.*, 2002). Thus, it is conceivable that the relatively short window of transgene expression used in this study (24h) did not provide enough substrate to saturate the hexokinase enzyme present. The second hypothesis is that the additional hexoses produced on sucrose cleavage in the AlcI lines are diverted from the glycolytic pathway. When considered alongside the facts that the rate of glycolysis does not increase in the AlcI lines but both maltose accumulation and the rate of maltose synthesis increase dramatically (discussed below), the second explanation would be favourable. However, despite this circumstantial evidence it must be noted that from the data presented here the precise reasons for these differences between the transgenics cannot yet be pinpointed.

Studying the levels of other metabolites in these lines also revealed that some patterns of change were conserved between the constitutive control and the AlcI lines but many that were not. The level of starch in the constitutive lines was significantly reduced, in accordance with findings of previous studies (Trethewey *et al.*, 1998; Hajirezaei *et al.*, 2000; Roessner *et al.*, 2001b), but this was observed in only one of the AlcI lines – and one exhibiting a relatively low invertase activity for that matter. Although this result can be rationalised by the fact that it is unlikely that a short term induction of an enzyme of the sucrose to starch pathway would have an impact on the level of starch which accumulates linearly with development (Burton, 1989), it is a striking observation nevertheless, since the reduction in starch content is one of the primary characteristics observed following the constitutive expression of invertase (Sonnewald *et al.*, 1997; Trethewey *et al.*, 1998). Both the constitutive transgenic control and the AlcI lines were characterised by similar changes in the levels of minor carbohydrates and sugar alcohols with sorbitol, galactitol, xylose and fucose decreasing in all transgenic lines studied suggesting that this was a rather direct effect of the expression of invertase. Similarly fatty acid 18:0, succinate and fumarate increased and dehydroascorbate decreased in all lines examined. However, further studies are required to fully understand the significance of these

conserved changes. Another metabolite that displayed a conserved pattern of change between the transgenics was maltose – which increased in both instances. The biochemical pathway of maltose synthesis in the tuber is presently unclear. It could be formed on the condensation of two glucose units (in either phosphorylated or not phosphorylated form) by the concerted action of a range of tuber enzymes including cytosolic α-1,4-glucan phosphorylase (Duwenig *et al.*, 1997) or be a product of starch degradation in the plastid (Fernie *et al.*, 2002a). The fact that it accumulates in the AlcI lines (in which glucose does not accumulate) suggests that the former is more likely. Furthermore, the clear increase in the rate of maltose synthesis in the AlcI lines is in keeping with this route predominating in the potato tuber. Interestingly, previous radiolabelling experiments of the constitutive invertase expressors revealed that maltose synthesis was dramatically higher following supply of [U$^{14}$C]glucose than [U$^{14}$C]fructose (which displays a higher incorporation rate into starch) and that subsequent supply of unlabelled glucose rapidly chased the radiolabel out of the maltose pool (Trethewey *et al.*, 1999). When taken together these evidences strongly suggest that maltose is formed in the potato tuber by the condensation of glucose units.

The pattern of change in amino acids in the AlcI lines is dramatically different (they generally decrease) from that previously observed on the constitutive expression of the yeast invertase (in which the majority of amino acids were found to increase; (Trethewey *et al.*, 1998; Roessner *et al.*, 2001b). This finding is the first instance in which it was observed that a genetic or environmental perturbation to the tuber sucrose level does not lead to a contrapuntal change in amino acid levels. This linkage was recently shown by demonstrating a high negative correlation between the total tuber sucrose and total tuber amino acid contents of 19 transgenic lines (Roessner-Tunali *et al.*, 2003). Whilst all these transgenes were expressed throughout the course of tuber development it cannot be claimed that the different trend in amino acid content is purely a matter of adaptation in the case of the constitutive lines since short term environmental perturbation of tuber sucrose content had similar effects on the content of several tuber amino acids (Roessner-Tunali *et al.*, 2003). This suggests that the normal mechanisms regulating the sucrose to amino acid balance of the tuber are somehow disrupted on the inducible expression of the yeast invertase. It has previously been suggested that amino acid content may be regulated contrapuntally to sucrose content as a mechanism of maintaining osmotic balance (Winter *et al.*, 1993; Geigenberger *et al.*, 1997; Hare *et al.*, 1998). It is possible that the accumulation of maltose in the AlcI lines could offset this requirement, however, it is worth noting that both maltose and amino acids accumulate in the lines that constitutively express invertase (Roessner *et al.*, 2001b).

Thus the reason for the different pattern of change in these metabolites remains elusive and will require further work to fully comprehend.

The changes observed in metabolite levels in the AlcI lines are largely mirrored by the intracellular metabolic fluxes that were determined. The AlcI lines were characterised by an increase in the rate of sucrose cycling and modified rates of starch synthesis but no change in the rate of glycolysis. This metabolic phenotype was in marked contrast to that observed following constitutive expression of the invertase which displayed elevated rates of glycolysis in addition to the increased sucrose cycling. When taken together with the observed increase in the activity of several enzymes of glycolysis this finding allows several important conclusions to be made. Enzymes of the glycolytic pathway were initially demonstrated to be induced following the constitutive expression of a yeast invertase (Trethewey *et al.*, 1998). Driven by compelling evidence from the yeast system (see Johnston, 1999) it was initially postulated that this induction may be caused by elevated levels of glucose. However, further experiments involving expression of a bacterial sucrose phosphorylase or xylose isomerase demonstrated that these enzymes could be induced in the absence of an increased glucose content (Trethewey *et al.*, 2001; Urbanczyk-Wochniak *et al.*, 2003b) and that the induction occurred at the transcript level (Urbanczyk-Wochniak *et al.*, 2003b). That the enzymes were again induced in the AlcI lines despite the lack of change in glucose in these lines adds further weight to the suggestion that fructose may be responsible for the glycolytic induction. Furthermore, the results of this study show that the induction of the glycolytic enzymes is relatively rapid but that it does not have an immediate impact on the rate of glycolysis. Therefore, although the increases in activity of cytosolic phosphoglucomutase, glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase are relatively small in the AlcI lines (and these enzymes do not vary independently), it looks unlikely that any of these enzymes play an important role in the control of glycolysis. The lack of increase in the rate of glycolysis in the AlcI lines is particularly interesting in comparison to the elevation observed on the constitutive expression of sucrose mobilising enzymes (both invertase and sucrose phosphorylase). It was previously postulated that the increase in glycolysis was caused by an increased ATP demand placed on the cell by the futile cycling of sucrose (Fernie *et al.*, 2002a) and furthermore given the clear demonstration that starch synthesis is highly dependent on adenylate supply (Tjaden *et al.*, 1998b; Loef *et al.*, 2001; Regierer *et al.*, 2002) it was hypothesised that this cycling was restricting starch synthesis. The results of the current work suggest that a relatively mild increase in the rate of sucrose synthesis over a short time period does not produce such a large strain on the cell as the

continuous large increase in resynthesis, observed on constitutive expression produced. Consequently this cycling does not greatly effect glycolysis or starch synthesis (discussed below) in the AlcI lines. It is worth noting that recently methods of the regulation of glycolysis that are independent of the transcript level have been demonstrated including differential proteolysis of pyruvate kinase (Tang *et al.*, 2003) and even microcompartmentation of the pathway (Giege *et al.*, 2003) it could be envisaged that such mechanisms would not be induced in the relatively short time scale of induction applied to the AlcI lines.

The increase in sucrose resynthesis in the AlcI lines is particularly intriguing given that it occurred without an increase in hexose phosphates, both substrates and activators (Winter and Huber, 2000) of the reaction catalysed by sucrose phosphate synthase. It is possible that a dramatic shift in the mass action ratio of the equilibrium reaction catalysed by sucrose synthase (Geigenberger and Stitt, 1993), that follows the induction of the invertase, favours the operation of sucrose synthase in the direction of sucrose synthesis. However, it seems unlikely that this in itself would be sufficient to account for the doubling of the sucrose biosynthetic flux especially when it is considered that in wild type potato tubers sucrose phosphate synthase accounts for the vast majority of this process. This opens up the possibility of the presence of a novel as yet undefined mechanism that regulates sucrose synthesis within the tuber. Recent studies on *Arabidopsis* have shown that sucrose phosphate synthase is a binding partner for 14-3-3 proteins (Cotelle *et al.*, 2000) and furthermore that this binding is temporally unlinked to light activation of the enzyme (Huber *et al.*, 2002). These proteins are therefore a possible candidate for mediating the elevated rate of sucrose cycling in these lines but as the possibility of a completely unknown mechanism being responsible for this phenomenon cannot be ruled out yet.

A major difference between the effects of expressing the invertase in a controlled rather than a constitutive fashion was the effect this had on the starch level. Although the results from the two feeding experiments presented here were not entirely in agreement with one another this could be expected since it was previously demonstrated that potato tubers exhibit a low internal oxygen environment which may restrict the operation of certain biosynthetic pathways (Geigenberger *et al.*, 2000). These results showed that in discs there was a slightly increased flux to starch but only in the weaker transgenic lines, whereas in the *in vivo* experiment, which most probably gives a more accurate representation of the actual fluxes in the tubers, there was a restriction of starch synthesis (again confined to the weaker expressing lines). Importantly, however there was little change in the steady

state levels of starch in the AlcI lines, suggesting that a longer term interval was required before any effect of the reduced flux to starch was visible in this large pool. An alternative explanation for the reduction in starch synthesis is the fact that the constitutive expression of sucrose mobilising enzymes has recently been shown to result in lower internal oxygen concentrations (Bologa *et al.*, 2003) which have previously been demonstrated to suppress starch synthesis. Although I did not directly test the internal oxygen concentrations of the AlcI lines their metabolic profiles are not reminiscent of very low oxygen conditions – which normally result in large increases in alanine.

**Conclusions and general implications of this research**

The data presented in this study demonstrate the importance of using chemically induced gene expression as a method for improving the understanding of temporal aspects of plant metabolism. It has been possible to identify changes in transgenic plants in which the transgene was driven by constitutive promoters that are conserved on the expression of the transgene at a unique timepoint such as the decrease in sucrose level and the increase in sucrose cycling observed in these lines, but also differences between the two transgenic systems were uncovered such as the lack of increase in flux through the glycolytic pathway, the reduction in various amino acid pool sizes, and the lack of considerable decreases in starch levels of the AlcI lines. Although the maximal invertase activity in the AlcI lines was only half that observed here in the constitutive transgenic control line it is important to note that this activity was comparable to that seen in other lines expressing the invertase constitutively and displaying very similar phenotype to the chosen control. For this reason it seems unlikely that the effects observed here are a simple function of the expression level, especially since some of the changes observed are contrapuntal to one another. Having excluded this possibility these results can best be interpreted as allowing the dissection of direct and secondary effects of the constitutive expression of invertase with direct effects being conserved irrespective of the promoter used to drive gene expression and the secondary, pleiotropic effects being those that were different between the different transformants. Such comparative studies will be very important at defining the precise role of an enzyme during a particular stage of development. This is particularly true given that the relative influence of metabolic enzymes often changes dramatically during development (see Roessner-Tunali *et al.* (2003) for an example). The recent adaptation of the Alc gene system to facilitate its use in RNA interference strategies (Chen *et al.*, 2003) finally allows this system to be used for strategies intent on

reducing expression of a given gene and in combination with the approaches described above should facilitate a higher temporal resolution of plant metabolism.

# Chapter 5. Vacuolar Invertase

## 5.1. Introduction

The factors governing the distribution of photoassimilates between the various number of sink organs (e.g. developing leaves, fruits, tubers) represent a central question in plant physiology, especially since sink organ productivity is of large importance for agriculture. In most plants, sucrose is the end product of photosynthesis and is transported via the phloem to the sink organs. The initial cleavage of sucrose in sink organs proceeds either by invertase or sucrose synthase. In the developing potato tuber, the vast majority of sucrose degradation is catalysed by sucrose synthase, while invertase plays only a significant role at the beginning of tuberisation (Appeldoorn *et al.*, 1997). As sucrose synthase activity is nearly entirely located in the cytosol of tuber cells (Farré, 2001), sucrose has to be translocated from the phloem to the cytosol of parenchyma cells. Some years ago it was still not totally clear whether sucrose unloading proceeds by direct cell to cell transfer through plasmodesmata (symplastic unloading) or via the extracellular space (apoplastic unloading). However, in a recent study (Viola *et al.*, 2001) showed that the phloem-mobile tracer carboxyfluorescein (CF) unloads from the phloem in tuberising, but not in non-tuberising stolons, while $^{14}C$ assimilates do so in both. Given that CF is large and hence membrane-impermeable whereas $^{14}C$ assimilates are membrane-permeable, this study strongly suggested a symplastic unloading process for tuberising stolons. The vacuole serves as a transient buffer for sugars and other metabolites (Marty, 1999) and the majority of the sucrose in the potato tuber is localised in the vacuole (Farré *et al.*, 2001). Therefore, the question remains as to whether the symplastic unloading of sucrose occurs directly via the plasmodesmata to the cytosol, suggesting that the symplastic continuum is complete, or whether sucrose is shifted endocytotically to the vacuole (described in Marty, 1999) where it is stored and later either transported to the cytosol or cleaved to hexoses in the vacuole which are then transported to the cytosol. So far, a vacuolar transporter translocating sucrose between the cytosol and the vacuole has been suggested, but no evidence was provided for its presence (Lalonde *et al.*, 1999). SUT1 (Riesmeier *et al.*, 1993), one of the three sucrose transporters that were cloned so far from potato, has been shown to be only active in veins of leaves and in the vasculature of sink leaves and sprouting tubers (Kühn, 2003). The second sucrose transporter from potato, SUT4, was only detected in leaves and in the ovaries of flowers

(Weise *et al.*, 2000). The third one, SUT2, has not been characterised yet (NCBI Accession AY291289).

Once within the vacuole, the metabolic fate of sucrose is unclear. Previously, it was shown that transgenic potato plants expressing a yeast invertase in the vacuole under the control of the constitutive 35S promoter showed similarities to plants under water stress (Büssis *et al.*, 1997). They were retarded in growth and accumulated hexoses and amino acids, especially proline. Here I generated transgenic potato plants overexpressing a yeast invertase in the vacuole under the control of a tuber-specific promoter. The plants surprisingly showed little changes in metabolism and little increase in glycolysis. Overall, they showed very similar characteristics to the plants expressing a yeast invertase in the tuber apoplasts (Sonnewald *et al.*, 1997).

## *5.2. Results*

### 5.2.1. Preparation and selection of transgenic lines

The aim of this section of work was to enhance understanding of the regulation of sucrose breakdown in the potato tuber. As the majority of the sucrose in the tuber is located in the vacuole, this is an interesting organelle in which to address this question. For this reason, I generated potato plants overexpressing a yeast invertase targeted to the vacuole of potato tubers using a vacuolar target sequence from the tuber storage protein patatin and the tuber-specific promoter from the same protein (Rocha-Sosa *et al.*, 1989). A total of 55 independently transformed potato lines were generated following an *Agrobacterium*-mediated gene transfer protocol (Dietze *et al.*, 1995). These lines were selected in sterile culture on kanamycin and subsequently transferred to the greenhouse. Tubers were formed in 2.5L pots and harvested from 10 week-old plants. The invertase activity in these lines varied from wild type levels to levels approximately 100-fold higher than those found in wild type (Figure 5.1). From this initial screening five vacI lines were selected, amplified in tissue culture, and the resultant clones grown in tissue culture. The invertase activity in these selected lines varied from 1 to 800 nmol sucrose cleaved $gFW^{-1}$ $min^{-1}$ (Table 5.1).

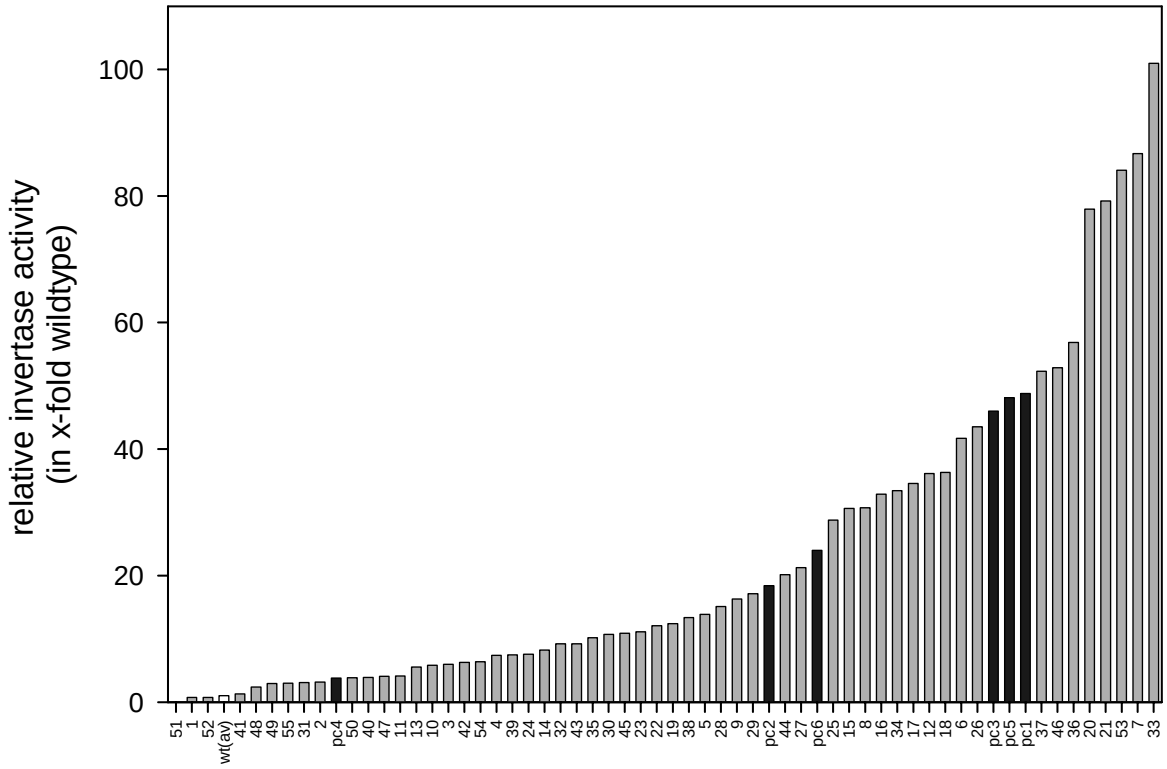



**Figure 5.1:** Screening of potato plants overexpressing a yeast invertase targeted to the vacuole of tubers. Tuber samples were taken from 10 week-old vacI primary transformants and desalted enzyme extracts were analysed for acid invertase activity. The wild type value (white bar) is the average of six independent samples. The tuber-specific, cytosolic invertase line U-IN2-30 served as a positive control (black bars, pc1-6).

**Table 5.1:** Acid invertase activity of selected vacI lines. Tuber samples were taken from 10-week-old vacI plants and desalted enzyme extracts were analysed for acid invertase activity. Activities are given in nmol $gFW^{-1}$ $min^{-1}$. Value represent mean ± SE of samples from 6 independent plants.

| line | invertase acivity | | |
|---|---|---|---|
| wt | 3.3 | ± | 1.5 |
| vacI-34 | 157.0 | ± | 24.2 |
| vacI-33 | 507.4 | ± | 14.5 |
| vacI-37 | 532.6 | ± | 34.8 |
| vacI-7 | 828.9 | ± | 30.7 |

The used targeting sequence for vacuolar localisation has been shown previously to be efficient in tobacco and potato leaves (Sonnewald *et al.*, 1991; Büssis *et al.*, 1997). To draw conclusions on the observed similarities between apoplastic and vacuolar expression (see below), it is essential that the

vacuolar targeting sequence is proved to be functional in the potato tuber as well. Therefore, I stained sections of potato tubers expressing a yeast invertase in the cytosol and in the vacuole for their invertase activity and photographed them through a microscope (Figure 5.3). The volume of a tuber cells consists to a large percentage of vacuoles, most of the remaining volume is filled with amyloplasts containing starch granules. It can clearly be seen that the invertase activity in the vacI plants is localised in the vacuole (overview: Figure 5.3 C, close-up: Figure 5.3 D). It is noticeable that only some cells are stained, while the cells around those seem to be empty. This is best explained by the fact that the solid starch granules can not be cut by the razor blade and therefore are pushed against the cell membranes and break them. The U-IN2-30 line expressing a yeast invertase in the cytosol showed the localisation of the activity in the area of the cytosol (Figure 5.3 B). It was particularly interesting to see that at one point (white arrow), the cytosol is stained but not the apoplast.

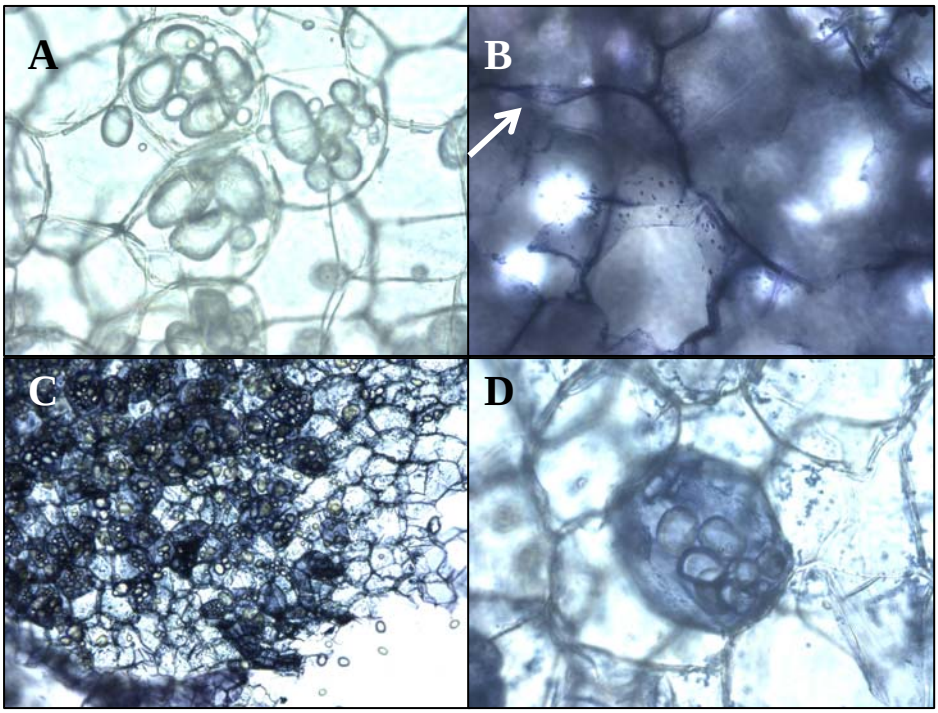



**Figure 5.3:** Staining of invertase activity in wild type (A), cytosolic invertase expressing line U-IN2-30 (B) and vacuolar invertase expressing line vacI-34 (C and D). Sections were cut from 10-week old tubers by hand with a razor blade. They were fixed with paraformaldehyde, washed several times in water in order to eliminate all endogenous sugars and then stained for glucose produced from sucrose by the invertase activity. The white arrow indicates an area where the cytosol is stained but not the apoplast (see text).

