ASPECTS OF SUCROSE METABOLISM IN TRANSGENIC TOBACCO

by

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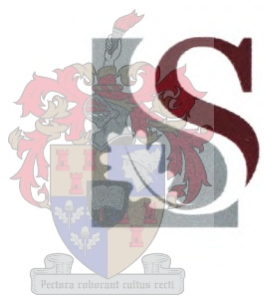

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

In most plants the efficiency of sucrose production and the systemic distribution thereof are the major determinants of growth, development and yield. The factors governing sugar partitioning co-ordinate its distribution in response to intrinsic and environmental signals. These factors include sugar transporters and invertases as well as metabolites, including sucrose and glucose, which function as signalling molecules to modulate gene expression.

The genetic transformation of plants and the subsequent development of transgenic lines with disturbed sugar metabolism have made an unprecedented impact on the study of sugar translocation and -partitioning. For instance, the transformation of plants with a yeast-derived invertase targeted to different subcellular compartments has led to the elucidation of several key aspects of sugar metabolism, including phloem loading mechanisms, the regulation of photosynthesis by sugars, the importance of sugar-metabolism compartmentation with regards to sucrose biosynthesis, storage and distribution, as well as the role of cell-wall invertase in phloem unloading and sink strength.

In this study, a similar strategy of transgenic plant analysis was employed to expand our insight into the regulation of sugar partitioning. The yeast-invertase *Suc2* gene, from *Saccharomyces cerevisiae*, was overexpressed in either the cytosol, vacuole or apoplast of transgenic tobacco plants. These transgenic lines displayed varying increases in invertase activity, altered sugar levels and consequently disturbed sink-source interactions and sugar partitioning. Transgenic lines overproducing the yeast-derived invertase in either the vacuole (Vac-Inv) or apoplast (Apo-Inv) were utilised to analyse the effect of the altered sugar levels in sink and source organs on the expression of sugar transporters, as well as the endogenous cell wall invertase and inhibitors in these plants.

Transcript levels of the sucrose transporter NtSUT1 and hexose transporter NtMST1 encoding genes increased significantly in the source leaves and roots of Vac-Inv lines, whereas increased *NtMst1* transcript levels were also detected in the roots of Apo-Inv lines. The increased mRNA levels could be correlated to the altered invertase activities and sugar levels in these tissues. It is concluded that NtSUT1 and NtMST1 are differentially regulated by sucrose and/or hexose content on a transcriptional level. Furthermore, the regulatory effect of the altered sugar levels on transporter expression depended on the subcellular compartment in which the yeast invertase was expressed. It would seem that the subcellular compartmentation of sugar metabolism is also fundamental to the regulation of sugar partitioning.

The transcription levels of the endogenous cell wall invertase (*Cwi*) and cell wall invertase inhibitor (*Cwi-Inh*) genes were examined in the various tissues of Apo-Inv and Vac-Inv lines at both the vegetative and flowering growth stages. In comparison with the control lines, the various tissues of the Apo-Inv and Vac-Inv lines

displayed altered *Cwi* and *Cwi-Inh* expression levels, depending on the sink-source status and growth stage. However, no obvious correlation between the *Cwi* and *Cwi-Inh* expression levels and soluble sugar content of these tissues was found. It is suggested that the post-transcriptional and post-translation control of these proteins by sugars might play an important role in their regulation. Analysis of the *Cwi:Cwi-Inh* mRNA ratio and growth observations of the various tissues of control as well as Apo-Inv and Vac-Inv lines indicated that this transcription ratio could be an accurate indicator of the sink strength of sink organs.

In addition, the influence of sink-source interactions on sugar partitioning was investigated. Reciprocal grafting between Apo-Inv and control lines resulted in scions with an altered sucrose metabolism in either the sink or source organs. These scions were subjected to biomass distribution, soluble sugar quantification and [^{14}C]-radiolabelling experiments. The latter revealed an unaltered state of sugar partitioning from the above-ground tissues of the Apo/GUS scions and a significant shift in sugar partitioning towards the roots of the GUS/Apo scions in comparison to the control GUS/GUS scions. Phenotypic changes, opposite to those observed in Apo-Inv lines expressing the heterologous invertase in both sink and source organs, could initially be observed in the GUS/Apo and Apo/GUS scions. However, no significant differences in phenotype or biomass distribution could be observed between the mature GUS/Apo, Apo/GUS and GUS/GUS scions seven weeks post-grafting. This inconsistency between phenotype and sugar partitioning might be explained by an increase in the respiration rate of the tissues as supported by the soluble sugar content. These results highlight the complexity and adaptability of sucrose metabolism and sugar partitioning. In addition, it confirms that sugar partitioning can be modulated by sink-source interactions and emphasise the importance of invertases in the regulation of sugar partitioning through its ability to alter sink strength.

This study forms part of the rapidly expanding initiative to unravel the control mechanisms of sugar partitioning. The results obtained in this study confirmed again that the introduction and expression of a single heterologous gene in transgenic plants could provide significant insight into the regulation of this process. It was shown here that the expression of sugar transporters is closely regulated by sugar levels and therefore fulfils a vital function in sugar sensing and consequently the regulation of sugar partitioning. The data presented in this study also demonstrated the intricate and flexible nature of the relationship that exists between sugar metabolism, partitioning and growth phenomena.

OPSOMMING

Die doeltreffendheid van sukroseproduksie, tesame met die sistemiese verspreiding daarvan, is die vernaamste faktore wat die groei, ontwikkeling en opbrengsvermoë van die meeste plante bepaal. Die faktore wat suikerverdeling beheer, funksioneer om suikerverspreiding te koördineer in reaksie op beide inherente- en omgewingsseine. Hierdie faktore sluit suikertransporters en invertases in, asook metaboliete soos sukrose en glukose wat funksioneer as seinmolekule in die modulering van geenuitdrukking.

Die genetiese transformasie van plante en die gevolglike daarstelling van transgeniese lyne met veranderde suikermetabolismes het 'n beduidende inwerking op die bestudering van suikervervoer en –verdeling gehad. Byvoorbeeld, die transformasie van plante met 'n gis-invertase geteiken na verskillende sub-sellulêre kompartemente, het tot die toeligting van verskeie aspekte van suikermetabolisme gelei, insluitende dié van floëemladingsmeganismes, die regulering van fotosintese deur suikers, die belang van kompartementalisering ten opsigte van sukrosebiosintese, -opberging en -verspreiding, en die rol van selwand-invertases in floëemontlaaiing en swelgpuntkrag.

In hierdie studie is van soortgelyke transgeniese plantontledings gebruik gemaak om 'n dieper insig tot die *regulering* van suikerverdeling te verkry. Die gis-invertase *Suc2* geen, afkomstig van *Saccharomyces cerevisiae,* is ooruitgedruk in òf die sitosol, vakuool òf apoplastiese ruimte van transgeniese tabakplante. Hierdie transgeniese lyne het wisselende toenames in invertase-aktiwiteite en veranderde suikervlakke getoon, asook gevolglike versteurde bron-swelgpunt interaksies en suikerverdeling. Transgeniese lyne met ooruitdrukking van die gis-invertase in òf die vakuool (Vac-Inv) òf die apoplast (Apo-Inv) is gebruik om die gevolg van die veranderde suikervlakke in bron- en swelgpuntorgane op die uitdrukking van suikertransporters, asook die endogene selwand-invertase en invertase-inhibitor in hierdie plante te bepaal.

Transkripsievlakke van die sukrosetransporter *NtSut1* en die heksose-transporter, *NtMst1*, het beduidend toegeneem in die bron-blare en wortels van die Vac-Inv lyne; 'n toename in *NtMst1* transkripsievlakke is ook in die wortels van Apo-Inv lyne bevestig. Die toenames in boodskapper RNA kon gekorreleer word met die veranderde invertase-aktiwiteite en suikervlakke in hierdie weefsels. Die gevolgtrekking word gemaak dat NtSUT1 en NtMST1 differensieël gereguleer word op transkripsionele vlak deur die sukrose en/of heksose inhoud van weefsels. Meer nog, die regulerende effek van die veranderde suikervlakke op transporter-uitdrukking het afgehang van die subsellulêre kompartement waarin die gis-invertase uitgedruk is. Dit wil dus voorkom dat die subsellulêre kompartementalisering van suikermetabolisme fundamenteel tot die deurgee en waarneming van suikerseine is, met 'n gevolglike eweneens belangrike rol in die regulering van suikerverdeling.

Die transkripsievlakke van beide die endogene selwand-invertase (CWI) en die selwand-invertase-inhibitor (CWI-Inh) enkoderende gene is in verskeie weefsels van die Apo-Inv en Vac-Inv lyne, tydens beide die vegetatiewe- en blomstadia, bestudeer. Die onderskeie weefsels van die Apo-Inv en Vac-Inv lyne het, in vergelyking met die kontrole lyne, veranderde *Cwi* en *Cwi-inh* transkripsievlakke getoon wat bepaal is deur bron-swelgpunt status en groeistadium. Geen duidelike korrelasie kon tussen beide *Cwi* en *Cwi-inh* uitdrukkingsvlakke en oplosbare suiker inhoud gevind word nie. Daar word voorgestel dat post-transkripsionele en post-translasionele beheer deur suikers 'n belangrike rol in die regulering van hierdie proteïne speel. Bestudering van die *Cwi:Cwi-Inh* mRNA verhouding, asook groei verskynsels van die onderskeie weefsels van kontrole en Apo-Inv en Vac-Inv lyne, dui daarop dat hierdie transkripsievlak-verhouding moontlik 'n akkurate aanwyser van die swelgpuntkrag van 'n swelgpuntorgaan kan wees.

Voorts is die invloed van bron-swelgpuntorgaan interaksies op suikerverdeling ondersoek. Omgekeerde enting tussen Apo-Inv en kontrole lyne het entlote met gemodifiseerde suikermetabolisme in òf hul bron- òf hul swelgpuntorgane tot gevolg gehad. Hierdie entlote is aan biomassaverspreidings-, oplosbare suiker kwantifisering en [^{14}C]-radiomerking eksperimente onderwerp. Hierdie resultate het gewys dat, in vergelyking met die kontrole (GUS/GUS) ente, daar geen verandering in die status van suikerverdeling vanaf die bogrondse plantdele in die Apo/GUS ente is nie, maar wel 'n beduidende verskuiwing in suikerverdeling na die wortels van die GUS/Apo ente. Fenotipiese veranderinge, wat teenoorgesteld van dié teenwoordig in die Apo-Inv lyne waar die heteroloë invertase in beide bron en swelgpuntorgane uitgedruk word, is aanvanklik in die GUS/Apo en Apo/GUS ente waargeneem. Geen verskille in fenotipe of biomassa-verspreiding kon egter sewe weke na die entings prosedures tussen die GUS/Apo, Apo/GUS and GUS/GUS ente gevind word nie. Dit mag verduidelik word deur 'n moontlike toename in respirasietempo in die betrokke weefsels; die oplosbare suikervlakke wat in die verskillende ente aangeteken is ondersteun dié moontlikheid. Hierdie resultate as geheel onderstreep die kompleksiteit en aanpasbaarheid van suikermetabolisme en -verdeling. Verder bevestig dit dat suikerverdeling beïnvloed kan word deur bron-swelgpunt interaksies, asook die belang van invertases in die regulering van suikerverdeling gegewe die vermoë om swelgpuntkrag te verander.

Hierdie studie vorm deel van 'n vinnig groeiende inisiatief om die beheer-meganismes van suikerverdeling te ontrafel. Die resultate verkry in hierdie studie bekragtig die belang van rekombinante DNA tegnologie in die bestudering van fundamentele plantprosesse. Die invoeging en uitdrukking van 'n geteikende gis-invertase in transgeniese plante het gelei tot veranderde suikervlakke en bron-swelgpunt interaksies in hierdie lyne met die gevolglike ontginning van waardevolle inligting ten opsigte van die regulering van suikerverdeling in reaksie tot interne seine. Daar is aangetoon dat suikertransporters onlosmaakbaar gekoppel is aan die deurgee en waarneming van suikerseine, spesifiek op die vlak van transkripsionele

regulering, en dus ook die regulering van suikerverdeling. Voorts wys die resultate op die komplekse en aanpasbare aard van die verhouding wat bestaan tussen suikermetabolisme, -verdeling en groeiverskynsels.

Hierdie proefskrif is opgedra
aan my Hemelse Vader

BIOGRAPHICAL SKETCH

Reinette (Esterhuizen) Champanis was born in Stellenbosch, South Africa, on 14 November 1974. She attended Stellenbosch Primary and matriculated with distinction from Stellenbosch High School in 1992. Reinette enrolled at Stellenbosch University in 1993 and obtained the BSc degree in Microbiology and Genetics in 1995. In 1996 she received the degree BScHons in Microbiology *cum laude*. In 1997 she enrolled at the Institute of Wine Biotechnology for an MSc degree in Wine Biotechnology, which was upgraded to a PhD degree in 1999. She subsequently registered for a PhD in Wine Biotechnology in 2000.

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PREFACE

This dissertation is presented as a compilation of seven chapters. Each chapter is introduced separately and is written according to the style of the Journal of Plant Physiology.

Chapter 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

Chapter 2 **LITERATURE REVIEW**
Sugar transport and partitioning in higher plants

Chapter 3 **RESEARCH RESULTS**
Transformation of tobacco and grapevine with a yeast-derived invertase (*Suc2*) targeted to either the cytosol, vacuole or apoplast
(Not to be submitted for publication)

Chapter 4 **RESEARCH RESULTS**
The effect of altered sugar levels on the expression of sugar transporters in transgenic tobacco
(To be submitted for publication in Journal of Plant Physiology)

Chapter 5 **RESEARCH RESULTS**
The expression of the endogenous cell wall invertase and invertase inhibitor in transgenic tobacco overexpressing a yeast-derived invertase
(Preliminary findings that will form part of a manuscript to be submitted for publication)

Chapter 6 **RESEARCH RESULTS**
Sugar partitioning in transgenic tobacco overexpressing a yeast-derived invertase in either the sink or source organs
(To be submitted for publication in Plant Molecular Biology)

Chapter 7 **GENERAL DISCUSSION AND CONCLUSIONS**

I hereby declare that I was the primary contributor with respect to the experiments conducted and the presentation and interpretation of data in the multi-author articles presented in Chapters 3, 4 and 6. Dr Huiqin Ma, a post-doctoral fellow at the Institute for Wine Biotechnology, was the main contributor with respect to the execution of the experiments presented in Chapter 5, while I was the primary contributor with respect to the presentation and interpretation of the data. My supervisors, Prof MA Vivier, Prof FC Botha, and Prof IS Pretorius, were involved in the conceptual development and continuous critical evaluation of the study.

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Plants are photoautotrophic organisms and thus able to produce the carbohydrates necessary to sustain their metabolism and to support their growth and development. Sucrose is synthesised in the cytosol of source organ-mesophyll cells from where it is exported to meet the requirements of the heterotrophic sink tissues. Source tissues include any exporting organ, typically a mature leaf that is capable of producing photosynthate in excess of its own needs, as well as storage organs during export. Sink tissues, on the other hand, are net carbon importers and include roots, tubers and immature leaves. It should be noted, however, that the sink or source status of tissues also depend on their diurnal, developmental and nutritional state. Thus in most plants, a net flow of photosynthate, primarily in the form of sucrose, occurs from the source tissues to the sink tissues via the phloem during the process of long-distance translocation (Ward et al., 1998).

The efficiency of carbohydrate production and the systemic distribution thereof, in conjunction with respiration, are the major determinants of plant growth, development and yield (Gifford et al., 1984). The distribution of photoassimilates, referred to as sugar partitioning, is co-ordinated at numerous levels in the plant to insure optimal photosynthate distribution in response to internal as well as external signals (Wardlaw, 1990). This is achieved through the allosteric regulation of metabolic enzymes as well as tissue-specific and temporal expression of the genes involved. These include genes encoding sugar transporters (Riesmeier et al., 1994) and invertases (Sturm, 1999), which are essential in sugar translocation and partitioning.

Long-distance translocation of sugars between source and sink tissues results from a combination of apoplastic and symplastic exchanges: delivery of sucrose to the phloem can be by symplastic diffusional transport via the plasmodesmata, or by apoplastic carrier-mediated transport where a disaccharide transporter mediates the transport of sucrose across the plasma membrane (Riesmeier et al., 1994; Ward et al., 1998). After long-distance transport, sucrose is unloaded from the sieve elements via a symplastic or apoplastic route. In the case of apoplastic unloading, sucrose can be imported into the sink cells directly from the apoplast via disaccharide transporters (Lemoine, 2000) or it can be hydrolysed to glucose and fructose by cell wall-bound invertases and taken up via monosaccharide transporters (Büttner and Sauer, 2000). Cell wall invertase plays a pivotal role in sugar partitioning in that the hydrolysis of sucrose at the site of phloem unloading steepens the pressure gradient, increasing translocation toward the sink organ (Sonnewald et al., 1997). Target sites for control over sugar partitioning can thus include loading and unloading from the phloem, uptake by the sink cells, as well as the conversion and compartmentation of sucrose within the sink cells.

In addition, sugar itself functions as a signalling molecule that initiates changes in gene expression (Sheen et al., 1999; Ho et al., 2001). The significance of

sugar as a signalling molecule in plants involves the integration of whole-plant carbon flux in order to match the production capacity of source tissues with the utilisation capacity of the sink tissues. By sensing changes in carbohydrate status, plants can respond by altering gene expression and/or enzyme activities, thereby controlling photosynthate production, partitioning, and subsequent utilisation (Koch, 1996). This is a valuable mechanism for plants to adjust to physiological, developmental and environmental change, as well as pathological events. Sugars can modulate the activity and expression of sugar transporters as well as different invertase isoforms, demonstrating that sugar signalling dynamically regulates phloem loading and unloading and thus sugar partitioning at the level of phloem translocation (Chiou and Bush, 1998; Vaughn et al., 2002).

There is evidence for at least two distinct modes of sugar signalling in plants, namely a hexose- (Jang and Sheen, 1994) and a sucrose-signalling pathway (Lalonde et al., 1999) and it is proposed that there are at least three possible sensing mechanisms: a hexokinase-sensing system (Jang and Sheen, 1994), a glucose-transport-associated sensor (Herbers et al., 1996), and a sucrose-transport-associated sensor (Lalonde et al., 1999; Barker et al., 2000). These signal transduction pathways have been shown to closely interact with developmental and environmental signals to form part of an intricate regulatory web (Smeekens, 2000). The mechanisms underlying the cross-talk between these pathways and subsequent gene regulation are not apparent. More information is necessary to improve our understanding of how intrinsic and environmental signals are sensed and translated between the various sink and source organs to ultimately result in physiological responses.

Physiological, biochemical and molecular approaches have been used to study sugar partitioning and sink-source relationships in plants. Recombinant DNA technology, together with the ability to genetically transform plants, made it possible to disturb sugar metabolism in plants at a particular step in order to answer fundamental questions regarding the processes being studied (Stitt and Sonnewald, 1995). In this regard, the transformation of plants with a yeast-derived invertase targeted to various cellular compartments has proved to be particularly successful as a model system. The introduction of the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to either the cytosol, vacuole, or apoplast, in a variety of species, resulted in the severe disturbance of sucrose metabolism, transport as well as sink-source interactions (Von Schaewen et al., 1990; Dickinson et al., 1991; Sonnewald et al., 1991; Heineke et al., 1992; Büssis et al., 1997; Weber et al., 1998; Ma et al., 2000). Information gained from these experiments included the elucidation of phloem loading mechanisms, repression of biosynthesis of the photosynthetic apparatus as caused by the accumulation of photosynthate, the central importance of sucrose compartmentation with respect to its biosynthesis, storage and distribution, as well as the role of invertases in sugar accumulation and sink strength.

Subsequent to these experiments, new-found knowledge on sugar signalling and sensing mechanisms as well as on the interaction between different plant signalling pathways, heralded a new era in the understanding of sugar metabolism and partitioning. The model system of invertase expressing tobacco lines can now be utilised to further our knowledge of these aspects by, for example, studying the influence of altered sugar content (due to the effective cleavage of sucrose into glucose and fructose by the yeast invertase) on gene expression and/or enzyme activities, and thereby sugar metabolism and partitioning. In this study, this approach of transgenic plant analysis has been taken whereby a yeast-derived invertase was overexpressed in either the cytosol, vacuole or apoplast of tobacco plants.

1.2 PROJECT AIMS

The objective of this study is to use the well-established method of transgenic plant analysis to expand our insight into how sugar signals are sensed and translated to modify sugar partitioning, as well as to investigate the role of sink/source interactions in this process. This will be accomplished by investigating growth phenomena at the level of sugar partitioning, as well as examining sugar-regulated gene expression of the proteins integrally linked to sugar metabolism and transport, such as plant sugar transporters, as well as endogenous invertases and invertase inhibitors. Furthermore, these transgenic lines, with disturbed sucrose metabolism, transport as well as sink-source interactions, will be utilised to investigate the complex interactions between sink-source relationships and sugar partitioning in order to facilitate the further exploration of the growth phenomena (including impaired root formation and stunted growth) that was observed in earlier studies involving invertase transgenic lines.

The research hypothesis is that in higher plants, sugar signals are sensed by proteins integrally linked to sugar transport and/or partitioning to ultimately result in physiological responses. To evaluate this hypothesis, the following specific aims were set:

i) to generate transgenic tobacco lines overexpressing the yeast-invertase *Suc2* gene in either the cytosol, vacuole, or apoplast to be used as a model system in subsequent analyses of sugar partitioning;
ii) to examine the effect of altered sugar levels in sink and source organs on the expression of sugar transporters in these plants;
iii) to evaluate the effect of altered sugar levels in sink and source organs on the expression of native tobacco invertase and invertase inhibitor;
iv) to investigate the influence of altered sucrose metabolism in either sink or source organs on sugar partitioning by means of reciprocal grafting between control and transgenic tobacco plants expressing the yeast invertase in the apoplast.

1.3 LITERATURE CITED

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CHAPTER 2

LITERATURE REVIEW

Sugar transport and partitioning in higher plants

2.1 INTRODUCTION

Light energy is converted into the chemical energy of organic molecules by plants through the process of photosynthesis. The resulting formation of sugars in the source leaves of higher plants produces the driving force for plant metabolism. This review will focus mainly on the *distribution* of these photoassimilates, referred to as sugar partitioning, as well as the *regulation* of this process in higher plants.

Sugar partitioning will be discussed as a function of sugar transport, the various phloem loading and unloading mechanisms, as well as the proteins integrally linked to these processes. Emphasis is placed on sugar transporters, which are responsible for the active transmembrane movement of sugars, as well as invertase with its role in the channelling of sucrose into sink metabolism. Moreover, the highly integrated nature of sugar partitioning throughout plant growth and development is highlighted, with specific reference to carbohydrate control of gene expression and sugar signalling. Finally, the transgenic approach to study sugar translocation and partitioning in higher plants is discussed by using transgenic plants expressing a targeted yeast-derived invertase as a model system.

2.2 SUGAR PARTITIONING IN HIGHER PLANTS

2.2.1 Principles of sugar partitioning

The distribution of photoassimilates, also known as sugar partitioning, initiates at the point of sugar production during photosynthesis (or the point of reserve remobilisation) and follows through to partitioning between the different competing sink organs. This phenomenon of sugar partitioning between the various heterotrophic sink tissues is one of the most important determining factors in plant growth, development and yield. These sink organs can be divided into two basic categories, namely metabolic and storage sinks. Metabolic sinks, such as meristems and roots, typically import carbohydrates to supply their high metabolic demand, whereas storage sinks, such as seeds and tubers, mainly import carbohydrates for accumulation.

Generally, these different types of sink organs employ different unloading mechanisms and have a different flux in carbon compounds. In metabolic sink organs, the majority of imported photoassimilate is utilised for growth, with only a small portion being stored temporarily. A steep sucrose gradient is sustained by the metabolic use of sucrose for respiration and the biosynthesis of cellular structures. Since respiration is the main metabolic activity of metabolic sink organs, it is thought that the rate of respiration will determine the rate of sucrose import into these organs (Gordon et al., 1985). In storage sink organs, most of the imported photoassimilate is stored as sugars (sucrose, glucose or fructose) or starch, although in certain sink organs it can be stored as lipids or proteins. Storage sinks usually store their

carbohydrate reserves in one form, although exceptions exist: tomato fruit store equal amounts of hexoses and starch during early stages of development, but eventually only accumulate hexoses (Johnson et al., 1988). It is thought that the storage process of storage sink organs may be the controlling step for assimilate import into these organs. The continuous cleavage and synthesis of sucrose in sugar storing organs such as sugarcane stems (Whittaker and Botha, 1997), and the activity of starch-synthesising enzymes in starch accumulating storage organs (Müller-Röber et al., 1992), may control the rate of accumulation.

2.2.2 Regulation of sugar partitioning

Despite the immense importance of the regulation of sugar partitioning in relation to crop yield, relatively little is known about this aspect. It has been shown, however, that the regulation of sugar partitioning is independent of photoassimilate production: experiments incorporating the antisense expression of cytosolic fructose-1,6-bisphosphatase (FBPase) (Zrenner et al., 1996a), the triose-P translocator (Riesmeier et al., 1993a; Kehr et al., 1998), and hexokinase (Veramendi et al., 1999) in potato, revealed that changes in the photosynthetic sucrose biosynthesis capacity can be compensated for without altering plant growth or tuber yield. These results highlight the complexity and flexibility of photosynthetic sucrose biosynthesis and illustrate that the distribution of photoassimilates towards sucrose formation in the light, does not influence sugar partitioning.

It is thought that the partitioning of fixed carbon between alternative sink organs is controlled by the relative sink strength of these tissues (Ho et al., 1987; Ho, 1988). Sink strength is defined as the intrinsic competitive ability of a heterotrophic organ to import, process, and store photoassimilates relative to other sink organs (Herbers and Sonnewald, 1998). Although the priority of sugar partitioning between these sink organs is consistent at each stage of plant development (Ho, 1984), the import rate into an individual sink can be changed by altering its sink strength or by changing the strength of a competing sink organ. Even though the potential sink strength of a sink organ is genetically determined, the actual sink strength is determined by factors that affect rate-limiting processes within the sinks during development.

The metabolisation of sucrose thus forms an integral part of sugar partitioning in most plant species. A stronger sink can deplete the sugar content of the sieve elements more readily and thus steepen the sucrose gradient, increasing translocation toward itself (Sonnewald et al., 1997). Therefore, enzymes involved in sucrose metabolism play a pivotal role in sugar partitioning (Ho, 1988). The hydrolysis of sucrose by cell wall invertase at the site of phloem unloading has been identified as one of the crucial steps involved in this process (Eschrich, 1980). When sucrose is removed by cell wall-bound invertase in the apoplastic space, the sucrose gradient is steepened and sink activity is increased, resulting in increased sink

strength. The increased sink strength, in turn, influences assimilate partitioning towards the sink. Thus, the carbohydrate demand of growing sink tissues can be satisfied by the up-regulation of the enzymes involved in phloem unloading, such as cell wall invertases. This was shown to be the case for specific cell wall invertase isoforms in tomato (Goetz et al., 2000) and *Chenopodium rubrum* (Roitsch, 1999), where these isoforms were induced in response to certain exogenous and endogenous stimuli to modulate source-to-sink translocation. Sucrose synthase also plays a role in the generation of sink strength in well-defined developmental stages in some sink organs; namely the storage phase of fava bean seeds (Weber et al., 1997b), during tomato fruit development (Wang et al., 1993; D'Aoust et al., 1999), as well as in potato (Zrenner et al., 1995).