To check the tissue-specificity of the B33 promoter, which has been reported to be leaky in some instances (Lytovchenko, personal communication; Rocha-Sosa *et al.*, 1989), enzymes extracts were made from leaf samples taken from 10 week-old plants, and the acid invertase activity was measured. In the lines vacI-7, -34 and -37 the mean invertase activity from 6 samples was approximately 2-fold higher than in the wild type control (1.2 compared to 0.6 nmol sucrose cleaved $gFW^{-1}$ $min^{-1}$). However, due to the large standard errors this increase was in no case significant. Also, this increase in activity is minor when compared to that measured in tubers (up to 800 nmol sucrose cleaved $gFW^{-1}$ $min^{-1}$, see Table 5.1).

## 5.2.2. Effect of vacuolar yeast invertase on tuber development

Apoplastic expression of a yeast invertase in the potato tuber leads to fewer tubers per plant, however since these are much bigger than those of the wild type plants, the consequence is an increased tuber yield per plant. In contrast, cytosolic localisation of the yeast invertase leads to plants that have a greater number, but smaller tubers, so that the yield per plant is unchanged (Sonnewald *et al.*, 1997). To determine the effect of vacuolar expression, the yield of the selected lines was measured. As a first experiment 12 plants of each line were grown alongside with 12 wild type plants under controlled greenhouse conditions; tubers were harvested from senescent 15 week-old plants, counted and weighed (Figure 5.4 A). The lines vacI-7, -33 and -37 produced significantly higher tuber numbers, while the tubers were in average significantly smaller, resulting in an unchanged yield per plant. Line vacI-34 produced slightly more tubers with a slightly increased average weight, resulting in a significantly increased yield per plant. The density was found to decrease slightly in the transgenics.

The experiment was next repeated on a larger scale growing 25 plants per line in the summer in an uncontrolled greenhouse (Figure 5.4 B). Total tuber number and average tuber weight did not change significantly in any line, whereas the total yield per plant decreased in the lines vacI-7, -33 and -37, but increased significantly in line vacI-34. The tuber density decreased significantly in all vacI lines compared to the wild type.

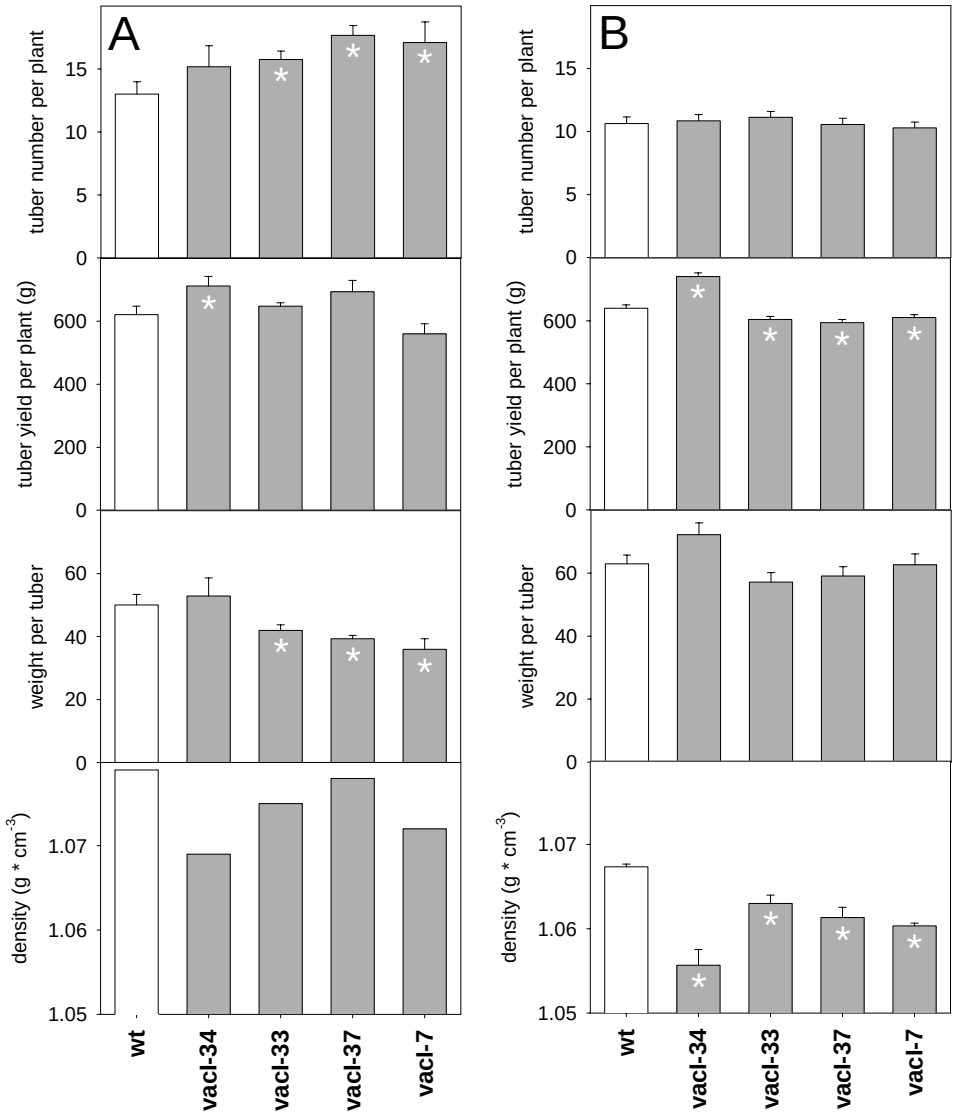



**Figure 5.4:** Yield experiment with vacI lines. Part A (part B in brackets): 12 (25) plants per line were grown in a controlled (uncontrolled) greenhouse for 15 (17) weeks, and all tubers per plant with a diameter of more than 20 mm were harvested, counted and weighed. For the density measurement, the tubers from 8-12 plants were combined, leading to 1 and 3 values for part A and part B, respectively. Values are represented as means ± SE. Significant changes from wild type are marked with an asterisk.

## 5.2.3. Effect of vacuolar yeast invertase on sugar and starch levels

The invertase cleaves sucrose to glucose and fructose, therefore I next determined the level of glucose with an enzyme-based assay (fructose is very low in the tuber and is measured more precisely by GC-MS). Figure 5.5 A shows that in the vacI lines, sucrose was found to decrease dramatically to around 20% in lines vacI-7, -33, -34, and -37, which is even lower than that observed in the cytosolic

line U-IN2-30 (see chapter 4.2.3.). Glucose increased close to 5-fold in the lines vacI-34 and -37 (Figure 5.5 B), which is about half the increase observed in the cytosolic line. Starch levels did not change significantly in any lines (Figure 5.5 C). The hexose phosphates Glc6P, Glc1P and Fru6P increased in all lines, but this increase was not significant for Glc1P in the line vacI-37 and for Glc6P in the lines vacI-33 and -37 (Figure 5.5 D, E and F). When the hexose phosphates were summed, this increase was significant in all vacI lines (data not shown).

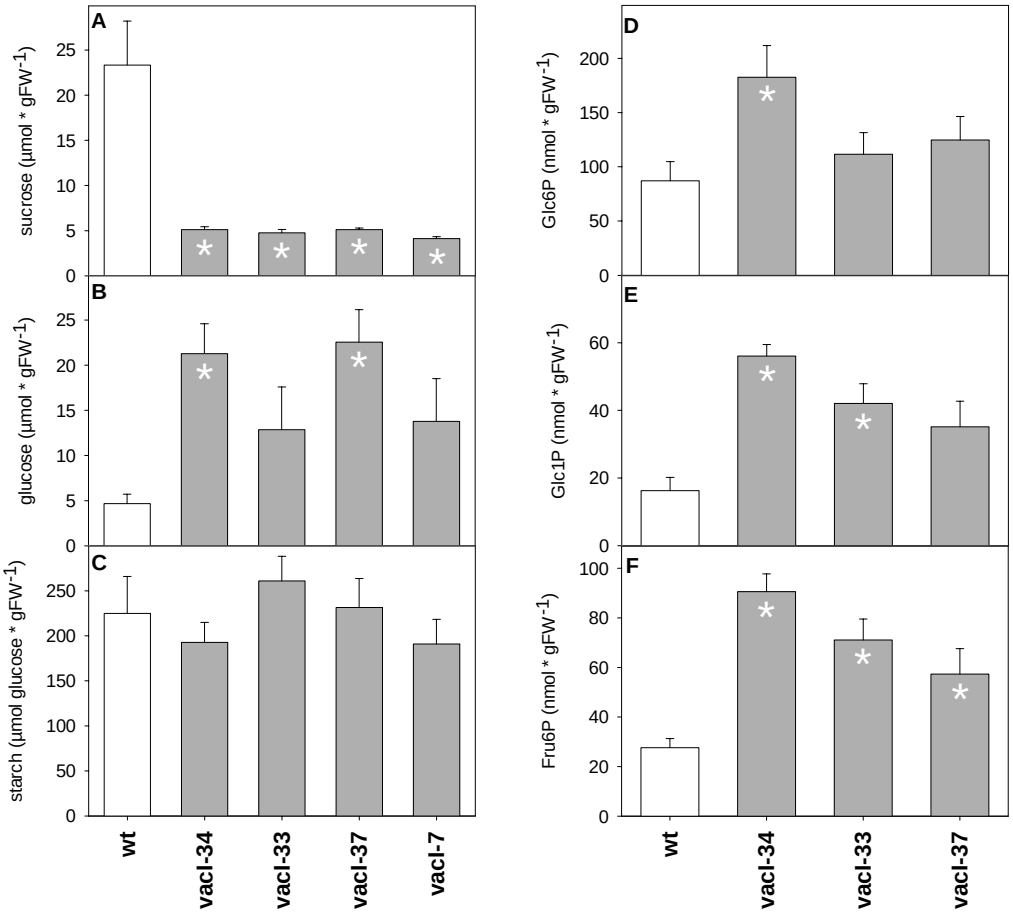



**Figure 5.5**: Carbohydrates and hexose phosphates in wild type and vacI plants. Sucrose (A), glucose (B), starch (C), Glc6P (D), Glc1P (E), Fru6P (F). Extracts were prepared from tuber discs (diameter 10 mm) of 10 week-old plants and carbohydrates and hexose phosphates were determined from six and four individual plants per line, respectively. Values are presented in µmol $gFW^{-1}$ and nmol $gFW^{-1}$ for carbohydrates and hexose phosphates, respectively, as means ± SE. Significant changes from the wild type are marked with an asterisk ($P < 0.05$ using the Student's t-test).

## 5.2.4. Effect of vacuolar yeast invertase on glycolytic enzymes

The cytosolic expression of a yeast invertase in the potato tuber results in an induction of glycolysis both at the level of enzyme activity and of flux (Trethewey *et al.*, 1998; Trethewey *et al.*, 1999). The exact mechanism behind this induction has thus far proven elusive, however, given that a similar induction is also observed following the expression of a bacterial sucrose phosphorylase it was previously postulated that the induction is linked to a signal coming either from the low sucrose content or from the additional energy demands placed on the cell by an induction of a cycle of sucrose degradation and (re)synthesis (Trethewey *et al.*, 1999; Fernie *et al.*, 2002a). In contrast, apoplastic expression of a yeast invertase does not perturb glycolysis, suggesting that the regulation of glycolysis is linked to cytosolic sucrose hydrolysis (Hajirezaei *et al.*, 2000). For these reasons I was interested to see the effect that the vacuolar expression of invertase had on the activities of the key enzymes of the glycolytic pathway (Figure 5.6). Desalted enzyme extracts were prepared from vacI lines and analysed for the activities of ten enzymes of glycolysis using assays that had been previously optimised for potato tuber extracts (Trethewey *et al.*, 1998; Veramendi *et al.*, 2002). The vacI lines generally showed a small decrease in glycolytic activities. The activity of PGM decreased significantly in all vacI lines compared to the wild type control (Figure 5.6 C), while aldolase and TPI decreased significantly in two out of four transgenic lines (Figure 5.6 F and G). Fructokinase, PFK, Enolase and PK showed a significant decrease in activity of about 30-40% compared to wild type only in the strongest invertase expressing line vacI-7 (Figure 5.6 B, D, I and J). The activities of hexokinase, PGI and GAPDH did not show any significant changes in the vacI lines (Figure 5.6 A, D and H).

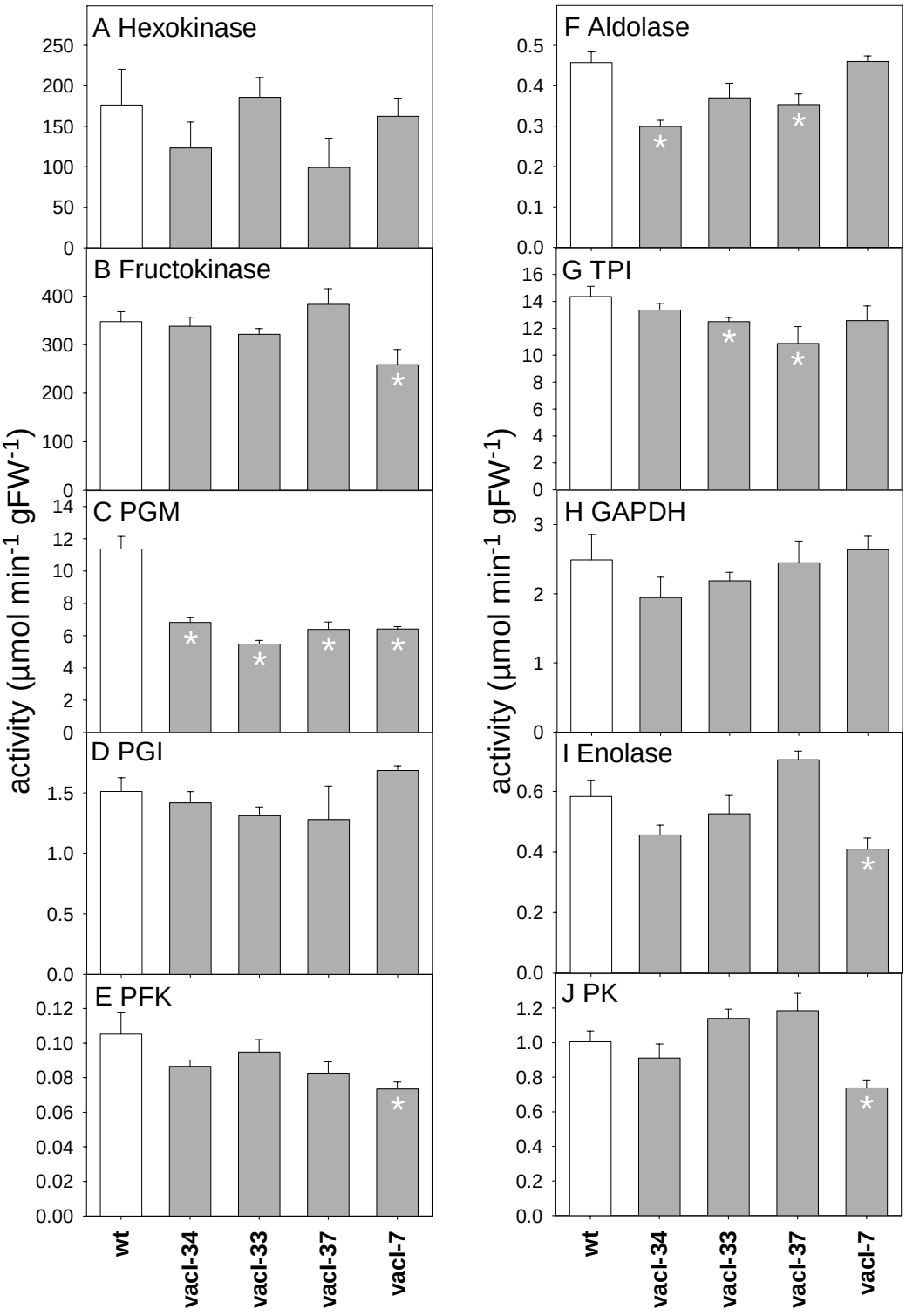



**Figure 5.6:** Maximal catalytic activities of enzymes of glycolysis in wild type and vacI plants. Values are presented in µmol $gFW^{-1}$ $min^{-1}$ as means ± SE. Significant changes from the wild type are marked with an asterisk ($P < 0.05$ using the Student's t-test). PGM, phosphoglucomutase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; TPI, triose phosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PK, pyruvate kinase.

## 5.2.5. Effect of vacuolar yeast invertase on other primary metabolites

I next evaluated the effect of the expression of the yeast invertase in the vacuole on a broad range of metabolites (Table 5.2). A total of 71 metabolites were measured by gas chromatography – mass spectrometry using a well-established protocol (Roessner *et al.*, 2001b). The metabolite measurements largely confirmed the results of the enzyme based tests presented in chapter 5.2.3 for sugars, showing that the sucrose level was reduced in the vacI lines whilst the glucose levels increased. Interestingly, the fructose levels were found to increase dramatically up to more than 30-fold compared to wild type. The hexose phosphates were slightly increased, but only significantly in one case (fructose-6-phosphate, vacI-7). These results were however somewhat different from those of the cycling-assay based analysis (Figure 5.5 D, E and F), possibly due to the fact that phosphorylated intermediates are not readily determined using the GC-MS protocol. Looking at amino and organic acids it becomes apparent that these metabolites were largely unchanged across the genotypes studied, with the exception of marginal decreases in citrate and increases in glycerate. In contrast, the transgenics showed many differences from the wild type with respect to sugar and sugar alcohol contents. Fructose, glucose, mannose, sorbitol/galactitol and trehalose were all observed to increase, whereas inositol, mannitol, melezitose, raffinose and sucrose all decreased in the transformants. Putrescine decreased significantly in all vacI lines to 40-70% of the levels found in wild type.