Significantly, current evidence suggests that sugar partitioning is controlled also by transmembrane transport events (Truernit et al., 1996). It was shown that sucrose can modulate the activity of sucrose-H^+ transporters, demonstrating that sucrose signalling can dynamically regulate phloem loading and thus, assimilate partitioning at the level of phloem translocation (Chiou and Bush, 1998; Vaughn et al., 2002). During the sink-to-source transition of leaves, sucrose-H^+ symporters might regulate other genes encoding proteins involved in carbohydrate metabolism through the regulation of the extracellular carbohydrate concentration (Truernit and Sauer, 1994). Antisense repression of sucrose transporters in potato and tobacco confirmed that these transporters are essential for phloem loading and sugar partitioning (Riesmeier et al., 1994; Bürkle et al., 1998; Schulz et al., 1998).

There is also growing evidence that the regulation of sugar partitioning by invertases and sugar transporters could be modulated by phytohormones. In C. *rubrum* the stimulation of growth and cell division by cytokinins relied on the expression of cell wall invertases and hexose transporters (Ehness and Roitsch, 1997). The co-ordinated induction of cell wall invertase and hexose transporters by this hormone, suggests that the regulation of sink strength, and thus sugar partitioning, might occur via hormonal control. It has also been shown that brassinosteroids stimulate growth by increasing the photoassimilate supply by tissue-specific up-regulation of cell wall invertases (Goetz et al., 2000).

Further investigation of the significance of these aspects is needed to complete the picture on the regulation of sugar partitioning. It is clear, however, that sugar partitioning is a highly integrated and orchestrated process throughout plant growth and development. Consequently, control can potentially be exerted at several levels in sink organs, including: photoassimilate unloading from the phloem, uptake by the sink cells, as well as the conversion and compartmentation inside the sink cells. Numerous other exogenous factors also affect partitioning, including temperature (for review, see Farrar, 1988), salinity (Gao et al., 1998), wounding (Roitsch, 1999), as well as virus and phytopathogenic fungi infection (Ehness et al., 1997; Lucas and Wolf, 1999). For the purpose of this study, sugar partitioning and its

regulation will be discussed further as a function of sugar transport and the proteins involved in this process.

2.3 SUGAR TRANSPORT IN HIGHER PLANTS

2.3.1 Pathways of sugar transport

Photosynthesis (for review, see Leegood, 2000) is the process by which green plants synthesise sugar molecules and oxygen from carbon dioxide and water in the presence of sunlight. As carbon is accumulated in the chloroplasts of mesophyll cells, triose phosphate (triose-P) is exported from the chloroplast by the triose-P antiporter, in exchange for inorganic phosphate (P_i), which is generated during the biosynthesis of sucrose in the cytosol. In most higher plants, sucrose is the main form in which photosynthate is translocated. It is exported from the producing source organs, transported and imported into the sink organs to allow for growth and development. Phloem transport is therefore directed by the relative locations of the areas of supply (sources) and utilisation (sinks) of the products of photosynthesis. The key processes involved in sugar translocation are: phloem loading (the movement of sucrose into the sieve elements), long-distance transport through the sieve elements, and phloem unloading (the movement of sucrose out of the sieve elements into the sink cells). The transport of solutes within the phloem is mainly regulated by mass flow and driving gradients created by the loading and unloading processes, according to the Münch hypothesis (Münch, 1927).

2.3.1.1 Sugar transport to the vascular tissues

Phloem loading actually describes the entire pathway that photosynthate follows from the mesophyll to the sieve element/companion cell complex (SE/CC-complex), including transfer between the mesophyll cells, transport from the mesophyll to the vein boundary and from the vein boundary into the SE/CC-complex. The latter is referred to as sieve element loading and represents the entrance of photosynthate into the sieve elements (Oparka and Van Bel, 1992). This loading process provides the driving force for translocation by generating turgor pressure in the sieve elements (Bush, 1999; Turgeon, 2000).

During the process of phloem loading, several boundaries have to be crossed until the photosynthate is loaded into the sieve elements and partakes in mass flow. The structural features of the various boundaries significantly contribute to the process. One of the most prominent of these includes the minor vein structure in source leaves. During the sink-to-source transition in leaves (Turgeon, 1989), assimilate import is ceased and export is initiated, which coincides with the maturation of minor vein phloem. The class-V minor vein network of dicotyledonous plants represents the principal loading sites for sucrose. These veins are the smallest

in the leaf and usually consist of a single xylem element, phloem parenchyma cells, and one or two sieve elements with their associated companion cells. During the development of sieve elements, selective degradation of organelles takes place while sieve area-pores form to link the individual sieve elements, thus forming a continuous membrane-bound conduit (Esau, 1969). The organelle- and cytoplasm-rich companion cells maintain the primary metabolism of their enucleated sieve element partners through the relatively large pores that exist between them. These plasmodesmata appear to be functionally distinct from those connecting mesophyll cells (Kempers and Van Bel, 1997). Companion cells are twice the diameter of sieve elements, probably because of their active role in sugar loading. The occurrence of plasmodesmata at the interface of mesophyll cells and the SE/CC-complex can vary greatly and the relevance thereof has been debated strongly, since it is across this boundary that photoassimilate accumulation occurs (Fisher, 1990). Evidence suggests that photoassimilate moves from the companion cells to the sieve elements by either diffusion or mass flow (Gunning et al., 1974; Gunning, 1976).

The other boundary of great importance occurs between the companion cells and the bundle sheath or phloem parenchyma. Species have been categorised according to the frequency of occurrence of plasmodesmata between these cells in the following manner: abundant plasmodesmatal connections (type-1), moderate (type-1-2a), sporadic (type-2a), and virtually no plasmodesmatal contacts (type-2b) (Gamalei, 1985; 1989). Companion cells that have very few plasmodesmata adjoining them to the surrounding cells, such as type 2b, have wall ingrowths to increase the surface area and the ability to load solute from the apoplast. These cells are termed transfer cells (Wimmers and Turgeon, 1991). In contrast, type-1 companion cells with high numbers of plasmodesmata and smooth walls are known as intermediary cells (Turgeon et al., 1975).

Pre-phloem transport. Sucrose that is synthesised in the mesophyll cells can reach concentrations of 25-100 mM in the cytosol (Heineke et al., 1994) and must be transported to the phloem. The movement of sucrose to the sieve elements of mature leaves involves several transport steps. Sucrose first undergoes short-distance transport when it moves from the mesophyll cells to the vicinity of the sieve elements. Sucrose is transported symplastically via plasmodesmata down a concentration gradient to the mesophyll cells close to the SE/CC-complex. This usually involves only a few cell diameters and types – adjacent mesophyll cells, bundle sheath cells, and phloem parenchyma or companion cells (Ward et al., 1998). Although this movement towards the phloem can theoretically also occur via the apoplast, the general consensus is that it is symplastic (Van Bel, 1993; Turgeon, 1996). Sucrose is then transferred to the SE/CC-complex, which represents the primary site of sucrose loading into the translocation system.

Sieve element loading mechanisms. There are two possible pathways for sucrose delivery to the phloem, namely symplastic cell-to-cell diffusional transport via the plasmodesmata and apoplastic carrier-mediated transport across the plasma membrane (Fig. 1) (Turgeon and Wimmers, 1988; Van Bel et al., 1992, 1994). These pathways depend on species-specific properties of tissues, including minor vein configuration (Gamalei, 1985, 1989; Orlich and Komor, 1989), and carbohydrate metabolism, including the translocation form of photosynthate (Gamalei, 1985; Turgeon et al., 1993; Flora and Madore, 1996). Although minor vein configuration alone is not sufficient to assess the nature of the phloem-loading pathway, it would seem that type-1 minor veins correspond to symplastic loading, whereas type-2 minor veins are associated with apoplastic loading (Gamalei and Pakhomova, 1981; Gamalei, 1985,1989,1991; Van Bel and Gamalei, 1992). Evidence indicates that these two diverse pathways do not necessarily occur mutually exclusively, but may be operational simultaneously or sequentially (Van Bel, 1993,1996; Turgeon, 1996).

Plants employing an apoplastic phloem-loading step generally exhibit low symplastic connectivity due to the lack of direct plasmodesmatal connections between mesophyll cells and the adjacent SE/CC-complex (Geiger et al., 1973; Evert et al., 1977). A high sucrose concentration gradient exists between the SE/CC-complexes and the surrounding mesophyll and phloem parenchyma (Geiger et al., 1973). Sucrose is loaded from the apoplast into the SE/CC-complex against this gradient by means of a sucrose-proton symporter (Riesmeier et al., 1994; Kühn et al., 1997). This sucrose-proton co-transport mechanism develops during the maturation of leaves (Lemoine et al., 1992). The metabolic energy required for loading against a chemical potential gradient, is provided by H^+-ATPases that are localised at the phloem plasma membrane (Giaquinta, 1979a; DeWitt et al., 1991a; Bouché-Pillon et al., 1994; DeWitt and Sussman 1995). Sucrose synthase, which is specifically localised in the phloem of source leaves, is involved in the catabolism of sucrose to provide ATP as substrate for the H^+-ATPases (Martin et al., 1993; Nolte and Koch, 1993). Sucrose/H^+ co-transport takes place, with potassium ion (K^+) movement occurring in response to the membrane potential (Van Bel and Van Erven, 1979). This is in accordance with chemical composition studies, which show that phloem has a high sucrose concentration (0.2-1.6 M), relatively low proton concentration (pH 7.5-8.5), and high K^+ concentration (100-200 mM) (Giaquinta, 1980).

Evidence for an apoplastic-loading pathway in different species has been obtained with experiments involving *p*-chloromercuribenzene sulfonic acid (PCMBS), a nonpermeable chemical modifier that inhibits apoplastic phloem loading exclusively (Eschrich and Fromm, 1994; Van Bel et al., 1994) through the analysis of transgenic plants expressing a yeast-derived invertase in the apoplast (Von Schaewen et al., 1990; Dickinson et al., 1991; Heineke et al., 1992) as well as antisense sucrose transporter experiments (Riesmeier et al., 1994; Bürkle et al., 1998; Schulz et al., 1998).

Observations regarding the frequency of plasmodesmatal connections and microscopical studies with fluorescent dyes have provided evidence for the existence of a symplastic phloem-loading pathway (Robards and Lucas, 1990). It would seem that this mechanism of loading is largely confined to plants displaying a high degree of connectivity between the mesophyll and the SE/CC-complex regarding the abundance of plasmodesmata (Van Bel et al., 1992), as well as those transporting raffinose-family oligosaccharides (Turgeon, 1996). Loading in these plants is not sensitive to PCMBS, which inhibits sucrose-proton co-transport from the apoplast (Turgeon and Gowan, 1990; Van Bel, 1992; Flora and Madore, 1993; Van Bel et al., 1994; Flora and Madore 1996). This indicates that an entirely symplastic phloem-loading pathway might exit for species that have intermediary cells, such as the cucurbits (Turgeon and Beebe, 1991). Although these plants exhibit type-1 minor vein phloem, they still accumulate sugar against a steep concentration gradient (Haritatos et al., 1996). Accumulation against this gradient has been shown to occur in the symplastically loading species *Coleus blumei* (Turgeon and Gowan, 1990).

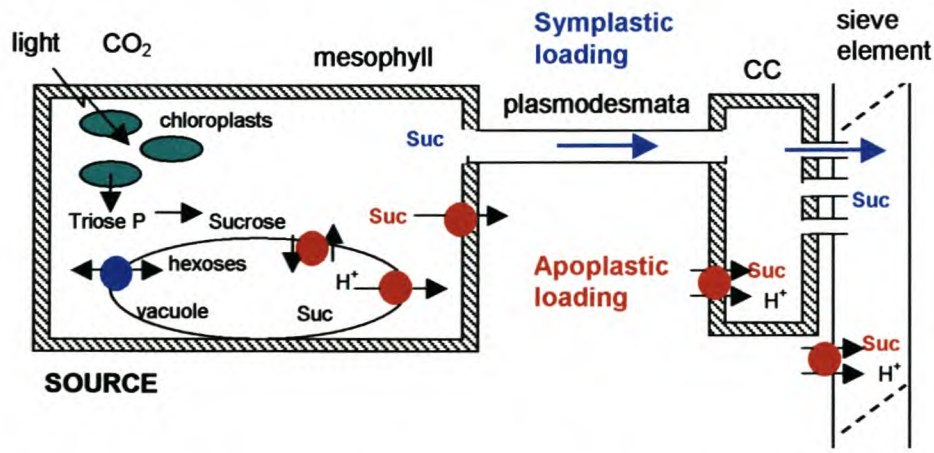

Figure 1. Diagram of symplastic and apoplastic phloem-loading mechanisms. The blue circle represents a hexose transporter and the red circles represent sucrose transporters. Abbreviations: CC, companion cell; P, phosphate; Suc, sucrose (adapted from Lalonde et al., 1999).

Various theoretical transport routes have been put forward (Tucker et al., 1989; Gamalei et al., 1994), none of which could expound the thermodynamic incompatibility of symplastic loading with the sugar concentrations found in the SE/CC-complex. By studying members of families with true intermediary cells such as the Cucurbitaceae (Turgeon et al., 1975; Schmitz et al., 1987), the polymer trap model arose (Turgeon, 1991). According to this mechanism, sucrose and galactinol that are synthesised in the mesophyll, diffuses into the companion cells where it is converted to raffinose, or eventually stachyose or verbascose (Holthaus and Schmitz, 1991; Beebe and Turgeon, 1992). The diameters of these sugars exceed the molecular exclusion limit of the plasmodesmata between companion cells and

mesophyll and, as a result, accumulate to high concentrations in these cells (Turgeon, 1991; Grusak et al., 1996). They are subsequently transferred into the sieve elements, possibly by diffusion, and translocated away by mass flow.

The exception to the phloem-loading mechanisms described above is the absence of phloem loading, as found in willow (Turgeon and Medville, 1998). No difference in solute potential could be demonstrated between the mesophyll and minor vein phloem. It is thought that photoassimilate diffuses from the mesophyll cells into the companion cells without an active transport step (Turgeon and Medville, 1998). This is achieved through the high sucrose concentration in the mesophyll cells, which provides a gradient for diffusion for sucrose into the minor vein phloem.

The different mechanisms of sieve element loading may have ecophysiological significance in that the species dedicated to either of the mechanisms, show a preference for specific climatic zones. It would seem that apoplastic loading prevails in species native to temperate and arid zones, whereas symplastic loading is associated with tropical rainforest species (Gamalei, 1991; Van Bel and Gamalei, 1992). This phenomenon could have an evolutionary origin since, hypothetically, symplastic-loading plants could have switched to an apoplastic loading mechanism due to temperature or water stress (Gamalei, 1991).

Sucrose export can be controlled on different levels, including the production and partitioning of photoassimilates, compartmentation thereof between and within cells, entry of sucrose into the apoplast and the loading mechanism employed (Giaquinta, 1983). The possible control mechanisms that operate at the loading sites include regulation of the sucrose transporters and sucrose concentration within the phloem. Turgor changes can also play an important role since information about the rate of unloading in the sink organs may be relayed to the source as a hydrostatic pressure change in the veinal network. Another possibility is that the rate of water entry into the sieve elements can change in response to changes in sink demand (Giaquinta, 1983).

2.3.1.2 Long distance transport of sugars

Following sieve element loading, sucrose and other solutes are translocated away from the source to the sink tissues, moving through the vascular system in long-distance transport. Translocation of these solutes is driven by mass flow, due to water entering the phloem during the loading process and thereby creating an osmotic pressure gradient along the phloem elements (Münch, 1927). The composition of phloem sap is highly variable from species to species. Dissolved in the phloem transport water are the translocated solutes, which in most plants, consist mainly of carbohydrates (Ward et al., 1998). This can constitute disaccharides (sucrose), oligosaccharides (raffinose, stachyose, verbascose) and sugar alcohols (sorbitol, mannitol), depending on the species.

Plants with a type-1 minor vein configuration translocate 20-80% of sugars in the form of raffinose-related compounds, whereas those with a type-2 configuration translocate sucrose almost exclusively (Gamalei, 1985). A steep concentration gradient is maintained in the SE/CC-complex by the symplastic isolation from surrounding cells along the conducting pathway (Van Bel and Kempers, 1991; Oparka et al., 1995; Ammerlaan et al., 1996). This, along with the high translocation rates found in phloem (0.5-3 $m.h^{-1}$), minimise the diffusion of molecules such as sucrose, which can reach concentrations of 0.2-1.6 M in the phloem sap (Kallarackal et al., 1989; Winzer et al., 1996). Active retrieval by sucrose transporters, however, is still necessary to reduce the loss of sucrose along the translocation path. The high build-up of sucrose in the phloem is made possible by its physicochemical properties: a low viscosity in concentrated solutions and its chemical stability as a nonreducing disaccharide. Glucose and fructose are linked through anomeric carbon groups disarming the functional reducing groups (Lucas and Madore, 1988), which renders it an ideal compound for long-distance translocation.

Other transported organic solutes include amino acids and amides, especially glutamate and aspartate (Riens et al., 1991), as well as most of the endogenous plant hormones such as auxin, gibberellins, cytokinins, and abscisic acid. Nucleotidephosphates and proteins with a molecular mass of 20-60 kD have also been found in phloem sap (Sjölund, 1997). It is thought that these proteins are synthesised and processed in the companion cells with subsequent movement via pores into the sieve elements (Fisher et al., 1992). Long-distance transport of low levels of endogenous mRNA has also been found, which has been interpreted as a possible gene-specific systemic-acquired silencing mechanism (Jorgensen et al., 1998). Inorganic solutes that move in the phloem include potassium, magnesium, phosphate, and chloride. Nitrate, calcium, sulphur, and iron are almost completely excluded from the phloem. Encapsulated virions (Nelson and Van Bel, 1998) are also frequently found in the phloem sap since practically all plant-infecting viruses utilise the phloem for their systemic spread throughout the plant (Leisner and Turgeon, 1993; Almon et al., 1997).

The direction and rate of phloem transport are dependent on several factors, including relative source and sink strength, proximity of source to sink, and the interconnections of the vascular system (Patrick, 1991), which all relate to the pressure gradient in the transport stream. These relationships are altered by physiological, developmental and pathological events, including temperature (Giaquinta and Geiger, 1973), light (Fondy et al., 1989), and osmotic conditions (Patrick, 1994).

2.3.1.3 Sugar transport from the vascular tissues

Phloem unloading of photosynthate is associated with a series of transport events, including sieve element unloading from the SE/CC-complex and post-sieve element

transport to recipient sink cells for storage or metabolism (Patrick and Offler, 1995). Sieve element unloading represents the exit of photosynthate from the sieve elements and thus plays an important role in regulating solute translocation to, and partitioning between, sinks (Wardlaw, 1990). It has to be taken into account that the different unloading pathways are, as in the case of phloem loading, not mutually exclusive, but may operate either simultaneously or successively in developing tissues.

Sieve element unloading mechanisms. As in the situation of phloem loading, two distinct pathways also exist for the unloading of photoassimilates from the sieve elements: an apoplastic and a symplastic mechanism (Fig. 2) (Ho, 1988). In the apoplastic-unloading pathway, imported sucrose is introduced directly into the apoplast followed by uptake by the sink cells with or without sucrose hydrolysis. In contrast, sucrose remains in the symplast and moves from the phloem to the sink cells through the plasmodesmata in the symplastic pathway.

The symplastic sieve-element unloading mechanism seems to be the most common pathway, whereas an apoplastic step at or beyond the sieve element boundary seems to be retained for specialised situations (Fisher and Oparka, 1996; Patrick, 1997). Although high plasmodesmal connectivity is associated with symplastic unloading, it should be noted that some plasmodesmata appear to be non-functional (Van Bel and Oparka, 1995). It is only in the complete absence of plasmodesmata that an apoplastic-unloading step is certain. The unloading mechanism employed appears to be influenced by sink development and function and is therefore a dynamic rather than static property of a particular sink (Patrick, 1990; Patrick and Offler, 1996). The unloading pathway can undoubtedly occur by both routes in many situations, depending on physiological and developmental control.

During symplastic unloading, no membranes are crossed *en route* to the sink cells. Movement through the plasmodesmata is passive, since transported sugars move from a high concentration in the sieve elements to a low concentration in the sink cells. The low concentration in the sink cells is maintained by continuous utilisation of sucrose, which is facilitated by soluble acid invertase activity in the vacuole and soluble neutral/alkaline invertase activity in the cytoplasm (Chapleo and Hall, 1989). Invertase thus plays an important role in phloem unloading and the utilisation of sucrose in sink cells, which is needed for respiration and the conversion into other growth-related compounds.

Sinks acting as permanent storage organs accumulate reserves continuously, have an irreversible assimilate import system, and usually employ symplastic unloading. Sink organs that utilise a symplastic-unloading mechanism include shoot and root apices, potato tubers, and *Agrobacterium tumefaciens*-induced tumours (Fisher and Oparka, 1996; Patrick, 1997; Pradel et al., 1999; Oparka and Santa Cruz, 2000). Even though symplastic unloading may be the primary unloading

pathway in these organs, sugars may still leak into the apoplasm from where it is retrieved by sugar transporters (Chapleo and Hall, 1989; Sauer et al., 1994). Symplastic unloading is primarily controlled by plasmodesmal conductivity and in the long term, through developmental shifts in plasmodesmal numbers (Patrick, 1997). Regulation by concentration or turgor differences is also fundamental in that the metabolic demands of the sink cells determine these gradients. The metaphloem of roots is isolated symplastically from surrounding tissue (Van Bel, 1993). Research by Schulz (1994) suggests that symplastic unloading from the protophloem is the most important pathway of assimilate unloading in roots. In developing sink leaves, phloem unloading of imported photoassimilate occurs primarily symplastically from large class-III veins (Turgeon, 1987, 1991). However, an apoplastic-unloading mechanism was found to operate in the developing leaves of *Zea mays* (Evert and Russin, 1993).

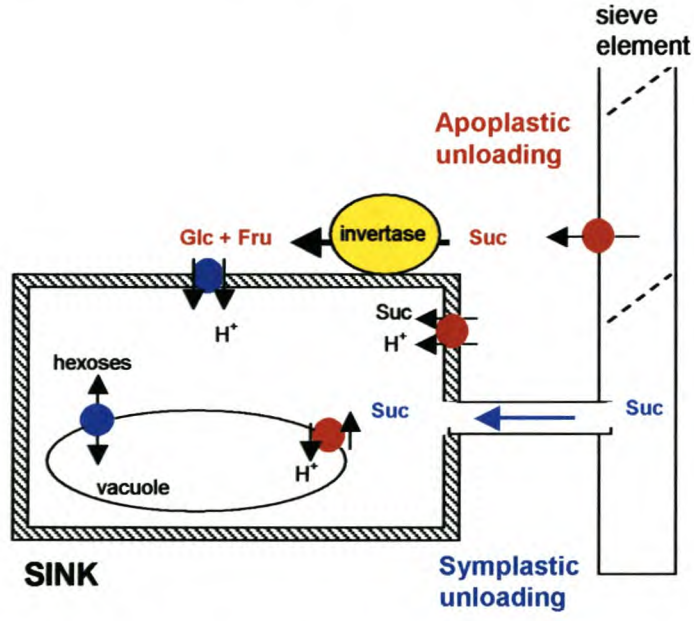

Figure 2. Diagram of symplastic and apoplastic phloem-unloading mechanisms. Blue circles represent hexose transporters and red circles represent sucrose transporters. Abbreviations: Fru, fructose; Glc, glucose; Suc, sucrose (adapted from Lalonde et al., 1999).

Apoplastic unloading from the phloem tissue can occur by diffusion, driven by sucrose gradients (Patrick, 1990) or possibly by sucrose transporter activity (Kühn et al., 2003). Metabolic conversions help to maintain the concentration gradient, which is mediated by the partial metabolisation of sucrose in the apoplast. Here it can be taken up as sucrose, or as glucose or fructose subsequent to hydrolysis by cell wall invertases. The importance of cell wall invertases in the sucrose metabolism of sink organs during the prestorage phase has been well documented (Sturm, 1999). The uptake of these sugars from the apoplast is carrier-mediated by a sugar/proton symport. Therefore, the apoplastic loading of sucrose into sink tissues is energy dependent. The uptake of monosaccharides into sink cells will lower the

H^+ concentration in the apoplast and activate phloem K^+ channels (Ache et al., 2001). These channels are involved in the control of the membrane potential and therefore sugar and K^+ translocation.

Apoplastic unloading occurs in specialised situations, which include the axial pathway in plants with a lowered source/sink ratio, sinks that accumulate photosynthate to high concentrations, and in solute passage into developing seeds (Patrick and Offler, 1996). During long distance transport, photoassimilates are unloaded along the entire length of the axial phloem path (Thorpe and Minchin, 1996). The apoplastic pathway is reversible in the axial pathway of the plant, but irreversible during the development of terminal sinks (Lucas et al., 1993). During direct sugar exchange from the SE/CC-complexes of the axial path, sucrose is unloaded down a steep concentration gradient. In this case, unloading is regulated by the difference between the rates of diffusion of sugar into the apoplast and its retrieval by sucrose/proton symport mechanisms (Patrick, 1997). Exchange during an apoplastic step, subsequent to symplastic transport from the SE/CC-complexes in terminal sinks, is governed by sucrose concentration gradients. These are maintained by the hydrolysis of sucrose by cell wall invertase (Damon et al., 1988; Ho, 1988; Sturm, 1999).

In terminal reproductive storage sinks such as developing seeds, a symplastic discontinuity exists between the maternal and filial tissues. It has been shown that developing embryos import sugars exclusively by an apoplastic mechanism (Weber et al., 1997a; Imlau et al., 1999), which is consistent with the lack of symplastic connections between the embryo and the maternal tissue. In anthers, symplastic post-phloem transport can proceed toward the cells of the middle layer, however, no further symplastic connections exist to the tapetal cells (Clément and Audran, 1995). Photoassimilate is typically delivered to the locular fluid by a plasma membrane transporter (Truernit et al., 1999).

At the subapical region of potato stolon tips, a terminal vegetative storage sink, a switch from apoplastic to symplastic phloem unloading occurs during tuberisation (Viola et al., 2001). Apoplastic phloem unloading predominates in stolons undergoing extension growth, whereafter, at the initiation of tuberisation, a switch to symplastic unloading takes place, with the concomitant decline in soluble invertase activity. Sink organs that remobilise their reserves, such as unmodified stems, display reversible import of assimilate and their unloading can switch from symplastic to apoplastic during certain stages of plant development. In stem elongation zones, both apoplastic- and symplastic-unloading pathways can be utilised depending on the whole plant source/sink ratio (Patrick and Offler, 1996). Rapid and reversible switching between symplastic- and apoplastic-unloading mechanisms along the axial pathway also seems to be regulated by source/sink ratios (Oparka and Prior, 1992; Patrick and Offler, 1996).

In terminal reproductive storage sinks such as fleshy fruits, different sequential unloading pathways exist. It has been found that a symplastic-unloading mechanism

is operable in developing tomato fruit, whereas an apoplastic unloading route develops with the switch from starch to soluble sugar accumulation (Johnson et al., 1988; Offler and Horder, 1992). In contrast, citrus fruit accumulates sugars to high concentrations and seem to employ an apoplastic-unloading mechanism throughout their development (Koch et al., 1986).