**Table 5.2:** Metabolite levels in developing tubers from 10 week-old wild type and vacI plants. Data are normalised to the mean response calculated for the wild type. Values presented are the mean ± %SE of six independent determinants. Those that are significantly different from wild type are identified in bold.

| | Metabolite | WT | vacI-34 | vacI-33 | vacI-37 | vacI-7 |
|---|---|---|---|---|---|---|
| 1 | alanine | 1.00 ± 0.23 | 0.93 ± 0.29 | 0.83 ± 0.20 | 0.65 ± 0.15 | 0.56 ± 0.10 |
| 2 | arginine | 1.00 ± 0.21 | 1.89 ± 0.35 | 1.32 ± 0.43 | 0.67 ± 0.28 | 1.54 ± 0.29 |
| 3 | asparagine | 1.00 ± 0.20 | 1.44 ± 0.21 | 1.60 ± 0.33 | 1.54 ± 0.38 | **2.04 ± 0.30** |
| 4 | aspartate | 1.00 ± 0.10 | 0.88 ± 0.10 | 0.87 ± 0.12 | 0.97 ± 0.10 | 0.88 ± 0.08 |
| 5 | β-alanine | 1.00 ± 0.18 | 0.72 ± 0.12 | 0.96 ± 0.13 | 0.82 ± 0.13 | 0.79 ± 0.06 |
| 6 | cysteine | 1.00 ± 0.23 | 0.97 ± 0.55 | 0.85 ± 0.14 | 0.74 ± 0.10 | 0.63 ± 0.19 |
| 7 | GABA | 1.00 ± 0.10 | 0.81 ± 0.06 | 0.91 ± 0.12 | 0.73 ± 0.09 | 0.95 ± 0.09 |
| 8 | glutamate | 1.00 ± 0.14 | 1.08 ± 0.13 | 1.04 ± 0.15 | 1.12 ± 0.13 | 1.28 ± 0.20 |
| 9 | glutamine | 1.00 ± 0.43 | 1.68 ± 0.41 | 1.25 ± 0.48 | 1.20 ± 0.43 | 2.16 ± 0.40 |
| 10 | glycine | 1.00 ± 0.20 | 0.68 ± 0.10 | 0.61 ± 0.09 | 0.60 ± 0.12 | 0.60 ± 0.06 |
| 11 | homoserine | 1.00 ± 0.10 | **0.59 ± 0.06** | 1.02 ± 0.24 | 1.00 ± 0.21 | **0.67 ± 0.09** |
| 12 | 4-hydroxy-proline | 1.00 ± 0.22 | 1.29 ± 0.23 | 1.11 ± 0.24 | 1.10 ± 0.32 | 1.45 ± 0.13 |
| 13 | isoleucine | 1.00 ± 0.25 | 0.96 ± 0.18 | 0.72 ± 0.25 | 0.55 ± 0.10 | 0.63 ± 0.17 |
| 14 | leucine | 1.00 ± 0.40 | 1.13 ± 0.29 | 0.89 ± 0.44 | 0.46 ± 0.09 | 0.73 ± 0.24 |
| 15 | lysine | 1.00 ± 0.30 | 0.76 ± 0.23 | 0.62 ± 0.29 | 0.43 ± 0.12 | 0.38 ± 0.12 |
| 16 | methionine | 1.00 ± 0.19 | 1.03 ± 0.18 | 1.15 ± 0.23 | 1.21 ± 0.24 | 1.30 ± 0.18 |
| 17 | norvaline | 1.00 ± 0.11 | 1.06 ± 0.14 | 1.47 ± 0.20 | 1.58 ± 0.29 | **1.58 ± 0.09** |
| 18 | O-acetyl-serine | 1.00 ± 0.44 | 1.08 ± 0.40 | 1.12 ± 0.43 | 0.92 ± 0.24 | 0.90 ± 0.26 |
| 19 | ornithine | 1.00 ± 0.27 | 1.65 ± 0.40 | 1.14 ± 0.39 | 1.15 ± 0.38 | 1.50 ± 0.25 |
| 20 | phenylalanine | 1.00 ± 0.25 | 1.04 ± 0.20 | 0.91 ± 0.29 | 0.64 ± 0.09 | 0.99 ± 0.19 |
| 21 | proline | 1.00 ± 0.20 | 1.08 ± 0.31 | 2.10 ± 0.67 | 1.19 ± 0.57 | 0.56 ± 0.12 |
| 22 | pyroglutamate | 1.00 ± 0.24 | 1.38 ± 0.22 | 1.19 ± 0.30 | 1.10 ± 0.28 | **1.75 ± 0.22** |
| 23 | serine | 1.00 ± 0.16 | 1.33 ± 0.20 | 1.09 ± 0.22 | 0.81 ± 0.21 | 1.00 ± 0.14 |
| 24 | threonine | 1.00 ± 0.19 | 1.16 ± 0.20 | 0.76 ± 0.16 | 0.59 ± 0.15 | 0.93 ± 0.14 |
| 25 | tryptophan | 1.00 ± 0.62 | 0.71 ± 0.25 | 0.98 ± 0.54 | 0.30 ± 0.08 | 0.89 ± 0.39 |
| 26 | tyrosine | 1.00 ± 0.36 | 0.90 ± 0.26 | 0.54 ± 0.26 | 0.34 ± 0.10 | 0.81 ± 0.28 |
| 27 | valine | 1.00 ± 0.14 | 0.91 ± 0.11 | 0.82 ± 0.16 | 0.72 ± 0.12 | 0.85 ± 0.13 |
| 1 | 16:0 fatty acid | 1.00 ± 0.03 | 0.92 ± 0.03 | **0.90 ± 0.03** | 0.93 ± 0.04 | 0.95 ± 0.03 |
| 2 | 18:0 fatty acid | 1.00 ± 0.04 | 0.92 ± 0.02 | 0.94 ± 0.02 | 0.97 ± 0.04 | 0.93 ± 0.02 |
| 3 | 9,12(Z,Z)-18:2 | 1.00 ± 0.09 | 1.00 ± 0.07 | 1.08 ± 0.08 | 1.25 ± 0.08 | 1.26 ± 0.13 |
| 4 | aconitate | 1.00 ± 0.48 | 0.14 ± 0.08 | 0.18 ± 0.08 | 0.17 ± 0.06 | 1.82 ± 1.34 |
| 5 | ascorbate | 1.00 ± 0.24 | **0.41 ± 0.08** | 0.87 ± 0.27 | 1.85 ± 1.34 | 1.09 ± 0.61 |
| 6 | benzoate | 1.00 ± 0.07 | **0.80 ± 0.03** | 0.86 ± 0.05 | **0.81 ± 0.05** | **0.80 ± 0.03** |
| 7 | citramalate | 1.00 ± 0.17 | 0.91 ± 0.15 | **1.90 ± 0.29** | **1.78 ± 0.26** | 1.23 ± 0.22 |
| 8 | citrate | 1.00 ± 0.07 | **0.78 ± 0.06** | 0.91 ± 0.04 | 0.95 ± 0.03 | **0.83 ± 0.01** |
| 9 | dehydroascorbate | 1.00 ± 0.12 | **0.69 ± 0.06** | 0.89 ± 0.10 | 0.86 ± 0.06 | **0.60 ± 0.04** |
| 10 | fumarate | 1.00 ± 0.18 | 0.77 ± 0.15 | 0.71 ± 0.13 | 0.85 ± 0.17 | 1.13 ± 0.13 |
| 11 | gluconate | 1.00 ± 0.06 | 0.99 ± 0.10 | 1.18 ± 0.13 | 1.31 ± 0.19 | **1.52 ± 0.17** |
| 12 | glycerate | 1.00 ± 0.13 | 1.21 ± 0.14 | 1.30 ± 0.14 | **1.45 ± 0.05** | **2.14 ± 0.13** |
| 13 | malate | 1.00 ± 0.26 | 0.62 ± 0.11 | 0.55 ± 0.06 | 0.88 ± 0.17 | **0.31 ± 0.02** |
| 14 | quinate | 1.00 ± 0.11 | 0.90 ± 0.11 | 1.27 ± 0.13 | 1.22 ± 0.16 | 0.78 ± 0.06 |
| 15 | shikimate | 1.00 ± 0.17 | 1.16 ± 0.31 | 1.53 ± 0.27 | 1.28 ± 0.26 | 1.16 ± 0.06 |
| 16 | succinate | 1.00 ± 0.25 | 0.64 ± 0.32 | 0.63 ± 0.18 | 0.66 ± 0.21 | 1.41 ± 0.36 |
| 17 | threonate | 1.00 ± 0.09 | 0.69 ± 0.11 | 0.83 ± 0.06 | 0.95 ± 0.10 | 0.93 ± 0.11 |
| 1 | fructose | 1.00 ± 0.23 | **19.31 ± 4.49** | **27.29 ± 6.93** | **32.23 ± 4.33** | **21.08 ± 6.24** |
| 2 | galactose | 1.00 ± 0.20 | 1.41 ± 0.38 | 0.99 ± 0.21 | 1.14 ± 0.21 | 0.58 ± 0.12 |
| 3 | glucose | 1.00 ± 0.31 | **4.53 ± 0.98** | **3.79 ± 1.03** | **5.62 ± 1.11** | 2.69 ± 1.03 |
| 4 | inositol | 1.00 ± 0.20 | **0.46 ± 0.06** | 0.91 ± 0.15 | 0.77 ± 0.16 | **0.38 ± 0.05** |
| 5 | maltitol | 1.00 ± 0.34 | 2.70 ± 0.83 | 2.65 ± 0.83 | 3.19 ± 0.95 | 0.32 ± 0.07 |
| 6 | maltose | 1.00 ± 0.16 | 1.12 ± 0.51 | 1.06 ± 0.13 | 1.29 ± 0.29 | 1.24 ± 0.16 |
| 7 | mannose | 1.00 ± 0.22 | **2.23 ± 0.47** | 1.64 ± 0.38 | 1.74 ± 0.30 | 0.91 ± 0.18 |
| 8 | mannitol | 1.00 ± 0.16 | **0.56 ± 0.09** | **0.42 ± 0.04** | **0.46 ± 0.04** | 0.64 ± 0.11 |
| 9 | melezitose | 1.00 ± 0.14 | **0.43 ± 0.06** | **0.45 ± 0.04** | **0.45 ± 0.07** | **0.34 ± 0.03** |
| 10 | raffinose | 1.00 ± 0.17 | 0.81 ± 0.13 | 0.71 ± 0.10 | 0.66 ± 0.18 | **0.44 ± 0.08** |
| 11 | ribose | 1.00 ± 0.10 | 1.03 ± 0.14 | 0.86 ± 0.17 | 1.01 ± 0.23 | 0.69 ± 0.16 |
| 12 | sorbitol/galactitol | 1.00 ± 0.06 | 1.71 ± 0.43 | **1.75 ± 0.27** | **1.92 ± 0.26** | **1.25 ± 0.09** |
| 13 | sucrose | 1.00 ± 0.13 | **0.15 ± 0.03** | **0.13 ± 0.02** | **0.14 ± 0.01** | **0.10 ± 0.01** |
| 14 | trehalose | 1.00 ± 0.07 | 1.77 ± 0.52 | **2.56 ± 0.67** | **2.50 ± 0.47** | 2.22 ± 0.58 |
| 15 | xylose | 1.00 ± 0.34 | 1.19 ± 0.26 | 1.28 ± 0.38 | 1.45 ± 0.47 | 0.76 ± 0.26 |
| 1 | fructose-6-P | 1.00 ± 0.17 | 1.66 ± 0.29 | 1.26 ± 0.07 | 1.24 ± 0.14 | **1.48 ± 0.09** |
| 2 | glucose-6-P | 1.00 ± 0.16 | 1.57 ± 0.26 | 1.24 ± 0.10 | 1.20 ± 0.15 | 1.38 ± 0.07 |
| 3 | glyceric-3-P | 1.00 ± 0.53 | 0.85 ± 0.32 | 0.83 ± 0.24 | 0.96 ± 0.32 | 0.98 ± 0.34 |
| 4 | glycerol-1-P | 1.00 ± 0.11 | 0.95 ± 0.08 | 1.04 ± 0.12 | 1.04 ± 0.11 | 0.86 ± 0.10 |
| 5 | inositol-1-P | 1.00 ± 0.07 | 0.78 ± 0.11 | 1.12 ± 0.04 | 1.16 ± 0.09 | 0.96 ± 0.09 |
| 6 | phosphate | 1.00 ± 0.03 | 0.84 ± 0.07 | 0.94 ± 0.04 | **0.80 ± 0.04** | 1.01 ± 0.03 |
| 1 | α-tocopherol | 1.00 ± 0.22 | 0.94 ± 0.10 | 0.91 ± 0.12 | 1.22 ± 0.11 | 0.94 ± 0.21 |
| 2 | erythritol | 1.00 ± 0.05 | **0.79 ± 0.06** | 0.90 ± 0.13 | 1.14 ± 0.13 | 1.01 ± 0.12 |
| 3 | glycerol | 1.00 ± 0.05 | 0.99 ± 0.09 | 0.97 ± 0.09 | **1.32 ± 0.11** | 1.04 ± 0.15 |
| 4 | putrescine | 1.00 ± 0.04 | **0.49 ± 0.07** | **0.65 ± 0.08** | **0.67 ± 0.07** | **0.41 ± 0.02** |
| 5 | spermidine | 1.00 ± 0.14 | 0.68 ± 0.16 | 0.72 ± 0.11 | 0.68 ± 0.18 | 0.69 ± 0.16 |
| 6 | tyramine | 1.00 ± 0.24 | **0.39 ± 0.08** | 0.65 ± 0.10 | **0.32 ± 0.05** | **1.91 ± 0.24** |

## 5.2.6. Metabolism of labelled glucose in tuber slices of vacI lines

In order to analyse the dominant fluxes of carbohydrate metabolism in the transgenic lines, freshly cut slices of growing potato tubers were incubated with 2 mM [$U^{14}C$]glucose (Figure 5.7). Labelled glucose was preferred to sucrose due to the large changes in sucrose content in the transformants. Furthermore, this choice of radiolabelled substrate provided the opportunity to measure the rate of sucrose resynthesis and allowed comparison of the results with those documented previously for wild type and cytosolic invertase lines (Trethewey *et al.*, 1998). There was no difference in the rate of [$U^{14}C$]glucose taken up by discs of the transgenics in comparison with the wild type (Figure 5.7 A). The proportion of radiolabel that was metabolised after uptake increased slightly but significantly in all vacI lines (Figure 5.7 B). Compared to the wild type control, in the vacI lines there was a smaller percentage of the label incorporated into starch (significantly so in line vacI-37; Figure 5.7 C), while there was an increase in the percentage of label in disaccharides and carbon dioxide (significant in two and three lines, respectively; Figure 5.7 D and E). The label incorporated into phosphate esters and amino acids did not change (Figure 5.7 F and H). The proportion of radioactivity recovered in organic acids decreased significantly only in line vacI-34 (Figure 5.7 G).

Labelling experiments are especially hard to interpret when treatments are compared that modify label uptake or turnover (Geigenberger *et al.*, 1997; Fernie *et al.*, 2001b). Therefore, I next determined the specific activity of the hexose phosphate pool (Figure 5.7 I) in order to estimate absolute fluxes of sucrose synthesis, starch synthesis and glycolysis. For this purpose, the label retained in the hexose phosphate pool was divided by the total carbon in the hexose phosphate pool. The specific activity of the hexose phosphate pool decreased significantly only in the line vacI-34. The higher the invertase activity in the vacI lines was, the smaller was the estimated flux to starch (Figure 5.7 J), a change that was however in no case significant. The sucrose synthetic flux increased 3-fold in the line vacI-34, but it did not change significantly in the other lines (Figure 5.7 K). There were also no significant differences between the wild type and the transgenics in the flux to glycolysis (Figure 5.7 L).

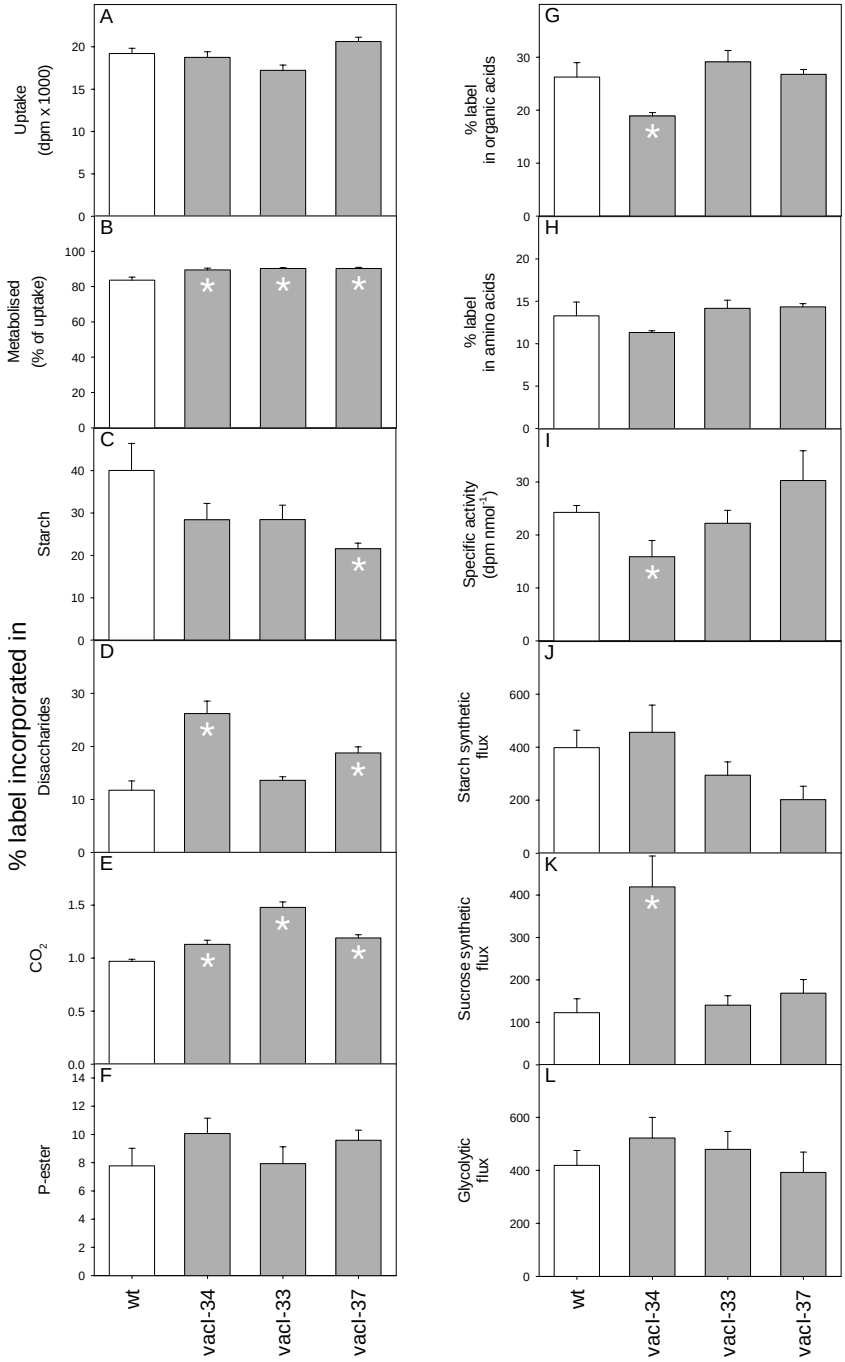



**Figure 5.7:** Metabolism of [U$^{14}$C]glucose in wild type and vacI tuber discs *in vitro*. 2 mM Glc including 7.4 GBq/ml [U$^{14}$C]glucose was supplied to discs taken randomly from the middle of tubers. [U$^{14}$C]glucose absorbed by the tissue (A); % of label that is metabolised to other compounds (B). Incorporation of $^{14}$C into starch (C); disaccharides (D); carbon dioxide (E); phosphoesters (F); organic acids (G); amino acids (H). The specific activity of the hexose-P pool (I) was estimated by dividing the label retained in the phosphate ester pool by the summed carbon of the hexose phosphates, and was used to calculate absolute fluxes to starch (J); sucrose (K); and glycolysis (L). Values represent the mean ± SE ($n$ = 4). Significant changes from the wild type ($P < 0.05$ using the Student's t test) are marked with an asterisk.

The technique of disc feeding is generally accepted and widely used, but the cutting out of slices and the suspension in buffer solution represent a strong perturbation of tuber morphology and thus could have large effects on metabolism. To employ a minimally invasive technique, the above experiment was therefore repeated under *in vivo* conditions. A fine borehole was made through tubers still attached to the mother plant and was filled with 65 mM [$U^{14}C$]glucose for a period of two hours. After the incubation, a concentric cylinder 10 mm in diameter was cut around the borehole and frozen in liquid nitrogen prior to extraction and fractionation. Surprisingly, the data from these analyses were quite different from the *in vitro* feeding experiment. The percentage of the uptaken label that was metabolised was, in contrast to the disc feeding experiment, significantly lower in the vacI lines compared to wild type (Figure 5.8 B). The redistribution of radiolabel to phosphate esters, organic acids and amino acids was similar to the disc feeding experiment (Figure 5.8 E, F and G); the first two did not show a significant change, and the latter exhibited a slight decrease in the line vacI-34. In contrast, the percentage of radiolabel that entered starch and disaccharides was markedly different from that observed in the *in vitro* experiment. While the levels of label incorporated into starch did not show significant changes in the *in vivo* experiment, the label incorporated into disaccharides was significantly lower in the lines vacI-33 and -37 (Figure 5.8 C and D). The specific activity of the hexose phosphate pool decreased significantly in all investigated vacI lines to between 20 and 40% of the values from wild type tubers (Figure 5.8 H. The starch synthetic flux and the glycolytic flux were 3- to 5-fold and 2- to 3-fold higher, respectively, than in the wild type control (Figure 5.8 I and K). The sucrose synthetic flux showed a similar picture than in the *in vitro* experiment: whilst there was an increase in the line vacI-34, this was not statistically significant (Figure 5.8 J).

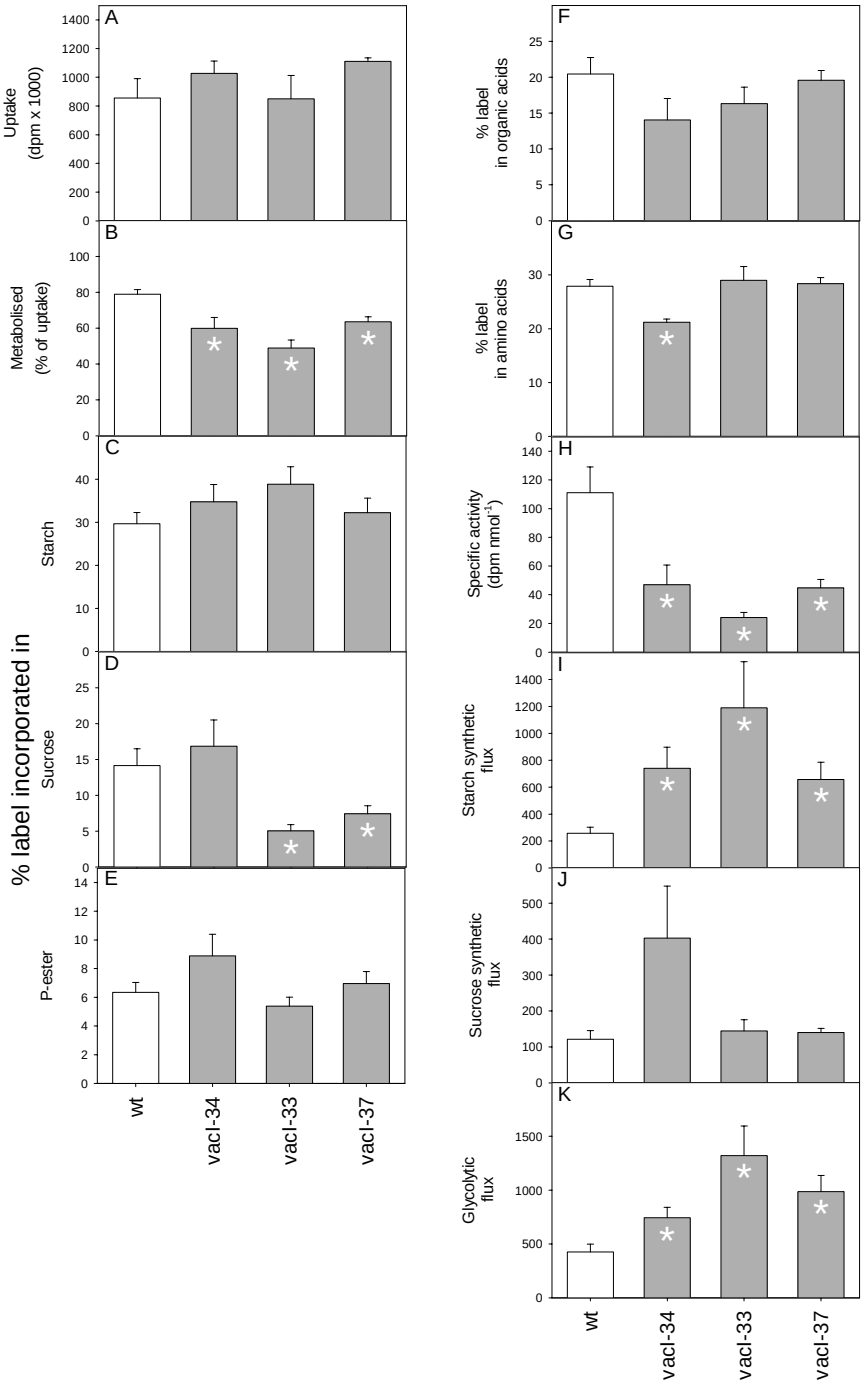



**Figure 5.8:** Metabolism of [U$^{14}$C]glucose in wild type and vacI tubers *in vivo*. [U$^{14}$C]glucose (specific activity: 11.5 GBq/mmol) was injected directly into intact tubers (*in planta*) into a fine borehole. [U$^{14}$C]glucose absorbed by the tissue (A); % of label that is metabolised to other compounds (B). Incorporation of $^{14}$C into starch (C); disaccharides (D); phosphoesters (E); organic acids (F); amino acids (G). The specific activity of the hexose phosphate pool (H) was estimated by dividing the label retained in the phosphate ester pool by the summed carbon of the hexose phosphates, and was used to calculate absolute fluxes to starch (I); sucrose (J); and glycolysis (K). Values represent the mean ± SE ($n$ = 4). Significant changes from the wild type ($P < 0.05$ using the Student's t test) are marked with an asterisk.