Post-phloem transport. Subsequent to sieve element unloading, assimilates are transported through a diverse range of non-phloem tissues in post-phloem transport (Fisher and Oparka, 1996). Continuity of either the tissue symplasm or apoplasm facilitates solute transport through these routes within sink organs. The cellular pathway employed for post-phloem sugar transport seems to be developmentally controlled and is associated with characteristic sugar transporter profiles. In tomato, a developmental shift of the cellular pathway for sugar post-phloem transport is displayed in the fruit (Ruan and Patrick, 1995). Subsequent to phloem unloading, the post-phloem transport pathway shifts from a symplastic route (throughout starch accumulation) to an apoplastic route (throughout hexose accumulation) during fruit development. Uptake of solutes from the apoplast into the sink cells relies on hexose or sucrose transporters, depending on the sink type. These transporters facilitate the active transfer of sugars from the apoplast across the plasma membrane in lateral transport. In sink cells that accumulate sugars to high concentrations, active transport across the tonoplast of the vacuole is also mediated by a sugar transporter.

It has been shown that cells of the post-phloem pathway are specialised for their transport function in that they possess plasmodesmata with a high molecular size exclusion limit, which facilitates rapid fluxes of solutes (Oparka et al., 1999; Fisher and Cash-Clark, 2000). This allows for the non-specific escape of a broad range of small macromolecules (<50 kD) from the phloem, which could be associated with the significant capacity of sink tissues for detoxification. Larger molecules may require recognition factors that facilitate a specific interaction with plasmodesmata (Fisher and Cash-Clark, 2000). Thus, the post-phloem pathway appears to be an active site of sequestration, with the products often being degraded or stored in the vacuoles (Fisher and Cash-Clark, 2000).

2.4 PROTEINS INTEGRALLY LINKED TO SUGAR TRANSPORT AND PARTITIONING IN HIGHER PLANTS

2.4.1 Plant sugar transporters

Sugar transporters play a crucial role in the distribution of sugars throughout the plant. Long distance transport of sugars between source and sink tissues results from a combination of apoplastic and symplastic exchanges. Apoplastic transport involves the uptake of sugars against a gradient across the plasma membrane, and involves co-transport with protons, which is driven by a proton-pumping ATPase

(Humphreys, 1988). The transport of carbohydrates across the plasma membrane is catalysed by two families of sugar carriers, namely monosaccharide transporters (MST) and disaccharide transporters (DST), both of which operate in conjunction with H^+-ATPases. A number of plasma membranes have to be crossed between the site of sucrose synthesis in a source cell, and the site of utilisation or storage in a sink cell. Several types of transporters might be responsible for the different types of transport, i.e. there are five predicted transport steps along the translocation path. The first step is the release of sucrose from the mesophyll cells of leaves, into the apoplastic space by a predicted facilitator or antiporter (Delrot, 1989). Sucrose is then taken up into the phloem by at least one transporter. The re-uptake of sucrose is also necessary along the translocation pathway (Minchin and Thorpe, 1987). In sink tissues, unloading can occur via the apoplastic pathway by means of sucrose efflux transporters, functioning as facilitators or antiporters (Walker et al., 1995) Sucrose in the apoplast can subsequently be taken up directly by a sucrose transporter, or by means of a hexose transporter, following the hydrolysis of sucrose into glucose or fructose. It should be noted, however, that only preliminary evidence exists for such efflux activities (Kühn et al., 2003).

2.4.1.1 Isolation and characterisation of plant sugar transporters

Physiological, biochemical and molecular approaches have revealed that plasma membranes contain several sugar transporters that have been characterised by kinetics, tissue localisation and sensitivity to sulfhydryl-modifying reagents. Plant MSTs and DSTs belong to a large superfamily of transmembrane facilitators that possibly evolved from a single ancestral transporter protein (Marger and Saier, 1993). On the amino acid level, the different MSTs and DSTs show a high degree of homology among themselves (Büttner and Sauer, 2000; Lemoine, 2000). The comparison of MSTs to DSTs, on the other hand, reveals that these two types of transporters are less closely related, with only 20% homology between them (Sauer et al., 1994). Despite this fact, their tertiary structures show a high degree of conservation. Typically, these sugar transporters contain several hydrophobic regions, which could be assigned to 12 membrane-spanning regions (Marger and Saier, 1993). These regions are separated by a large central loop and contain conserved structural features. The first and second halves of the protein display a high degree of homology (Sauer and Stolz, 1994). Although there is no three-dimensional structural data available for the sugar transport proteins, topological models exist, based on the identification of functional regions (Fig. 3) (Büttner and Sauer, 2000).

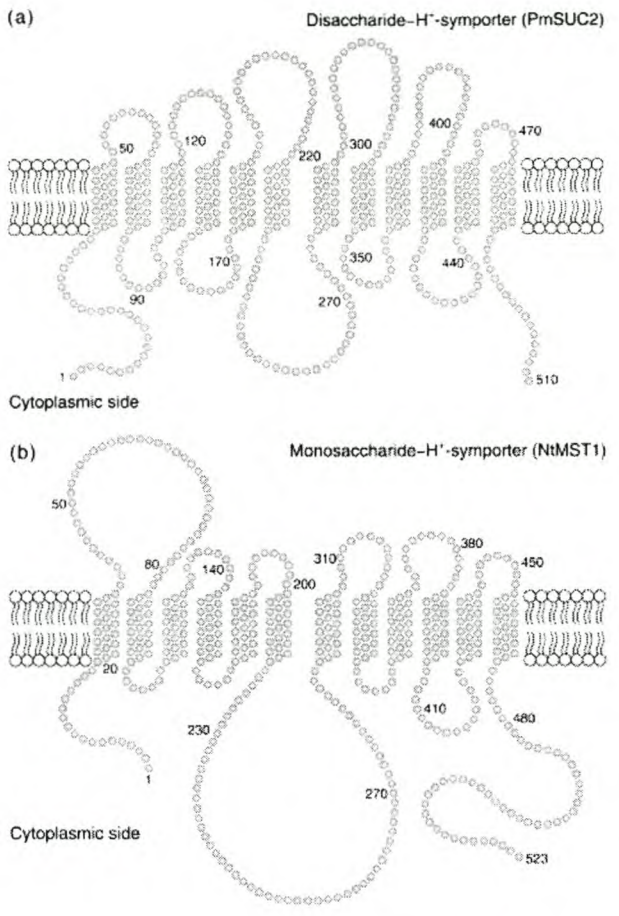

Figure 3. Topological models of a disaccharide transporter (a) PmSUC2 from *Plantago major* and a monosaccharide transporter (b) NtMST1 from tobacco (Williams et al., 2000).

Isolation and characterisation of this large family of sugar transporters is accomplished by means of functional complementation in yeast strains, such as *Schizosaccharomyces pombe* (Sauer et al., 1990) and *Saccharomyces cerevisiae* (Riesmeier et al., 1992). These strains are modified by the deletion of the secreted invertase encoding gene, as well as the addition of sucrose synthase activity, enabling the yeast cells to metabolise ingested sucrose. Electrophysical characterisation of the transporters is done by expression in oocytes of *Xenopus laevis* (Boorer et al., 1992). Sugar transporters commonly consist of 42 to 62-kD polypeptides, with pH optima in the range of pH 5.0-6.0. Active sucrose transport in higher plants results from the simultaneous operation of at least two distinct mechanisms. The first is a saturable mechanism, which displays a high affinity for sucrose and is H^+-mediated (Lalonde et al., 1999). This mechanism has a pH optimum of 5.5 and is sensitive to sulfhydryl-modifying reagents (Lemoine, 2000). The second mechanism is nonsaturable, independent of H^+ movement and has a low sucrose affinity (Lalonde et al., 1999; Weise et al., 2000). This mechanism is relatively insensitive to sulfhydryl-modifying reagents, and sucrose uptake is not pH-dependent.

Most of the sucrose transporters studied employ the saturable mechanism of sucrose transport and have K_m values for sucrose of 0.4-1.5 mM (Table 1). They are sensitive to sulfhydryl-modifying reagents, as well as uncouplers such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Riesmeier et al., 1992). The sucrose transporters of spinach, potato, tomato, tobacco and *Arabidopsis* all belong to this group. The transporters of potato, spinach, *Plantago major* and *Arabidopsis thaliana* also mediate the uptake of maltose, although with a substantially lower affinity (Riesmeier et al., 1993b; Sauer and Stolz, 1994). In contrast, the sucrose transporter of sugar beet is highly specific for sucrose (Bush, 1989).

Table 1. List of various characterised high-affinity disaccharide transporters (DSTs) (Adapted from Lemoine, 2000).

DST	Species	K_m (mM)	Acc number	References
AgSUT1	Celery	0.14	4091891	Noiraud et al. 2000
AtSUC1	*Arabidopsis*	0.45	481132	Sauer and Stolz, 1994
AtSUC2	*Arabidopsis*	0.53	407092	Sauer and Stolz, 1994
AtSUTX1	*Arabidopsis*		2160188	Vystskaia et al. unpub
AtSUTX2	*Arabidopsis*		3287687	Rousley et al. unpub
AtSUTX3	*Arabidopsis*		3810593	Rousley et al. unpub
AtSUTX4	*Arabidopsis*		3461813	Vystskaia et al. unpub
BvSUT1	Sugar beet		633172	Westram et al. unpub
DcSUT1a/b	Carrot	0.5	2969889	Shakya and Sturm, 1998
DcSUT2	Carrot	0.7	2969884	Shakya and Sturm, 1998
LeSUT1	Tomato		575299	Bürkle and Frommer, unpub
NtSUT1	Tobacco		575351	Bürkle et al. 1998
NtSUT3	Tobacco		149981	Lemoine et al. 1999
OsSUT1	Rice		2723471	Hirose et al. 1997
PmSUC1	*Plantago*	0.3	1086253	Gahrtz et al. 1996
PmSUC2	*Plantago*	1.0	415988	Gahrtz et al. 1994
RcSCR1	Castor bean	2.0	542020	Weig and Komor, 1996
SoSUT1	Spinach	1.5	549000	Riesmeier et al. 1992
StSUT1	Potato	1.0	542087	Riesmeier et al. 1993b
VfSUT1	Broad bean	1.4	Z93774	Weber et al. 1997a

It has been shown that a large portion of substrate recognition by sucrose transporters may arise from the interaction by the hydrophobic portion of the sucrose molecule and the hydrophobic region of the transporter-binding site (Hitz et al., 1986). The potato StSUT1 symporter was expressed in *Xenopus* oocytes to investigate the exact transport mechanism utilised (Boorer et al., 1996). Based on the kinetic data obtained from these experiments, the following model was proposed: a proton binds to the external region of the negatively charged transporter, prior to sucrose binding. The fully loaded transporter then undergoes a conformational change and releases the ligands at the cytoplasmic surface. The binding sites subsequently return to the external surface to complete the transport cycle.

The activities of the neutral sucrose transporters, AtSUC1 and PmSUC1 are unchanged between pH 4.0-6.0, but decrease to 50% at pH 7.0 (Sauer and Stolz, 1994; Gahrtz et al., 1996). This rather pH-insensitive transport mechanism

distinguishes them from other acid sucrose transport proteins such as AtSUC2, SoSUT1, and StSUT1, which display increasing transport rates with decreasing extracellular pH. There is an indication that all the acid sucrose transporters are localised in the phloem and that their core function is the loading of sucrose into the phloem: the DSTs of *Plantago* and *Arabidopsis* are localised in the companion cells (Stadler et al., 1995; Stadler and Sauer, 1996), whereas SUT1 from potato, tobacco and tomato is detected in the plasma membranes of sieve elements only (Kühn et al., 1997). It is thought that transcription of the encoding genes occurs within the companion cells and that either the mRNA or the protein is transported via the plasmodesmata to the sieve element.

Recently, AtSUC5 was characterised as a dual biotin and sucrose transporter, with transport properties typical of SUT1 transporters (Ludwig et al., 2000). It is postulated that biotin-H^+ symport may be a general feature of all higher plant sucrose transporters. A number of sucrose transporter cDNAs from monocotyledonous plants have been obtained, including *OsSut1-5* from rice (Hirose et al., 1997; Aoki et al., 2003), *ZmSut1* from maize (Aoki et al., 1999) and *HvSut1* from barley (Weschke et al., 2000). OsSUT1 is ~78% homologous to dicotyledonous sucrose transporters at the amino acid level and contains most of the common sugar transporter characteristics. mRNA from the first isolated sucrose transporter from an oligosaccharide-translocating species, AmSUT1, has been detected in the phloem sap of *Alonsoa meridionalis* by Knop et al. (2001). Although the structural properties of this plant are typical of a symplastic sieve element-loading mechanism, a sucrose transporter may be involved in the loading of sucrose as it is postulated that more than one loading mechanism is operable in certain plants. Another possibility is that AmSUT1, whose expression is higher in stem tissue than source leaves, functions in sucrose retrieval during long-distance transport.

Low-affinity sucrose transporters display a nonsaturable mechanism of sucrose uptake, with a K_m for sucrose of 11-12 mM. These transporters include the 62-kD sucrose binding protein (SBP) of soybean (Overoorde et al., 1996) and SUT4 of *Arabidopsis*, tomato, and potato (Weise et al., 2000). According to the deduced amino acid sequence, the SBP is not a typical integral membrane protein, although studies revealed that this hydrophilic protein is tightly associated with the membrane, with the only putative transmembrane domain present at the N-terminus (Overoorde and Grimes, 1994). It is hypothesised that the SBP could be located on the external surface of the plasma membrane to function as an external sucrose concentration sensor in the apoplast (Overoorde and Grimes, 1994).

Sucrose transporter-like proteins, such as SUT2 from tomato and *Arabidopsis*, have, in addition to the predicted 12 transmembrane domains, extended N-terminal and central loop domains of 30-50 amino acids longer respectively, than other DST members (Barker et al., 2000). These loops are localised intercellularly to allow for interaction with cytoplasmic proteins. AtSUT2 has a K_m for sucrose of 1.9 mM, low expression levels of the encoding gene, and a high sensitivity to CCCP and PCMBS

(Meyer et al., 2000). They are more strongly detected in sink than in source leaves and are inducible by sucrose (Schulze et al., 2000).

Most of the characterised MSTs (Table 2) are energy-dependent H^+-symporters, which mediate the high-affinity transport of a range of hexoses and pentoses, with K_m values of 10-200 μM for the preferred substrate (Büttner and Sauer, 2000). Substrates that are efficiently transported by MSTs include, β-D-glucose, 3-O-methyl β-D-glucose, 2-deoxy β-D-glucose, α-D-mannose and β-D-fructose. β-L-glucose and β-D-ribose are, however, not included (Gogarten and Bentrup, 1989).

Table 2. List of various characterised monosaccharide transporters (MSTs) (adapted from Büttner and Sauer, 2000).

MST	Species	Transported substrates	K_m (μM)	References
AtSTP1	*Arabidopsis*	Glc>>Gal>>Fru	Glc: 20	Sauer et al. 1990
AtSTP2	*Arabidopsis*	Gal	Gal: 50	Truernit et al. 1999
AtSTP3	*Arabidopsis*	Glc	Glc: 2000	Büttner et al. 2000
AtSTP4	*Arabidopsis*	Gal	Glc: 15	Truernit et al. 1996
AtSTP5-14	*Arabidopsis*			Büttner et al. 2000
BvMST1	Sugar beet			Chiou and Bush, 1996
BvMST2	Sugar beet			Chiou and Bush, 1996
BvMST3	Sugar beet			U43629
LeMST1	Tomato			AJ010942
MtST1	*Medicago truncatula*	Glc		Harrison, 1996
NtMST1	Tobacco	Glc>>Gal=Xyl		Sauer and Stadler, 1993
OsMST2-3	Rice			Toyofuku et al. 2000
OsMST5	Rice			Ngampanya et al. 2003
PaMST1	*Picea abies*			Z83829
PhPMT1	Petunia			Ylstra et al. 1998
RcHEX1	Castor bean			Weig et al. 1994
RcHEX3	Castor bean	Glc	Glc: 80	Weig et al. 1994
RcHEX6	Castor bean			Weig et al. 1994
SspSGT2	Sugarcane			Bugos and Thom, 1993
VfSTP1	Broad bean	Glc	Glc: 30	Weber et al. 1997a
VvSTP1	Grape			AJ001061

MSTs exist in large, differentially regulated families of hexose transporters. *A. thaliana* contains at least 14 hexose transporters (Büttner et al., 2000), *Ricinus communis* contains eight (Weig et al., 1994), and *C. rubrum* possesses a family of seven MSTs (Roitsch and Tanner, 1994). Many of the characterised MST multigene families display a very specific mode of expression in sink organs, e.g. *AtStp1* is found in the ovaries of *Arabidopsis* flowers, *AtStp2* in anthers, *AtStp3* in the mesophyll cells of leaves, and *AtStp4* in the anthers and roots (Truernit et al., 1996; Truernit et al., 1999; Büttner et al., 2000). AtSTP1, from *Arabidopsis*, was the first characterised sugar transporter of a higher plant (Sauer et al., 1990). It was shown to have a K_m for D-glucose of 20 μM. D-xylose and D-galactose are also substrates of this transport protein. The kinetic mechanism of monosaccharide-H^+ symport for AtSTP1 involves a sequential sugar uptake mechanism (Boorer et al., 1994). This

means that protons and sugar molecules are imported in two separate steps, rather than simultaneously. In addition, studies with beetroot and maize protoplasts revealed the existence of a low-affinity transport mechanism (Lin et al., 1984; Getz et al., 1987). It was, however, only recently that the first MST belonging to this group, AtSTP3, was cloned (Büttner et al., 2000).

Multigene MST families have also been characterised in monocotyledonous plants. For example, four MSTs were characterised in *Oryza* spp. (Toyofuku et al., 2000; Ngampanya et al., 2003) The isolated OsMST2, OsMST3 and OsMST5 were characteristic of other MSTs reported so far, whereas a fourth MST, OsMST1, was inactive (Toyofuku et al., 2000; Ngampanya et al., 2003).

2.4.1.2 Regulation of plant sugar transporters

Transport by sucrose transporters of the plasma membrane can be controlled directly through regulatory mechanisms imparted on the transporters, or indirectly via the regulation of the H^+- ATPase activity (for review, see Sze et al., 1999). Only direct regulation will be discussed here. Direct regulation of sugar transporters includes transcriptional and post-translational control (for review, see Delrot et al., 2000). Transcriptional regulation includes developmental expression, diurnal control, environmental signals, and nutritional control. It has been proposed that the differential expression of sugar transporters is due to tissue-specific promoters, regulation through substrate induction, or both.

Sucrose transporters that are under developmental regulation include *PmSuc1*, whose expression is limited to developing ovules, where the transporter loads sucrose into the developing seed (Gahrtz et al., 1996). In addition, the expression of *VfSut1* and *VfStp1* is detected at different stages of cotyledon differentiation in fava bean seeds (Weber et al., 1997a). The V*vSuc11*, *VvSuc12* and *VvSuc27* genes of *V. vinifera* also display developmental regulation with distinct expression levels during the different stages of berry development (Davies et al., 1999). MSTs displaying developmental regulation in sink tissues include *VvHt1* of *V. vinifera*, which displays a biphasic expression pattern, and *VvHt2*, whose expression is closely related to the pattern of sugar accumulation in the ripening grape berry (Fillion et al., 1999). The expression of some sucrose transporters, such as *AtSuc2* and *StSut1*, follows the sink-to-source transition of leaves, which correlates with the development of sucrose transport in leaves upon maturation, with the highest expression in mature leaves (Lemoine et al., 1992). Additionally, the expression of the tobacco *NtSut3* transporter gene is restricted to late pollen development, pollen germination and pollen tube growth (Lemoine et al., 1999), indicating that pollen is supplied not only with glucose, but also with sucrose through a specific sucrose transporter.

In addition to developmental regulation, sucrose transporters are also subject to diurnal regulation. For example, SUT1 sucrose transporters are diurnally regulated

at both the mRNA and protein levels (Kühn, et al., 1997). Environmental signals also seem to play a role in symporter expression and activity. The impact of wounding and ageing on transporter mRNA and protein abundance has been illustrated by the work done by Sakr et al. (1997), where both MST and DST transcripts increased in response to the stimuli. However, only wounding stimulated transporter activity. The sink-specific MST of *Arabidopsis*, AtSTP4, is also regulated by environmental stress, such as wounding, elicitors, and pathogen attack (Truernit et al., 1996). The co-ordinated increase in cell wall invertases in response to stress (Sturm and Chrispeels, 1990) confirms the close interaction of these enzymes and MSTs during stress response.

In *C. rubrum*, it was found that levels of the MST, *Cst2*, were highly elevated by the cytokinin, zeatin (Ehness and Roitsch, 1997). This was also accompanied by an increase of cell wall invertase transcripts. It has also been shown that *Sut1* expression can be regulated by phytohormones (Harms et al., 1994).

The regulation of sugar transporter genes in response to sugars, appear to be a general feature in many organisms. However, the sugar sensitivity of sugar transporter expression varies from transporters being not sensitive (Stadler and Sauer, 1996), repressed (Weber et al., 1997a; Chiou and Bush, 1998; Vaughn et al., 2002), or stimulated (Hilgarth et al., 1991; Atanassova et al., 2003) by the presence of sugars. A sucrose-dependent signal transduction pathway, which regulates the sucrose symporter in mature leaf tissue, was identified by Chiou and Bush (1998). They reported a decrease in sucrose symporter mRNA levels in leaves due to the exogenous feeding of sucrose. These results indicate a sink regulation pathway: a decrease in sink demand leads to increased sucrose concentrations in the phloem, which, in turn, down-regulates the sucrose symporter (Chiou and Bush, 1998; Vaughn et al., 2002). An equivalent regulatory mechanism exists for photosynthetic genes (Krapp et al., 1993), and invertases (Roitsch et al., 1995). The first example of induction of a MST by sugar was reported for *VvHt1* of grape (Atanassova et al., 2003). This MST was found to be induced by its own substrate, glucose, as well as sucrose. It was concluded that *VvHt1* is regulated by glucose and glucosyl-containing disaccharides.

Post-translational regulation includes phosphorylation and protein turnover. Regulation by phosphorylation is the most common type of post-translational control. Roblin et al. (1998) concluded that phosphorylation inactivates sucrose transporters. Turnover rates of transporter proteins are also an important regulatory mechanism. Studies have shown that the half-life of transporter proteins is in the range of only a few hours, rendering this a relatively responsive system (Kühn, et al., 1997). Active transport of sucrose can be inhibited by sulfhydryl-modifying reagents (Giaquinta, 1976), such as PCMBS and *N*-ethylmaleimide (NEM), histidine reagents, such as diethyl pyrocarbonate (DEPC), and $HgCl_2$. DEPC, but not PCMBS-binding, is substrate protectable, implicating binding at or near the active site of the transporter (Bush, 1993). The PCMBS-sensitive residue is therefore not associated with the

substrate-binding site. DEPC binding can be partially reversed by hydroxylamine, indicating that a histidine residue, at or near the active site, may play an essential role in the reaction mechanism. Further analysis proved that the histidine at position 65 is conserved, and that this residue is involved in the rate-limiting step in the transport reaction (Lu and Bush, 1998). Another strong direct inhibitor is sulphite, which can inhibit transport activity, and thus phloem loading at 1 mM (Maurousset et al., 1992). This accounts for the observed reduction in growth of plants exposed to sulphur dioxide, a major air pollutant.

The differential regulation of sugar transporters, through external and internal signals, constitutes an important link between environmental and developmental information and plant growth responses.

2.4.1.3 Functional analysis of plant sugar transporters

The function of sugar transporters in sugar transport and partitioning is critical. Sucrose transporters play a significant role in primarily phloem loading, but could also be involved in unloading into sink tissues, the retrieval of sucrose leaking from the phloem, and transport across the tonoplast (Fig. 4). To elucidate the function of transporters in assimilate partitioning, transgenic plants with altered transporter gene expression were examined. Transgenic potato and tobacco plants with reduced *Sut1* mRNA levels, due to antisense gene repression, showed a dramatic increase in leaf carbohydrate content and reduced development of roots and tubers, which is consistent with the assumption that these sucrose transporters are essential for phloem loading (Riesmeier et al., 1994; Bürkle et al., 1998; Schulz et al., 1998). The light period and light intensity had a strong influence on the observed phenotype. Sucrose and starch showed a 5-10 times increase in comparison to that produced in wild type plants, and hexoses even more. Starch grains were prominent in the mesophyll and bundle sheath cells, thus indicating a build-up of assimilates at the SE/CC-complex border.

The results of these experiments provide direct evidence for an apoplastic pathway in phloem loading. The localisation of StSut1 and NtSut1 to the minor veins of phloem, specifically to the sieve-element plasma membranes, also supports a role for these transporters in phloem loading. By way of a sucrose-sensing pathway, plants can sink-regulate phloem loading via its sucrose symporters. It would seem that a reduction in sink demand for sucrose leads to a build-up of sucrose in the phloem, which in turn down-regulates sucrose symporter activity, and thus phloem loading (Chiou and Bush, 1998; Vaughn et al., 2002).

Many of the DSTs occur in both source and sink organs. The expression of genes encoding sucrose transporters, such as NtSUT1 and AtSUC2, in sink tissues was an unexpected finding (Stadler and Sauer, 1996; Bürkle et al., 1998). Expression of sucrose transporter-encoding genes has been detected in roots, flowers, and developing seeds. It is postulated that these transporters might play a role in sucrose

retrieval along the translocation path and possibly phloem unloading and/or post-phloem transport. *OsSut1* from rice was found to be expressed mostly in source organs, although it was also detected in sink organs, such as germinating seeds. This companion cell-specific transporter was thought to play a role in phloem loading. However, antisense experiments concluded that it plays no important role in carbon metabolism in leaves of the vegetative growth stage, and that either a symplastic-loading pathway is operable, or that another sucrose transporter might function in these tissues (Ishimaru et al., 2001).

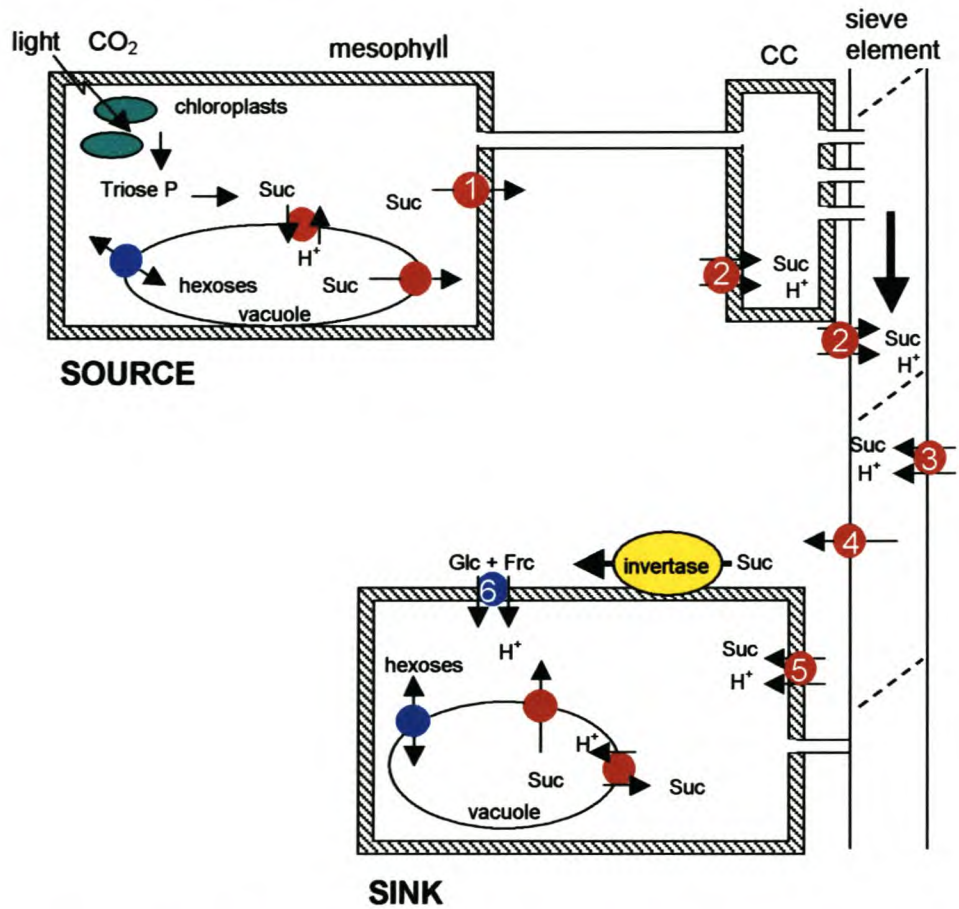

Figure 4. A diagram that illustrates the involvement of sugar transporters in sugar transport. From its point of synthesis in the mesophyll, sucrose may be loaded into the SE/CC complex either through plasmodesmata or via the apoplast. The apoplastic-loading mechanism requires sucrose export (1) from the mesophyll or the vascular parenchyma and re-uptake (2) into the SE/CC complex. Passive leakage can take place and re-uptake (3) can occur along the path of the phloem. Apoplastic phloem or post-phloem unloading necessitates a sucrose exporter at the sink tissue (4). Import of sucrose into sink tissue may occur through plasmodesmata or sucrose transporters (5). In addition, sucrose can be hydrolysed in the apoplast by cell wall invertase and taken up as hexoses (6). The vacuolar transport system could consist of a H^+/sucrose antiporter for uptake and a uniporter for release. Abbreviations: CC, companion cell; Fru, fructose; Glc, glucose; P, phosphate; Suc, sucrose (adapted from Lalonde et al., 1999).

Some DSTs are expressed exclusively in sink organs, such as *PmSuc1* of *P. major,* which is specific for flowers in this plant (Gahrtz et al., 1996). The *DcSut2* transporter from carrot is expressed mainly in the parenchyma cells of the vascular tissue of the developing taproot, indicating that it might be involved in the sucrose loading of these cells (Shakya and Sturm, 1998). Furthermore, a sucrose transporter from tobacco, *NtSut3*, is exclusively expressed in symplastically isolated pollen cells (Lemoine et al., 1999).

In sink tissues, sucrose transporters seem to function in direct sucrose loading into sink cells or in sucrose retrieval, playing an important part in controlling sink strength. It has been postulated that the induction of sucrose transporters in fruits may regulate its sink strength and carbohydrate import by the retrieval of sugars from the apoplastic space, hence, establishing it as the dominant sink of the plant (Ho, 1992; Ruan et al., 1997). The results obtained by Davies et al. (1999) and Ageorges et al. (2000) also indicate a role for grapevine's sucrose transporters in the ripening-associated transport of sucrose, leading to the accumulation of hexoses in the berry vacuole.

The role of sugar transporters is also vital in seed development. Developing embryos of seeds are symplastically isolated from the maternal tissues, however, a high hexose to sucrose ratio is necessary in the promotion of mitotic activity (Weber et al., 1996). This challenge is overcome through the activity of invertases that cleave sucrose to its hexose constituents, which are then taken up by a MST, such as VfSTP1, to supply the mitotically active parenchyma of the embryo. During later stages of seed development, a DST, such as VfSUT1 in *Vicia faba*, replaces the MST in order to provide substrate for the synthesis of storage compounds (Weber et al., 1997a). HvSUT1 has been associated with sucrose accumulation and starch biosynthesis within the endosperm of barley (Weschke et al., 2000). Results obtained by Bick et al. (1998), showed that the DST, RcSUT1 of *R. communis*, is active in phloem loading of the sucrose released from the endosperm upon germination.

Recently, preliminary evidence for a role for StSUT1 in phloem unloading has been found in potato tubers (Kühn et al., 2003). The presence of the StSUT1 protein in the sieve elements of potato tubers, together with results obtained from antisense experiments, suggested that this transporter might be responsible for phloem unloading during early tuber development. It was concluded that NtSUT1 is either directly involved in phloem unloading or indirectly by regulating the apoplasmic osmolarity via its retrieval function. Further investigation, including electrophysical analysis and actual gradients for sucrose, protons and membrane potential was proposed to evaluate this possibility.

The low affinity transporter SBP is expressed in sink leaves where it is strongly associated with the plasma membrane of several cell types that are actively transporting sucrose. These include the mesophyll cells of young sink leaves, the cotyledon sink cells, and the SE/CC-complexes (the companion cells more so than the sieve elements) of mature phloem, (Grimes et al., 1992). Co-localisation with

sucrose/H^+ symporters in *V. faba*, indicated that SBP may have a regulatory role in phloem sucrose transport through interaction with SUT1 (Harrington et al., 1997). Based on structure, as well as expression studies, it was proposed that SBP, and its homologues, function as external sucrose concentration sensors in the apoplast (Pedra et al., 2000).

The SUT4 transporters are expressed predominantly in minor veins of source leaves, where high-capacity sucrose transport is needed for phloem loading (Weise et al., 2000). StSUT4 and LeSUT4 are localised to the sieve elements of the phloem and require, like SUT1, macromolecular trafficking of either the mRNA or protein to the enucleate sieve elements. AtSUT2 is found in the major veins of mature leaves, as well as in sink organs such as sepals, anthers, the pistil of flowers, and young siliques (Schulze et al., 2000). The high occurance of this protein in these tissues indicates function as a sucrose sensor in sugar partitioning. The extremely low transport rate by this DST is linked to its possible function as a flux sensor, which measures sucrose through conformational changes during transport, instead of substrate binding. The LeSUT2 of tomato co-localises with high- and low-affinity sucrose transporters in the sieve elements of tomato petioles (Barker et al., 2000). It is thought that SUT2 may also function as a receptor for extracellular sucrose and regulates the activities of the high-affinity SUT1 transporters and the low-affinity SUT4 transporters. This could be achieved through protein turnover or signal transduction, resulting in transcriptional control in the companion cell. Transporters of different affinities may be necessary for optimal sucrose affinity and uptake into the sieve elements (Lalonde et al., 1999). In addition to a possible sugar-sensing function, AtSUT2 may also have a possible role in funnelling sucrose from the mesophyll to the phloem (Schulze et al., 2000).

Another essential step in sucrose metabolism is the storage of this sugar in the vacuole. A sugar transporter, cloned from sugar beet, is the first transporter shown to be localised in the tonoplast (Chiou and Bush, 1996). It is expressed in all vegetative tissues, with increasing expression in developing leaves. It is proposed that this sugar transporter mediates partitioning between the cytoplasm and the vacuole.

The OsMST3 of rice is highly restricted to the sclerenchyma and xylem cells in the young root (Toyofuku et al., 2000). It is proposed that OsMST3 supplies monosaccharides as substrate for the formation of cellulose (for lignin synthesis in the cell) during cell wall thickening. The *Arabidopsis AtStp2* (Truernit et al., 1999) and petunia *PhPmt1* genes (Ylstra et al., 1998) are exclusively expressed in pollen. These MSTs supply the cytoplastically isolated pollen with carbohydrates during early pollen development. The induced expression of *AtStp4* upon mechanical stress and pathogen attack could also be to supply hexoses to stressed cells (Truernit et al., 1996).

In addition to the role of MSTs in nutritional carbohydrate uptake, they may also fulfil an important function in phloem unloading. In this scenario, nonregulated

glucose-uptake, together with regulated pH-dependent invertase control phloem unloading (Caspari et al., 1994). MSTs are located mainly in sink tissues where they catalyse the import of glucose and fructose into the sink cells, subsequent to sucrose cleavage by cell wall invertase. *AtStp1* of *Arabidopsis* is expressed in the seedling root of germinating seeds where it may be responsible for the uptake of glucose from the apoplast; this glucose is liberated by invertase activity subsequent to sucrose transport from the cotyledons (Sherson et al., 2000). AtSTP4 is found only at the root tip of *Arabidopsis* plants, most likely in the root meristem (Truernit et al., 1996), indicating that an apoplastic step is involved in post-phloem transport. AtSTP4 mediates the import of hexoses generated by cell wall invertase subsequent to phloem unloading.

In addition to sink organs, lower levels of MST expression are found in other plant tissues, e.g. low levels of tobacco *Mst1* have also been detected in source leaves (Sauer and Stadler, 1993). Among the hexose transporters, *AtStp1* is the most strongly expressed in source leaves (Sauer et al., 1990). It is possible that this transporter might be responsible for the retrieval of sugars that are lost into the apoplast, in source tissues.

After the transition from light to dark, the synthesis of triose-P and its conversion to hexose-P in the cytosol is ceased (Gerhardt et al., 1987). In the dark, sucrose synthesis is continued by means of starch degradation in the chloroplast. Evidence suggests that the products of starch breakdown are exported to the cytosol for sucrose synthesis, via a hexose transporter located in the chloroplast envelope (Trethewey and Ap Rees, 1994). A plastidic glucose transporter was cloned by Weber et al. (2000) from tobacco, spinach, potato, *Arabidopsis* and maize.

It is clear that these DST's and MST's fulfil a crucial function in sugar transport and partitioning of higher plants.

2.4.2 Plant invertases

Sucrose is central to most of the physiological activities and biochemical needs of plants. This nonreducing disaccharide is channeled into various pathways in different subcellular compartments: it can enter glycolysis and the tricarboxylic acid cycle for the production of ATP and NADH; it can be used for the biosynthesis of primary metabolites necessary for tissue growth and development; it may be converted into polymers, such as starch, for long-term storage; or it can be converted to secondary compounds involved in defence against pests and predators or other environmental challenges. A number of these processes may be in operation simultaneously in a cell and therefore sucrose allocation to the various processes must be efficient and under precise control.

The utilisation of sucrose as a carbon source depends on its cleavage into glucose and fructose. In plants this reaction is catalysed by either sucrose synthase (EC 2.4.1.13) or invertase (EC 3.2.1.26). The isoforms of the cytoplastic enzyme

sucrose synthase are glycosyl transferases, which catalyse the readily reversible conversion of sucrose, in the presence of UDP, into UDP-glucose and fructose (Geigenberger and Stitt, 1993). Invertase, on the other hand, is a hydrolase and exists in several isoforms with different biochemical properties and subcellular locations (for review, see Sturm, 1996; Tymowska-Lalanne and Kreis, 1998; Sturm, 1999). Although both invertase and sucrose synthase play a role in sucrose metabolism, only the former has a direct involvement in sucrose unloading and partitioning (Eschrich, 1980; Tang and Sturm, 1999) and will therefore be discussed further for the purpose of this review.

2.4.2.1 Isolation and characterisation of plant invertases

A number of different invertase isoforms have been cloned and characterised from a variety of plants (Tymowska-Lalanne and Kreis, 1998). These isoforms are distinct in their expression patterns during development and show significant structural differences. Most of the characterised invertases fall within two categories. Firstly, soluble, glycosylated invertases that have acidic pI values and acid pH optima (pH 3.0-5.0), which are typically located in the lumen of vacuoles (vacuolar invertases), although some may be cytosolic (Fahrendorf and Beck, 1990; Sturm and Chrispeels, 1990). The second group constitutes insoluble invertases. These nonglycosylated isoforms with basic pI values and neutral pH optima (pH 7.0-7.8) are found in the cytosol (neutral invertases), whereas those with acid pH optima (pH 4.0-5.3) are ionically bound to the cell wall (cell wall invertases). The acid pH optima of cell wall invertases might represent an acid-controlled switch for sugar partitioning and the high pI probably facilitates immobilisation of the enzyme in the cell wall by ionic interactions (Eschrich, 1980). Vacuolar and cell wall acid invertases both cleave sucrose from the fructose-residue side, rendering them β-fructofuranosidases. These enzymes are also capable of hydrolysing other β-fructose-containing oligosaccharides such as raffinose and stachyose. Neutral invertases, however, appear to be sucrose-specific in their cleavage activity (Sturm, 1999).

Acid invertases have a K_m for sucrose in the 1-8 mM range and the mature polypeptides are N-glycosylated, with the majority having molecular masses between 55 and 70 kD. Proteolytic fragments have been reported under denaturing and reducing conditions, which appear to be under developmental control (Arai et al., 1991; Unger et al., 1992, 1994). Amino acid sequences reveal a conserved pentapeptide NDPNG, the βF-motif, which is located close to the N-terminus and a Cys residue closer to the C-terminus. Neutral invertase polypeptides are homotetramers, except for the invertase from carrot (Lee and Sturm, 1996), and are composed of subunits with a molecular mass of 54 and 65 kD. The mature polypeptides are not N-glycosylated, except for the sugarcane isoform (Del Rosario and Santisopasri, 1977) and hydrolyse sucrose with a K_m of 10-20 mM (Lee and Sturm, 1996; Ross et al., 1996).

2.4.2.2 Regulation of plant invertases

Despite the central role of invertases in plants, comparatively little is known about their mode of regulation. At present, cell wall invertases are known to be under developmental control (Sturm et al., 1995; Davies and Robinson, 1996; Godt and Roitsch, 1997; Haouazine-Takvorian et al., 1997), are regulated at both the transcriptional and posttranscriptional levels by sugars (Xu et al., 1995); are expressed under wounding and pathogen attack (Sturm and Chrispeels, 1990; Benhamou et al., 1991; Zhang et al., 1996; Rosenkranz et al., 2001); and are possibly controlled by heavy metal ions (Lee and Sturm, 1996; Vorster and Botha, 1998), oxygen levels (Zeng et al., 1999), plant hormones (Morris and Arthur, 1984; Estruch and Beltran, 1991; Wu et al., 1993; Ehness and Roitsch, 1997; Goetz et al., 2000), alkaloids (Rojo et al., 1997), as well as by proteinacious inhibition (Isla et al., 1992; Weil et al., 1994).

The presence of the different isoforms is dependent on the type of tissue and its sink/source status (Masuda and Sugawara, 1980; Nakagawa et al., 1980). Analysis of isoform-specific steady-state transcript levels showed markedly different organ- and development-stage-specific expression patterns for carrot and tomato (Sturm et al., 1995; Godt and Roitsch, 1997). It was also found that the two soluble invertases from *Arabidopsis* showed an organ-specific pattern of expression (Haouazine-Takvorian et al., 1997). Furthermore, the two vacuolar invertase genes of *V. vinifera*, *Gin1* and *Gin2*, are expressed in a variety of tissues, but with different expression patterns (Davies and Robinson, 1996). These findings suggest that the small gene family of acid invertases is expressed independently in a temporally- and spatially-specific pattern during plant development.

The different invertase isoforms show contrasting responses to various carbohydrates (Xu et al., 1995). The activity of acid invertases can be inhibited by their reaction products, with glucose acting as a non-competitive inhibitor and fructose as a competitive inhibitor. Neutral invertases are strongly inhibited by fructose and glucose (Lee and Sturm, 1996; Voster and Botha, 1998). In maize two gene classes for vacuolar invertases were identified with contrasting sugar responses (Xu et al., 1996). The one class was up-regulated by increasing sugar supply, whereas the second class was repressed under these conditions and up-regulated only by depletion of this resource. Roitsch et al. (1995) found that the neutral and vacuolar invertases of *C. rubrum* were not affected by sugars, whereas the cell wall invertase's expression was induced by glucose, fructose and sucrose. Their results indicated, however, that the strong effects of sucrose on enzyme activity and expression level resulted from sucrose cleavage and transcriptional activation by the resulting glucose. Nevertheless, the presence of sucrose had a greater effect on enzyme activity compared to the effect of hexoses, whereas the mRNA levels were the same. This suggests that sucrose may have a positive effect on enzyme activity. Cell wall invertase was also shown to be up-regulated by sucrose in tomato (Godt

and Roitsch, 1997; Sinha et al., 2002). In carrot, however, the expression of the acid invertase genes was found not to be regulated by sugars at all (Sturm, 1999). The differential sugar modulation of invertase genes aids the adjustment of plant metabolism in response to relative sugar availability.

Cell wall invertases can also be induced by wounding and pathogen attack (Sturm and Chrispeels, 1990). Mechanical wounding of carrot and sugar beet taproots resulted in increased invertase mRNA levels, followed by increased cell wall and vacuolar invertase activity respectively (Sturm and Chrispeels, 1990; Rosenkranz et al., 2001). A correlation was also found between pathogen infection and acid invertase activity in carrot taproots upon infection with *Erwinia carotovora*. Gene expression was restricted to the site of infection and the observed induction appeared not to spread systemically (Benhamou et al., 1991).

Acid invertases are inhibited by heavy metal ions such as Hg^{2+} and Ag^{+} suggesting the presence of a sulfhydryl group in the catalytic site. With the possible exception of a sugarcane isoform (Vorster and Botha, 1998), neutral invertases are not inhibited by heavy metal ions, unlike the acid invertases (Lee and Sturm, 1996). This indicates marked differences between the catalytic sites of neutral/alkaline and acid invertases. Invertase genes are also strongly repressed by low oxygen levels (Zeng et al., 1999). This response could aid in the conservation of sugar and ATP, and concomitantly affect sugar signalling.

It has been shown that invertase secretion and activity could be under the control of hormones such as auxin (Morris and Arthur, 1984), gibberellic acid (Estruch and Beltran, 1991; Wu et al., 1993), cytokinins (Ehness and Roitsch, 1997; Godt and Roitsch, 1997), and brassinosteroids (Goetz et al., 2000). Experiments conducted with the synthetic auxin-like compound, benzothiazole-2-oxyacetic acid (BTOA), showed that the expression of grapevine vacuolar invertase genes was modulated by auxin levels (Davies et al., 1997). Cultured cells of *C. rubrum* showed an increase of cell wall invertase transcripts along with glucose transporter mRNA in the presence of cytokinin (Ehness and Roitsch, 1997). Furthermore, brassinosteroid growth responses were linked to the induction of cell wall invertase mRNA in tomato suspension cultures, although intracellular invertase activity was not affected by the addition of this steroid hormone (Goetz et al., 2000). However, direct regulation of invertase genes by plant hormones is still speculative since the hormone-stimulated cells could create a stronger sink for sucrose resulting in higher concentrations of this sugar.

Alkaloids also appear to be effectors of plant invertase activity, as indicated by the results of Rojo et al. (1997). It was shown that sucrose hydrolysis might be changed by the actions of these compounds, with the physiological significance depending on the alkaloid, its vacuolar concentration and the sensitivity of the corresponding invertase.

Weil et al. (1994) identified a 17-kD polypeptide that inhibits cell wall invertase of tobacco; this inhibition was shown to be pH-dependent. There was also evidence

for the co-localisation of the inhibitor and cell wall invertase in the cell wall (Weil and Rausch, 1994), which suggested that the polypeptide might bind to and regulate invertase *in situ*. Another invertase inhibitor was similarly identified in the cell wall of potato (Isla et al., 1992). This protein, however, binds to the vacuolar invertase isoform, excluding *in vivo* inhibition of the vacuolar invertase. There appear to be three factors affecting binding of the inhibitor to cell wall invertase. Firstly, in the case of tobacco it was found that sucrose binding at the catalytic site of cell wall invertase prohibited binding of the inhibitor (Weil et al., 1994). It is therefore suggested that cell wall invertase should become susceptible to inhibition only after *in situ* substrate depletion. The same observations were also made for the binding of the potato inhibitor to the vacuolar invertase (Anderson et al., 1980). The second factor influencing binding is the protective effects of the divalent metal ions Ca^{2+}, Mg^{2+} and Zn^{2+}. Evidence indicates that the protective effect of these metal ions may be due to binding to the inhibitor, cell wall invertase, or both (Weil et al., 1994). It was shown that binding of the inhibitor to potato vacuolar invertase was significantly inhibited in the presence of 100 mM $MgCl_2$ (Anderson et al., 1980). Finally, the variability of the apoplastic pH (4.5-6.5) could also affect the binding of the inhibitor to the invertase enzyme. It was shown in maize (Jaynes and Nelson, 1971), potato (Anderson et al., 1980) and tobacco (Weil et al., 1994) that inhibition of the invertases was most effective at pH 5.5, 4.8, and 4.5 respectively, with minor or no inactivation at pH 6.5. Greiner et al. (1998) proved that inhibitor and cell wall invertase expression was not coordinated and that the proteins probably only associate in the apoplastic space and not during their transit through the secretory pathway. The apoplastic inhibitor seems to act as a regulatory switch of cell wall invertase activity (Krausgrill et al., 1998).

Research done by Sander et al. (1996) has indicated significant differences in the inhibition mechanisms for cell wall invertases and vacuolar invertases. It was shown that a different binding mechanism might exist for the vacuolar isoforms and that sucrose concentration does not control this inactivation process. This is probably due to the different properties of cell wall invertases and vacuolar invertases and indicates that these invertases are regulated differentially in their physiological context (with different sucrose concentrations typically occurring in the vacuole and apoplastic space).

2.4.2.3 Functional analysis of plant invertases

In view of the fact that sucrose plays a variety of roles in plants, including acting as a nutrient, an osmoticum and a signal molecule, it is evident that invertases also have several different functions in the plant by hydrolysing sucrose to its hexose constituents (Fig. 5) (Roitsch et al., 2003). Sucrose must be converted to glucose and fructose to provide for energy needed in respiration and the synthesis of growth-related compounds. Insoluble cell wall-bound invertases function in this capacity to cater for the high demand for hexoses associated with rapidly growing tissues such

as developing roots (Ricardo and Ap Rees, 1970), elongating stems (Morris and Arthur, 1985) and meristems. It has also been reported that cell wall invertase may be involved in the hormone-mediated modulation of cell division in these tissues (Ehness and Roitsch, 1997; Herbers and Sonnewald, 1998). Tissue growth is also dependent on hexoses for osmotic pressure and an increase in cell wall extensibility to drive cell elongation (Gibeaut et al., 1990).

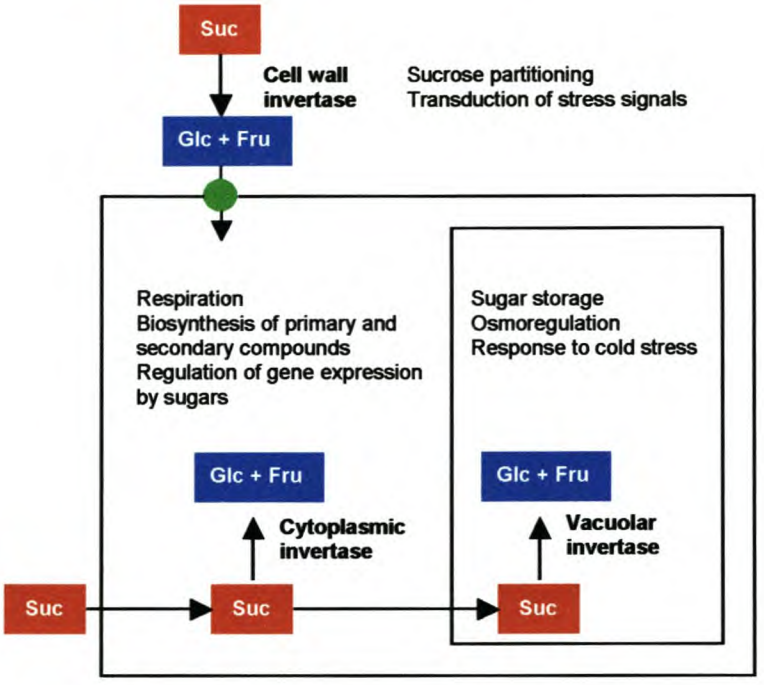

Figure 5. Subcellular locations and proposed functions of plant invertase isoforms. Abbreviations: Fru, fructose; Glc, glucose; Suc, sucrose (adapted from Sturm, 1999).

Cell wall invertases are induced by wounding and pathogenic attack leading to hexose production (Matsushita and Uritani, 1974; Sturm and Chrispeels, 1990; Zhang et al., 1996). It is thought that the wound meristem forms a strong local sink and the induction of cell wall and vacuolar invertases mobilise energy resources (Rosenkranz et al., 2001). It has also been proposed that extracellular sugars and cell wall invertases may play a role in stress signalling in plant defence responses (Herbers et al., 1996, 2000; Roitsch et al., 2003). They are involved in phloem unloading (Eschrich, 1989; Miller and Chourey, 1992; Ruan and Patrick, 1995; Weber et al., 1995), sugar partitioning (Weil and Rausch, 1990; Roitsch et al., 1995; Ehness and Roitsch, 1997; Roitsch, 1999), osmoregulation (Meyer and Boyer, 1981; Wyse et al., 1986; Perry et al., 1987), and gravitropism (Wu et al., 1993). They also seem to play an important role in maize (Miller and Chourey, 1992; Carlson and Chourey, 1999), fava bean (Weber et al., 1995) and rice (Hirose et al., 2002) seed development with regards to unloading, sugar partitioning, and control of storage functions during seed development. The overexpression of a yeast-derived invertase in transgenic plants confirmed the important role of cell wall-bound invertase in sugar

partitioning, as well as to suggest a role for invertase in the regulation of photosynthesis (Von Schaewen et al., 1990; Sonnewald et al., 1991).

Acid invertase gene expression has been associated with vascular tissues in barley and pea leaves (Kingston-Smith and Pollock, 1996; Zhang et al., 1996), carrot (Ramloch-Lorenz et al., 1993) and potato (Hedley et al., 2000). Their expression indicates a role for these enzymes in the transfer of photoassimilate to sink organs. The role of cell wall invertases is also important in light of its cleavage products: hexoses taken up from the apoplastic space may act as metabolic signals, which strongly affects the expression of other genes such as those involved in response to wounding and infection, as well as photosynthesis (Herbers et al., 1996; Koch, 1996; Jang et al., 1997).

Soluble acid invertases are often found in fruits and tuberous roots (Ricardo, 1974; Ricardo and Sovia, 1974). This class of invertases is involved in fruit ripening (Elliott et al., 1993) and is likely to be important in the regulation of hexose levels in mature tissues and the utilisation of sucrose stored in the vacuoles (Lingle and Dunlop, 1987). Sugar content, along with vacuolar invertase activity, was measured in different developing *Cucumis* fruit (Schaffer et al., 1987). Sucrose accumulated in the maturing fruit of sweet melon, while non-sweet melon and cucumber had a low sucrose content. Invertase activity decreased radically in sweet melon at the onset of sucrose accumulation, whereas significant activity was retained in mature non-sweet melon and cucumber. This trend has been reported in a number of sucrose storing tissues: sugar beet (Giaquinta, 1979b), citrus fruit (Kato and Kubota, 1978), carrot (Ricardo and Ap Rees, 1970), and sugar cane stem (Hatch and Glasziou, 1963). In contrast, storage tissues that do not accumulate sucrose retain their vacuolar invertase activity, for example, radish and turnip roots (Ricardo and Sovia, 1974), and botanical relatives of sugar cane (Hatch and Glasziou, 1963). It has been shown that invertase also controls the composition of sugars in tomato fruit. Different levels of vacuolar invertase have been found in two species of tomato, differing in the type of sugar stored. *L. esculentum* accumulates hexoses and has high levels of vacuolar invertase activity, whereas *L. chmielewskii* stores sucrose and contains low levels of invertase activity (Yelle et al., 1991; Stommel, 1992; Ohyama et al., 1995; Klann et al., 1996). In ripening grape berries, high levels of glucose and fructose are accumulated within the vacuoles after *véraison* (Davies and Robinson, 1996). Although soluble invertases are not the trigger, they are essential for the accumulation of these hexoses and responsible for differences in sucrose levels between varieties (Takayanagi and Yokotsuka, 1997). In sugarcane, sucrose accumulation is correlated with the down-regulation of vacuolar invertase activity in stem storage tissue (Zhu et al., 1997). In cold-stored potato tubers it was found that inhibition of vacuolar invertase led to a decreased hexose and an increase in sucrose content (Zrenner et al., 1996b). It was also shown that vacuolar invertase does not control the total amount of soluble sugars in the cold-stored tubers, but is involved in the regulation of the ratio between hexoses and sucrose. Altered sucrose-to-hexose

levels also caused undifferentiated growth in carrot where antisense vacuolar and cell wall invertase were expressed (Tang et al., 1999). Impaired organ formation in these plants showed that these enzymes play an important role in early plant development, probably through the control of sugar composition and metabolic fluxes. Later in plant development, both isoenzymes appear to have essential functions in sucrose partitioning.