## *5.3. Discussion*

**The expression of a yeast invertase in the tuber vacuole**

The transgenic lines expressing a yeast invertase in the potato tuber vacuole were generated for several reasons. It has been previously shown that expression of a yeast invertase in the cytosol leads to a large decrease in sucrose coupled to an increased glucose content (Sonnewald *et al.*, 1997; Trethewey *et al.*, 1998). Expression of the same enzyme in the apoplast caused similar changes in glucose and sucrose (Sonnewald *et al.*, 1997). Interestingly, these two transgenic lines exhibited a different phenotype: while the cytosolic expression of yeast invertase was responsible for smaller and more numerous tubers, the apoplastic expression of the same enzyme yielded less but larger tubers. This effect was due to an increased cell number, but the mechanism of regulation for this process remained unknown (Sonnewald *et al.*, 1997). In another study, a bacterial glucokinase was overexpressed in the cytosol of tubers with an apoplastic yeast invertase background (Fernie *et al.*, 2000). Due to the observations made during this study, the authors suggested that the glucose produced by the apoplastic invertase could either (i) be transported through endocytotic-like mechanisms to the vacuole over the tonoplast, from where it would be released into the cytosol, or (ii) be delivered directly into the cytosol by specific glucose transporters in the plasma membrane (Fernie *et al.*, 2000). To answer this question, I chose the vacuolar expression of the invertase. The targeting sequence used here has been previously shown to yield specific vacuolar expression in tobacco (Sonnewald *et al.*, 1991; Heineke *et al.*, 1994) and potato leaves (Büssis *et al.*, 1997). The potato plants used in the latter study were expressing the yeast invertase under the control of the 35S-promoter conferring expression in all parts of the plants, thus it would not be possible to allocate changes in these plants in tuber carbohydrate partitioning to the invertase in the tubers. In contrast, these changes could be pleiotropic effects that have their sources in changes in leave metabolism. For this reason, in this study a tuber specific B33 promoter was used. It was shown again that this promoter is not active in leaves, and that the targeting sequence really yields invertase activity localised in the vacuole.

**Consequences of vacuolar expression of yeast invertase on tuber yield**

The expression of a yeast invertase in the apoplast of potato tubers led to an increased tuber size, whereas the cytosolic expression of the same enzyme led to a decreased tuber size (Sonnewald *et al.*, 1997). In this study, the vacuolar expression of invertase seemed in part A of the yield experiment

(Figure 5.4) to result in an increase in tuber number together with a decreased tuber weight. These results were not reproducible in part B of the experiment, where more plants were analysed per line; neither tuber number nor tuber weight showed significance differences in the vacI lines compared to wild type. These results demonstrate that in spite of the similarities in metabolites between potato plants expressing a yeast invertase in the apoplast and in the vacuole (discussed below), the effects of these two transgenes on tuber morphology are quite different. This again suggests successful vacuolar targeting of the protein since the default pathway of an aberrant vacuolar targeting would be the apoplast.

**Metabolic consequences of vacuolar expression of invertase in the developing tuber**

The constitutive cytosolic expression of a yeast invertase in potato tubers resulted in dramatic increases in the levels of the majority of other metabolites of primary metabolism (Roessner *et al.*, 2001b), whereas the apoplastic expression of a yeast invertase resulted in a quite distinct metabolic phenotype with only a few major changes, e.g. fructose increasing up to 260-fold and hexose-phosphates increasing 2- to 3-fold (Roessner *et al.*, 2001a).

The vacI plants expressing a yeast invertase in the vacuole of potato tubers exhibited a very similar metabolic phenotype to the plants expressing the same enzyme in the apoplast. In both transgenics, sucrose strongly decreased while glucose increased, however, this increase in glucose was higher in the apoplastic invertase expressing lines (Sonnewald *et al.*, 1997) than in those expressing the same enzyme in the vacuole. An explanation for this might be that in the vacuole, the free glucose produced by invertase is more accessible for further metabolism than if it is located in the apoplast. Starch levels were largely unaffected in both the plants with an overexpressed vacuolar and those with an apoplastic yeast invertase (Hajirezaei *et al.*, 2000). The increase of hexose phosphates was also similar in the apoplastic and vacuolar invertase expressing lines (Fernie *et al.*, 2000; Roessner *et al.*, 2001a). Studying the levels of other metabolites as measured by GC-MS revealed that the two transgenics (vacuolar and apoplastic invertase) behave very similarly: there were only minor changes in amino and organic acids, there was a very strong increase in fructose accompanied by an intermediate increase in glucose, sucrose strongly increased and hexose phosphates slightly increased (Roessner *et al.*, 2001a). The results of the metabolite analyses by GC-MS for the expression of a cytosolic, apoplastic or vacuolar invertase can be visualised by Principle Component Analysis (PCA). PCA uses a multi-dimensional vector approach to separate samples on the basis of the cumulative

correlation of all metabolite data and then identifies the vector that yields the greatest separation between samples. The results from the chosen vectors were then displayed in two dimensions (Figure 5.9). The cytosolic invertase expressing lines constitute a single cluster, while apoplastic and vacuolar invertase expressing lines cluster together. All these observations underline the similarity between plants expressing a yeast invertase in the apoplast with those expressing the same enzyme in the vacuole, and the dissimilarity between these two transgenics to plants expressing the same enzyme in the cytosol (Sonnewald *et al.*, 1997; Trethewey *et al.*, 1998; Roessner *et al.*, 2001a). This fact suggests that the vacuole and the apoplast of potato tubers are closely linked, and hence provides circumstantial support for the previously suggested endocytotic uptake mechanism from the apoplast into the vacuole (Oparka and Prior, 1988).

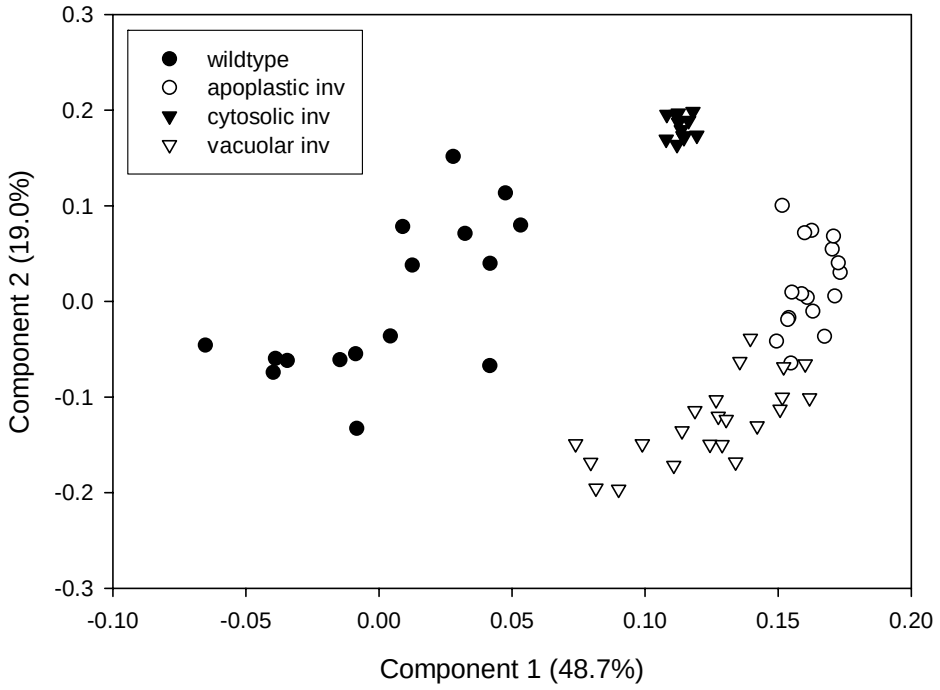



**Figure 5.9**: Principal component analysis (PCA) of transgenic line expressing a yeast invertase in three different compartments. The data from the GC-MS analysis (vacuolar invertase: this study; apoplastic and cytosolic invertase: Roessner *et al.*, 2001a) was analysed with PCA algorithms included in the statistical software package "R". PCA vectors span a multi-dimensional space to give the best samples separation, with each *point* or *triangle* representing a linear combination of all metabolites of an individual sample. *Vectors 1* and *2* were chosen for the best visualisation of differences between genotypes and between nutrient supplies and include 67.7% of the information derived from the metabolic variances.

Cytosolic expression of a yeast invertase leads to a severe increase in glycolysis (Trethewey *et al.*, 1998), whereas the expression of the same enzyme in the apoplast is not accompanied by an induction in glycolysis (Fernie *et al.*, 2000). In the vacI lines from this study, there was an increase in hexose phosphates, but no increase in the glycolytic intermediate 3-PGA. Also, activities of glycolytic enzymes generally were slightly decreased, and in the disc feeding experiment no increase in the glycolytic flux could be detected. Taken together, these results show that similarly to the apoplastic invertase lines but differently from the cytosolic invertase lines, in the vacuolar invertase lines there is no increase in glycolysis.

**Conclusion**

The data presented in this study on the vacuolar expression of a yeast invertase in the potato tuber is in most cases very similar to those previously reported for the apoplastic expression of the same enzyme (Sonnewald *et al.*, 1997; Fernie *et al.*, 2000). These similarities strongly suggest that the photoassimilate sucrose that arrives at the tuber cell is taken up by endocytosis-like mechanisms into the vacuole, where it is stored and later either directly transported to the cytosol or cleaved to hexose units that are then delivered to the cytosol for further metabolism. These results brings further understanding of sucrose metabolism in the potato tuber.

# Chapter 6. Kinetic Model of Sucrose Breakdown

## 6.1. Introduction

The potato tuber is a widely accepted model system for sink organs. Sucrose is transported from photosynthetic tissues via the phloem to the tuber, where it is degraded in the cytosol to hexose phosphates, which are mainly utilised for starch synthesis in the amyloplasts. The pathway of sucrose degradation is well defined (see for example Stitt and Sonnewald, 1995; Fernie *et al.*, 2002b; Geigenberger, 2003). In the last decade, a large portfolio of transgenic plants overexpressing (sense construct) or inhibiting (antisense construct) most of the genes have been generated and analysed in detail, in the case of overexpression also with many heterologous genes from other species (Fernie *et al.*, 2002b). These approaches have greatly contributed to improve the understanding of this pathway, but also led to some ambiguous experimental results which provoked further discussion and hypotheses rather than evident explanations. One reason for this may be that these studies are largely reductionist and it may be prudent to adopt pathway- or even systems-based approaches. However, to date there have been few attempts into this direction regarding the sucrose-to-starch pathway. One exception is the work of Tiessen *et al.* (2002), who calculated the mass-action ratios (i.e. the ratio between the *in vivo* concentration of the products and the substrates) for every reaction step between Suc and ADP-Glc. With this approach, the authors found that the unique site for starch synthesis being inhibited on tuber detachment is the enzyme ADP-glucose pyrophosphorylase. As the pathway of sucrose breakdown is a very dense net with many possible routes including substrate cycles and multiple outputs to other pathways, a model is needed to facilitate understanding of the processes. Such models have previously been built for sucrose synthesis in sugar cane (Rohwer and Botha, 2001), glycolysis in the potato tuber (Thomas *et al.*, 1997b), photosynthesis in tobacco leaves (Poolman *et al.*, 2001) and some other pathways (for a review see Morgan and Rhodes, 2002). In this Chapter, a kinetic model for sucrose degradation in the potato tuber is presented: it starts at sucrose and ends at the hexose phosphate pool from which the pathways of glycolysis and starch synthesis obtain their substrates. The model contains detailed compartment-specific kinetic equations for each enzyme, including all known terms for allosteric inhibition. The maximal catalytic activities are in all cases specific for the potato tuber, whereas $K_m$ and $K_i$ constants were taken, if not available for potato,

from the nearest taxonomical neighbour species possible. The model allows to simulate the steady state concentrations and fluxes in a wild type potato tuber. It also enables to qualitatively simulate the effects of overexpression of further enzymes catabolising sucrose.

## *6.2. Methods*

### 6.2.1. Metabolic system delineation

The subject of this study is the sucrose degradation pathway in developing potato (*Solanum tuberosum*) tubers. The photosynthetic product sucrose is transported from the leaves to the tubers, where it is unloaded from the phloem, ultimately transported to the cytosol (see Chapter 5), and cleaved to fuel the hexose phosphate pool. This pool constitutes the metabolic juncture of many different pathways, including glycolysis, starch synthesis and the pentose phosphate cycle (Figure 6.1). The former two were modelled as single reactions, whereas the latter was included in glycolysis. As about 20% of Fru6P is used in the pentose phosphate cycle (ap Rees and Beevers, 1960), glycolysis was therefore modelled to yield only 29 instead of 36 ATP, which corresponds to 80%.

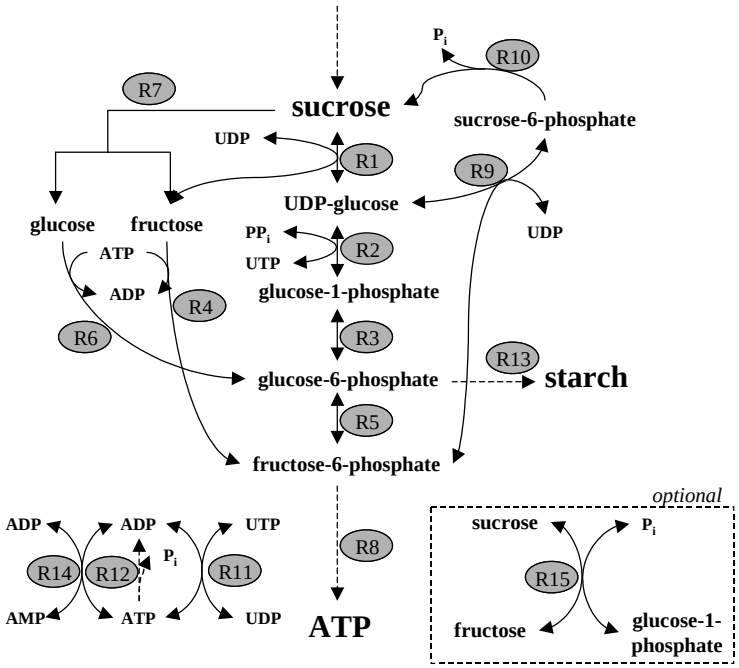



**Figure 6.1:** The sucrose breakdown pathway as it was modelled with Gepasi. The numbers denote the following reactions (the reactions in *italics* are summarising more than one enzyme in the model): (R1) sucrose synthase; (R2) UDPglucose pyrophosphorylase; (R3) phosphoglucomutase; (R4) fructokinase; (R5) phosphoglucose isomerase; (R6) hexokinase; (R7) invertase; *(R8) glycolysis*; (R9) sucrose phosphate sythase; (R10) sucrose phosphate phosphatase; (R11) NDP kinase; *(R12) ATP consumption by other cellular processes*; *(R13) starch synthesis*; (R14) adenylate kinase; (R15) sucrose phosphorylase.

## 6.2.2. Simulation, steady state calculation and metabolic control analysis

The whole modelling procedure including simulation, steady state calculation and metabolic control analysis was done using the computer program Gepasi (Mendes, 1993). The version 3.30 was used, which is freely available at the website http://www.gepasi.org.

## 6.2.3. Kinetics

**R1: Sucrose synthase**

SuSy activity was modelled with a reversible ordered bi-reactant mechanism, with UDP-glc binding first and UDP dissociating last from the enzyme (Rohwer and Botha, 2001). The model calculated the flux through Reaction 1 to proceed in the direction of sucrose degradation, therefore the sign of the rate law from Rohwer and Botha (2001) was reversed:

$$v = v_f \frac{[Suc][UDP] - \frac{[Fru][UDPglc]}{K_{eq}}}{[Suc][UDP]\left(1+\frac{[Fru]}{K_{i\,Fru}}\right) + K_{m\,Suc}\left([UDP]+K_{i\,UDP}\right) + K_{m\,UDP}[Suc] + \frac{v_f}{v_r K_{eq}} \times \left[[Fru]K_{m\,UDPglc}\left(1+\frac{[UDP]}{K_{i\,UDP}}\right) + [UDPglc]\left\{K_{m\,Fru}\left(1+\frac{K_{m\,UDP}[Suc]}{K_{i\,UDP}K_{m\,Suc}}\right) + [Fru]\left(1+\frac{[Suc]}{K_{i\,Suc}}\right)\right\}\right]}$$

**R2: UDP-glucose pyrophosphorylase**

UGPase was modelled with the "ordered Bi-Bi" kinetic type implemented in Gepasi, with UDPglc binding first and UTP dissociating last from the enzyme. This kinetic type contains product inhibition by UDPglc and pyrophosphate on the reverse reaction and by Glc1P on the forward reaction.

**R3 and R5: Phosphoglucomutase and phosphoglucose isomerase**

PGM and PGI were modelled without inhibition as "Uni-Uni" type reaction by using the rate law implemented in Gepasi.

**R4: Fructokinase**

FK was modelled as irreversible random-order bi-reactant kinetic, including competitive inhibition by ADP and non-competitive inhibition by Fru (Rohwer and Botha, 2001):

$$v = \frac{v_{\max}}{1+\frac{[Fru]}{K_{i\,Fru}}} \times \frac{\frac{[Fru][ATP]}{K_{m\,Fru}K_{m\,ATP}}}{1+\frac{[Fru]}{K_{m\,Fru}}+\frac{[ATP]}{K_{m\,ATP}}+\frac{[Fru][ATP]}{K_{m\,Fru}K_{m\,ATP}}+\frac{[ADP]}{K_{i\,ADP}}}$$

**R6: Hexokinase**

HK was simulated as an irreversible random order bi-reactant mechanism as done by Rohwer and Botha (2001). In potato, hexokinase is not able to efficiently phosphorylate fructose because it has a $K_{m\ Fru}$ that is several orders of magnitude higher than the $K_{m\ Glc}$ (Renz and Stitt, 1993), so the fructose-phosphorylating kinetics as well as the competitive inhibition by fructose were omitted from the rate law used by Rohwer and Botha (2001):

$$v = v_{\max}\frac{\frac{[Glc][ATP]}{K_{m\,Glc}K_{m\,ATP}}}{\left(1+\frac{[ATP]}{K_{m\,ATP}}\right)\left(1+\frac{[Glc]}{K_{m\,Glc}}+\frac{[Glc6P]}{K_{i\,Glc6P}}\right)}$$

**R7: Neutral (cytosolic) invertase**

Invertase activity was modelled as an irreversible Michaelis-Menten mechanism with competitive inhibition by fructose and non-competitive inhibition by glucose. As the mechanism of inhibition has not been determined in potato tubers, I assumed it to be the same as for carrot neutral invertase (Lee and Sturm, 1996):

$$v = \frac{v_{\max}}{1+\frac{[Glc]}{K_{i\,Glc}}} \times \frac{[Suc]}{K_{m\,Suc}\left(1+\frac{[Fru]}{K_{i\,Fru}}\right)+[Suc]}$$

**R8: Glycolysis**

Glycolysis was modelled as a single reaction proceeding from Fru6P, as an irreversible random order bi-reactant mechanism, including competitive inhibition by ATP. As about 20% of Fru6P is metabolised in the pentose phosphate cycle (ap Rees and Beevers, 1960), glycolysis was supposed to yield 29 instead of 36 ATP.

$$v = v_{\max} \frac{\frac{[Fru6P][ADP]}{K_{m\,Fru6P} K_{m\,ADP}}}{\left(1+\frac{[ADP]}{K_{m\,ADP}}\right)\left(1+\frac{[Fru6P]}{K_{m\,Fru6P}}+\frac{[ATP]}{K_{i\,ATP}}\right)}$$

**R9: Sucrose phosphate synthase**

SPS activity was modelled with a reversible ordered bi-reactant mechanism, with UDP-glucose binding first and UDP dissociating last from the enzyme. The enzyme activity was defined to be positive in the direction of sucrose phosphate synthesis. $P_i$ was modelled as a competitive inhibitor with respect to Fru6P (Rohwer and Botha, 2001):

$$v = v_f \frac{[Fru6P][UDPglc] - \frac{[Suc6P][UDP]}{K_{eq}}}{[Fru6P][UDPglc]\left(1+\frac{[Suc6P]}{K_{i\,Suc6P}}\right)+K_{m\,Fru6P}\left(1+\frac{[P_i]}{K_{i\,P_i}}\right)\left([UDPglc]+K_{i\,UDPglc}\right)+K_{m\,UDPglc}[Fru6P]+\frac{v_f}{v_r K_{eq}}\times \left[[Suc6P]K_{m\,UDP}\left(1+\frac{[UDPglc]}{K_{i\,UDPglc}}\right)+[UDP]\left\{K_{m\,Suc6P}\left(1+\frac{K_{m\,UDPglc}[Fru6P]}{K_{i\,UDPglc}K_{m\,Fru6P}\left(1+[P_i]/K_{i\,P_i}\right)}\right)+[Suc6P]\left(1+\frac{[Fru6P]}{K_{i\,Fru6P}}\right)\right\}\right]}$$

**R 10: Sucrose phosphate phosphatase**

SPP activity was modelled as an irreversible Michaelis-Menten mechanism with non-competitive inhibition by sucrose.

$$v = v_{\max} \frac{[Suc6P]}{\left[K_{m\,Suc6P}\left(1+\frac{[Suc]}{K_{i\,Suc}}\right)+[Suc6P]\left(1+\frac{[Suc]}{K_{i\,Suc}}\right)\right]}$$

**R11 and R14: NDP and adenylate kinases**

NDP kinase and AdK activities were modelled with the "reversible mass action" kinetic type implemented in Gepasi.