It is thought that alkaline invertase, along with sucrose synthase, may fulfil the function of hydrolysing sucrose to meet metabolic needs during vacuolar sucrose accumulation in storage tissue (Schaffer et al., 1987). However, contradicting results were found by Ma et al. (2000) in cultured sugarcane cells. Neutral invertase activities were low in meristematic cells of high sucrose storing sugarcane varieties, but increased concomitantly with the increase in sugar accumulation in storage tissue (Hatch and Glasziou, 1963). It has been suggested that this enzyme might play an important role in the control of hexose levels in the cytosol of sugarcane stem cells, which could affect the expression of sugar responsive genes (Vorster and Botha, 1998). A neutral invertase from carrot (Sturm et al., 1999) was detected in all plant organs at all developmental stages analysed, which suggests that this enzyme has a general and possibly growth-related function in carrot sucrose metabolism.

Overall, the information obtained from various plant species confirmed that invertases have multiple functions that directly or indirectly (through sugar signalling pathways) affect different processes, such as the control of metabolic fluxes, downstream sucrose partitioning, osmoregulation, as well as the response to wounding and infection. In light of this, the importance of invertases and their involvement in the control of cell differentiation and plant development is evident.

2.5 CARBOHYDRATE CONTROL OF GENE EXPRESSION IN HIGHER PLANTS

2.5.1 Sugar as a signalling molecule

Sugars not only function as substrates for growth in higher plants, but can, in addition act as signalling molecules that initiate changes in gene expression (Sheen et al., 1999). The significance of sugar as a signalling molecule in plants involves the integration of whole-plant carbon flux in order to match the production capacity of source tissues with the utilisation capacity of the sink tissues. By sensing changes in carbohydrate status, plants can respond by altering gene expression and/or enzyme activities, thereby controlling photosynthate production, partitioning, and subsequent utilisation (Koch, 1996). This is a valuable mechanism for plants to adjust to environmental change. The effects of sugars, specifically glucose and sucrose on gene expression and plant development are characteristic of classic plant hormone actions (Kende and Zeevaart, 1997). Signalling and sensing of these sugars are performed at millimolar concentrations through interaction with sugar binding

enzymes or transporters (Jang and Sheen, 1997; Smeekens and Rook, 1997; Chiou and Bush, 1998; Lalonde et al., 1999; Vaughn et al., 2002). These sugars can qualify as plant hormones with dual functions operating as signalling molecules as well as intermediary metabolites. The abundance and depletion of these carbohydrates can both induce or repress the expression of genes (Koch, 1996; Farrar et al., 2000).

Carbohydrate abundance leads to the induction of utilisation and storage processes whereas synthesis is reduced. Carbohydrate depletion, on the other hand, results in utilisation and storage processes being reduced or inactivated while photosynthesis, reserve mobilisation and export processes are induced. These changes are reflected in the expression of the genes whose products are integral to these processes (for review, see Koch, 1996; Smeekens, 1998; Sheen et al., 1999; Yu, 1999). Elevated sugar levels positively modulate the genes for carbon storage and starch biosynthesis, secondary product pathways, utilisation and import. High sugar levels induce the genes for storage-related (Nakamura et al., 1991; Peña-Cortes et al., 1992; Thomas et al., 1995; Goldschmidt and Koch, 1996) and starch biosynthetic (Müller-Röber et al., 1990; Kossman et al., 1991; Krapp et al., 1993) proteins in both sink and source tissues. A number of defence, as well as pigment genes are also positively regulated by high carbohydrate levels (Kim et al., 1991). Expression of a grapevine anthocyanin gene, leucoanthocyanidin dioxygenase (*LDOX*), for instance, is induced by sucrose (Gollop et al., 2001).

Conversely, sugar depletion up-regulates the genes for photosynthesis as well as those for remobilisation of sugars and other molecules from polymers and vacuoles. Storage proteins are degraded in source tissues, whereas genes for carbohydrate, lipid, and protein remobilisation, amino acid synthesis (Lam et al., 1994), and sucrose formation are induced in sink tissues (Klein et al., 1993; Hesse et al., 1995). Respiratory and sugar metabolism genes, such as invertases (see Section 2.4.2. on the regulation of plant invertases) and sucrose synthase genes, are affected to varying degrees by sugars (Koch et al., 1992; Roitsch et al., 1995; Sturm et al., 1995).

There is evidence of at least two distinct modes of sugar signalling, namely a hexose and a sucrose-signalling pathway. The hexose signalling mechanism supersedes light activation and is coupled to developmental and environmental control. Photosynthetic genes are repressed by various hexoses as part of this feedback control mechanism (Krapp et al., 1993; Sheen, 1994). Experiments involving transgenic plants overexpressing a yeast-derived invertase showed that the physiological and metabolic status, as well as the photosynthetic capacity of the leaf cells control the level of sugar repression (Stitt et al., 1990; Sonnewald et al., 1991; Büssis et al., 1997). Further analysis of these transgenics revealed that hexose sensing in the secretory pathway is crucial for mediating the activation of defence-related genes and thus systemic acquired resistance (SAR) (Herbers et al., 1996). For example, wounding or pathogen attack up-regulates the expression of cell wall invertase (Sturm and Chrispeels, 1990), which leads to elevated levels of glucose

and fructose. These hexoses are taken up by the adjacent cells to cause repression of the photosynthetic genes. A common mechanism for the repression of photosynthetic genes and the induction of defence responses is suggested (Jang and Sheen, 1994). A coordinate accumulation of antifungal proteins and hexoses was also correlated with pathogen resistance in ripening grape berries (Salzman et al., 1998). Glucose acts as the signal molecule modulating a developmentally regulated defence mechanism. It is clear that a complex interaction exists between the enzymes providing the hexose signal (sucrose synthase, invertase and α-amylase), and those that are regulated by it, such as the photosynthetic genes, as well as the genes encoding for the enzymes providing the signal in the first place.

Evidence for a sucrose-signalling pathway (for review, see Lalonde et al., 1999) that operates independently of a hexose-signalling pathway, was provided by Wenzler et al. (1989). It was shown that sucrose, as well as glucose and fructose could induce the expression of the potato tuber-specific class-I patatin gene *PS20* in transgenic tobacco. Induction by sucrose, however, was approximately 20 times higher compared to the induction by hexoses at equal concentrations. In storage sink tissues a number of genes expressing storage proteins, such as the patatin genes of potato (Wenzler et al., 1989), as well as starch biosynthetic enzymes, including ADP-glucose pyrophosphorylase (Müller-Röber et al., 1990; Weber et al., 1998; Borisjuk et al., 2002) and sucrose synthase (Salanoubat and Belliard, 1989; Koch et al., 1992; Weber et al., 1998) are induced in response to high sucrose levels. Transcription of the storage-specific class-I patatin and defence genes, such as proteinase inhibitor II, can be induced in source tissues by elevated sucrose levels (Johnson and Ryan, 1990; Wenzler et al., 1989). This illustrates that sugar induction can dominate other developmental signals. Rook et al. (1998) confirmed the presence of a sucrose-specific signalling system in plants by observing sucrose-specific repression of the *Arabidopsis* b-ZIP transcription factor gene *Atb2*. Sucrose was also revealed to be the signal molecule in the signal transduction pathway that regulates sucrose-H^+ symporter activity (Chiou and Bush, 1998; Vaughn et al., 2002). It was shown that the sucrose-sensing pathway could modulate transport activity as a result of changing sucrose concentrations and thus also influences assimilate partitioning. Sucrose synthase gene expression in potato cell cultures was also shown to be induced by sucrose, but not by glucose. This occurred in conjunction with regulation by the SNF1-like protein, SNF1-related protein kinase-1 (SnRK1) (Salanoubat and Belliard, 1989; Fu and Park, 1995). Furthermore, phenotypes observed with the yeast-derived SUC2 mutants of *Arabidopsis* might be due to the reduced levels of sucrose as a signal, rather than as a nutrient (Gottwald et al., 2000).

It has also been shown that sugar regulates the expression of H^+-ATPase gene family members in tomato (Mito et al., 1996). H^+-ATPases play a critical role in plants by establishing electrical potential across the plasma membrane and thereby facilitating transport of various molecules, including sugars. Sugar signalling thus plays a pivotal role in plant development since total H^+-ATPase levels may be directly

correlated with growth. Evidence suggests that plant seed development is solely triggered by a sugar signal (Offler et al., 1997), establishing an important role for invertase (Weber et al., 1996) during this developmental process. It would seem that the regulation of soluble invertase genes by sugars could provide a mechanism for integrating and transducing information on photosynthate levels to fertilised ovules in fruit and seed-set responses to the environment. It was also shown that sucrose and glucose co-ordinately and differentially regulate growth- and stress-related gene expression through various control mechanisms and complex signal transduction pathways (Ho et al., 2001). It is clear that sugar signalling is integral to plant growth and development (Borisjuk et al., 2002).

2.5.2 Sugar sensing mechanisms

Although the elucidation of sugar-sensing mechanisms is still in its infancy, it is proposed that there are at least three possible mechanisms operable in plants: a hexokinase-sensing system, a glucose-transport-associated sensor, and a sucrose-transport-associated sensor.

Hexokinase sensing system. Hexokinase (HK) may have a dual function as a catalytic protein kinase with regulatory functions. Evidence indicates that this enzyme is capable of sensing sugar fluxes, which could result, for example, in the repression of photosynthetic (Fig. 6) (Jang and Sheen, 1994) and glyoxylate cycle genes (Graham et al., 1994). Jang and Sheen (1994) found that glucose transport across the plasma membrane is necessary, but not sufficient to trigger gene repression. They proposed that subsequent phosphorylation of glucose by HK may be required and that this enzyme may act as the sugar sensor responsible for sugar sensing in the hexose pathway, but not the sucrose pathway.

It is thus proposed that HK is the intracellular hexose sensor that mediates the first step of the signal transduction pathway (Jang and Sheen, 1994; Umemura et al., 1998). Evidence in this regard includes the fact that various hexoses and glucose analogues, which can be phosphorylated by HK, are able to trigger repression. Also, carbon flux, related to the rate of hexose phosphorylation, is the critical signal in the pathway, as opposed to steady-state hexose-P levels and further metabolism of sugar phosphates is not necessary to cause repression. Moreover, mannoheptulose, a competitive inhibitor of HK is capable of blocking repression (Jang and Sheen, 1994). Repression caused by glucose could not be mimicked by a non-metabolisable glucose analogue (6-deoxyglucose), which indicates that glucose *per se* interacts with a putative receptor involved in signal transduction and transcriptional activation.

Further evidence for HK as a sugar sensor was provided by analysing transgenic *Arabidopsis* and tomato plants (Jang et al., 1997; Dai et al., 1999). *Arabidopsis* plants overexpressing HK (*AtHxk*) displayed sugar hypersensitivity, whereas those expressing antisense *AtHxk*, exhibited sugar hyposensitivity. A variety

of effects on *Arabidopsis* seedling development, as well as inhibited growth, reduced photosynthetic capacity and rapid senescence are displayed by tomato plants overexpressing *AtHxk* and are attributed to the specific sugar sensing effects mediated by HK. It is hypothesised that a conformational change of HK upon binding and/or phosphorylation of hexoses controls its interaction with downstream regulatory elements to trigger the signalling cascade for the repression. It has been speculated that plant fructokinases might also act as sugar sensors in plants, however, proof in this regard is lacking (Pego and Smeekens, 2000).

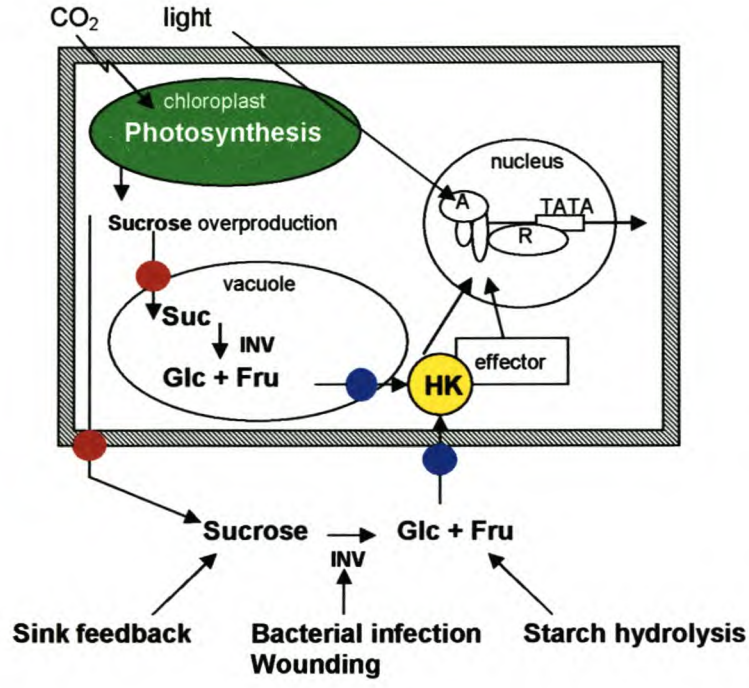

Figure 6. A model for sugar repression of photosynthetic gene transcription in higher plants. Blue circles represent hexose transporters and red circles represent sucrose transporters. Abbreviations: A, transcriptional activator; Fru, fructose; Glc, glucose; HK, hexokinase; INV, invertase; R, transcriptional repressor; Suc, sucrose (adapted from Jang and Sheen, 1994).

Glucose-transport-associated sensing system. Hexokinases are not involved in all plant sugar response pathways as proven by the findings of Herbers et al., (1996). They propose that sensing mechanisms that are independent of HK, could be located at the secretory membrane system, conceivably at the endoplasmic reticulum or Golgi apparatus. This is based on the finding that elevated hexose levels in the cytosol of transgenic plants overexpressing a yeast invertase in the cytosol, did not lead to the accumulation of pathogenesis related (PR) proteins, and thus SAR (Sonnewald et al., 1991). Results obtained by Tang et al. (1999) support the notion that sugar signalling from the apoplast and the endomembrane system feed into the same sensing pathways (Fig. 7). A different regulatory pathway, excluding the HK reaction, was also proposed for the sugar induction of cell wall invertase and sucrose synthase in *C. rubrum* cell cultures (Godt et al., 1995; Roitsch et al., 1995). Antisense

repression of hexokinase 1 in potatoes led to no significant changes in tuber carbohydrate metabolism, which indicated that this enzyme is not involved in glucose sensing in potato (Veramendi et al., 1999). Furthermore, experiments with cell suspension cultures of maize led to the conclusion that the 3' untranslated region of the cell wall invertase gene (*Incw1*) acts as the regulatory sensor of sugar depletion and that this occurs independently from the HK pathway (Cheng et al., 1999). Other important role players in hexose sensing include the hexose transporters. These transport proteins are located at strategic positions in sink tissues and could act, in conjunction with invertases, as glucose sensors (Lalonde et al., 1999).

It would seem that different regulatory pathways are responsible for the regulation of plant genes by hexoses. Irrespective of the mechanism employed, sucrose-cleaving enzymes, such as invertase, play a fundamental role in controlling cell differentiation and development since they have the potential to increase sugar perception by the hexose sensing systems (Sturm and Tang, 1999).

Sucrose-transport-associated sensing system. The first evidence that the transcript level of a sucrose-H^+ symporter is specifically regulated by sucrose was provided by Chiou and Bush (1998). Regulation of sucrose-H^+ symporter activity was controlled by sucrose concentration and was not influenced by equal molar concentrations of hexoses or the HK inhibitor mannoheptulose. It was suggested that a sucrose sensor is localised in the phloem (Chiou and Bush, 1998; Vaughn et al., 2002). Repression of the *Arabidopsis Atb2* gene by sucrose was not influenced by the intracellular sucrose concentration (Rook et al., 1998). This led to the conclusion that sucrose sensing is associated with its transport across the plasma membrane (Fig. 7) (Lalonde et al., 1999; Barker et al., 2000). Evidence indicates a role for SBP in extracellular sucrose sensing (Pedra et al., 2000). SUT2 transporter-like proteins also have a possible involvement in sucrose sensing by regulating both high- and low-affinity transport activities (Schulze et al., 2000). The SUT2 sucrose transporter-like proteins structurally resemble the yeast SNF3 and RGT2 sugar sensors, since they also contain intercellular extended loop domains. The latter are pivotal in a possible sugar sensing function, since it allows interaction with the cytoplasm. Through structure-function analysis it was revealed that the fructose moiety of the disaccharide is required for sensing (Loreti et al., 2000).

Recently, it was shown that the grape hexose transporter, VvHT1, is induced by glucose, as well as sucrose, although induction by the latter did not require transport (Atanassova et al., 2003). It was also shown that the glucosyl moiety was necessary for sucrose sensing in this instance. Induction by palitinose, a non-metabolisable and non-transported isomer of sucrose, indicated that sensing occurred through a sucrose sensor located at the plasma membrane. The data also suggested that the *VvHt1* promoter may be stimulated by two independent signalling pathways, i.e. a hexose as well as a sucrose pathway.

2.5.3 Sugar signal transduction

Specific components of the protein kinase cascade that transmits signals to the nucleus have not yet been identified. It was suggested that mitogen-activated protein (MAP) kinases might be involved in sugar signalling pathways (Ehness et al., 1997), although evidence to the contrary has been found in tomato (Sinha et al., 2002). Downstream components of the signal transduction pathway(s), involved in regulation of genes by sugars, might involve a distinct group of the protein kinase superfamily that are functional homologues to the yeast SNF1 protein kinase (Celenza and Carlson, 1986; Halford et al., 2003). This family includes RKIN1 of rye (Alderson et al., 1991), NPK5 of tobacco (Muranaka et al., 1994), SnRK1 of potato (Man et al., 1997) and others from barley (Halford et al., 1992), wheat (Le Guen et al., 1992) and *Arabidopsis* (Sano and Yousseflan, 1994). These homologues have been classified into three subgroups (SnRK1-SnRK3) according to sequence similarities at the amino- and carboxy-terminal regions (Halford and Hardie, 1998).
It has been shown that SnRK1 is subject to transcriptional regulation, such as developmental regulation, and post-transcriptional regulation, including phosphorylation (Man et al., 1997; Sugden et al., 1999). The PRL1 protein has been shown to interact with plant SNF-like proteins and is postulated to function as a negative regulator of these proteins (Bhalerao et al., 1999). The identification of SnRK1 substrates and the genes that they regulate, provided evidence that this kinase is involved in the control of carbon metabolism. It is postulated that SnRK1 is activated by high intracellular sucrose and/or low intracellular glucose levels (Halford and Dickinson, 2001).

A role for these proteins in plant signalling cascades (Fig. 7) is supported by evidence that members of the SnRK1 subfamily can complement the yeast *snf1* mutant (Alderson et al., 1991; Muranaka et al., 1994; Bhalerao et al., 1999). Furthermore, experiments involving antisense repression of the SNF1-like protein kinase of potato resulted in the loss of sugar-inducible expression of sucrose synthase (Purcell et al., 1998). Experiments that involved calcium ion channel inhibitors and calmodulin suggested that calcium might have a potential function as a second messenger in the sugar-signalling pathway (Ohto and Nakamura, 1995). This was supported by the identification of a calcium-dependent protein kinase in maize that was active only in sink tissues (Barker et al., 1998), as well as a calcium-dependent protein kinase in tobacco that is sucrose-inducible (Iwata et al., 1998). Moreover, Vitrac et al. (2000) proved the involvement of calcium in the up-regulation of anthocyanin biosynthesis by sugars in grape cell cultures. This was disputed, however, by Jang and Sheen (cited in Sheen et al., 1999), illustrating yet again that multiple sensors are operable in the diverse sugar responses found in plants.

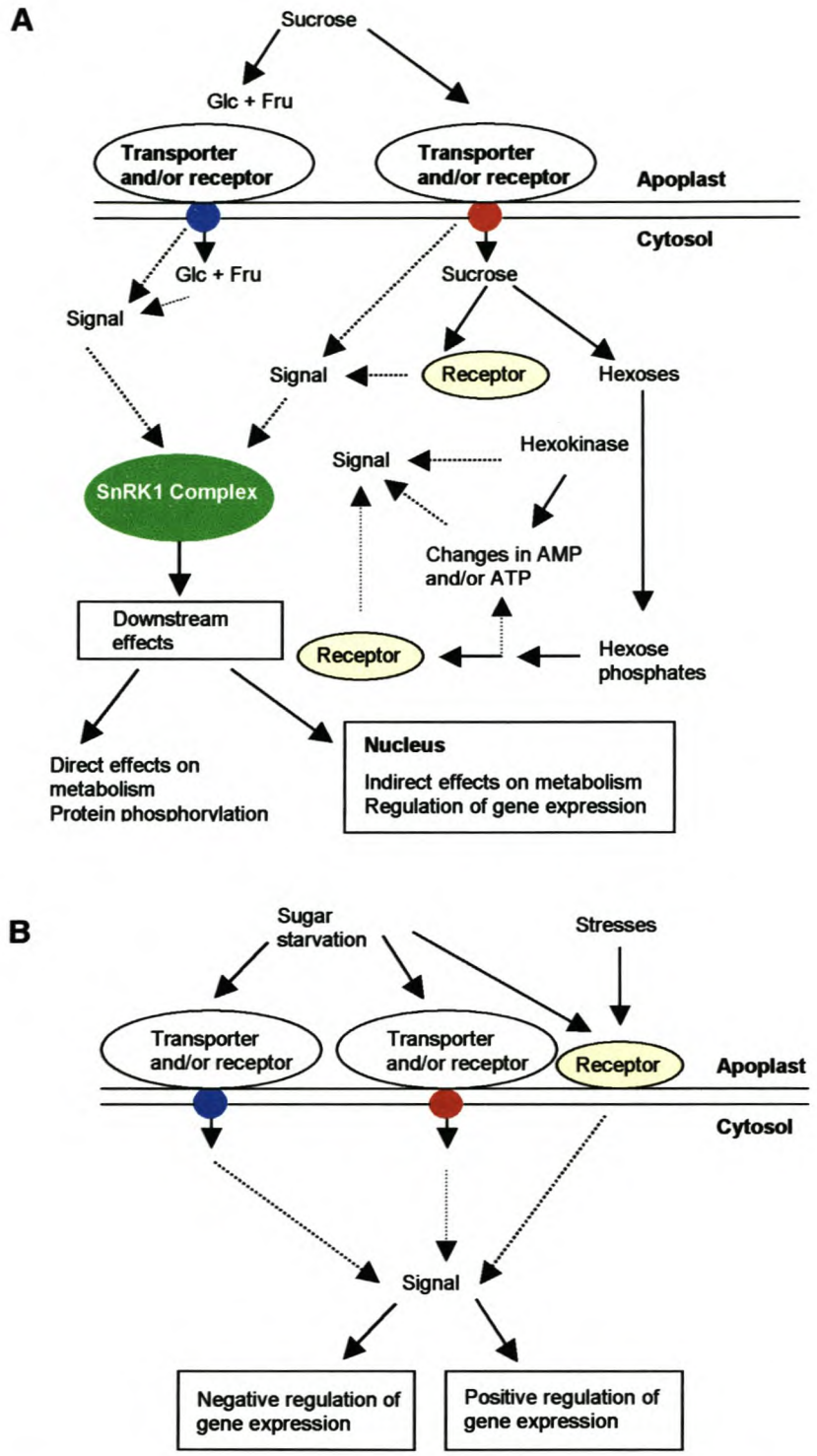

Figure 7. A model of sugar signal transduction pathways for differential and coordinated regulation of gene expression. (a) Signal transduction in sugar-provided cells. Sucrose may be sensed by the receptor directly, or it may be converted to glucose and fructose and sensed by a hexokinase-dependent or hexokinase-independent pathway. (b) Signal transduction in sugar-starved cells. The signals of sugar starvation and environmental stresses may be perceived by the same receptor or by different receptors but the signal pathways converge downstream. Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; Fru, fructose; Glc, glucose (adapted from Halford et al., 1999; Ho et al., 2001).

Due to the fact that sugar-mediated gene expression is affected by many diverse signal transduction pathways, distinct regulatory DNA elements and transcription factors are likely to be employed (Sheen, 1990; Sadka et al., 1994; Sheen, 1994). *Cis*-sequences and *trans*-acting factors responsible for metabolic and developmental regulation have been identified in promoter analysis of the patatin class-I gene (Grierson et al., 1994). Beside the association of such general elements as G and GATA motif sequences (Toyofuku et al., 1998), a TATCCA element has been shown to serve as a common putative *cis*-regulatory element responsible for sugar repression. This element is present upstream of the transcriptional start sites of α-amylase genes (Lu et al., 1998; Yu, 1999), as well as other sugar-repressible genes such as malate synthase (Graham et al., 1989), sucrose synthase (Koch et al., 1992) and isocitrate genes (Reynolds and Smith, 1995).

2.5.4 Interaction between different signalling pathways

Evidence shows that sugar-signalling pathways closely interact with developmental and environmental signals imparted by hormones (DeWald et al., 1994; Zhou et al., 1998), phosphate (Sadka et al., 1994) nitrogen metabolism (Coruzzi and Zhou, 2001), light (Sheen, 1990), pathogen infection (Herbers et al., 1996), as well as wounding and anaerobiosis (Salanoubat and Belliard, 1989). Cross-talk between sugar and hormonal signal transduction pathways was illustrated by the ability of auxin (DeWald et al., 1994) and cytokinin (cited in Jang et al., 1997) to block certain sugar responses. It has been found that sugars can activate the biosynthesis of ethylene (Sonnewald et al., 1995), whilst repressing the expression of brassinolides (Szekeres et al., 1996). A role for abscisic acid (ABA) in sugar-induced regulation was implied by ABA-deficient mutants (Huijser et al., 2000; Laby et al., 2000). Data obtained by Rook et al. (2001) suggested that ABA plays a crucial role in the sugar induction of starch biosynthetic genes, the repression of seedling development and concurrent induction of photosynthetic gene expression by means of controlling the way in which tissues respond to sugar signals. Characterisation of a *gin1* mutant (deficient in glucose sensing and downstream signalling) revealed an unexpected cross-talk between glucose and ethylene signalling pathways (Fig. 8) (Zhou et al., 1998). The interaction of sugar, ethylene signalling and ABA pathways was further demonstrated by the work of Gazzarrini and McCourt (2001). It would appear that hormone- and sugar-responsive elements overlap, indicating that the signalling pathways could interact at a point upstream of the promoter elements (Smeekens, 2000).

It has been shown that sugar signals can coordinate the uptake of nitrogen (N) through its regulatory relationship with genes encoding for nitrate transporters, nitrate reductase, asparagine synthase, and glutamine synthase (Koch, 1996; Jang and Sheen, 1997; Lam et al., 1998; Lejay et al., 1999). The emerging picture suggests that sucrose metabolism is intimately linked to that of inorganic and organic N

through a matrix effect in which sugar responses are dependent on N status and *vice versa* (Coruzzi and Zhou, 2001).

The interaction between light and sugar signalling is multifaceted – sugar and light have opposite effects on photosynthesis, but a synergistic effect on anthocyanin accumulation and defence gene expression. Regulation of the starch granule-bound starch synthase I gene of sweet potato was shown to be regulated by two independent pathways involving both light and sucrose (Wang et al., 2001). Anaerobiosis induces a rapid change in the regulation of invertases and sucrose synthases, which immediately impacts on sucrose cleavage to reduce concomitant hexose sugar signalling (Zeng et al., 1999).

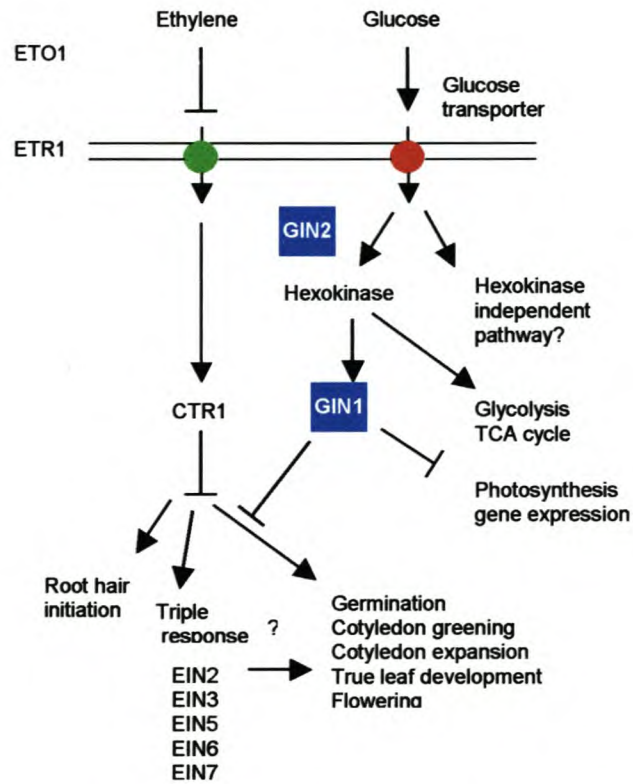

Figure 8. Interaction between glucose and ethylene signalling pathways determines plant growth and development. The convergent point between the two pathways is downstream of GIN1 and GIN2 in the glucose-signalling pathway, and downstream of ETR1, CTR1, and EIN2 in the ethylene-signalling pathway (adapted from Sheen et al., 1999).

It is clear that the pathways by which plants respond to sugars and other hormonal and nutrient signals form part of an intricate regulatory web. The mechanisms underlying the cross-talk between these pathways and gene regulation are not apparent. The ultimate significance of sugar-modulated gene expression is the induction of changes in whole-plant morphology. More information is necessary to improve our understanding of how intrinsic and environmental signals are translated into physiological responses.

2.6 THE TRANSGENIC APPROACH TO STUDY SUGAR TRANSLOCATION IN HIGHER PLANTS: THE USE OF A YEAST-DERIVED INVERTASE AS A CASE STUDY

2.6.1 Transgenic plants expressing a yeast-derived invertase as a model system

Studies involving sink-source relationships have been conducted in the past by means of various physiological approaches. Classically, phloem transport was inhibited by cold girdling or detaching leaves (Shaw et al., 1986), as well as by altering the sink-source balance in whole plants. This was achieved through several different experimental approaches, including the removal of potential sinks (Shaw et al., 1986), inhibiting sink metabolism (Bagnall et al., 1988), increasing the supply of light or CO_2 to the source leaves (Cave et al., 1981), or by removing other competing source leaves (Sasek et al., 1985). These studies, however, could not establish clear causal relationships. Also, many of the treatments were unspecific, and could also affect movement of nitrogen, minerals, and hormones.

In the last decade, it became possible to alter plant metabolism on the genetic level in a fairly controlled and directed manner by means of DNA recombinant technology (Stitt and Sonnewald, 1995). The ability to transform plants with various genes opened up vast new possibilities to manipulate plants to have enhanced characteristics and targeted mutations, which could be analysed on the physiological, biochemical and phenotypical level in order to answer fundamental questions regarding the processes being studied. Recombinant biotechnology, for example, made it possible to disturb sugar metabolism in plants at a particular step by overexpression of endogenous genes, introduction of antisense genes, or expression of heterologous genes in transgenic plants. The introduction of heterologous genes into plant genomes enabled researchers to overcome most of the problems experienced with the classical methods of studying sink-source relationships. The introduction of a yeast-derived invertase into a plant genome, for example, results in the severe disturbance of sucrose metabolism and transport, as well as sink-source interactions. Consequently, valuable insights into the principles governing these processes and interactions can be gained.

Disturbing sugar metabolism by means of a targeted yeast-derived invertase proved to be particularly successful for two reasons. Firstly, yeast invertases share little homology with plant invertases and should therefore escape homology-dependent gene silencing. It should also not be inhibited by plant-endogenous invertase inhibitors (Stitt and Sonnewald, 1995). Secondly, yeast invertase is active over a broad pH spectrum, which is critical for expression of the gene in the different subcellular compartments with their respective pH ranges (Goldstein and Lampen, 1975).

The heterologous invertase enzyme employed is derived from the yeast *S. cerevisiae.* The yeast cleaves sucrose into glucose and fructose by means of its periplasmic invertase that is encoded by the *Suc2* gene (Carlson and Botstein, 1982). The *Suc2* gene encodes two forms of the invertase enzyme (β-D-fructofuranoside fructohydrolase). The one form is a secreted, glycosylated enzyme, whose synthesis is modulated by glucose repression, whereas the other is an intracellular, nonglycosylated constitutively produced enzyme. The *Suc2* gene encodes two RNAs (1.8 and 1.9 kb) that differ at their 5' ends. The larger, regulated, mRNA contains the initiation codon for the signal sequence required for synthesis of the secreted, glycosylated form of invertase, whereas the smaller, constitutively transcribed mRNA initiates within the coding region of the signal sequence, resulting in the synthesis of the intracellular enzyme. The coding region of the intracellular form of the *Suc2* gene is used in fusions with different promoters and targeting signals that are functional in plants. Targeting of the SUC2 protein to the vacuole requires positive sorting information and is achieved by fusion to the entire targeting signal of the proteinase inhibitor II (a vacuolar protein) from potato (Keil et al., 1986). Apoplastic targeting of SUC2 is achieved by a chimeric protein fusion with a 230 bp N-terminal portion of the potato proteinase inhibitor II, which is efficiently secreted by transformed cells due to the absence of positive sorting information (Von Schaewen et al., 1990). The heterologous protein is secreted via the default pathway, as is known for mammals and yeast. The absence of a targeting signal, on the other hand, will ensure a cytosolic localisation of the invertase protein.

The success of this approach was illustrated by the work of Von Schaewen et al. (1990) in which a yeast invertase (SUC2) was introduced into the cell wall of tobacco plants. The presence of the invertase led to decreased sucrose export, accumulation of carbohydrates, and inhibition of photosynthesis. The export of photosynthate was impaired by the hydrolysis of sucrose to glucose and fructose after entering the apoplast. The free hexoses cannot be taken up into the phloem as efficiently as sucrose and are retrieved by the mesophyll cells, rephosphorylated by hexokinase and fructokinase and reconverted to sucrose (Fig. 9) (Maynard and Lucas, 1982). This inhibition of sucrose export from the source leaves led to severe changes in sink-source interactions. Dramatic changes in the development and phenotype of the invertase mutants could also be observed: stunted growth due to a reduction of internodal distances, impaired root formation, as well as bleached areas and necrotic lesions on older leaves. The latter phenotype followed a strict developmental pattern, spreading from the tip and/or rims towards the base of the leaves. The phenomena of stunted growth and reduced root formation was linked to the impaired sucrose export of the leaves, which would be reflected in the development of the sink organs. The bleaching and development of necrotic lesions on older leaves could be explained by an apoplastic phloem-loading step: sucrose present in the apoplast is hydrolysed by the yeast invertase into fructose and glucose, which are not taken up into the phloem and ultimately inhibit photosynthesis.

The results obtained in these experiments demonstrated that the introduction and expression of a single heterologous gene in transgenic plants could alter sink-source relationships and therefore sugar partitioning. Similar successes have been obtained with the introduction of a heterologous invertase targeted to either the cytoplasm, vacuole, or apoplast in *Arabidopsis* (Von Schaewen et al., 1990), tobacco (Von Schaewen et al., 1990; Sonnewald et al., 1991), tomato (Dickinson et al., 1991), potato (Heineke et al., 1992; Büssis et al., 1997), *Vicia narbonensis* (Weber et al., 1998), and sugarcane (Ma et al., 2000).

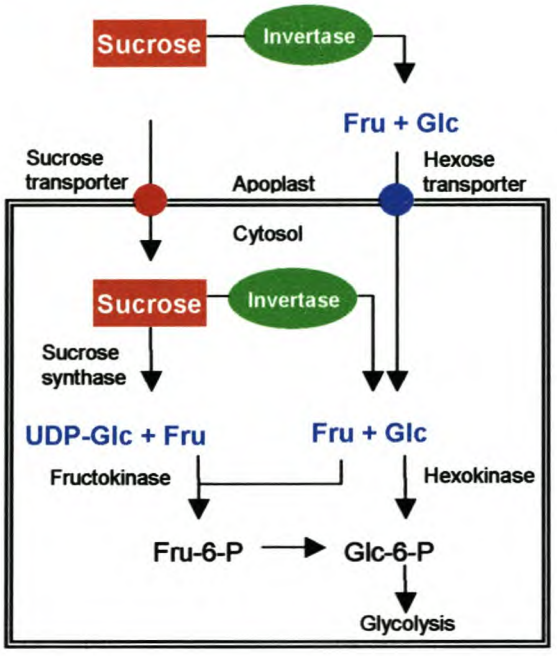

Figure 9. Typical sucrose metabolism in plant sink cells. Sucrose can be cleaved by invertase in the apoplast or intracellularly. The resulting hexoses will be converted to hexose phosphates by fructokinase and hexokinase in the cytosol. Alternatively, upon cell entry, sucrose can be cleaved by sucrose synthase, yielding UDP-glucose and fructose. Following phosphorylation, the resulting hexose phosphates can be further metabolised through glycolysis. Abbreviations: Fru, fructose; Fru-6-P, fructose-6-phosphate; Glc, glucose; Glc-6-P, glucose-6-phosphate (adapted from Pego and Smeekens, 2000).

2.6.2 Information gained from this experimental model

The research of Von Schaewen et al. (1990), continued by Stitt et al. (1990), concluded that the accumulation of photosynthate led to a decrease in the level of Calvin-cycle enzymes and thus, to an inhibition of photosynthesis. A yeast-derived invertase was expressed in the cytoplasm and vacuole, in addition to the apoplast of tobacco by Sonnewald et al. (1991). The results achieved in these experiments proved the central importance of the compartmentation of sucrose with respect to its biosynthesis, storage and distribution. By utilising a nonaqueous fractionation technique, the subcellular distribution of sucrose, hexoses and amino acids were

analysed in the transgenic mutants (Heineke et al., 1994). It was found that vacuoles contain transporters for active uptake of glucose and fructose against a high concentration gradient and that the decrease in photosynthesis was due to a long-term dedifferentiation of the leaf cells and not through the alteration of osmotic conditions as in the case of potato (Heineke et al., 1992). The dedifferentiation is due to the repression of biosynthesis of the photosynthetic apparatus as caused by the accumulation of photosynthate.

The hypothesis that the accumulation of photosynthetic products causes oxidative stress was investigated by Polle (1996). It was found that the transgenic plant cells had an increase in available reductant, while at the same time suffering from increased oxidative stress. Fukushima et al. (2001) examined salt tolerance in tobacco plants expressing yeast invertase in the apoplast and found a marked increase in the sugar concentration in the chloroplasts, protecting the photosynthetic apparatus under these conditions. Plants expressing a cytosolic or apoplastic yeast invertase showed distinctive patterns of phenylpropanoids (Baumert et al., 2001), which could in part explain the observed resistance response of cell wall invertase expressing mutants toward potato virus Y infection (Herbers et al., 1996).

Dickinson et al. (1991) expressed yeast invertase in the apoplast of tomato. Their results confirmed the interpretation that cell wall invertase prevents photosynthate export from source leaves and that phloem loading in tomato includes an apoplastic step.

In potato the enzyme was targeted to the cell wall and led to the conclusion that in potato leaves, phloem loading occurs apoplastically and that its presence affected the partitioning of photosynthate between carbohydrates and amino acids, leading to an increase in the protein to starch ratio in the potato tubers (Heineke et al., 1992). The study by Büssis et al. (1997), where yeast invertase was overexpressed in the different cellular compartments, led to the conclusion that the causes of reduction in CO_2 assimilation were different in the various lines due to the existence of more than one signal transduction pathway acting reduce the rate of photosynthesis under seemingly similar circumstances. Sonnewald et al. (1997) expressed yeast invertase in either the cytosol or apoplast of the potato tubers using a tuber-specific promoter, addressing the importance of sucrose cleavage for sink strength. They found tuber fresh weight to be increased up to threefold, a decrease in tuber number per plant and increase in total tuber yield of up to 30%. These results point towards the possibility of manipulating sink organ size in agriculturally important crop plants. Further analysis of these constructed transgenic plants (Hajirezaei et al., 2000), together with results obtained by Trethewey et al. (1999) suggested that regulation of glycolysis is linked to cytosolic sucrose hydrolysis. Another relevant topic in research on potato is the effect of cold-induced sweetening of potato tubers. The enzymes thought to be involved in this process were identified, but their relative contribution and their *in vivo* regulation was still largely unknown until Greiner et al. (1999) expressed a tobacco invertase inhibitor homologue in potato.

In *V. narbonensis* a yeast-derived invertase was expressed in the apoplast of maturing embryos under the control of the *LeB4* promoter, which confers cotyledon-specific expression during the mid to late stage of development (Weber et al., 1998). Their results strongly suggest a sucrose specific sensing mechanism acting on the level of the storage parenchyma cells sensing sucrose and initiating storage-associated differentiation. These transgenic lines were also utilised by Neubohn et al. (2000) to verify that sugars control the subcellular organisation of the vacuolar system and cell differentiation in maturing transgenic cotyledon cells, thus confirming the important role of sugars in legume seed development.

In sugarcane the yeast invertase gene, as well as an antisense orientation vacuolar invertase gene was expressed to elucidate the role of invertase in the sucrose accumulation process (Ma et al., 2000). It was concluded that hexose uptake, rather than hexose availability, may be the limiting factor in sucrose accumulation. Also, co-ordinated expression of cell wall invertase activity and hexose transporters plus decreasing vacuolar invertase activity may be the key biochemical factors required for maximising sucrose storage.

2.6.3 Information to be obtained from this model

This well-established approach of mutant analysis facilitates the elucidation of fundamental sink-source related questions at a molecular level. In the past decade, this method has been employed extensively, although not exhaustively, to gain insight into sugar metabolism, transport, and sink-source related interactions (see Section 2.6.2. on information gained from this model). Nevertheless, several of the observations made in previous studies were only noted and not examined further. For example, the phenomena of impaired root formation and stunted growth was linked directly to impaired sucrose export from the source leaves, which would result in the "starvation" of sink tissues without further investigation (Von Schaewen et al., 1990; Sonnewald et al., 1991). Also, Sonnewald et al. (1991) speculated on the expression of sucrose transporter-encoding genes in the invertase transformants with increased sucrose concentrations and further investigation was proposed.

Futhermore, new-found knowledge on sugar signalling and sensing mechanisms as well as on the interaction between different plant signalling pathways, has opened new avenues for exploration regarding sugar metabolism and partitioning. However, more information is necessary to understand how these signals are sensed and translated to influence sugar partitioning. Invertase expressing mutant plant lines display disturbed sugar levels due to the effective cleavage of sucrose into glucose and fructose. These changes in carbohydrate status result in the alteration of gene expression and/or enzyme activities (Section 2.5), thereby regulating plant sugar metabolism and partitioning (Koch, 1996). This system can now be utilised further to examine the effect of the altered sugar levels in the different cellular compartments on the expression of the proteins integrally linked

to sugar translocation and partitioning, such as plant sugar transporters, as well as endogenous invertases and invertase inhibitors.

This system is also valuable as a defined experimental system to study the sink regulation of long-distance sucrose transport and sugar signalling (as achieved with photosynthetic metabolism, Stitt et al., 1990). Important information can be obtained by examining the effects of altered sucrose and hexose levels in sink tissues on co-ordinately and differentially regulated growth- and stress-related gene expression (Borisjuk et al., 2002).

The invertase expressing transgenic lines would also provide a useful model system to study the effect of heterologously overexpressed genes on native enzyme systems and the expression of their encoding genes in the host, with similar functions as the introduced genes.

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CHAPTER 3

RESEARCH RESULTS

Transformation of tobacco and grapevine with a yeast-derived invertase (*Suc2*) targeted to either the cytosol, vacuole or apoplast

Transformation of tobacco and grapevine with a yeast-derived invertase (*Suc2*) targeted to either the cytosol, vacuole or apoplast

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In order to establish a system of mutant plant lines with disturbed sink-source interactions, a yeast-derived invertase, encoded by the *Saccharomyces cerevisiae Suc2* gene, targeted to either the cytosol (Cyt-Inv), vacuole (Vac-Inv) or apoplast (Apo-Inv), was transformed into tobacco and grapevine plants using an *Agrobacterium*-mediated transformation system. These transformations generated only transgenic tobacco lines expressing the heterologous invertase and these plants showed increased invertase activity as well as symptoms characteristic of an inhibition of sucrose translocation. The Apo-inv lines also displayed a reduction in growth and development. The overall developmental performance of these transgenic plants was correlated with increased hexose levels and lowered chlorophyll content.

3.1 INTRODUCTION

Sucrose is the main product of photosynthesis in C3 plants and plays a central role in the metabolism of these plants. It is synthesised in the cytosol of source-organ mesophyll cells, is transiently stored in the vacuoles and can move via the apoplast during phloem loading and unloading. Excess carbon is transported in the form of sucrose from the carbon source organs (net exporters), such as mature leaves, via the phloem to the carbon sink tissues (net importers), which include roots, tubers and immature leaves, to allow for growth and development. Moreover, plant development is accompanied by dynamic changes in the sink/source status and relationships of the various sink and source organs.

Plants expressing a yeast-derived invertase targeted to various cellular compartments have been utilised with great success in the past to study sink-source relationships (Von Schaewen et al., 1990). Chimeric yeast invertase genes with different targeting signals to direct the invertase enzyme to the apoplast, vacuole or cytosol have been constructed and widely used to transform different plant species (Von Schaewen et al., 1990; Dickinson et al., 1991; Sonnewald et al., 1991; Heineke et al., 1992; Büssis et al., 1997; Weber et al., 1998; Ma et al., 2000).

Although much research has been done involving these transgenic plants, new avenues for exploration regarding sugar metabolism and partitioning have arisen

with advances in the fields of sugar signalling and sensing. The invertase expressing transgenic lines have disturbed sink-source relationships due to the hydrolysis of sucrose to its constituents, glucose and fructose, in the various cellular compartments. These lines can now be utilised to examine the effect of the altered sugar levels in the different cellular compartments on the expression of the proteins integrally linked to sugar translocation and partitioning, such as sugar transporters, as well as endogenous invertases and invertase inhibitors.

Furthermore, several of the observations made in previous studies (e.g., stunted growth and impaired root formation) were only noted and not examined further. For example, the phenomena of impaired root formation and stunted growth were linked directly to starvation of the sink tissues, due to impaired sucrose export from the source leaves, without experimental verification (Von Schaewen et al., 1990; Sonnewald et al., 1991). Also, Sonnewald et al. (1991) speculated on the expression of genes encoding for proteins needed in sucrose transport in the invertase transformants with emphasis on the need for further investigation.

In this study, both tobacco and grapevine were transformed with expression cassettes containing the coding region of the mature yeast invertase fused to different targeting signals. The grapevine transformations were intended to generate lines for analysis to identify the phloem loading mechanism operable in *Vitis vinifera* as well as to study sink-source relationships in this plant species. The tobacco transformations yielded plant lines that could be used to investigate certain unexplored aspects of sugar metabolism. These include the effect of disturbed sugar levels in different cellular compartments of the various plant tissues on gene expression and sugar partitioning (Chapters 4, 5, and 6).

Phenotypic, molecular and biochemical analyses verified the generation of suitable tobacco transgenic lines, which compared well with previous work (Von Schaewen et al., 1990; Sonnewald et al., 1991; Dickinson et al., 1991; Heineke et al., 1992; Büssis et al., 1997; Weber et al., 1998; Ma et al., 2000).

3.2 MATERIALS AND METHODS

3.2.1 Reagents

DNA restriction and modification enzymes were obtained from Bioline (London, UK), New England Biolabs (Danvers, USA), Promega (Madison, USA), Roche Molecular Biochemicals (Ingelheim, Germany) and Stratagene (California, USA). Chemical reagents were purchased from either Amersham Biosciences (Buckinghamshire, UK), GIBCO/BRL Life Technologies (California, USA), Saarchem (Gauteng, RSA) or Sigma (St Louis, USA). Synthetic oligonucleotides were synthesised by Roche Molecular Biochemicals using an Oligo 2000 synthesiser. D-glucose/D-fructose assay kits were obtained from Roche Molecular Biochemicals.

3.2.2 Media, plant material and bacterial strains

Media used to cultivate *in vitro* plants and bacteria are listed in Table 1. *In vitro* tobacco (*Nicotiana tabacum* cv. Petit Havanna SR-1) plants were grown in tissue culture under a 16 h-light: 8 h-dark regime (50 μmol.m^{-2}.s^{-1}) at a temperature of 28°C on solidified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Shoot and root formation was induced by transferring plant material to shooting and rooting media respectively (Table 1).

Table 1. Media used for transformation and regeneration of plant material.

Media	Description	Reference
Tobacco media		
MS	2% (w/v) sucrose and 1% (w/v) agar, pH 5.8	Murashige and Skoog, 1962
Shooting	MS containing 1 mM 6-benzylamino-purine (BAP), 400 mg·L^{-1} cefotaxime and 120 mg·L^{-1} kanamycin, pH 5.8	Murashige and Skoog, 1962
Rooting	MS containing 1 mM α-naphthalene-acetic acid (NAA), 400 mg·L^{-1} cefotaxime and 120 mg·L^{-1} kanamycin, pH 5.8	Murashige and Skoog, 1962
Grapevine media		
Embryo selection	GS1CA containing 1% (w/v) agar, 100 mg·L^{-1} kanamycin and 1000 mg·L^{-1} timentin, pH 6.2	Franks et al. 1998
Embryo germination	MS containing 6% (w/v) sucrose, 0.25% (w/v) activated charcoal and 100 mg·L^{-1} kanamycin, pH 6.2	Murashige and Skoog, 1962
Shooting	MS containing 1.5% (w/v) sucrose and 10 μM BAP, pH 5.7	Murashige and Skoog, 1962
Rooting	MS containing 1.5% (w/v) sucrose and 0.5 μM NAA, pH 5.7	Murashige and Skoog, 1962

Embryogenic cell lines from *V. vinifera* cv. Chardonnay were derived according to the method of Iocco et al. (2001) and used in the grapevine transformation experiments. Embryogenic callus was maintained on GS1CA medium (Franks et al., 1998) and transformed somatic embryos were selected on GS1CA medium supplemented with antibiotics (Table 1). Embryo germination took place on embryo germination medium and shoot and root formation on shooting and rooting media, respectively. Plant regeneration was performed according to Franks et al. (1998). *In vitro* tobacco and grapevine plants were subcultured every four weeks. Plants used for molecular and biochemical analysis were grown in a 2:1 mixture of

soil and peat moss in a growth chamber (50 μmol.m^{-2}.s^{-1}) under a 16 h-light: 8 h-dark cycle, at 24°C. Plants were watered every second day and fed every two weeks with a 1:300 dilution of organic plant food (8% N, 2% P and 5.8% K).

The sources and relevant genotypes of bacterial strains and plasmids used in this study are listed in Table 2.

Table 2. Strains and plasmids used in this study.

Strain or plasmid(s)	Description	Source or reference
***E. coli* strains**		
DH5α	supE44 Δ*lac*U169[ϕ80*lacZ*ΔM15	Sambrook et al. 1989
	*hsd*R17 *rec*A1 *gyr*A96 *thi*-1*rel*Al	GIBCO/BRL Life Technologies
HB101	Containing helper plasmid pRK2013	Ditta et al. 1980
***A. tumefaciens* strains**		
EHA 105	Disarmed, succinomopine-type strain	Hood et al. 1993
Plasmids		
pGEM T-Easy	pGEM5Zf(+)-based PCR cloning vector	Promega
pBluescript	Cloning vector	Stratagene
pART 7	Cloning vector, CaMV 35S promoter, OCS 3' terminator	Gleave, 1992
pART 27	Plant expression vector, kanamycin resistance marker	Gleave, 1992
pART7-Apo	pART7 containing 1.9 kb *Suc2* fragment with targeting sequence to the apoplast	This study
pART7-Cyto	pART7 containing 1.5 kb *Suc2* fragment	This study
pART7-GUS	pART7 containing 1.9 kb *Gus* fragment	This study
pART7-Vac	pART7 containing 2.9 kb *Suc2* fragment with targeting sequence to the vacuole	This study
pART27-Apo	pART27 containing 4.1 kb pART7-Apo cassette	This study
pART27-Cyto	pART27 containing 3.7 kb pART7-Cyto cassette	This study
pART27-GUS	pART27 containing 4.1 kb pART7-GUS cassette	This study
pART27-Vac	pART27 containing 5.1 kb pART7-Vac cassette	This study

Escherichia coli strain DH5α was cultivated on Luria Bertani (LB) medium and HB101 on LB containing 50 mg·L^{-1} kanamycin and handled using standard techniques (Sambrook et al., 1989). E. coli DH5α transformants, containing the binary plasmids, were grown under the same conditions except for the addition of 20 mg·L^{-1} kanamycin. Agrobacterium tumefaciens strain EHA105 was cultivated on LB containing 0.1% (w/v) glucose, 50 mg·L^{-1} streptomycin and 10 mg·L^{-1} rifampicin at 28°C. A. tumefaciens strains containing the binary plasmids were grown under the same conditions with the supplementation of 20 mg·L^{-1} kanamycin.

3.2.3 DNA manipulation and plasmid construction

Standard methods for plasmid DNA isolation, purification, manipulation and cloning of DNA fragments and agarose gel electrophoresis were used as described in Sambrook et al. (1989). The cytosolic invertase construct, pART7-Cyto (Fig. 1A), was

prepared by subcloning a 1530 bp *Bam*HI/*Eco*RI DNA fragment, containing the sequence of the intracellular invertase *Suc2* gene (Taussig and Carlson, 1983), into the *Bam*HI/*Eco*RI sites of pART7. The apoplastic invertase construct, pART7-Apo (Fig. 1B), was generated through the subcloning of a 1930 bp *Sph*I/Klenow/*Eco*RI DNA fragment, containing the *Suc2* gene and 230 bp of the signal sequence of the patatin proteinase inhibitor II (PI-II) gene (Von Schaewen et al., 1990), into the *Sma*I/*Eco*RI sites of pART7. The vacuolar invertase construct was obtained through the subcloning of a 2900 bp *Sma*I/*Ecl*136II DNA fragment into the *Sma*I site of pART7, rendering pART7-Vac (Fig. 1C). The DNA fragment contains a 1188 bp targeting fragment, which consists of parts of the untranslated leader, the signal sequence, and 597 bp of the coding region of the mature PI-II protein of potato, which allows for vacuolar targeting of fusion proteins. A control plasmid, pART7-GUS, was constructed by subcloning a 1940 bp *Sma*I/*Hin*dIII β-glucuronidase (Gus) DNA fragment into the *Sma*I/HindIII sites of pART7 (Fig. 1D).