**R12: ATP consumption**

ATP consumption by other cellular processes was simulated as irreversible constant flux using the corresponding pre-defined kinetic type in Gepasi.

**R13: Starch synthesis**

StaSy was modelled as a single reaction proceeding from Glc6P. It was modelled as an irreversible Ping-pong Bi-Bi mechanism in order to make the reaction ATP sensing, i.e. if there is not enough ATP available, the reaction will slow down.

$$v = v_{\max} \frac{[G6P][ATP]}{[G6P][ATP] + K_{m\,G6P}[ATP] + K_{m\,ATP}[G6P]}$$

**R15: Sucrose phosphorylase from *Pseudomonas saccharophila***

This enzyme was only included into the model in Chapter 6.3.2., that means it is absent under wild type conditions. It was modelled as a ping-pong Bi-Bi kinetic type (Mieyal and Abeles, 1972) with the pre-defined rate law in Gepasi.

## 6.2.4. Model parameters

The kinetic parameters used for the model are summarised in Table 6.1. The values were taken from the literature on potato tubers, or from the nearest taxonomical neighbour from which the parameter was available. If no data were available, the parameters were estimated as indicated in the table. One exception was the maximal catalytic activity of SPP, which was measured in 20 enzyme extracts according to the Material and Methods section of this thesis. The units of the system are mM and s. For conversion, the density of tubers of 1.16 g/ml and the percentage of the cytosol compared to

the whole cell of 12% were used (Farré, 2001). When values from the literature were given normalised to protein content, the tuber protein content of 2.1 mg/gFW was used (measured on 50 samples with the method from Bradford, 1976) to calculate a reaction velocity in mM/s. In order to be able to calculate a steady state for the model, the concentrations of the initial and terminal metabolites (Suc and starch) needed to be fixed. Additionally, $P_i$ and PP were fixed, mainly because it is still unclear how pyrophosphate is regenerated in the cytosol after plastidial AGPase and pyrophosphatase reactions (Farré *et al.*, 2000). The fixed metabolite concentrations are summarised in table 6.2, which contrasts the simulated and experimental metabolite concentrations.

**Table 6.1:** Modelling parameters. Columns: 1, parameter; 2, value; 3, organism; 4, reference and/or comment. "% cytosol" means that this percentage of the overall activity is located in the cytosol.

| | | | |
|---|---|---|---|
| *Reaction 1: sucrose synthase* | | | |
| % cytosol | 91 | potato | (Farré, 2001) |
| $V_f$ | 0.035 | potato | (Farré, 2001) |
| $V_r$ | 0.08 | | ratio from (Rohwer and Botha, 2001) |
| $K_{eq}$ | 0.36 | potato | range 0.15-0.56 (Tiessen *et al.*, 2002) |
| $K_{m\ Suc}$ | 50 | various | (Rohwer and Botha, 2001) |
| $K_{m\ UDP}$ | 0.058 | potato | estimate based on UDP concentration |
| $K_{m\ Fru}$ | 7.8 | maize | (Doehlert, 1987) |
| $K_{m\ UDPglc}$ | 0.076 | maize | (Doehlert, 1987) |
| $K_{i\ UDP}$ | 0.058 | potato | estimate based on UDP concentration |
| $K_{i\ Fru}$ | 7.8 | maize | assumed equal to $K_{m\ Fru}$ |
| $K_{i\ Suc}$ | 40 | potato | (Stitt and Steup, 1985) |
| *Reaction 2: UDP-glucose pyrophosphorylase* | | | |
| % cytosol | 100 | potato | (Farré, 2001) |
| $V_f$ | 1.31 | potato | (Sowokinos *et al.*, 1993) |
| $V_r$ | 0.78 | potato | ratio $V_f/V_r$ from (Katsube *et al.*, 1991) |
| $K_{eq}$ | 3.2 | potato | (Tiessen *et al.*, 2002) |
| $K_{m\ UDPglc}$ | 0.137 | potato | averaged from (Nakano *et al.*, 1989; Katsube *et al.*, 1991; Sowokinos *et al.*, 1993) |
| $K_{m\ PP}$ | 0.127 | | |
| $K_{m\ Glc1P}$ | 0.16 | | |
| $K_{m\ UTP}$ | 0.142 | | |
| $K_{i\ UDPglc}$ | 0.137 | potato | J.R. Sowokinos, pers. communication |
| $K_{i\ PP}$ | 0.127 | potato | J.R. Sowokinos, pers. communication |

| | | | |
|---|---|---|---|
| $K_{i\ Glc1P}$ | 0.16 | potato | J.R. Sowokinos, pers. communication |
| $K_{i\ UTP}$ | 0.142 | potato | J.R. Sowokinos, pers. communication |

*Reaction 3: Phosphoglucomutase*

| | | | |
|---|---|---|---|
| % cytosol | 5 | potato | (Farré, 2001) |
| $V_{max}$ | 0.023 | potato | averaged from (Trethewey *et al.*, 1998; Sweetlove *et al.*, 1999; Farré, 2001) |
| $K_{eq}$ | 19 | potato | (Tiessen *et al.*, 2002) |
| $K_{m\ Glc1P}$ | 0.06 | potato | (Thomas *et al.*, 1997b) |
| $K_{m\ Glc6P}$ | 0.5 | potato | (Thomas *et al.*, 1997b) |

*Reaction 4: Fructokinase*

| | | | |
|---|---|---|---|
| % cytosol | 61 | potato | (Farré, 2001) |
| $V_{max}$ | 0.058 | potato | (Farré, 2001) |
| $K_{m\ Fru}$ | 0.077 | | |
| $K_{m\ ATP}$ | 0.026 | potato | averaged from (Renz and Stitt, 1993; Dai *et al.*, 1997; Veramendi *et al.*, 1999) |
| $K_{i\ Fru}$ | 5.9 | | |
| $K_{i\ ADP}$ | 0.078 | | |

*Reaction 5: Phosphoglucose isomerase*

| | | | |
|---|---|---|---|
| % cytosol | 12 | | (Farré, 2001) |
| $V_{max}$ | 0.041 | potato | (Burrell *et al.*, 1994) |
| $K_{eq}$ | 2 | potato | (Tiessen *et al.*, 2002) |
| $K_{m\ Fru6P}$ | 0.15 | potato | (Thomas *et al.*, 1997b) |
| $K_{m\ Glc6P}$ | 0.27 | potato | (Thomas *et al.*, 1997b) |

*Reaction 6: Hexokinase*

| | | | |
|---|---|---|---|
| % cytosol | 61 | | assuming equal subcellular distribution as for FK |
| $V_{max}$ | 0.015 | potato | (Trethewey *et al.*, 1998) |
| $K_{m\ Glc}$ | 0.13 | potato | (Renz and Stitt, 1993) |
| $K_{m\ ATP}$ | 0.185 | potato | range 0.09-0.28 (Renz and Stitt, 1993) |
| $K_{i\ Glc6P}$ | 4.1 | potato | (Renz and Stitt, 1993) |

*Reaction 7: Invertase*

| | | | |
|---|---|---|---|
| % cytosol | 80 | potato | (Farré, 2001) |
| $K_{m\ Suc}$ | 41 | | estimate based on Suc concentration |
| $K_{i\ Glc}$ | 31 | | estimate based on Glc concentration |
| $K_{i\ Fru}$ | 0.01 | | estimate based on Fru concentration |

*Reaction 8: Glycolysis*

| | | | |
|---|---|---|---|
| $V_{max}$ | 0.01 | | estimate |
| $K_{m\ Fru6P}$ | 0.14 | | estimate based on Fru6P concentration |
| $K_{m\ ADP}$ | 0.018 | | estimate based on ADP concentration |
| $K_{i\ ATP}$ | 0.21 | | estimate based on ATP concentration |

*Reaction 9: Sucrose phosphate synthase*

| | | | |
|---|---|---|---|
| % cytosol | 100 | | J. Lunn, pers. communication |
| $V_f$ | 0.0077 | potato | (Sweetlove *et al.*, 1999) |
| $V_r$ | 0.0041 | | ratio $V_f/V_r$ taken from (Rohwer and Botha, 2001) |
| $K_{eq}$ | 10 | potato | (Kruger, 1997) |
| $K_{m\ Fru6P}$ | 0.3 | potato | (Reimholz *et al.*, 1994) |
| $K_{m\ UDPglc}$ | 4.6 | potato | (Reimholz *et al.*, 1994) |
| $K_{m\ UDP}$ | 0.3 | various | (Rohwer and Botha, 2001) |
| $K_{m\ Suc6P}$ | 0.41 | | estimate based on Suc6P concentration |
| $K_{i\ Suc6P}$ | 0.07 | | estimate based on Suc6P concentration |
| $K_{i\ Fru6P}$ | 0.14 | | estimate based on Fru6P concentration |
| $K_{i\ P}$ | 3 | potato | (Reimholz *et al.*, 1994) |

*Reaction 10: Sucrose phosphatase*

| | | | |
|---|---|---|---|
| % cytosol | 100 | | J. Lunn, pers. communication |
| $V_{max}$ | 0.0025 | potato | measured in this study |
| $K_{m\ Suc6P}$ | 0.1 | various | range 0.045-0.15 in (Quick and Schaffer, 1996) |
| $K_{i\ Suc}$ | 41 | | estimate based on Suc concentration |

*Reaction 11: NDP kinase*

| | | |
|---|---|---|
| $k_1 = k_2$ | 0.1 | estimate |

*Reaction 12: ATP consumption by other cellular processes*

| | | |
|---|---|---|
| flux | 0.1 | estimate |

*Reaction 13: Starch synthesis*

| | | |
|---|---|---|
| $V_{max}$ | 0.03 | estimate |
| $K_{m\ Glc6P}$ | 0.74 | estimate based on Glc6P concentration |
| $K_{m\ ATP}$ | 0.21 | estimate based on ATP concentration |

*Reaction 14: Adenylate kinase*

| | | |
|---|---|---|
| $k_1 = k_2$ | 1 | estimate |

*Reaction 15: Sucrose phosphorylase (from Pseudomonas saccharophila), only included in chapter 6.3.2.*

| | | | |
|---|---|---|---|
| % cytosol | 100 | | assumed |
| $V_f$ | 0.029 | potato | (Trethewey *et al.*, 2001) |
| $V_r$ | 0.02 | potato | (Trethewey *et al.*, 2001) |
| $K_{eq}$ | 21 | | calculated with Haldane equation (Sinnott *et al.*, 1998) |
| $K_{m\ P}$ | 1.8 | *P.saccharophila* | (Mieyal and Abeles, 1972) |
| $K_{m\ Suc}$ | 1.71 | *P.saccharophila* | (Mieyal and Abeles, 1972) |
| $K_{m\ Glc1P}$ | 2.3 | *P.saccharophila* | (Mieyal and Abeles, 1972) |
| $K_{m\ Fru}$ | 13 | *P.saccharophila* | (Mieyal and Abeles, 1972) |
| $K_{i\ P}$ | 33.4 | | estimate based on P concentration |
| $K_{i\ Fru}$ | 0.01 | | estimate based on Fru concentration |

## *6.3. Results*

The pathway of sucrose breakdown in the potato tuber can be divided into different blocks. At first, sucrose can be cleaved by the action of invertase or sucrose synthase. The resulting hexoses or hexose phosphates can then be used for starch synthesis, glycolysis or other pathways (which are not included in the presented model). The energy in form of ATP generated in glycolysis can be used in starch synthesis, in sucrose cycling, or by other cellular processes. From this information, futile cycles and optimal conversion pathways from sucrose to starch can be deduced (Geigenberger and Stitt, 1993). A detailed structural analysis of these cycles and pathways, which are also called *elementary flux modes* in Metabolic Control Analysis or *T-invariants* in Petri net theory (see Chapter 1.5), is described elsewhere (Koch *et al.*, 2004). However, a structural analysis cannot ascertain which factors will influence the flux distribution in a metabolic network from one elementary flux mode to another, since it considers only stoichiometric information. Yet, metabolic behaviour depends not only on stoichiometry, but also on thermodynamics and kinetics. To better understand the dynamics of sucrose degradation, I combined the known kinetic and thermodynamic information of the enzymes in a detailed kinetic model.

### 6.3.1. Steady state properties of the kinetic model

Having established the kinetic model of sucrose breakdown in the potato tuber with the computer program Gepasi, the steady state metabolite concentrations were first simulated and then compared to the experimentally determined metabolite concentrations from the literature (Table 6.2).

Most of the metabolites did not vary more than 2-fold between the measured and simulated concentration, with the exception of ADP and AMP (both 4-fold higher in the simulation), Suc6P (10-fold lower) and Glc (4 orders of magnitude lower in the simulation, respectively). While the differences in adenylate and Suc6P levels were not so drastic and do not have a greater impact on the model, the difference in Glc requires some explanation. The low glucose can be best explained by the fact that starch degradation (yielding glucose) was not included in this model. Starch metabolism may be characterised by a continuous cycle of synthesis and degradation (Sweetlove *et al.*, 1996b). To simulate starch metabolism, one would need to separate the model into two compartments (cytosol and plastid) and include some reactions of the plastidial metabolism (pPGM, AGPase), starch synthesis and starch degradation. This would lead to a much more complex model, which would not be as easy to handle as the model described in this study.

**Table 6.2**: Cytosolic metabolite concentrations as taken from experiments described in the literature, from simulation with wild type conditions, and from simulation after increasing invertase activity. Concentrations are given in mM. Abbreviations: Ref., reference; sim., simulated; exp., experimental; wt, wild type; Inv., yeast invertase; SuPho, *Pseudomonas* sucrose phosphorylase.

| Metabolite | experimental | Ref | simulated (wildtype) | Factor sim. to exp. | simulated (Invertase) | Factor wt to Inv. | simulated (SuPho) | Factor wt to SuPho |
|---|---|---|---|---|---|---|---|---|
| Suc | 41.00 | a | 41.00 | 1.0 | 41.00 | 1.0 | 41.00 | 1.0 |
| Suc6P | 0.01 | b | 0.001 | 0.1 | 0.01 | 7.8 | 0.02 | 21.0 |
| Glc | 31.00 | a | 0.004 | 0.0001 | 6.51 | 1695 | 0.002 | 0.5 |
| Fru | 0.01 | c | 0.01 | 1.3 | 0.04 | 3.4 | 0.06 | 4.9 |
| UDPglc | 0.83 | a | 0.54 | 0.7 | 0.79 | 1.4 | 1.12 | 2.1 |
| Glc6P | 0.74 | a | 0.35 | 0.5 | 1.90 | 5.5 | 2.89 | 8.3 |
| Glc1P | 0.05 | a | 0.05 | 1.0 | 0.12 | 2.3 | 0.50 | 10.1 |
| Fru6P | 0.14 | a | 0.19 | 1.4 | 1.19 | 6.3 | 1.86 | 9.8 |
| ATP | 0.21 | a | 0.12 | 0.6 | 0.21 | 1.7 | 0.22 | 1.8 |
| ADP | 0.02 | a | 0.07 | 4.0 | 0.02 | 0.3 | 0.02 | 0.2 |
| AMP | 0.01 | d | 0.04 | 4.1 | 0.002 | 0.1 | 0.001 | 0.03 |
| UTP | 0.40 | a | 0.72 | 1.8 | 0.49 | 0.7 | 0.16 | 0.2 |
| UDP | 0.06 | a | 0.03 | 0.5 | 0.01 | 0.3 | 0.01 | 0.3 |
| PP | 0.02 | a | 0.02 | 1.0 | 0.02 | 1.0 | 0.02 | 1.0 |
| P | 33.40 | d | 33.40 | 1.0 | 33.40 | 1.0 | 33.40 | 1.0 |

a: Farré *et al.* (2001)

b: S. Chen and F. Börnke, personal communication

c: estimated; Fru was below the detection limit in the cytosol in Farré *et al.* (2001)

d: A. Tiessen, personal communication

The steady state fluxes, summarised in Table 6.3, are much higher than those determined experimentally (e.g. starch to flux calculated from chapter 4 = 5 x $10^{-5}$ mM/s). One reason for this might be that Gepasi takes only the pure kinetic reaction of an enzyme into account, neglecting the much greater time it takes for the substrates to approach the enzyme by diffusion. This would be supported by the theory that the cytosol's gel-like structure is responsible for limited diffusion (Clegg, 1992). Another reason might be that that the *in vitro* and *in vivo* kinetics are different due to additive and competing effects of the complex mixture of the cell. However, the ratios between the fluxes were in very close accordance to values reported in the literature. It was experimentally determined that from the hexose phosphates, 61% enter starch synthesis, 6% enter sucrose biosynthesis and 33% enter glycolysis (Trethewey *et al.*, 1999). From these values, a ratio between the flux to glycolysis and the flux to starch of 0.54 can be calculated, which is very close to the value of the simulation (0.59). Furthermore, it was reported that sucrose synthase contributes 40-80% (averaged: 60%) to sucrose biosynthesis (Geigenberger and Stitt, 1993), that means 40% is covered by SPS/SPP, which corresponds to 2.4% of the flux from hexose phosphates. This values is again very close to the value from the model (2.0% calculated by the formula $2*J_{SPS} / (2*J_{SPS} + J_{StaSy} + J_{Glyc})$ ). Also, it can be calculated from the simulation that the endogenous neutral invertase in the potato tuber contributes only 3% ( $=J_{Inv} / (J_{SuSy} + J_{Inv})$ ) to sucrose breakdown.

**Table 6.3**: Simulated fluxes with wild type conditions and after virtual overexpression of a yeast invertase and of a bacterial sucrose phosphorylase. Values are given in µM/s.

| Enzyme | wildtype | Inv | Factor wt to Inv | SuPho | Factor wt to SuPho |
|---|---|---|---|---|---|
| SuSy | 4.89 | 1.38 | 0.3 | 0.88 | 0.2 |
| UGPase | 4.79 | 0.88 | 0.2 | 0.07 | 0.02 |
| PGM | 4.79 | 0.88 | 0.2 | 8.83 | 1.8 |
| FK | 5.05 | 9.04 | 1.8 | 9.70 | 1.9 |
| PGI | 1.27 | 4.11 | 3.2 | 4.65 | 3.7 |
| HK | 0.16 | 7.66 | 48.8 | 0.07 | 0.5 |
| Inv | 0.16 | 7.66 | 48.8 | 0.07 | 0.5 |
| Glyc | 3.68 | 4.43 | 1.2 | 4.25 | 1.2 |
| SPS | 0.10 | 0.50 | 5.1 | 0.80 | 8.1 |
| SPP | 0.10 | 0.50 | 5.1 | 0.80 | 8.1 |
| NDP kin. | -4.79 | -0.88 | 0.2 | -0.07 | 0.02 |
| ATP cons. | 100 | 100 | 1.0 | 100 | 1.0 |
| StaSy | 6.22 | 12.65 | 2.0 | 13.55 | 2.2 |
| AdK | 0.00 | 0.00 | 0.0 | 0.00 | 0.0 |
| SuPho | - | - | - | 8.76 | - |

## 6.3.2. Effect of manipulating enzyme activities

In the previous section, it was illustrated that the model is in close accordance at the level of metabolites and flux ratios to the experimentally determined values from the literature. Thus, a change in the model should reflect those measured on experimentation. The next step was to simulate overexpression of a yeast invertase simply by increasing the $v_{max}$ of this enzyme in the model to 460 nmol $gFW^{-1}$ $min^{-1}$ (corresponds to 0.06 mM/s in the cytosol), an activity that was experimentally determined in the strongest inducible AlcI line from Chapter 4. The effect of this change in activity on the metabolite levels is shown in Table 6.2. The most notable effect is the major increase in glucose (1700-fold) compared to the model with wild type conditions, whereas the increase in fructose is only minor (3-fold). Hexose-phosphates increase 2- to 6-fold, Suc6P increases 8-fold, and there is a slight shift from uridyl-phosphates, AMP and ADP to ATP.

The effect of an increased invertase maximal catalytic activity ($v_{max}$) on fluxes is included in Table 6.3. As expected, the flux through invertase and the flux through hexokinase increase strongly (50-fold). The invertase becomes responsible for 85% of sucrose breakdown, compared to 3% under wild type conditions. The flux through SuSy was much smaller than under simulated wild type conditions. By imposing an even higher increase in invertase activity this flux can even be forced in the backward direction (data not shown). The sucrose cycling flux through SPS and SPP was 5-fold, and the flux to starch was 2-fold higher compared to the fluxes simulated for wild type invertase activity, while the flux through glycolysis was only marginally increased.

In a second sucrose catabolism overexpression scenario, the reaction of a sucrose phosphorylase was included into the model, as recently performed *in planta* by Trethewey *et al.* (2001). In comparison to the simulation under wild type conditions, hexose phosphates increased 8- to 10-fold, fructose increases 5-fold, while glucose decreases 2-fold (Table 6.2). Suc6P increased 21-fold, and there was similarly to the invertase overexpression scenario a slight shift from uridyl-phosphates, AMP and ADP to ATP.

Under these circumstances sucrose phosphorylase accounted for 90% of sucrose breakdown, whereas sucrose synthase and invertase were only responsible for 9% and 1% of sucrose breakdown, respectively (Table 6.3). Very similarly to the simulated invertase overexpression, the flux to starch increased more than 2-fold, while the flux through glycolysis was less than 20% higher than under simulated wild type conditions. The sucrose cycling flux through SPS and SPP was increased more than 8-fold.

### 6.3.3. Metabolic Control Analysis

Metabolic control analysis is a theory originally described in two seminal works by Kacser and Burns (1973) and Heinrich and Rapoport (1974) to quantitatively analyse the control of fluxes and concentrations in metabolic systems. The system response is described with control coefficients (C), while elasticity coefficients (ε) are used to describe local properties which ultimately generate the system properties. In the study presented here I will concentrate on the flux control coefficient, which states that

$$C^{J_{E_i}} = \frac{\frac{\delta J}{J}}{\frac{\delta E_i}{E_i}} = \frac{\delta J}{\delta E_i} \cdot \frac{E_i}{J}$$

where $\delta J/J$ is the fractional change of a pathway flux which results from the fractional change $\delta E_i/E_i$ of the amount of enzyme in question. If a single enzyme controls flux through a pathway, there is a direct linear relationship between the enzyme amount and the pathway flux (C = 1) and the slope of a normalised plot of J vs. $E_i$ will equal unity. If the enzyme exerts no control a change in the amount of the enzyme will have no effect on the pathway flux (C = 0) and the slope of a normalised J vs. $E_i$ plot will be zero. In between these two extremes are enzymes that exhibit partial or shared control. They will have control plots in which the slope exhibits a value which is somewhere between 0 and 1. In a simple linear pathway, the control coefficients of the constituent enzymes sum to unity.

The computer program Gepasi calculates the flux control coefficient $C^J$ that each enzyme has on the flux through another enzyme. These control coefficients are interesting also for biotechnological applications, because they give hints which enzymes might be suitable candidates for increasing intermediates, products or the fluxes through the pathway under study. As an example it can be seen from Figure 6.2, that ATP consumption has got the highest positive flux control coefficient on glycolysis, that means an increase in ATP consumption is increasing the flux through glycolysis, which is intuitively explainable. The block of glycolytic reactions in the model has a large negative control coefficient on the flux through SPS, so does the step defined as starch synthesis, whereas NDPkinase positively influences the flux through this reaction. The flux into starch synthesis is negatively controlled by glycolysis, and positively by NDPkinase.

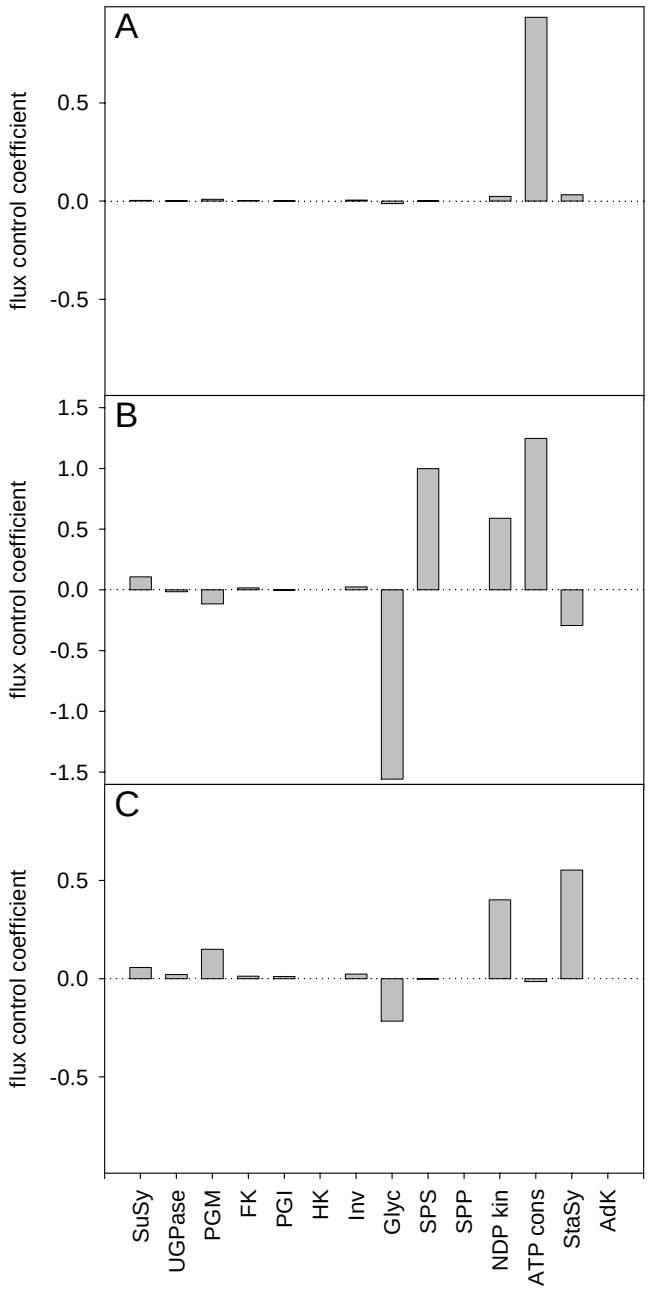



**Figure 6.2**: Selected flux control coefficients of all reactions of the model on the flux through glycolysis (A), sucrose cycling via SPS (B) and starch synthesis (C), calculated from the simulation under wild type conditions. The control coefficients for one reaction always sum up to unity.

## *6.4. Discussion*

This chapter describes the generation of a kinetic model for simulation and control analysis of sucrose breakdown in developing potato tubers. To make this project achievable, certain boundaries had to be set to the model. The starting point is cytosolic sucrose; sucrose import was not considered in the model because it is not yet fully understood how sucrose enters the cytosol, if it is directly from the apoplast or via the vacuole (see also Chapter 5). Further on, it is probable that the simulation would behave very similarly if internal sucrose was allowed to vary and sucrose import was included in the model. The input of the model has to be fixed to a certain concentration, and in this case it would be external sucrose that would have to be fixed. Glycolysis was modelled as a single reaction because the upper part of glycolysis is a generally linear pathway and therefore easy to characterise, and lower glycolysis (TCA cycle) with its branch points to amino acid synthesis and other pathways would add too much complexity to the model. Also for reasons of simplification, starch synthesis was modelled as a single reaction, because this strategy avoids the necessity of adding a second compartment. Modelling more than one compartment requires calculating in volumes with fixed amounts of metabolites that have to be known for each compartment separately, whereas assuming a single compartment only requires concentrations of metabolites. More importantly, modelling more than one compartment would require to include all transport processes between these compartment. These transport processes are in the case of the potato tuber poorly understood. Nevertheless, this model should be regarded as a starting point for an expansion that should address at first the central pathways of starch synthesis, starch degradation, glycolysis, TCA cycle, oxidative phosphorylation and probably the pentose phosphate cycle and amino acid synthesis.

Many of the steps in the model were taken from the sugar cane model from Rohwer and Botha (2001). There are, however, three main differences to that model. First, in sugar cane the major flux is from hexoses to sucrose, whereas in the potato tuber the major flux is from sucrose to hexose phosphates and then to starch synthesis and glycolysis. The second difference is that the sugar cane model was simplified by summarising Glc1P, Glc6P, Fru6P and UDPglc as “hexose phosphates”. Because all the enzymes interconverting these metabolites in the potato tuber are well characterised, there was no reason for this simplification here. The third and most important difference is that part of the nucleotide phosphate metabolism was included in the model presented here: ATP, ADP, AMP, UTP and UDP were allowed to vary whereas they were artificially fixed in the sugar cane model. The reactions interconverting these metabolites (NDP kinase, AdK), as well as ATP production by

glycolysis and its consumption by other cellular processes were included. The reasons to add this level of complexity to the model are numerous. At first, nucleotide phosphates represent the high-energy constituents of the cell, and therefore they are central to metabolism and essential for all cellular processes. Furthermore, glycolysis is driven by ATP demand (Koebmann *et al.*, 2002), and other processes in the sucrose breakdown pathway like sucrose cycling by SPS/SPP and hexose phosphorylation are requiring ATP. By including nucleotide phosphate metabolism, the model can sense if there is not enough ATP, and then shift the flux from the ATP-consuming process (starch synthesis) to the ATP-generating process (glycolysis). However, the most important reason is that previously an increase in starch content was achieved in potato tubers by alterations in adenylate metabolism, for example after changing the activity of the plastidial adenylate kinase (Regierer *et al.*, 2002) and after overexpression of the adenylate translocator (Tjaden *et al.*, 1998a). This suggests that adenylate metabolism, and therefore nucleotide phosphate metabolism, plays an essential role in the regulation of the sucrose-to-starch pathway, and are therefore imperative for a model simulating sucrose breakdown.

As described, the model reaches a steady state for each of the three scenarios simulated here (wild type, invertase overexpressor, sucrose phosphorylase overexpressor) with concentrations similar to those observed in experiments. Whilst some of the absolute fluxes are too high in the model, the relative ratios between the fluxes are reasonably close to the values represented in the literature. When looking at the simulated fluxes, it is noticeable that some enzymes appear to be coupled, because their fluxes at steady state are always the same: UGPase and PGM, Invertase and HK, SPS and SPP. The reason for this is very simple: in every possible path that can be taken through the model, there is either none or both of the enzymes from one of these couples present. Or, in other words, if for example glucose is produced by invertase, in the model it has no other choice than to be phosphorylated by HK.

When manipulating enzyme activities in the model, most of the resulting changes in metabolites and fluxes were at least qualitatively the same as described from experiments in the literature. Overexpression of a yeast invertase results in a large increase in glucose and only a minor increase in fructose (Sonnewald *et al.*, 1997), however the glucose increase was much more drastic in the model (1700-fold) than in the experiment (15-fold; Sonnewald *et al.*, 1997). This can be best explained by the fact that such a drastic increase of a metabolite is unlikely to take place because the real cell has got much more buffering capacity than the model. For example, any metabolite accumulating could be

utilised by other reactions that are not incorporated in the model, or the reactions producing this metabolite could be downregulated by a mechanism that does not directly effect the rate law (such as transcriptional regulation or post-translational modification). The changes in hexose phosphates after invertase overexpression (Table 6.2) are astonishingly close to those described by Trethewey *et al.* (1998): (simulated vs. experimental) Glc6P 5.5 vs. 4.1, Glc1P 2.3 vs. 2.8, Fru6P 6.3 vs. 4.6. Also, the flux to starch and the sucrose cycling via SPS and SPP was increased in the model as in experiments (Trethewey *et al.*, 1999). However, the large increase in glycolysis reported by Trethewey *et al.* (1999) was not observed in the model. There are two possibilities to explain this inconsistency: at first, as the model is built on parameters determined *in vitro*, it simply might not reflect the *in vivo* conditions. Second and more likely, the increase in glycolysis observed by Trethewey *et al.* (1999) might be a late effect of the expression of the invertase early in potato tuber development, or an effect that builds up over time after the high invertase activity has been present for a large timeframe, rather than an immediate metabolic response to invertase expression. This possibility is supported by the finding that the flux through glycolysis is not increased after temporally controlled induction of the yeast invertase (Chapter 4).

The effects of an overexpression of a bacterial sucrose phosphorylase is also very close in the simulation and experimental determinations. Trethewey *et al.* (2001) reported that glucose decreases up to 2-fold upon sucrose phosphorylase expression, while all three hexose phosphates increase 9-fold in the strongest line. The situation is exactly the same in the model. Similarly, the flux through sucrose cycling via SPS and SPP, as well as the starch synthetic flux increased in both the model and the experiment (Trethewey *et al.*, 2001). Similar to the effects observed upon simulated and experimental overexpression of the invertase, glycolysis again showed only a 1.2-fold increase in the model while it increases nearly 3-fold in the experiment. A reason for this might be again that in the experiment, this increase is a result of regulations that are not included in the model.

The flux control coefficients reveal that NDP kinase has a strong effect on all major fluxes: glycolysis, starch synthesis and sucrose cycling. A simple explanation for this fact might be that the reaction was somehow limiting because its kinetic constants (which were estimated due to missing experimentally determined values) were too low. In contrast to that, the adenylate kinase had no effect at all on any fluxes, whereas its manipulation in experiments led to one of the few successful increases in tuber starch content (Regierer *et al.*, 2002). This suggests that also the kinetic of the AdK probably does not represent the *in vivo* situation. From the flux control coefficient from PGM on the flux to

starch one could conclude that this would be a good target to improve the flux to starch, especially as sucrose cycling should decrease because the $C^J$ is negative. Experiments are in progress to test this hypothesis (A. Lytovchenko, A.R. Fernie, pers. communication).

In conclusion, the model at its present state is able to give a good picture of the processes experimentally described for wild type and transgenic potato tubers. The case studies identify that it should additionally have utility as a predictive tool. The model should be refined by determining more of the kinetic constants from potato tubers, and it should be extended to other pathways central to potato tuber metabolism.

# Chapter 7. General Discussion

The aim of the work presented in this thesis was to improve the understanding of sucrose breakdown in the potato tuber. Chapter 1 described the importance of the potato tuber as a model system for sink organs. Carbohydrate metabolism and its regulation was summarised and the molecular biological, biochemical and computational tools used during this study were presented. In Chapter 3 it was shown that the alc-gene expression system in combination with induction by acetaldehyde is a useful tool to study metabolism of the potato tuber. In Chapter 4 these finding were applied to express a yeast invertase in a organ-specific, temporally regulated manner, and it was found that the differences between transient and constitutive expression can be quite drastic. Conclusions from this chapter were that probably maltose is produced from glucose rather than from starch, and that apparently glycolysis is driven by ATP demand, because sucrose cycling but not glycolysis increased in the inducible lines. Chapter 5 provided some new hints for the hypothesis that sucrose is transported by endocytosis-like mechanisms through the vacuole to the cytosol of parenchyma cells in potato tubers rather than by direct uptake to the cytosol. In the bioinformatical Chapter 6 a kinetic model was presented that is able to predict some observed changes in metabolism upon invertase expression in an astonishingly precise manner.

To optimally characterise the effect of a transgene on plant metabolism, the highest degree of spatial and temporal resolution is necessary. Whilst it is possible for a long time to express a transgene in a specific plant organ, tissue, or cell compartment, the temporal resolution was rarely assessed. The choice of the appropriate inducible promoter depends on the biological question. Generally, the ideal inducible promoter should exhibit a very low basal expression level, high inducibility, high specificity with respect to inducers, a high dynamic range of response with respect to inducer concentrations, a fast response upon induction, and a rapid switch-off following inducer withdrawal (Zuo and Chua, 2000). Further on, the inducer should be absent from plant metabolism the inducing agent should be non-toxic and have no physiologic effects on plants. The alc gene expression system from the fungus *Aspergillus nidulans* used in this work is a good compromise that fulfils most of these criteria. As I showed in Chapter 3, the basal expression is very low and the inducibility comes close to the levels of the constitutive B33 promoter. It can be induced directly or indirectly by some different chemicals, but with the exceptions of very bulky tissues (very large tubers) the levels of these chemicals do not

become high enough to mediate transcription. Other inducible systems are less advantageous. Heat induction for example will have obviously strong effects on metabolism, so do other systems like the salicylic acid inducible promoter because they are involved in plant pathogen response (Martini *et al.*, 1993). The glucocorticoid-inducible gene expression system by Schena *et al*. (1991) (Schena *et al.*, 1991) is rapid and highly-inducible, but it was shown that the presence of the promoter itself causes severe growth defects and induces defence-related genes (Kang *et al.*, 1999). Also, the toxicity of the inducer plays an important role for cultivation outside greenhouses, for example in field trials or for commercial applications. It is obvious that systems using mammalian hormones are out of question for such a use. In contrast, ethanol is characterised by a relatively low toxicity to human beings and the environment, and the toxicity of acetaldehyde is only two-fold higher (Budavari, 2001). Further on, the finding of Chapter 3 that acetaldehyde mediates faster transgene induction than ethanol suggests that this inducer should be routinely used with the alc system.

The results from the AlcI plants strongly suggest that maltose is formed in the potato tuber by the condensation of two glucose units rather than as a product of starch degradation. In a recent study, a new maltose transporter was discovered that is essential for starch degradation in *Arabidopsis* leaves at night (Niittylä *et al.*, 2004). The authors also show similarities to an expressed sequence tag (EST) from potato. This suggests that in leaves maltose is a major product of starch degradation. However, these findings are not in contrast to those from the AlcI plants, but they are suggesting that the maltose pool is generated from different sources in heterotrophic compared to photosynthetic tissues. The finding that there was no induction of glycolysis in the AlcI lines while glycolysis was reported to strongly increase upon constitutive expression of the same enzyme (Trethewey *et al.*, 1999) is particularly striking because the computer model of sucrose breakdown presented in Chapter 6 also did not show an increase in glycolysis upon simulated invertase overexpression. This suggests that the increase in glycolysis observed by Trethewey *et al.* (1999) was either a pleiotropic effect of the invertase expression that has its cause back in earlier developmental stages of the tuber or an effect of additional regulation that takes place upon long-term presence of the metabolic changes provoked by the high invertase activity.

Also the invertase activity in the vacI plants was the highest shown so far in potato, the changes in metabolites, enzyme activity and fluxes was quite small. The fact that most of the sucrose is stored in the vacuole (Farré, 2001) lets these minimal changes seem even more surprising. An explanation for these findings would be that the vacuole is metabolically inert compartment which has primarily a

storage function, which was previously discussed (Farré, 2001). The similarities between apoplastic and vacuolar expression of a yeast invertase are present on all biochemical levels that were measured. These data provide circumstantial evidence for the endocytotic trafficking of sucrose between the apoplast and vacuole.

In Chapter 6 a kinetic model of sucrose breakdown and predictions for metabolites and fluxes in wild type and invertase-overexpressing potato tubers was described. Even though much input for the model, especially many of the kinetic laws, was taken from a kinetic model of sucrose synthesis that was developed for sugar cane culm (Rohwer and Botha, 2001), the model presented here was able to give an astonishingly precise picture of sucrose breakdown in the potato tuber. In order to provide a basis for models not only in the potato tuber, but also in other heterotrophic tissues producing starch from sucrose, compartmentation was deliberately set aside. Thus, with only minor changes in kinetic parameters, this model could be adapted for the pericarp in cereal grains, in which ADP-glucose, and not Glc6P, is transported from the cytosol to the apoplast. One minor drawback of the model is that fluxes are by far too large, even though the relative distribution of fluxes is similar to data from laboratory experiments. The most likely reason for this is that while the kinetic laws of the model a based on the assumption of free diffusion, the actual situation in the cell is more likely that the diffusion is limited because of a gel-like texture of the cytosol (Clegg, 1992). Moreover, the model is based on kinetic data from *in vitro* studies which are not necessarily reflecting *in vivo* conditions, because with some enzymes there are quite drastic differences between the *in vitro* velocities under ideal conditions ($v_{max}$) and under limited conditions ($v_{sel}$). To date, values for fluxes are relatively rare because they require tedious radioactive labelling experiments which can only report the distribution of the label into different metabolite groups like amino acids or organic acids. Recently, a new technique using the non-radioactive label $^{13}C$ in combination with gas chromatography-mass spectrometry has been established that allows localisation of the label in individual metabolites (Roessner-Tunali *et al.*, 2004).

Another model that is related to the sucrose breakdown model presented here is the model of potato tuber glycolysis (Thomas *et al.*, 1997a, b). The authors first show that the changes in glycolysis after the overexpression of Phosphofructokinase (PFK), which was supposed to be a regulatory step of glycolysis, are surprisingly small. With a detailed kinetic model of glycolysis and the application of Metabolic Control Analysis they found that this experimental data could be explained by a small control coefficient of PFK on the flux through glycolysis. Their conclusion that activation of a single

enzyme early in the pathway will be of limited effectiveness in achieving flux increases is now generally accepted (Morandini and Salamini, 2003). The model of sucrose breakdown presented in Chapter 6 is representing a much more complex situation. Firstly, the model contains more reactions (16 compared to 9 in the glycolysis model) with inhibition being included for many more enzymes, and secondly the pathway of sucrose breakdown is highly branched and contains several cycles, which means that the behaviour upon overexpression of a single enzyme is hard to predict without a model. It was shown that the model presented here can predict the effects of invertase overexpression on metabolites and fluxes. The detailed analysis of the glycolysis model by Thomas *et al.* (1997b) gives a hint in which direction the sucrose breakdown model can be extended.

**Conclusion**

The study presented here is the first detailed case study of metabolism following temporally and spatially controlled transgene expression. The use of the *alc* gene expression system to find differences in the effects of constitutive and inducible transgene expression can be easily adapted to other enzymes, pathways and organisms. The expression of a yeast invertase in potato tuber vacuoles improved the understanding of how sucrose enters tuber metabolism. Also, the use of kinetic models to predict changes in metabolism was shown to be able to improve the understanding of complex, dense pathways like sucrose breakdown. Inducible promoters and kinetic pathway models are likely to play a key role in the future of pathway analysis.

# Chapter 8. References

**Aoyama, T. and Chua, N.-H.** (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J.* 11: 605-612.

**ap Rees, T. and Beevers, H.** (1960) Pathways of glucose dissimilation in carrot slices. *Plant Physiol.* 35: 830-838.

**ap Rees, T.** (1988) Hexose phosphate metabolism by non-photosynthetic tissues of higher plants. In: *The biochemistry of plants*. (Preiss, J., eds.), pp. 1-33, Academic Press, New York.