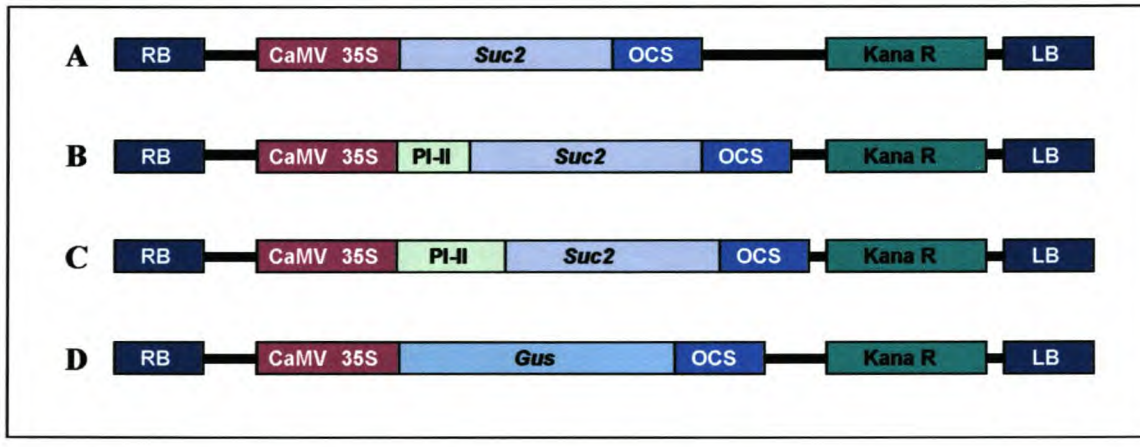

Figure 1. A schematic diagram of the pART27 chimeric invertase cassettes transformed into *Nicotiana tabacum* and *Vitis vinifera*. The *Suc2* gene of *Saccharomyces cerevisiae* was fused to different targeting signals resulting in (A) a cytosolic invertase construct, (B) an apoplastic invertase construct, and (C) a vacuolar invertase construct. An *Escherichia coli* β-glucuronidase (*Gus*) gene, with no targeting signal was prepared as a control construct (D). LB and RB correspond to the T-DNA left and right borders, respectively, PI-II to the signal sequence of the patatin proteinase inhibitor II gene, CaMV 35S to the Cauliflower Mosaic Virus 35S promoter, OCS to the *Agrobacterium* octopine synthase terminator, and Kana R to the pnos-*ntpII*-nos3' kanamycin resistance cassette.

These expression cassettes were excised from the constructed pART7-plasmids by *Not*I digestions and subcloned into the corresponding site of pART27, yielding pART27-Apo, -Cyto, -GUS and -Vac, respectively. The presence of the corresponding DNA fragments was confirmed through sequencing, using an ABI Prism 377 automated DNA sequencer from PE Biosystems (results not shown).

3.2.4 Tobacco transformation

The plant expression cassettes were mobilised into *A. tumefaciens* strain EHA105 by means of triparental mating as described by Pen et al. (1992). Transformation of tobacco plants was carried out using the *A. tumefaciens* leaf disk technique as described by Rosahl et al. (1987). After co-cultivation with *A. tumefaciens* for two days, the leaf disks were placed on shooting medium containing kanamycin and cefotaxime. The regenerated shoots were then placed onto rooting media containing the appropriate antibiotics.

3.2.5 Grapevine transformation

Grapevine somatic embryos were transferred to fresh GS1CA medium a week before exposure to transformation treatments. Grapevine transformations were done according to the method of Iocco et al. (2001). The somatic embryos were co-cultivated with *A. tumefaciens* EHA 105 containing either the engineered plasmid pART27-Apo, -Cyto, -Vac or -GUS. Co-cultivation took place for two days at 22°C on GS1CA medium. The embryos were rinsed and transferred to GS1CA medium containing 1 $mg \cdot L^{-1}$ timentin for one week at 28°C. Selection proceeded for at least two to three months on medium containing 100 $mg \cdot L^{-1}$ kanamycin before plant regeneration was initiated. Putatively transformed somatic embryos were placed onto kanamycin-containing germination media (GS1CA media without hormones) to allow for the germination of the somatic embryos. Germinating embryos were transferred to shoot-induction medium after the primordial roots were truncated. Selection pressure was removed at this stage since the presence of the antibiotics severely retards the organogenesis of the developing plantlet. Shoot formation took place in light conditions (16 h-light: 8-h dark). The developing shoots were excised and transferred onto rooting media.

3.2.6 DNA isolation and screening of transformants

Genomic DNA (gDNA) from 50 mg *in vitro* tobacco and grapevine leaf tissue was extracted according to the protocol described by McGarvey and Kaper (1991). A modified extraction buffer was used for tobacco, consisting of 3% (w/v) cetyl trimethyl ammonium bromide (CTAB), 1.4 M NaCl, 20 mM ethylene-diamine tetra-acetic acid (EDTA) and 1 M Tris-HCl (pH 8.0). One percent (v/v) β-mercaptoethanol, 1% (v/v) Triton X-100, and 8.5% (w/v) polyvinylpolypyrrolidone (PVPP) were added to the extraction buffer used for grapevine.

Approximately 20 ng of tobacco and grapevine genomic DNA was used as template for polymerase chain reaction (PCR) screening reactions. Primers Cyto-inv-forward and Cyto-inv-reverse were used for amplifying a 1.44 kb cytosolic invertase fragment, PI-II-forward and Cyto-inv-reverse for a 1.77 kb apoplastic invertase

fragment, wheras Vac-inv-forward and Vac-inv-reverse were used for the detection of a 2.9 kb vacuolar invertase fragment (Table 3). Primers GUS-forward and GUS-reverse were utilised for the amplification of a 1.8 kb *Gus* fragment. The PCR amplifications were performed using the BioTaq™ DNA polymerase and buffer supplied by Bioline).

PCR amplifications were performed in 50 µl reaction mixtures typically consisting of 1× BioTaq™ PCR buffer without $MgCl_2$, 200 nM of each primer, 20 ng template DNA, and $MgCl_2$ added to the optimal concentration. Typical reaction conditions consisted of an initial DNA denaturation step at 95°C for 2 min, followed by cycles of denaturation at 95°C for 10 sec, primer annealing according to the specific primer melting temperatures for 30 sec, and elongation at 68°C, allowing 40 sec per 1 kb amplified. Amplification reactions proceeded for 30 cycles in a BioRad PCR thermal cycler.

Table 3. List of primers used in this study. Primers were designed according to published sequences.

Primer name	Primer sequence	Reference
Cyto-inv-forward	5'-AGC GAT AGA CCT TTG GTC CA-3'	Taussig and Carlson, 1983
Cyto-inv-reverse	5'-CCG GTG GTC ATG AAG TAG GT-3'	Taussig and Carlson, 1983
PI-II-forward	5'-GAT CTC TAG ACA TGG ATG TTC ACA AGG A-3'	Keil et al. 1986
Vac-inv-forward	5'-CGA TGA GCT CAA TCT GCA AAA TGG C-3'	Rosahl et al. 1986
Vac-inv-reverse	5'-CGA TCC CGG GCT CGT TGC TAA AGC C-3'	Taussig and Carlson, 1983
GUS-forward	5'-GGCCGAGCTCTTACGTCCTGTAGAAACCC-3'	Jefferson et al. 1986
GUS-reverse	5'-GGCCGAGCTCATTGTTTGCCTCCCTGCTG-3'	Jefferson et al. 1986

3.2.7 Southern blot analysis

Confirmation of transgene integration was achieved through Southern blot analysis. Approximately 0.5 µg DNA of the putative tobacco and grapevine transformants were digested with *Xba*I, subjected to agarose gel electrophoresis and blotted onto Hybond N nylon membranes (Amersham Biosciences). These membranes were subjected to hybridisation reactions, employing the DIG system as described by the supplier (Roche Molecular Biochemicals). The presence of the invertase cassettes was confirmed by probing the membranes with a 1440 bp *Suc2* gene fragment, which was PCR labelled using the DIG dNTP labelling mixture (Roche Molecular Biochemicals) according to the manufacturer's specifications.

3.2.8 RNA isolation and Northern blot analysis

Total RNA was isolated from 100 mg of tobacco leaf tissue using the TRIzol® Reagent (GIBCO/BRL Life Technologies) according to the manufacturer's specifications. Total grapevine mRNA was isolated using the method of Davies and Robinson (1996). The protocol was modified for small scale RNA isolation: six frozen leaf disks were ground to a powder and 1 mL of extraction buffer added. The tubes were vortexed for 10 min and centrifuged for 10 min at 10000 *g*. The supernatant was extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and once with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). RNA was precipitated by adding 0.6 volume of 100% isopropanol. Subsequent to sedimentation, the pellets were rinsed, dried and resuspended in 25 μL DEPC treated water.

Total RNA was size fractionated by electrophoresis in a 1.2% (v/v) formaldehyde containing agarose gel and blotted onto Hybond-N nylon membranes (Amersham Biosciences) using standard techniques as described by Sambrook et al. (1989). Hybridisation of the DNA probe to the immobilised RNA was performed at 50°C overnight in a standard DIG hybridisation buffer (Roche Molecular Biochemicals) containing 50% (v/v) formamide. The non-specifically bound probe was removed by washing the membrane twice with 2 × tri-sodium citrate (SSC) and 0.1% (w/v) sodium dodecyl sulphate (SDS) for 15 min at room temperature and twice with 0.5 × SSC and 0.1% (w/v) SDS for 15 min at 65°C. Chemiluminescent signal detection proceeded according to the manufacturer's specifications. Transcript sizes were confirmed by comparing co-migration with a Gibco 0.24-9.5 kb RNA ladder (GIBCO/BRL Life Technologies).

3.2.9 Invertase activity assays

Total protein extracts were prepared from 100 mg of fresh leaf tissue homogenised in liquid nitrogen (from hardened-off transgenic tobacco plantlets). Aliquots of 200 μL ice-cold 100 mM potassium phosphate extraction buffer (pH 7.0) containing 10% (v/v) glycerol; 2% (w/v) PVPP; 1 mM EDTA, pH 8.0; 10 mM 1.4-dithiothreitol (DTT); 0.5 mM Pefabloc SC 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) were transferred to the homogenised leaf tissue. The homogenate was centrifuged at 14000 *g* (15 min, 4°C). The supernatant was filtered through Miracloth (Interscience, Canada) and directly used for invertase activity assays. Invertase activity was determined by incubating 5 μL of the sample at 30°C for 15 min in 50 mM Na acetate pH 5.0 and 100 mM sucrose in a final reaction volume of 100 μL. Reactions were terminated by the addition of 50 mM Tris-HCl, pH 8.0. Reducing sugars were measured using a D-glucose/D-fructose kit (Roche Molecular Biochemicals), an enzyme system coupled to nicotinamide adenine dinucleotide phosphate (NADP). Specific activities were linear over time and proportional to the amount of protein

being assayed. Protein concentration was quantified using the Bradford dye-binding assay according to the manufacturer's instructions (Bio-Rad, Richmond, CA). Invertase activity was expressed in nmol sucrose hydrolysed per minute per mg protein ($nmol.min^{-1}.mg^{-1}$ prot).

3.2.10 Soluble sugars assay

Sugars were extracted from 25 mg ground leaf tissue by incubation with 160 μL of 100% ethanol at 68°C for 15 min. An aliquot of 40 μL water was then added and the mixture incubated as before. This was followed by the addition of 100 μL water and 200 μL chloroform and centrifugation at 12000 *g* for 5 min. The supernatant was dried in a vacuum drier and dissolved in 200 μL water. Glucose and fructose were detected with the D-glucose/D-fructose kit (Roche Molecular Biochemicals). Sucrose was hydrolysed by yeast invertase (Sigma) and the resulting hexoses were assayed with the previously mentioned kit.

3.2.11 Chlorophyll assay

Chlorophyll (Chl) was extracted and assayed according to the method of Lichtenthaler (1987) with the following modification: two leaf discs (ϕ=1.1 cm) were collected from the middle of each leaf and ground in liquid nitrogen, after which 400 μL of 85% (v/v) methanol (containing 1% (w/v) Na_2CO_3) was added and incubated on ice for one hour. A 100 μL aliquot of the extract was then removed and added to 900 μL of 95% (v/v) ethanol and extracted for another hour. The Chl content ($\mu g.mL^{-1}$) was determined spectrophotometrically using the absorbency measured at 664.2 nm and 648.6 nm in the following equations: Chl *a* $=13.36.\Delta OD_{664.2}-5.19.\Delta OD_{648.6}$ and Chl *b* $=27.43.\Delta OD_{648.6}-8.12.\ \Delta OD_{664.2}$. Total Chl *a/b* $=5.24.\ \Delta OD_{664.2}+22.24.\Delta OD_{648.6}$.

3.2.12 Statistical analysis

A non-parametrical bootstrap was used in the statistical analysis of the data. This method was more suitable than ANOVA due to the nature of the experimental layout: little variance was observed between the control (GUS) lines in most of the analyses conducted and therefore less replications were used for these lines to ensure that more invertase transformants could be examined.

3.3 RESULTS

3.3.1 Construction of expression cassettes

The coding region of the intracellular form of the *Suc2* gene of *S. cerevisiae* was fused to different portions of the targeting signal of the proteinase inhibitor II (a

vacuolar protein) from potato (Keil et al., 1986). Targeting to the vacuole requires positive sorting information and was achieved through fusion to the complete signalling sequence, whereas apoplastic targeting of SUC2 was achieved by fusion to a 230 bp N-terminal portion of the potato proteinase inhibitor II signalling sequence. A cytosolic localisation of the invertase protein was ensured by the absence of a targeting signal. Similar constructs have previously been shown to result in vacuolar, apoplastic and cytosolic localisation of the invertase enzyme (Von Schaewen et al., 1990; Sonnewald et al., 1991). A construct containing the *E. coli* β-glucuronidase (*Gus)* reporter gene with no targeting signals was prepared for use in control transformations. The chimeric genes were inserted into a plant expression cassette between the constitutive CaMV 35S promoter and the 3' termination element of the octopine synthase gene (OCS). The constructed invertase plasmids, containing the expression cassettes with different targeting signals, were mobilised into *A. tumefaciens* for the transformation of both tobacco and grapevine plants.

3.3.2 Regeneration of putative transgenic lines

The grapevine transformations yielded 20 putative transgenic grapevine plants transformed with the apoplastic (Apo-Inv) yeast invertase expression cassette, whereas calli transformed with the cytosolic (Cyt-Inv) and vacuolar (Vac-Inv) yeast invertase cassettes failed to germinate and could therefore not be regenerated. The putative Apo-Inv grapevine plants were subjected to PCR, Southern and Northern blot analysis, which revealed that these plants were false positive transformants and did not contain the heterologous invertase gene (results not shown).

However, approximately 10, 40 and 50 independent putative transgenic tobacco plants were regenerated for the Cyt-Inv, Apo-Inv and Vac-Inv expression cassettes, respectively. The putative Cyt-Inv transformants had a very low survival rate and therefore only the putative Apo-Inv and Vac-Inv transgenic tobacco lines were subsequently subjected to further analyses (Section 3.3.4 to 3.3.8).

3.3.3 Effect of yeast-invertase expression on phenotype and growth

The *in vitro* Cyt-Inv tobacco plants displayed severely reduced shoot and root growth (Fig. 2A). The Apo-Inv transformants displayed a less dramatic phenotype than the Cyto-Inv lines, although they still displayed stunted growth and bleached regions in the interveinal leaf tissue (Fig. 2B). No obvious phenotypic differences could be observed between the Vac-Inv transformants (Fig. 2C) and control (GUS) plants (Fig. 2D). The growth rates of these plants were similar to that of the control plants and no bleached regions could be observed.

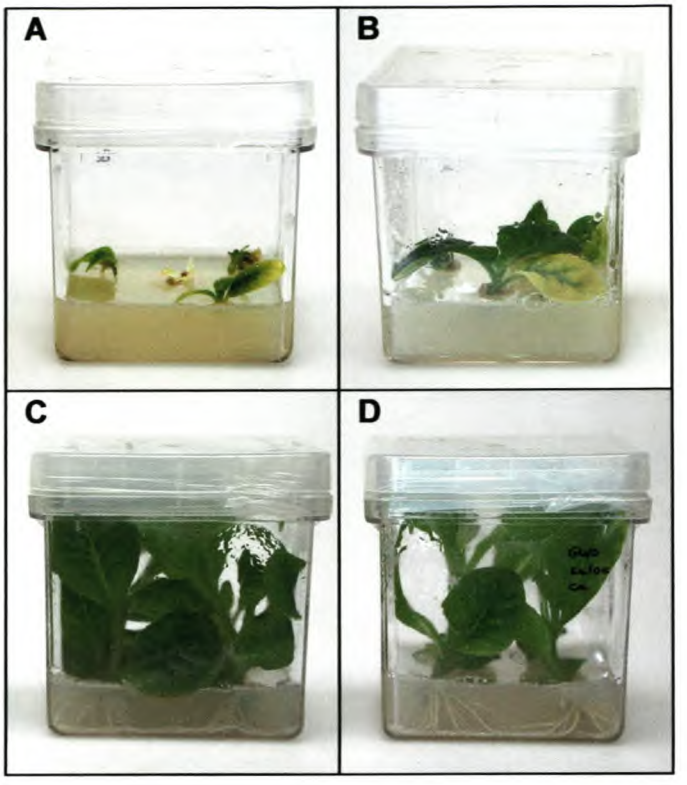

Figure 2. Examples of phenotypical appearance of *in vitro* tobacco plantlets of lines transformed with the *Saccharomyces cerevisieae* invertase (*Suc2*) gene targeted to the (A) cytosol, (B) apoplast and (C) vacuole and (D) control lines transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene.

When the transgenic tobacco plants were transferred to soil and grown in a growth room, the phenotypes observed *in vitro* persisted. No Cyto-Inv transformants survived the transfer to soil due to severely reduced root formation. The Apo-Inv transformants displayed a significantly reduced height and root formation with considerable variation between the different transformants (Fig. 3A). Reduction in root formation varied from plants with almost no roots to plants with roots that were approximately one third of the control plant roots in volume. The height of these transformants varied between 3 and 60 cm, the latter being the height of the control plants. Bleached and necrotic regions appeared in the interveinal tissues of leaves, which followed the sink-source transitional pattern of development as described by Von Schaewen et al. (1990) (Fig. 3B). The leaves of some of the Apo-Inv transformants exhibited a tendency to curl downward as observed in potato plants expressing a yeast invertase targeted to the apoplast (Heineke et al., 1992). The phenotype of transgenic tobacco plants with a yeast invertase located in the vacuole was mostly indistinguishable from the control plants. Some Vac-Inv transformants, however, displayed reduced height (smallest, 15 cm) and bleached regions in the old leaves.

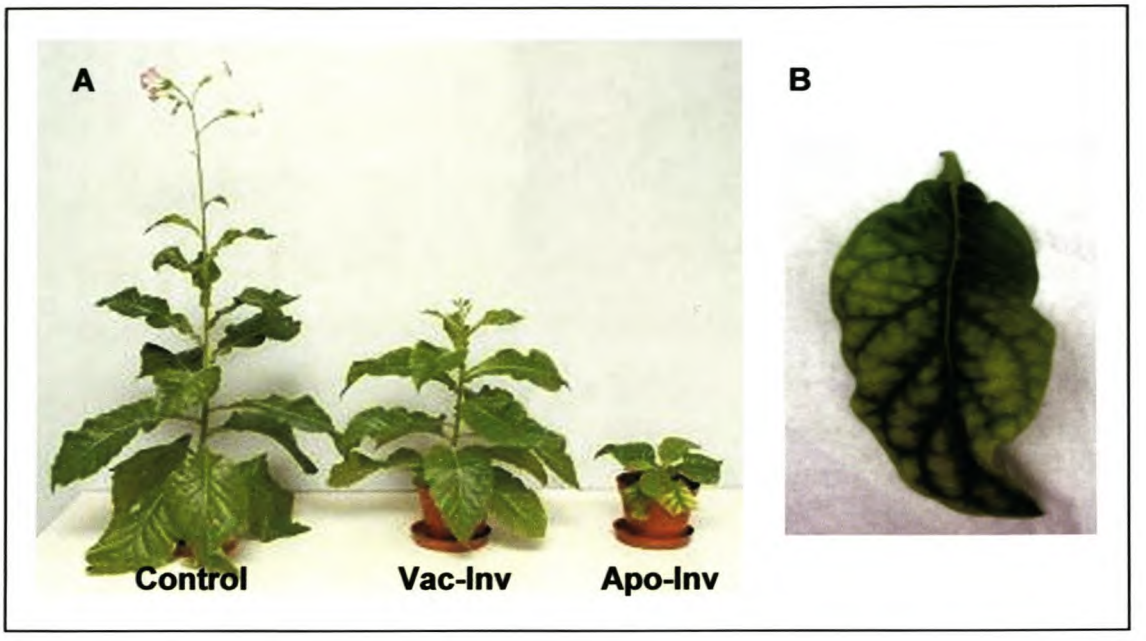

Figure 3. (A) Examples of phenotypical appearance of a soil-grown tobacco control plant transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene and tobacco lines of the same age transformed with the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the vacuole (Vac-Inv) and apoplast (Apo-Inv). (B) Phenotype of a typical mature leaf from the Apo-Inv lines.

3.3.4 Confirmation of transgene integration

The putative transgenic tobacco plants were screened for the presence of the recombinant invertase gene by means of PCR amplification. As expected for the different constructs, a 1.7 kb, 2.9 kb and 1.8 kb fragment was amplified for the positive Apo-Inv, Vac-Inv (Fig. 4) and GUS transformants, respectively.

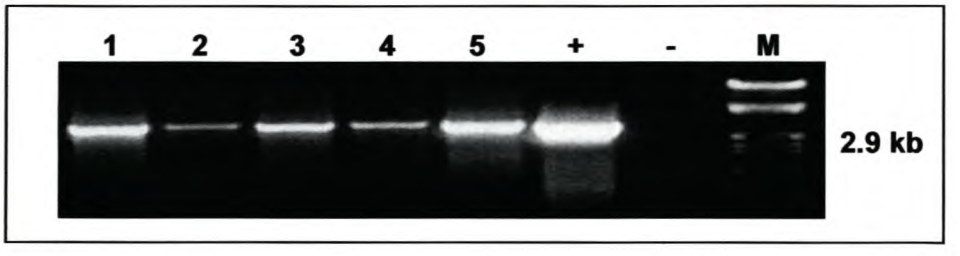

Figure 4. Confirmation of the presence of the heterologous invertase gene in tobacco lines transformed with the *Saccharomyces cerevisiae Suc2* gene targeted to the vacuole (Vac-Inv). Lanes 1-5 represent amplification reactions using gDNA templates from independent Vac-Inv plants. A 2910 bp PCR product indicated positive Vac-Inv tobacco transformants. A positive (+) (plasmid DNA) and negative (–) (untransformed tobacco gDNA) control as well as a λ (*Eco*RI/*Hin*dIII) marker (M) is indicated.

Integration of the heterologous invertase gene into the plant genome was confirmed by Southern blot analysis. Genomic DNA was digested with *Xba*I, which

recognises one site within the yeast invertase gene and one at the carboxyl terminus. This results in a hybridisation signal representing 1 kb of the invertase gene fragment, as well as additional signals representing the number of integrated gene copies when probed with a 1440 bp digoxigenin-labelled fragment corresponding to the coding region of the yeast *Suc2* gene.

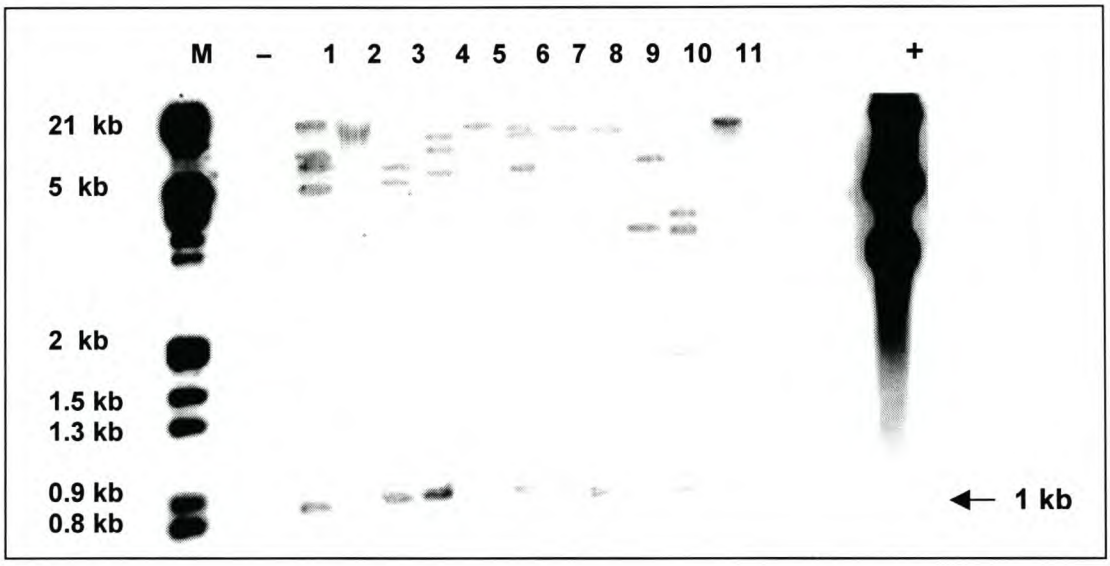

Figure 5. Southern blot analysis of genomic DNA, digested with *Xba*I, of independent tobacco plants transformed with the *Saccharomyces cerevisieae* invertase (*Suc2*) gene targeted to the vacuole (Lanes 1-11). The λ (*Eco*RI/*Hin*dIII) marker (M), with fragment sizes indicated in kb, as well as a positive (+) (plasmid DNA) and negative (–) (untransformed tobacco gDNA) control are indicated.

Between one and four copies of the expression cassettes integrated into the tobacco genome of the 23 positive Apo-Inv and 37 positive Vac-Inv transformants. A representative example of a Southern blot analysis performed on 11 Vac-Inv transformants is depicted in Figure 5. Eleven plants of each of the Apo-Inv and Vac-Inv transgenic lines, containing an intact copy of the chimeric invertase gene, were subjected to Northern blot analysis. Integration of the *Gus* expression cassette, as well as expression and functionality was confirmed by means of positive histological GUS assays according to the method of Jefferson et al. (1987) (results not shown).

3.3.5 Verification of transgene transcription

Northern blot analysis revealed the active transcription of the pART27 expression cassettes in all the transgenic tobacco lines analysed. The membranes were probed with the same 1440 bp labelled *Suc2* fragment as used in the Southern blot analysis. A hybridisation signal at 2 kb was observed for the Vac-Inv transformants, which was smaller than the expected 2.9 kb chimeric invertase fusion sequence (Fig. 6). This could be due to an inaccurate size estimation of the mRNA gel. Positive invertase

expressing lines were subsequently transferred to soil and grown in a growth room for further characterisation. No yeast invertase transcripts were detected in the GUS control plants (results not shown).

Figure 6. Northern blot analysis of total RNA isolated from independent tobacco plants transformed with the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the vacuole (Lanes 1-11). A negative control (–) (untransformed tobacco RNA) is indicated.