**ap Rees, T. and Morrell, S.** (1990) Carbohydrate metabolism in developing potatoes. *Am. Potato J.* 67: 835-847.

**ap Rees, T.** (1992) Synthesis of storage starch. In: *Carbon partitioning within and between organism.* (Pollock, C.J., Farrar, J.F. and Gordon, A.J., ed.), pp. 115-131, Bios Scientific Publishers, Oxford.

**Appeldoorn, N.J.G., deBruijn, S.M., KootGronsveld, E.A.M., Visser, R.G.F., Vreugdenhil, D. and vanderPlas, L.H.W.** (1997) Developmental changes of enzymes involved in conversion of sucrose to hexose-phosphate during early tuberisation of potato. *Planta* 202: 220-226.

**Avigad, G.** (1982) Sucrose and other disaccharides. In: *Plant Carbohydrates I.* (Loewus, F.A., ed.).

**Beck, E. and Ziegler, P.** (1989) Biosynthesis and degradation of dtarch in higher plants. *Annu. Rev. Plant Physiol. Mol. Biol.* 40: 95-117.

**Böhner, S., Lenk, I., Rieping, M., Herold, M. and Gatz, C.** (1999) Transcriptional activator TGV mediates dexamethasone-inducible and tetracycline-inactivatable gene expression. *Plant J.* 19: 87-95.

**Bologa, K.L., Fernie, A.R., Leisse, A., Ehlers Loureiro, M. and Geigenberger, P.** (2003) A bypass of sucrose synthase leads to low internal oxygen and impaired metabolic performance in growing potato tubers. *Plant Physiol.* 132: 2058-2072.

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

**Bradford, M.M.** (1976) A rapid and sensitive method for quantification of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.

**Bruce, W., Folkerts, O., Garnaat, C., Crasta, O., Roth, B. and Bowen, B.** (2000) Expression profiling of the maize flavonoid pathway genes controlled by estradiol-inducible transcription factors CRC and P. *Plant Cell* 12: 65-79.

**Budavari, S.** (2001) The Merck Index: an encyclopedia of chemicals, drugs and biologicals. Rathway, NJ, USA, Merck.

**Burrell, M.M., Mooney, P.J., Blundy, M., Carter, D., Wilson, F., Green, J., Blundy, K.S. and apRees, T.** (1994) Genetic manipulation of 6-phosphofructokinase in potato tubers. *Planta* 194: 95-101.

**Burton, W.G.** (1989) The Potato. Singapore, Longman.

**Büssis, D., Heineke, D., Sonnewald, U., Willmitzer, L., Raschke, K. and Heldt, H.-W.** (1997) Solute accumulation and decreased photosynthesis in leaves of potato plants expressing yeast-derived invertase either in the apoplast, vacuole or cytosol. *Planta* 202: 126-136.

**Caddick, M.X., Greenland, A.J., Jepson, I., Krause, K.-P., Qu, N., Riddell, K.V., Salter, M.G., Schuch, W., Sonnewald, U. and Tomsett, A.B.** (1998) An ethanol inducible gene switch for plants used to manipulate carbon metabolism. *Nat. Biotechnol.* 16: 177-180.

**Chen, S., Hofius, D., Sonnewald, U. and Börnke, F.** (2003) Temporal and spatial control of gene silencing in transgenic plants by inducible expression of double-stranded RNA. *Plant J.* 36: 731-740.

**Clegg, J.S.** (1992) Cellular infrastructure and metabolic organization. *Cell. Regul.* 33: 3-14.

**Cotelle, V., Meek, S.E.M., Provan, F., Milne, F.C., Morrice, N. and MacKintosh, C.** (2000) 14-3-3s regulate global cleavage of their diverse binding partners in sugar-starved *Arabidopsis* cells. *EMBO J.* 19: 2869-2876.

**Creaser, E.H., Porter, R.L., Britt, K.A., Pateman , J.A. and Doy, C.H.** (1985) Purification and preliminary characterization of alcohol dehydrogenase from *Aspergillus nidulans*. *Biochem. J.* 225: 449-454.

**Dai, N., Schaffer, A., Petreikov, M. and Granot, D.** (1997) Potato (*Solanum tuberosum* L.) fructokinase expressed in yeast exhibits inhibition by fructose of both *in vitro* enzyme activity and rate of cell proliferation. *Plant Sci.* 128: 191-197.

**Dancer, J., Hatzfeld, W.-D. and Stitt, M.** (1990) Cytosolic cycles regulate the turnover of sucrose in heterotrophic cell-suspension cultures of *Chenopodium rubrum* L. *Planta* 182: 223-231.

**Deblaere, R., Bytebier, B., Degreve, H., Deboeck, F., Schell, J., Vanmontagu, M. and Leemans, J.** (1985) Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene-transfer to plants. *Nucleic Acids Res.* 13: 4777-4788.

**Dietze, J., Blau, A. and Willmitzer, L.** (1995). In: *Gene transfer to plants xxii*. (Potrykus, I. and Spangenberg, G., eds.), pp. 24-29, Springer, Berlin.

**Dieuaide-Noubhani, M., Raffard, G., Canioni, P., Pradet, A. and Raymond, P.** (1995) Quantification of compartmented metabolic fluxes in maize root-tips using isotope distribution from 13C- labeled or 14C-labeled glucose. *J. Biol. Chem.* 270: 13147-13159.

**Doehlert, D.C. and Huber, S.C.** (1983) Regulation of spinach leaf sucrose phosphate synthase by glucose-6-phosphate, inorganic phosphate, and pH. *Plant Physiol.* 73: 989-994.

**Doehlert, D.C. and Felker, F.C.** (1987) Characterization and distribution of invertase activity in developing maize (*Zea mays*) kernels. *Physiol. Plant.* 70: 51-57.

**Doehlert, D.C.** (1987) Substrate inhibition of maize endosperm sucrose synthase by fructose and its interaction with glucose inhibition. *Plant Sci.* 52: 153-157.

**Doehlert, D.C.** (1990) Distribution of enzyme activities within the developing maize (*Zea mays*) kernel in relation to starch, oil and protein accumulation. *Physiol. Plant.* 78: 560-567.

**Doehlert, D.C. and Choury, P.S.** (1991) Possible roles of sucrose synthase in sink function. In: *Recent advances in phloem transport and assimilate compartmentation*. (Bonnemain, J.L., Delrot, S., Lucas, W.J. and Dainty, J., eds.), pp. 187-195, Quest Editions, Nantes.

**Duwenig, E., Steup, M., Willmitzer, L. and Kossmann, J.** (1997) Antisense inhibition of cytosolic phosphorylase in potato plants (*Solanum tuberosum* L.) affects tuber sprouting and flower formation with only little impact on carbohydrate metabolism. *Plant J.* 12: 323-333.

**Entwistle, G. and ap Rees, T.** (1990) Lack of fructose-1,6-bisphosphatase in a range of higher plants that store starch. *Biochem. J.* 271: 467-472.

**Farré, E.M., Geigenberger, P., Willmitzer, L. and Trethewey, R.N.** (2000) A possible role for pyrophosphate in the coordination of cytosolic and plastidial carbon metabolism within the potato tuber. *Plant Physiol.* 123: 681-688.

**Farré, E.M., Tiessen, A., Roessner, U., Geigenberger, P., Trethewey, R.N. and Willmitzer, L.** (2001) Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars, organic acids, amino acids and sugar alcohols in potato tubers using a non-aqueous fractionation method. *Plant Physiol.* 127: 685-700.

**Farré, E.M.** (2001). Compartmentation in potato tubers, PhD thesis, Potsdam University.

**Fernie, A.R., Riesmeier, J.W., Martiny, A., Ramalingam, S., Willmitzer, L. and Trethewey, R.N.** (2000) Consequences of the expression of a bacterial glucokinase in potato tubers, both in combination with and independently of a yeast-derived invertase. *Aust. J. Plant Physiol.* 27: 827-833.

**Fernie, A.R., Roessner, U., Trethewey, R.N. and Willmitzer, L.** (2001a) The contribution of plastidial phosphoglucomutase to the control of starch synthesis within the potato tuber. *Planta* 213: 418-426.

**Fernie, A.R., Roscher, A., Ratcliffe, R.G. and Kruger, N.J.** (2001b) Fructose 2,6-bisphosphate activates pyrophosphate: fructose-6-phosphate 1-phosphotransferase and increases triose phosphate to hexose phosphate cycling in heterotrophic cells. *Planta* 212: 250-263.

**Fernie, A.R. and Willmitzer, L.** (2001) Molecular and biochemical triggers of potato tuber development. *Plant Physiol.* 127: 1-7.

**Fernie, A.R., Roessner, U. and Geigenberger, P.** (2001c) The sucrose analog palatinose leads to a stimulation of sucrose degradation and starch synthesis when supplied to discs of growing potato tubers. *Plant Physiol.* 125: 1967-1977.

**Fernie, A.R., Tiessen, A., Stitt, M., Willmitzer, L. and Geigenberger, P.** (2002a) Altered metabolic fluxes result from shifts in metabolite levels in sucrose phosphorylase-expressing potato tubers. *Plant Cell Environ.* 25: 1219-1232.

**Fernie, A.R., Willmitzer, L. and Trethewey, R.N.** (2002b) Sucrose to starch: a transition in molecular plant physiology. *Trends Plant Sci.* 7: 35-41.

**Fiehn, O., Kopka, J., Dörmann, P., Altmann, T., Trethewey, R.N. and Willmitzer, L.** (2000) Metabolite profiling for plant functional genomics. *Nat. Biotechnol.* 18: 1157-1161.

**Flipphi, M., Mathieu, M., Cirpus, I., Panozzo, C. and Felenbok, B.** (2001) Regulation of the aldehyde dehydrogenase gene (*aldA*) and its role in the control of the coinducer level necessary for induction of the ethanol utilization pathway in *Aspergillus nidulans*. *J. Biol. Chem.* 276: 6950-6958.

**Gatz, C., Frohberg, C. and Wendenburg, R.** (1992) Stringent repression and homogeneous de-repression by tetracycline of a modified CaMV 35S promoter in intact transgenic tobacco plants. *Plant J.* 2: 397-404.

**Gatz, C.** (1997) Chemical control of gene expression. *Annu. Rev. Plant Physiol. Mol. Biol.* 48: 89-108.

**Geigenberger, P. and Stitt, M.** (1991) A futile cycle of sucrose synthesis and degradation is involved in regulating partitioning between sucrose, starch and respiration in cotyledons of germinating *Ricinus communis* L. seedlings when phloem transport is inhibited. *Planta* 185: 81-90.

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

**Geigenberger, P., Krause, K.-P., Hill, L.M., Reimholz, R., MacRae, E., Quick, P.W., Sonnewald, U. and Stitt, M.** (1995) The regulation of sucrose synthesis in the leaves and tubers of potato

plants. In: *Sucrose metabolism, biochemistry, physiology and molecular biology*. (Pontis, H.G., Salerno, G.L. and Echeverria, E.J., eds.), pp. 14-24, American Society of Plant Physiologists, Rockville.

**Geigenberger, P., Reimholz, R., Geiger, M., Merlo, L., Canale, V. and Stitt, M.** (1997) Regulation of sucrose and starch metabolism in potato tubers in response to short-term water deficit. *Planta* 201: 502-518.

**Geigenberger, P., Geiger, M. and Stitt, M.** (1998) High-temperature perturbation of starch synthesis is attributable to inhibition of ADP-glucose pyrophosphorylase by decreased levels of glycerate-3-phosphate in growing potato tubers. *Plant Physiol.* 117: 1307-1316.

**Geigenberger, P., Reimholz, R., Deiting, U., Sonnewald, U. and Stitt, M.** (1999) Decreased expression of sucrose phosphate synthase strongly inhibits the water stress-induced synthesis of sucrose in growing potato tubers. *Plant J.* 19: 119-129.

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

**Geigenberger, P.** (2003) Regulation of sucrose to starch conversion in growing potato tubers. *J. Exp. Bot.* 54: 457-465.

**Geigenberger, P., Stitt, M. and Fernie, A.R.** (2004) Metabolic control analysis and regulation of the conversion of sucrose to starch in growing potato tubers. *Plant Cell Environ.* 27: 655-673.

**Gibon, Y., Vigeolas, H., Tiessen, A., Geigenberger, P. and Stitt, M.** (2002) Sensitive and high throughput metabolite assays for inorganic pyrophosphate, ADPGlc, nucleotide phosphates, and glycolytic intermediates beased on a novel enzymic cycling system. *Plant J.* 30: 221-235.

**Giege, P., Heazlewood, J.L., Roessner-Tunali, U., Millar, A.H., Fernie, A.R., Leaver, C.J. and Sweetlove, L.J.** (2003) Enzymes of glycolysis are functionally associated with the mitochondrion in *Arabidopsis* cells. *Plant Cell* 15: 2140-2151.

**Gilbertson, L.** (2003) Cre-*lox* recombination: Cre-active tools for plant biotechnology. *Trends Biotechnol.* 21: 550-555.

**Godt, D.E., Riegel, A. and Roitsch, T.** (1995) Regulation of sucrose synthase expression in *Chenopodium rubrum* - Characterization of sugar induced expression in photoautotrophic suspension-cultures and sink tissue-specific expression in plants. *J. Plant Physiol.* 146: 231-238.

**Graham, I.A.** (1996) Carbohydrate control of gene expression in higher plants. *Res. Microbiol.* 147: 572-580.

**Hajirezaei, M.-R., Takahata, Y., Trethewey, R.N., Willmitzer, L. and Sonnewald, U.** (2000) Impact of elevated cytosolic and apoplastic invertase activity on carbon metabolism during potato tuber development. *J. Exp. Bot.* 51: 439-445.

**Hajirezaei, M.-R., Börnke, F., Peisker, M., Takahata, Y., Lerchl, J., Kirakosyan, A. and Sonnewald, U.** (2003) Decreased sucrose content triggers starch breakdown and respiration in stored potato tubers (*Solanum tuberosum*). *J. Exp. Bot.* 54: 477-488.

**Halford, N.G., Purcell, P.C. and Hardie, D.G.** (1999) Is hexokinase really a sugar sensor in plants? *Trends Plant Sci.* 4: 117-120.

**Hare, P.D., Cress, W.A. and van Staden, J.** (1998) Dissecting the roles of osmolyte accumulation during stress. *Plant Cell Environ.* 21: 535-553.

**Hargreaves, J.A. and ap Rees, T.** (1988) Turnover of Starch and Sucrose in Roots of *Pisum sativum*. *Phytochemistry* 27: 1627-1629.

**Hatzfeld, W.D. and Stitt, M.** (1990) A study of the rate of recycling of triose phosphates in heterotrophic *Chenopodium rubrum* cells, potato tubers, and maize endosperm. *Planta* 180: 198-204.

**Heineke, D., Wildenberger, K., Sonnewald, U., Willmitzer, L. and Heldt, H.-W.** (1994) Accumulation of hexoses in leaf vacuoles: studies with transgenic tobacco plants expressing yeast-derived invertase in the cytosol, vacuole or apoplasm. *Planta* 194: 29-33.

**Heiner, M., Ventre, G. and Wikarski, D.** (1994) A petri net based methodology to integrate qualitative and quantitative analysis. *Inf. Softw. Technol.* 36: 435-441.

**Heinrich, R. and Rapoport, T.A.** (1974) A linear steady-state treatment of enzymatic chains. General properties, control and effector strength. *Eur. J. Biochem.* 42: 89-95.

**Hill, S.A. and ap Rees, T.** (1995) The effect of hypoxia on the control of carbohydrate metabolism in ripening bananas. *Planta* 197: 313-323.

**Hirose, T. and Ohsugi, R.** (1998) Sucrose transport pathways in source and sink tissues of plants and crops. *Jpn. J. Crop Sci.* 67: 129-135.

**Hoefgen, R. and Willmitzer, L.** (1990) Biochemical and genetic analysis of different patatin isoforms expressed in various organs of potato (*Solanum tuberosum*). *Plant Sci.* 66: 221-230.

**Huber, S.C. and Huber, J.L.** (1991) Regulation of maize leaf sucrose-phosphate synthase by protein phosphorylation. *Plant Cell Physiol.* 32: 319-326.

**Huber, S.C., MacKintosh, C. and Kaiser, W.M.** (2002) Metabolic enzymes as targets for 14-3-3 proteins. *Plant Mol. Biol.* 50: 1053-1063.

**Hübschmann, H.-J.** (1996) Handbuch der GC-MS, VCH Verlagsgesellschaft mbH.

**Initiative, T.A.G.** (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* 408: 796-815.

**Jang, J.C., Leon, P., Zhou, L. and Sheen, J.** (1997) Hexokinase as a sugar sensor in higher plants. *Plant Cell* 9: 5-19.

**Jang, J.C. and Sheen, J.** (1997) Sugar sensing in higher plants. *Trends Plant Sci.* 2: 208-214.

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

**Jepson, I., Martinez, A. and Sweetman, J.P.** (1998) Chemical-inducible gene expression systems for plants - a review. *Pestic. Sci.* 54: 360-367.

**Johnston, M.** (1999) Feasting, fasting and fermenting - glucose sensing in yeast and other cells. *Trends Genet.* 15: 29-33.

**Kacser, H. and Burns, J.A.** (1973) The control of flux. *Symp. Soc. Exp. Biol.* 27: 65-104.

**Kammerer, B., Fischer, K., Hilpert, B., Schubert, S., Gutensohn, M., Weber, A. and Flügge, U.I.** (1998) Molecular characterization of a carbon transporter in plastids from heterotrophic tissues: The glucose 6-phosphate phosphate antiporter. *Plant Cell* 10: 105-117.

**Kanehisa, M. and Goto, S.** (2000) KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucl. Acids Res.* 28: 27-30.

**Kang, H.-G., Fang, Y. and Singh, K.B.** (1999) A glucocorticoid-inducible transcription system causes severe growth defects in *Arabidopsis* and induces defense-related genes. *Plant J.* 20: 127-133.

**Katsube, T., Kazuta, Y., Tanizawa, K. and Fukui, T.** (1991) Expression in *Escherichia coli* of UDP-glucose pyrophosphorylase cDNA from potato tuber and functional asessment of the five lysyl residues located at the substrate-binding site. *Biochemistry* 30: 8546-8551.

**Keeling, P.L., Wood, J.R., Tyson, R.H. and Bridges, I.G.** (1988) Starch biosynthesis in developing wheat-grain - Evidence against the direct involvement of triose phosphates in the metabolic pathway. *Plant Physiol.* 87: 311-319.

**Koch, I., Junker, B.H. and Heiner, M.** (2004) Application of Petri net theory for modelling and validation of the sucrose breakdown pathway in the potato tuber. *Bioinformatics*: in press.

**Koch, K.E.** (1996) Carbohydrate-modulated gene expression in plants. *Annu. Rev. Plant Physiol. Mol. Biol.* 47: 509-540.

**Koebmann, B.J., Westerhoff, H.V., Snoep, J.L., Nilsson, D. and Jensen, P.R.** (2002) The glycolytic flux in *Escherichia coli* is controlled by the demand for ATP. *J. Bacteriol.* 184: 3909-3916.

**Komor, E.** (2000) Source physiology and assimilate transport: the interaction of sucrose metabolism, starch storage and phloem export in source leaves and the effects on sugar status in phloem. *Aust. J. Plant Physiol.* 27: 497-505.

**Kosegarten, H. and Mengel, K.** (1994) Evidence for a glucose-1-phosphate translocator in storage tissue amyloplasts of potato (*Solanum tuberosum*) suspension-cultured cells. *Physiol. Plant.* 91: 111-120.

**Kossmann, J., Müller-Röber, B., Dyer, T.A., Raines, C.A., Sonnewald, U. and Willmitzer, L.** (1992) Cloning and expression analysis of the plastidic fructose-1,6-bisphosphatase coding sequence from potato - Circumstantial evidence for the import of hexoses into chloroplasts. *Planta* 188: 7-12.

**Kossmann, J. and Lloyd, J.** (2000) Understanding and influencing starch biochemistry. *Crit. Rev. Plant Sci.* 19: 171-226.

**Kruger, N.J.** (1997) Carbohydrate synthesis and degradation. In: *Plant Metabolism.* (Dennis, D.T., Turpin, D.H., Lefebvre, D.D. and Layzell, D.B., eds.), pp. 83-104, Longman, Essex.

**Kühn, C., Quick, W.P., Schulz, A., Riesmeier, J.W., Sonnewald, U. and Frommer, W.B.** (1996) Companion cell-specific inhibition of the potato sucrose transporter SUT1. *Plant Cell Environ.* 19: 1115-1123.

**Kühn, C.** (2003) A comparison of the sucrose transporter systems of different plant species. *Plant Biol.* 5: 215-232.

**Lalonde, S., Boles, E., Hellmann, H., Barker, L., Patrick, J.W., Frommer, W.B. and Ward, J.M.** (1999) The dual function of sugar carriers: transport and sugar sensing. *Plant Cell* 11: 707-726.

**Laufs, P., Coen, E., Kronenberger, J., Traas, J. and Doonan, J.** (2003) Separable roles of UFO during floral development revealed by conditional restoration of gene function. *Development* 130: 785-796.

**Lee, H.-S. and Sturm** (1996) Purification and characterization of neutral and alkaline invertase from carrot. *Plant Physiol.* 112: 1513-1522.

**Liu, L.J., Prat, S., Willmitzer, L. and Frommer, W.B.** (1990) *Cis* regulatory elements directing tuber-specific and sucrose-inducible expression of a chimeric class I patatin promoter/GUS-gene fusion. *Mol. Gen. Genet.* 223: 401-406.

**Lockhart, D.J., Dong, H.L., Byrne, M.C., Follettie, M.T., Gallo, M.V., Chee, M.S., Mittmann, M., Wang, C.W., Kobayashi, M., Horton, H. and Brown, E.L.** (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol.* 14: 1675-1680.

**Loef, I., Stitt, M. and Geigenberger, P.** (2001) Increased levels of adenine nucleotides modify the interaction between starch synthesis and respiration when adenine is supplied to discs from growing potato tubers. *Planta* 212: 782-791.

**MacRae, E., Quick, W.P., Benker, C. and Stitt, M.** (1992) Carbohydrate metabolism during postharvest ripening in kiwifruit. *Planta* 188: 314-323.

**Martini, N., Egen, M., Runtz, I. and Strittmatter, G.** (1993) Promoter sequence of a potato pathogenesis-related gene mediate transcriptional activation selectively upon fungal infection. *Mol. Gen. Genet.* 236: 179-186.

**Marty, F.** (1999) Plant vacuoles. *Plant Cell* 11: 587-599.

**Mendes, P.** (1993) GEPASI: a software package for modelling the dynamics, steady states and control of biochemical and other systems. *Comput. Applic. Biosci.* 9: 563-571.

**Merlo, L., Geigenberger, P., Hajirezaei, M.-R. and Stitt, M.** (1993) Changes of carbohydrates, metabolites and enzyme activities in potato tubers during development, and within a single tuber along a stolon-apex gradient. *J. Plant Physiol.* 142: 392-402.

**Mett, V.L., Lochhead, L.P. and Reynolds, P.H.S.** (1993) Copper-controllable gene expression system for whole plants. *Proc. Natl. Acad. Sci. USA* 90: 4567-4571.

**Mett, V.L., Podivinsky, E., Tennant, A.M., Lochhead, L.P., Jones, W.T. and Reynolds, P.H.S.** (1996) A system for tissue-specific copper-controllable gene expression in transgenic plants: Nodule-specific antisense of asparate aminotransferase-P-2. *Transgenic Res.* 5: 105-113.

**Mieyal, J.J. and Abeles, R.H.** (1972) Disaccharide phosphorylases. In: *The Enzymes*. (Boyer, P.D., eds.), pp.,

**Miller, J.F., Dower, W.J. and Tompkins, L.S.** (1988) High-voltage electroporation of bacteria - Genetic transformation of *Campylobacter jejuni* with plasmid DNA. *Proc. Natl. Acad. Sci. USA* 85: 856-860.

**Morandini, P. and Salamini, F.** (2003) Plant biotechnology and breeding: allied for years to come. *Trends Plant Sci.* 8: 70-75.

**Morgan, J.A. and Rhodes, D.** (2002) Mathematical modeling of plant metabolic pathways. *Metab. Eng.* 4: 80-89.

**Motulsky, H.** (1995) Intuitive Biostatistics. Oxford, Oxford University Press.

**Müller, L.A., Zhang, P.F. and Rhee, S.Y.** (2003) AraCyc: A biochemical pathway database for *Arabidopsis*. *Plant Physiol.* 132: 453-460.

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

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

**Naeem, M., Tetlow, I.J. and Emes, M.J.** (1997) Starch synthesis in amyloplasts purified from developing potato tubers. *Plant J.* 11: 1095-1103.

**Nagao, R.T. and Gurley, W.B.** (1999) Use of heat-shock promoters to control gene expression in plants. In: *Inducible Gene Expression in Plants*. (Reynolds, P.H.S., ed.), CABI Publishing, Wallingford, UK.

**Nakano, K., Omura, Y., Tagaya, M. and Fukui, T.** (1989) UDP-glucose pyrophosphorylase from potato tuber: purification and characterization. *J. Biochem.* 106: 528-532.

**Niittylä, T., Messerli, G., Trevisan, M., Chen, J., Smith, A.M. and Zeeman, S.C.** (2004) A previously unknown maltose transporter essential for starch degradation in leaves. *Science* 303: 87-89.

**Oparka, K.J. and Wright, K.M.** (1988) Influence of cell turgor on sucrose partitioning in potato tuber storage tissues. *Planta* 175: 520-526.

**Oparka, K.J. and Prior, D.A.M.** (1988) Movement of lucifer yellow CH in potato tuber storage tissues: a comparison of symplastic and apoplastic transport. *Planta* 176: 533-540.

**Oparka, K.J., Viola, R., Wright, K.M. and Prior, D.A.M.** (1992) Sugar transport and metabolism in the potato tuber. In: *Carbon partitioning between and within organism*. (Pollock, C.J., Farrar, J.F. and Gordon, A.J., eds.), pp. 91-111, Bios Scientific Publishing, Oxford.

**Padidam, M.** (2003) Chemically regulated gene expression in plants. *Curr. Opin. Plant Biol.* 6: 169-177.

**Pego, J.V., Weisbeek, P.J. and Smeekens, S.C.M.** (1999) Mannose inhibits *Arabidopsis* germination via a hexokinase-mediated step. *Plant Physiol.* 119: 1017-1023.

**Plaxton, W.C.** (1990) Glycolysis. In: *Methods in Plant Biochemistry: Enzymes of Primary Metabolism*. (Lea, P.J., eds.), pp. 145-173, Academic Press, San Diego.

**Poolman, M.G., Ölçer, H., Lloyd, J.C., Raines, C.A. and Fell, D.A.** (2001) Computer modelling and experimental evidence for two steady states in the photosynthetic Calvin cycle. *Eur. J. Biochem.* 268.

**Preiss, J. and Sivak, M.N.** (1996) Starch synthesis in sinks and sources. In: *Photoassimilate distribution in plants and crops, source-sink relationships*. (Zamski, E. and Schaffer, A.A., eds.), pp. 63-96, Marcel Dekker, New York.

**Quick, P.W. and Schaffer, A.A.** (1996) Sucrose metabolism in sources and sinks. In: *Photoassimilate Distribution in Plants and Crops: Source-Sink Relationships*. (Zamski, E. and Schaffer, A.A., eds.), Marcel Dekker Inc., New York.

**Rao, A.G. and Flynn, P.** (1992) Microtiter plate-based assay for ß-D-glucoronidase: a quantitative approach. In: *GUS protocols: using the GUS gene as a reporter of gene expression*, eds.), Academic Press, San Diego.

**Reeve, R.M., Timm, H. and Weaver, M.L.** (1973) Parenchyma cell growth in potato tubers. 2. Cell divisions vs. cell enlargement. *Am. Potato J.* 50: 71-78.

**Regierer, B., Fernie, A.R., Springer, F., Perez-Melis, A., Leisse, A., Koehl, K., Willmitzer, L., Geigenberger, P. and Kossmann, J.** (2002) Starch content and yield increase as a result of altering adenylate pools in transgenic plants. *Nat. Biotechnol.* 20: 1256-1260.

**Reimholz, R., Geigenberger, P. and Stitt, M.** (1994) Sucrose-phosphate synthase is regulated via metabolites and protein phosphorylation in potato tubers, in a manner analogous to the enzyme in leaves. *Planta* 192: 480-488.

**Renz, A., Merlo, L. and Stitt, M.** (1993) Partial purification from potato tubers of three fructokinases and three hexokinases which show differing organ and developmental specificity. *Planta* 190: 156-165.

**Renz, A. and Stitt, M.** (1993) Substrate specificity and product inhibition of different forms of fructokinases and hexokinases in developing potato tubers. *Planta* 190: 166-175.

**Riesmeier, J., Hirner, B. and Frommer, W.B.** (1993) Potato sucrose transporter expression in minor veins indicates a role in phloem loading. *Plant Cell* 5: 1591-1598.

**Rocha-Sosa, M., Sonnewald, U., Frommer, U., Stratmann, M., Schell, J. and Willmitzer, L.** (1989) Both developmental and metabolic signals activate the promoter of a class I patatin gene. *EMBO J.* 8: 23-29.

**Roessner, U., Willmitzer, L. and Fernie, A.R.** (2001a) High-resolution metabolic phenotyping of genetically and environmentally diverse potato tuber systems. Identification of phenocopies. *Plant Physiol.* 127: 749-764.

**Roessner, U., Luedemann, A., Brust, D., Fiehn, O., Linke, T., Willmitzer, L. and Fernie, A.R.** (2001b) Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. *Plant Cell* 13: 11-29.

**Roessner-Tunali, U., Urbanczyk-Wochniak, E., Czechowski, T., Kolbe, A., Willmitzer, L. and Fernie, A.R.** (2003) *De novo* amino acid biosynthesis in potato tubers is regulated by sucrose levels. *Plant Physiol.* 133: 683-692.

**Roessner-Tunali, U., Liu, J.L., Leisse, A., Balbo, I., Melis-Perez, A., Willmitzer, L. and Fernie, A.R.** (2004) Kinetics of labelling of organic and amino acids in potato tubers by gas chromatography-mass spectrometry following incubation in 13C labelled isotopes. *Plant J.* 39: 668-679.

**Rohwer, J.M. and Botha, F.C.** (2001) Analysis of sucrose accumulation in the sugar cane culm on the basis of *in vitro* kinetic data. *Biochem. J.* 358: 437-445.

**Roslan, H.A., Salter, M.G., Wood, C.D., White, M.R.H., Croft, K.P., Robson, F., Coupland, G., Doonan, J., Laufs, P., Tomsett, A.B. and Caddick, M.X.** (2001) Characterization of the ethanol-inducible *alc* gene expression system in *Arabidopsis thaliana*. *Plant J.* 28: 225-235.

**Ross, H.A. and Davies, H.V.** (1992) Sucrose metabolism in tubers of potato (*Solanum tuberosum* L.) - Effects of sink removal and sucrose flux on sucrose-degrading enzymes. *Plant Physiol.* 98: 287-293.

**Sablowski, R.W.M. and Meyerowitz, E.M.** (1998) A homolog of *NO APICAL MERISTEM* is an immediate target of the floral homeotic genes *APETALA3/PISTILLATA*. *Cell* 92: 93-103.

**Salter, M.G., Paine, J.A., Riddell, K.V., Jepson, I., Greenland, A.J., Caddick, M.X. and Tomsett, A.B.** (1998) Characterisation of the ethanol-inducible *alc* gene expression system for transgenic plants. *Plant J.* 16: 127-132.

**Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F. and Coupland, G.** (2000) Distinct roles of CONSTANS target genes in reproductive development of *Arabidopsis*. *Science* 288: 1613-1616.

**Sambrook, J., Fritsch, E.F. and Maniatis, T.** (1989) Molecular cloning: A laboratory manual; 2nd edition. New York, Cold Spring Harbor Laboratory Press.

**Schena, M., Lloyd, A.M. and Davis, R.W.** (1991) A steroid-inducible gene expression system for plant cells. *Proc. Natl. Acad. Sci. USA* 88: 10421-10425.

**Schott, K., Borchert, S., Müller-Röber, B. and Heldt, H.W.** (1995) Transport of inorganic phosphate and C3-sugar and C6-sugar phosphates across the envelope membranes of potato tuber amyloplasts. *Planta* 196: 647-652.

**Schuster, S. and Hilgetag, C.** (1994) On elementary flux modes in biochemical reaction systems at steady state. *J. Biol. Syst.* 2: 165-182.

**Schuster, S., Dandekar, T. and Fell, D.A.** (1999) Detection of elementary flux modes in biochemical networks: a promising tool for pathway analysis and metabolic engineering. *Trends Biotechnol.* 17: 53-60.

**Shevchenko, A., Jensen, O.N., Podtelejnikov, A.V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Boucherie, H. and Mann, M.** (1996) Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. *Proc. Natl. Acad. Sci. USA* 93: 14440-14445.

**Siegl, G., Mackintosh, C. and Stitt, M.** (1990) Sucrose phosphate synthase is dephosphorylated by protein phosphatase-2a in spinach leaves - Evidence from the effects of okadaic acid and microcystin. *FEBS Lett.* 270: 198-202.

**Sinnott, M., Garner, C.D., First, E. and Davies, G.** (1998) Comprehensive biological catalysis - A mechanistic reference. London, Academic Press.

**Smeekens, S.** (2000) Sugar-induced signal transduction in plants. *Annu. Rev. Plant Physiol. Mol. Biol.* 51: 49-81.

**Smith, C.J.** (1999) Carbohydrate Biochemistry. In: *Plant Biochemistry and Molecular Biology*. (Lea, P.J. and Leegood, R.C., eds.), pp. John Wiley & Sons, West Sussex, England.

**Sonnewald, U., Brauer, M., von Schaewen, A., Stitt, M. and Willmitzer, L.** (1991) Transgenic tobacco plants expressing yeast-derived invertase in either the cytosol, vacuole or apoplast: a powerful tool for studying sucrose metabolism and sink/source interactions. *Plant J.* 1: 95-106.

**Sonnewald, U., Hajirezaei, M.-R., Kossmann, J., Heyer, A., Trethewey, R.N. and Willmitzer, L.** (1997) Increased potato tuber size resulting from apoplastic expression of a yeast invertase. *Nat. Biotechnol.* 15: 794-797.

**Sowokinos, J.R., Spychalla, J.P. and Desborough, S.L.** (1993) Pyrophosphorylases in *Solanum tuberosum*. Purification, tissue localization, and physicochemical properties of UDP-glucose pyrophosphorylase. *Plant Physiol.* 101: 1073-1080.

**Steup, M.** (1990) Starch degrading enzymes. In: *Methods in plant biochemistry*. (Dey, P.M., ed.), pp. 103-128.

**Stitt, M. and Steup, M.** (1985) Starch and sucrose degradation. In: *Higher plant cell respiration*. (Douce, R. and Day, D.A., eds.), pp. 347-390, Springer-Verlag, Heidelberg.

**Stitt, M., Lilley, R.M.C., Gerhardt, R. and Heldt, H.-W.** (1989) Metabolite levels in specific cells and subcellular compartments of plant leaves. *Methods Enzymol.* 174: 518-552.

**Stitt, M. and Sonnewald, U.** (1995) Regulation of metabolism in transgenic plants. *Annu. Rev. Plant Physiol. Mol. Biol.* 46: 341-368.

**Sweetlove, L.J., Burrell, M.M. and ap Rees, T.** (1996a) Characterization of transgenic potato (*Solanum tuberosum*) tubers with increased ADPglucose pyrophosphorylase. *Biochem. J.* 320: 487-492.

**Sweetlove, L.J., Burrell, M.M. and ap Rees, T.** (1996b) Starch metabolism in tubers of transgenic potato (*Solanum tuberosum*) tubers with increased ADPglucose pyrophosphorylase. *Biochem. J.* 320: 493-498.

**Sweetlove, L.J., Müller-Röber, B., Willmitzer, L. and Hill, S.A.** (1999) The contribution of adenosine 5 '-diphosphoglucose pyrophosphorylase to the control of starch synthesis in potato tubers. *Planta* 209: 330-337.

**Sweetman, J.P., Chu, C., Qu, N., Greenland, A.J., Sonnewald, U. and Jepson, I.** (2002) Ethanol vapor is an efficient inducer of the *alc* gene expression system in model and crop plant species. *Plant Physiol.* 129: 943-948.

**Tang, G.Q., Hardin, S.C., Dewey, R. and Huber, S.C.** (2003) A novel C-terminal proteolytic processing of cytosolic pyruvate kinase, its phosphorylation and degradation by the proteasome in developing soybean seeds. *Plant J.* 34: 77-93.

**Tauberger, E., Fernie, A.R., Emmermann, M., Renz, A., Kossmann, J., Willmitzer, L. and Trethewey, R.N.** (2000) Antisense inhibition of plastidial phosphoglucomutase provides compelling evidence that potato tuber amyloplasts import carbon from the cytosol in the form of glucose-6-phosphate. *Plant J.* 23: 1-11.

**Thomas, S., Mooney, P.J.F., Burrell, M.M. and Fell, D.A.** (1997a) Finite changes analysis of glycolytic intermediates in tuber tissue of lines of transgenic potato (*Solanum tuberosum*) overexpressing phosphofructokinase. *Biochem. J.* 322: 111-117.

**Thomas, S., Mooney, P.J.F., Burrell, M.M. and Fell, D.A.** (1997b) Metabolic control analysis of glycolysis in tuber tissue of potato (*Solanum tuberosum*): explanation for the low control coeffecient of phosphofructokinase over respiratory flux. *Biochem. J.* 322: 119-127.

**Tiessen, A., Hendriks, J.H.M., Stitt, M., Branscheid, A., Gibon, Y., Farre, E.M. and Geigenberger, P.** (2002) Starch synthesis in potato tubers is regulated by post-translational redox modification of ADP-glucose pyrophosphorylase: A novel regulatory mechanism linking starch synthesis to the sucrose supply. *Plant Cell* 14: 2191-2213.

**Tjaden, J., Mohlmann, T., Kampfenkel, K., Henrichs, G. and Neuhaus, H.E.** (1998a) Altered plastidic ATP/ADP-transporter activity influences potato (*Solanum tuberosum* L.) tuber morphology, yield and composition of tuber starch. *Plant J.* 16: 531-540.

**Tjaden, J., Schwoppe, C., Mohlmann, T., Quick, P.W. and Neuhaus, H.E.** (1998b) Expression of a plastidic ATP/ADP transporter gene in *Escherichia coli* leads to a functional adenine

nucleotide transport system in the bacterial cytoplasmic membrane. *J. Biol. Chem.* 273: 9630-9636.

**Trethewey, R.N., Geigenberger, P., Riedel, K., Hajirezaei, M.-R., Sonnewald, U., Stitt, M., Riesmeier, J.W. and Willmitzer, L.** (1998) Combined expression of glucokinase and invertase in potato tubers leads to a dramatic reduction in starch accumulation and a stimulation of glycolysis. *Plant J.* 15: 109-118.

**Trethewey, R.N., Riesmeier, J.W., Willmitzer, L., Stitt, M. and Geigenberger, P.** (1999) Tuber-specific expression of a yeast invertase and a bacterial glucokinase in potato leads to an activation of sucrose phosphate synthase and the creation of a sucrose futile cycle. *Planta* 208: 227-238.

**Trethewey, R.N., Fernie, A.R., Bachmann, A., Fleischer-Notter, H., Geigenberger, P. and Willmitzer, L.** (2001) Expression of a bacterial sucrose phosphorylase in potato tubers results in a glucose-independent induction of glycolysis. *Plant Cell Environ.* 24: 357-365.

**Urbanczyk-Wochniak, E., Luedemann, A., Kopka, J., Selbig, J., Roessner-Tunali, U., Willmitzer, L. and Fernie, A.R.** (2003a) Parallel analysis of transcript and metabolic profiles: a new approach in systems biology. *EMBO Rep.* 4: 989-993.

**Urbanczyk-Wochniak, E., Leisse, A., Roessner-Tunali, U., Lytovchenko, A., Riesmeier, J., Willmitzer, L. and Fernie, A.R.** (2003b) Expression of a bacterial xylose isomerase in potato tubers results in an altered hexose composition and a consequent induction of metabolism. *Plant Cell Physiol.* 44: 1359-1367.

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

**Veramendi, J., Fernie, A.R., Leisse, A., Willmitzer, L. and Trethewey, R.N.** (2002) Potato hexokinase 2 complements transgenic *Arabidopsis* plants deficient in hexokinase 1 but does not play a key role in tuber carbohydrate metabolism. *Plant Mol. Biol.* 49: 491-501.

**Vervliet, G., Holsters, M., Teuchy, H., Vanmontagu, M. and Schell, J.** (1975) Characterization of different plaque-forming and defective temperate phages in *Agrobacterium* strains. *J. Gen. Virol.* 26: 33-48.

**Viola, R., Davies, H.V. and Chudeck, A.R.** (1991) Pathways of starch and sucrose biosynthesis in developing tubers of potato (*Solanum tuberosum* L.) and seeds of faba bean (*Vicia faba* L.) - Elucidation by 13C-nuclear-magnetic-resonance spectroscopy. *Planta* 183: 202-208.

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

**Weber, H., Heim, U., Golombek, S., Borisjuk, L., Manteuffel, R. and Wobus, U.** (1998) Expression of a yeast-derived invertase in developing cotyledons of *Vicia narbonensis* alters the carbohydrate state and affects storage functions. *Plant J.* 16: 163-172.

**Weiner, H., Stitt, M. and Heldt, H.W.** (1987) Subcellular compartmentation of pyrophosphate and alkaline pyrophosphatase in leaves. *Biochim. Biophys. Acta* 893: 13-21.

**Weinmann, P., Gossen, M., Hillen, W., Bujard, H. and Gatz, C.** (1994) A chimeric transactivator allows tetracycline-responsive gene expression in whole plants. *Plant J.* 5: 559-569.

**Weise, A., Barker, L., Kühn, C., Lalonde, S., Buschmann, H., Frommer, W.B. and Ward, D.C.** (2000) A new subfamily of sucrose transporters, SUT4, with low-affinity/high capacity localised in sieve elements of plants. *Plant Cell* 12: 1345-1355.

**Wendler, R., Veith, R., Dancer, J., Stitt, M. and Komor, E.** (1991) Sucrose storage in cell-suspension cultures of *Saccharum* sp (sugarcane) is regulated by a cycle of synthesis and degradation. *Planta* 183: 31-39.

**Winter, H., Robinson, D.G. and Heldt, H.W.** (1993) Subcellular volumes and metabolite concentrations in barley leaves. *Planta* 191: 180-190.

**Winter, H. and Huber, S.C.** (2000) Regulation of sucrose metabolism in higher plants: Localization and regulation of activity of key enzymes. *Crit. Rev. Biochem. Mol. Biol.* 35: 253-289.

**Wischmann, B., Nielsen, T.H. and Möller, B.L.** (1999) *In vitro* biosynthesis of phosphorylated starch in intact potato amyloplasts. *Plant Physiol.* 119: 455-462.

**Wittich, P.E. and Vreugdenhil, D.** (1998) Localization of sucrose synthase activity in developing maize kernels by *in situ* enzyme histochemistry. *J. Exp. Bot.* 49: 1163-1171.

**Yu, J., Hu, S.N., Wang, J., Wong, G.K.S., Li, S.G., Liu, B., Deng, Y.J., Dai, L., Zhou, Y., Zhang, X.Q., *et al.*** (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp indica). *Science* 296: 79-92.

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

**Zuo, J. and Chua, N.-H.** (2000) Chemical-inducible systems for regulated expression of plant genes. *Curr. Opin. Biotech.* 11: 146-151.