3.3.6 Increased invertase activity in transgenic tobacco plants

Protein extracts from hardened-off transgenic tobacco source leaves were subjected to invertase activity assays at pH 5.0 for optimal activation of the invertase enzyme (Goldstein and Lampen, 1975). As described in section 3.3.3, Cyto-Inv transformants could not be hardened off and therefore the invertase activity of these lines was not determined. Invertase activities of more than 1.5 nmol.min^{-1}.mg^{-1} protein (prot) (measured for the control (GUS) plants) were detected in the Apo-Inv and Vac-Inv transformants (Fig. 7).

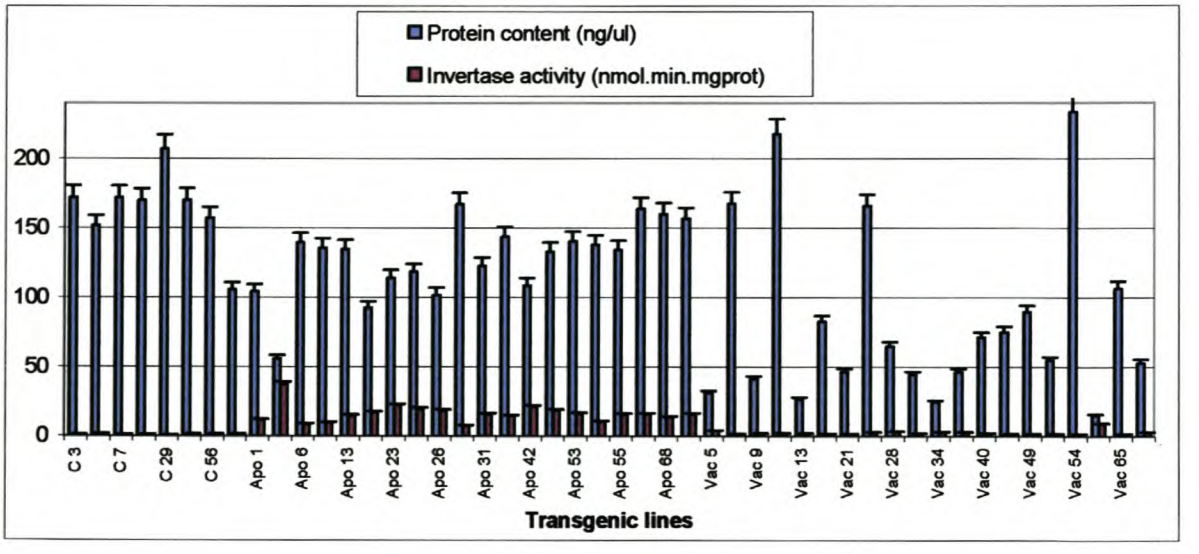

Figure 7. Protein content (ng/μl) and invertase activity (nmol.min^{-1}.mg^{-1}prot) of independent transgenic tobacco lines expressing the *Suc2* invertase gene of *Saccharomyces cerevisiae*, targeted to the apoplast (Apo) or vacuole (Vac) in comparison with control (C) plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene. The error bars indicate the standard error.

The Apo-Inv transformants exhibited invertase activities ranging from 7.5 to 37 $nmol.min^{-1}.mg^{-1}$ prot, which were significantly higher than the control plants ($P<0.01$). The Vac-Inv transformants displayed a trend of higher invertase activity than the control plants ($P=0.15$) with invertase activities ranging from 1.5 to 9 $nmol.min^{-1}.mg^{-1}$ prot. The results of these assays confirmed the functionality of the heterologous yeast protein in the transformed plants. No obvious correlation could be found between the levels of invertase activity and protein content in both the control and invertase transformants.

3.3.7 Expression of yeast-invertase leads to altered soluble sugar levels

The levels of both the substrate (sucrose) and products of the invertase reaction (glucose and fructose) were determined in the sink leaves, source leaves and roots of control (GUS) and transgenic lines expressing a yeast invertase in the apoplast or vacuole (Fig. 8). In the sink leaves, both the Apo-Inv and Vac-Inv lines displayed a 2.4-fold ($P=0.03$) decrease in Suc content in comparison to the control plants. A concomitant increase in both the Glc and Fru levels were also measured in these lines: the Apo-Inv lines exhibited a 1.2-fold and fivefold ($P<0.01$) increase in Glc and Fru levels, respectively, whereas the Vac-Inv lines displayed a 1.4-fold and 2.8-fold ($P=0.02$) increase in Glc and Fru amounts, respectively.

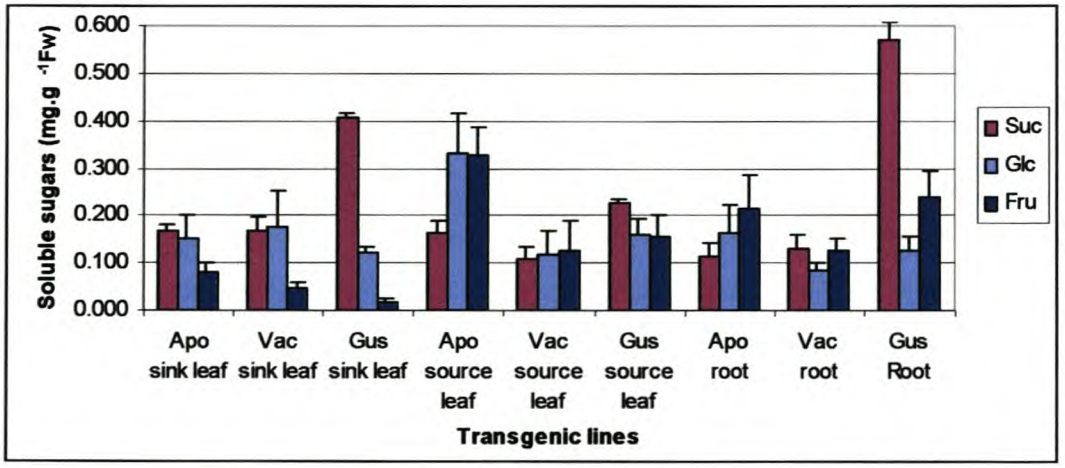

Figure 8. Sucrose (Suc), glucose (Glc) and fructose (Fru) levels in sink leaves, source leaves and roots of control tobacco lines (Gus) transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene and tobacco lines expressing the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the apoplast (Apo) or vacuole (Vac). The error bars indicate the standard error.

The source leaves of both the Apo-Inv and Vac-Inv lines exhibited a less pronounced decrease in Suc levels. Although not statistically significant, a 1.4-fold decrease in the average Suc content and a doubling in both the average Glc and Fru content, were measured in the Apo-Inv lines. In comparison to the control lines, a significant twofold ($P=0.04$) decrease in Suc levels were displayed by the Vac-Inv lines. The

source leaves of the Vac-Inv lines were further characterised by a 1.4-fold and 1.3-fold decrease in the average Glc and Fru levels, respectively. Sucrose levels were significantly reduced in the roots of the invertase transformants, with the Apo-Inv lines and Vac-Inv lines displaying a fivefold ($P<0.01$) and 4.4-fold ($P<0.01$) decrease, respectively, when compared to the control lines. No significant differences in Glc and Fru levels were observed between the control and Apo-Inv lines. In comparison to the control plants, a 1.5-fold and 1.9-fold decrease in the averages of the Glc and Fru levels, respectively, were measured in the Vac-Inv lines.

3.3.8 Decreased chlorophyll content in Apo-Inv lines

The Chl content of successive leaves of invertase expressing tobacco plants, as well as GUS control plants were measured (Fig. 9). In the Apo-Inv lines, the Chl content was threefold lower in all the leaves tested when compared to the Vac-Inv or control GUS plants. The loss in Chl follows the trend of leaf development as found in the control plants. The Vac-Inv lines displayed no significant difference in Chl content in comparison to the control GUS lines.

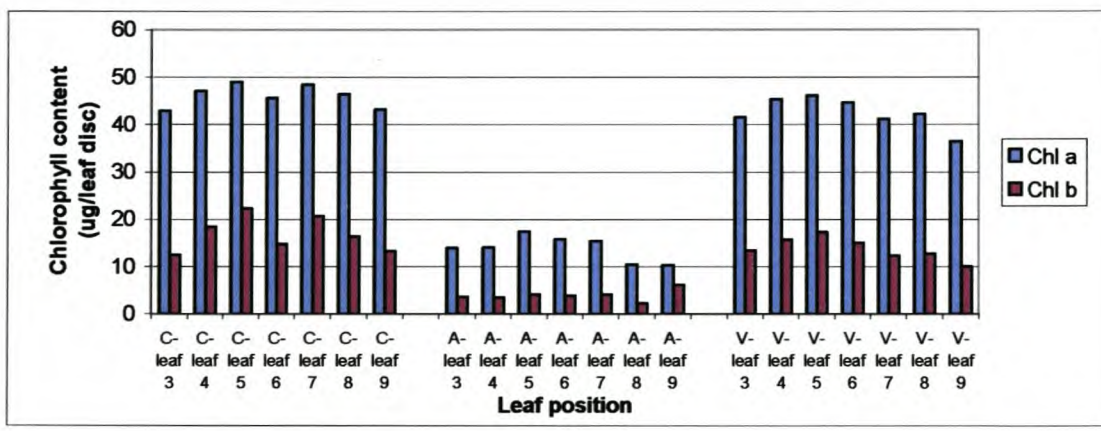

Figure 9. Chl *a* and Chl *b* content of successive leaves of control (C-leaf) tobacco plants, transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene, and transgenic tobacco lines expressing the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the apoplast (A-leaf) and vacuole (V-leaf). Leaf numbers 3 to 4 represent young leaves, 5 to 7 fully-expanded leaves, and numbers 8 to 9 old leaves.

3.4 DISCUSSION

In this study it was attempted to transform both tobacco and grapevine plants with a yeast-derived invertase targeted to either the cytoplast, vacuole or apoplast.

Grapevine was transformed in order to elucidate the underlying mechanisms of sugar translocation and sink-source relationships in *V. vinifera*. Unfortunately, none of the regenerated grapevine plants contained the transgene. This was surprising since the transformed calli were regenerated on selective media for an

extensive period of time (at least three months) to attempt to reduce the risk of false positive and chimeric plants. A possible explanation could be that habitualisation occurred whereby the calli developed resistance to the selection agent. Transformation of grapevine with a yeast-derived invertase will be attempted again, since expanding our understanding of the mechanisms involved in sugar translocation at both the physiological and biochemical levels will help to target, isolate and manipulate genes involved in this process.

The transformation of tobacco was done in order to establish transgenic plant lines, affected in their sink-source relationships, which would be comparable to previously established lines (Von Schaewen et al., 1990; Sonnewald et al., 1991). Following the same trend as the previously established systems, an increase in invertase activity was observed with both the Vac-Inv and Apo-Inv lines when compared to the control plants. This average increase was approximately 2.5-fold for the Vac-Inv transformants and 10-fold for the Apo-Inv transgenic lines. These values are, however, lower than the values reported by Von Schaewen et al. (1990) and Sonnewald et al. (1991). A baseline invertase activity was also established for the control tobacco plants (ca. 1.5 $nmol.min^{-1}.mg^{-1}$ prot) in this study, whereas no invertase activity for the control plants was reported by Von Schaewen et al. (1990). This is probably due to variety-specific differences between the explant material used (Samsun NN vs Petit Havanna SR1). Furthermore, as observed by Von Schaewen et al. (1990) and Sonnewald et al. (1991), the highest invertase activity was measured in the Apo-Inv lines, whereas the invertase activity in the Vac-Inv lines was low in comparison with these lines. This could possibly indicate a lower stability of the fusion protein in the vacuole (Sonnewald et al., 1991).

Another difference between results obtained here and previous experiments relates to the phenotype of *in vitro* plantlets. In previous experiments, no obvious phenotypical differences could be observed between the *in vitro* transformants and control plants (Von Schaewen et al., 1990; Sonnewald et al., 1991). Dramatic phenotypical differences could, however, be observed between *in vitro* as well as soil-grown transgenic plants containing the different chimeric gene constructs, and the control plants, in this study. These dissimilarities could possibly be related to variety-specific differences or changes in growth and irradiation conditions. The Cyto-Inv transgenic plants were most severely affected, followed by the Apo-Inv lines with less dramatic phenotypes, and the Vac-Inv plants with almost no visible phenotype. The affected lines displayed varying degrees of stunted growth, reduced root formation and the development of bleached leaf areas.

From these observations it is clear that the sub-cellular compartmentation of the yeast invertase, and the resulting site of altered sugar levels, have profound effects on plant physiology. This might be explained by differences in sensitivity towards alterations in sugar status by the various subcellular compartments as a result of sub-cellular sugar metabolism specialisation. For example, invertase expressed in the cytosol of source leaf cells would hydrolyse sucrose as it is

synthesised and therefore adversely affect sucrose formation, as well as export, and consequently whole-plant development and growth (Sonnewald et al., 1991). The apoplastic location of the heterologous invertase had a less severe effect on plant physiology since the disturbance in sugar metabolism occurred at the site of export only. Plant growth and development is affected by reduced phloem loading as well as the accumulation of hexoses, as a result of sucrose hydrolysis in the apoplastic space (Von Schaewen et al., 1990). Vacuolar expression of the yeast invertase had the least effect on plant morphology, when compared to the other localisations. It is thought that sucrose is only transiently stored in the vacuole of source cells and that the hydrolysis of sucrose in this compartment would not affect sucrose synthesis and export directly. However, lower levels of invertase activity were measured in these lines. It is also possible that plant cells are better equipped to regulate gene expression in response to altered sugar levels in the vacuole.

As with the previous studies, the heterologous invertase action in the various sub-cellular compartments led to altered sucrose, glucose and fructose levels. Analysis of the sugar levels in the tissues of the invertase transformants revealed varying degrees of reduced sucrose levels. Reduced sucrose levels were also measured in plants exposed to low irradiances by Sonnewald et al. (1991). An increase in sucrose levels was, however, reported for transgenic lines grown at higher irradiances (300 $\mu mol.m^{-2}.s^{-1}$) (Von Schaewen et al., 1990; Stitt et al., 1990; Sonnewald et al., 1991). In accordance with previous results, the hydrolysis of sucrose by invertase resulted in increased hexose content in the Apo-Inv lines. The Vac-Inv lines, however, displayed a reduction in hexose levels.

By sensing these changes in carbohydrate status, plants can respond by altering gene expression and/or enzyme activities, thereby controlling photosynthate production, partitioning, and subsequent utilisation (Koch, 1996). Photosynthetic genes are repressed by various hexoses as part of this feedback control mechanism (Sheen 1990; Krapp et al., 1993; Jang and Sheen, 1994; Sheen, 1994). Previous experiments involving transgenic plants overexpressing a yeast-derived invertase showed that the metabolic status of source leaf cells controls the level of sugar repression (Stitt et al., 1990; Sonnewald et al., 1991; Büssis et al., 1997) and that the produced hexoses are responsible for the long-term repression of the photosynthetic genes (Heineke et al., 1994; Stitt et al., 1995). A causal relation between the increase in hexoses and a lowered rate of photosynthesis and decreased Chl content was established by Von Schaewen et al. (1990). Stitt et al. (1990) have shown that the decrease in photosynthesis, because of Calvin cycle inhibition, will decrease the demand for ATP and NADPH, which in turn will result in the loss of Chl. In this study, transgenic tobacco transformed with a yeast invertase targeted to the apoplast displayed an up to threefold reduction in Chl content. This reduction in Chl content coincided with the visible phenotype: the decrease in Chl content followed the development of the visible symptoms and inhibition was most severe in the mature leaves of Apo-Inv plants where bleached areas were observed.

In conclusion, we have successfully transformed tobacco with a yeast-derived invertase gene targeted to either the cytosol, vacuole or apoplast. The overexpression of this enzyme led to dramatic changes in the growth and development of these plants, especially the Cyto-Inv and Apo-Inv lines. These changes, specifically the reduction in Chl content (related to reduced photosynthetic capacity) were clearly linked to the disturbed sink-source relationships caused by the invertase activity and resulting increase in hexose levels. These results are consistent with previous experiments involving the overexpression of a yeast-derived invertase in tobacco. This valuable model system can now be utilised to gain additional fundamental knowledge concerning sugar metabolism, specifically gene regulation and sugar partitioning, in tobacco.

3.5 ACKNOWLEDGEMENTS

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CHAPTER 4

RESEARCH RESULTS

The effect of altered sugar levels on the expression of sugar transporters in transgenic tobacco

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The effect of altered sugar levels on the expression of sugar transporters in transgenic tobacco

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It has been shown previously that sugars can have varied effects on the expression of sugar transporters, thus forming part of the complex regulatory web that governs sugar transport and consequently sugar partitioning in higher plants. In order to examine the *in vivo* role of sugars on sugar transporter expression, a yeast-derived invertase was targeted to either the vacuole or apoplast of *Nicotiana tabacum* to alter soluble sugar content. Transcript levels of tobacco sucrose (NtSUT1) and hexose (NtMST1) transporter encoding genes increased in the source leaves and roots of transformants expressing a yeast-derived invertase in the vacuole. A significant increase in *NtMst1* transcript levels were also detected in the roots of transgenic lines expressing the heterologous invertase in the apoplast. From these data it is evident that the NtSUT1 and NtMST1 transporters analysed here are differentially regulated at the transcriptional level by sucrose and/or hexose content. In addition, it would seem that the regulatory effect of the altered sugar levels on transporter expression depended on the subcellular compartment to which the yeast invertase was targeted.

4.1 INTRODUCTION

The growth and development of higher plants are dependent on the energy gained during the process of photosynthesis, as well as the systemic distribution of photosynthate among the various plant organs. In most species, photosynthate is transported primarily in the form of sucrose during long-distance transport through the phloem. Sucrose translocation is controlled by the concentrations of this product in the areas of supply (sources) and utilisation (sinks) and therefore follows a strict source-to-sink movement.

The processes of phloem loading at the source organs (Grusak et al., 1996) and unloading at the sink organs (Patrick, 1997) are pivotal in sucrose translocation. The delivery of sucrose to the phloem can be by symplastic cell-to-cell diffusional transport via the plasmodesmata, or by apoplastic carrier-mediated transport where a disaccharide transporter (DST) mediates the transport of sucrose across the plasma membrane (Riesmeier et al., 1994; Ward et al., 1998). After long-distance transport through the vascular system, the transported sugar is unloaded from the sieve elements via a symplastic or apoplastic route. In the case of apoplastic

unloading, sucrose (Suc) can be imported directly from the apoplast via DSTs (Lemoine, 2000) or it can be hydrolysed to glucose (Glc) and fructose (Fru) by cell wall-bound invertases and taken up via monosaccharide transporters (MSTs) (Büttner and Sauer, 2000).

Experiments involving transgenic tobacco plants expressing a yeast-derived invertase in the apoplast (Von Schaewen et al., 1990), as well as antisense transporter experiments (Bürkle et al., 1998) concluded that an apoplastic-loading pathway is functional in tobacco. Tobacco phloem unloading mechanisms, on the other hand, have been identified utilising the heterologous production of a jellyfish green fluorescent protein (GFP) (Imlau et al., 1999). A symplastic-unloading pathway was identified in sink leaves and root tips, whereas an apoplastic unloading mechanism is operable in developed roots. Three sugar transporters, which play an essential role in these loading and unloading pathways, have been identified in tobacco: two Suc transporters, NtSUT1 (Bürkle et al., 1998) and NtSUT3 (Lemoine et al., 1999), as well as a hexose transporter NtMST1 (Sauer and Stadler, 1993). A plastidic Glc transporter NtGlcT, which mediates the export of Glc from plastids, was also identified (Weber et al., 2000).

Sugar transporters can be regulated by transcriptional (Truernit et al., 1996; Sakr et al., 1997; Delrot et al., 2000) and posttranslational control (Sakr et al., 1997; Roblin et al., 1998; Delrot et al., 2000), with transcriptional regulation including developmental expression, diurnal control, and regulation by plant hormones (Sauer and Stadler, 1993; Heineke et al., 1994; Bürkle et al., 1998; Lemoine et al., 1999; Weber et al., 2000). In addition, sugars have been shown to have a varied influence on sugar transporter expression, ranging from transporters being repressed (Weber et al., 1997; Chiou and Bush, 1998), stimulated (Hilgarth et al., 1991; Atanassova et al., 2003), or unaffected (Stadler and Sauer, 1996) by the presence of sugars. These significant findings strongly indicate that sugars, i.e. Suc as well as hexoses, play an important regulatory role in sugar transporter activity and thus sugar partitioning.

This study focussed on the influence of altered sugar levels on assimilate partitioning by examining the effect on sugar transporter expression. To this end, transgenic tobacco plants overexpressing a yeast-derived invertase, targeted to either the vacuole (Vac-Inv) or apoplast (Apo-Inv), have been created (Chapter 3) and shown to have altered sugar levels in their various tissues. These plants were analysed for the levels of sugar transporter mRNA in sink and source tissues, which were correlated to the invertase activities and altered sugar levels in these tissues.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

See Section 3.2.1 of Chapter 3.

4.2.2 Selection of transgenic lines

Hardened-off transgenic tobacco lines containing a yeast-derived invertase (SUC2) targeted to either the vacuole or apoplast, as well as control lines transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene, were prepared and selected as described in Chapter 3. The lines were screened by determining the invertase activity in the mature leaves (Section 3.2.9 of Chapter 3). Four plants from each of the low, medium and high invertase activity Apo-Inv and Vac-Inv lines, as well as control plants, were selected for analysis.

4.2.3 RNA isolation and Northern blot analysis

The soil-grown transgenic lines were harvested after five weeks of growth in the growth chamber. Plant material from fully expanded source leaves (ca. 10 cm) and roots was harvested after 4-6 hours of light exposure and ground to a fine powder using liquid nitrogen. Excess material was stored at –80°C. Total RNA was isolated from 100 mg source leaves and roots, respectively, using the TRIzol® Reagent (GIBCO/BRL Life Technologies, California, USA) according to the manufacturer's specifications. Total RNA was size fractionated by electrophoresis in 1.2% (v/v) formaldehyde containing agarose gels and blotted onto Hybond-N nylon membranes (Amersham Biosciences, Buckinghamshire, UK) using standard techniques (Sambrook et al., 1989). Hybridisation of cDNA probes to immobilised RNA was performed at 50°C overnight in a standard DIG hybridisation buffer (Roche Molecular Biochemicals, Ingelheim, Germany) containing 50% (v/v) formamide. The non-specifically bound probe was removed by washing the membrane twice with 2 × tri-sodium citrate (SSC) and 0.1% (w/v) sodium dodecyl sulphate (SDS) for 15 min at room temperature and twice with 0.5 × SSC and 0.1% (w/v) SDS for 15 min at 65°C. Chemiluminescent signal detection proceeded according to the manufacturer's specifications (Roche Molecular Biochemicals). Transcript sizes were confirmed by comparing co-migration with a Gibco 0.24-9.5 kb RNA ladder (GIBCO/BRL Life Technologies). Subsequent to hybridisation with the transporter probes, the membranes were stripped according to the manufacturer's specifications (Roche Molecular Biochemicals) and re-hybridised with an 18S rRNA probe, following the same procedure as previously described. The transporter autoradiograms, and their related rRNA autoradiograms, were scanned on a digital camera (AlphaImager 1220; Alpha Innotech Corporation, California, USA), and the signal intensities were analysed with AlphaEase v5.5 densitometry software (Alpha Innotech Corporation). To normalise the intensity values of the hybridisation signals, they were divided by the intensity values of the total rRNA in the corresponding lane. The resulting ratios allowed comparison between the intensities of different hybridisation signals within the same autoradiogram.

The following cDNA fragments were used as DIG-probes after polymerase chain reaction (PCR) labelling according to the manufacturer's specifications (Roche Molecular Biochemicals): the complete cDNAs of *N. tabacum Sut1* and *pGlcT*, as well as a partial gDNA fragment of *Mst1*. The following primer pair was used to amplify *NtMst1* from genomic *N. tabacum* DNA: MST1 forward: 5'-GATCGGATCC(*Bam*HI)GTTCAGGA-AGCAAAAGGCAG-3' and MST1-reverse: 5'-GATCGAGCTC(*Sac*I)CACTCGCC-ACGACTAAATCA-3' (Sauer and Stadler, 1993). Amplification was achieved with the following conditions: an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and elongation at 72°C for 2 min. The PCR fragments obtained were ligated into the vector pGEM T-Easy (Promega, Madison, USA). The presence of a DNA fragment corresponding to the sequence of *NtMst1* was confirmed through sequencing, using an ABI Prism 377 automated DNA sequencer from PE Biosystems. The mentioned PCR primers were used to PCR DIG label the *NtMst1* fragment, whereas the primer pair T7 and T3 was used for *NtSut1* and *NtpGlcT* from pBluescript (Stratagene, California, USA). The 18S rRNA probe was labelled with a DIG RNA labelling mix according to the manufacturer's specifications (Ambion, Texas, USA).

4.2.4 Soluble sugars assay

Excess plant material harvested during the isolation of RNA was used in the determination of soluble sugars. Sugars from source leaves and roots were extracted and measured as described in Section 3.2.10 of Chapter 3.

4.2.5 Statistical analysis

See Section 3.2.12 of Chapter 3.

4.3 RESULTS

4.3.1 Analysis of transformants expressing a chimeric invertase

Subsequent to *Agrobacterium tumefaciens* transformation procedures, 40 to 50 independent putative Apo-Inv and Vac-Inv lines, as well as control lines transformed with the *E. coli* β-glucuronidase (*Gus*) gene, were regenerated (Chapter 3). These putative transformants were analysed by means of PCR, Southern- and Northern blot analyses and the positive lines hardened off for further characterisation (Chapter 3). The observed phenotypes of the hardened-off plants are described in Section 3.3.3 of Chapter 3.

A range of invertase activities was found among the mature leaves of the transformed plants, which may be ascribed to chromosomal positional effects and/or

integration patterns of the transgenes, as is typical of plants transformed with *A. tumefaciens* (Kuhlemeier et al., 1987). The results of the invertase assays confirmed that the chimeric yeast genes are functionally expressed in the transformed plants. Transformants with low, medium and high invertase activities (ranging from approximately 1.5 to 37 nmol.min^{-1}.mg^{-1}prot) were identified (Fig. 1). Four plants from the medium invertase activity Apo-Inv and Vac-Inv lines, as well as control plants, were selected for further characterisation.

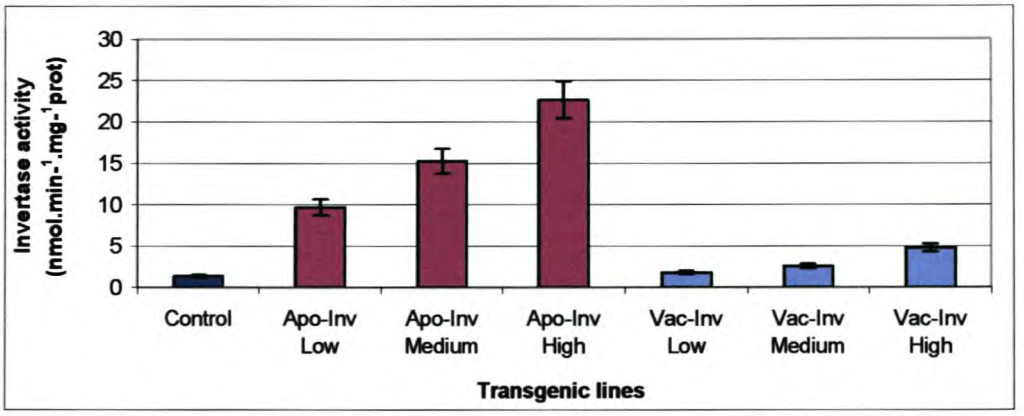

Figure 1. Averages of invertase activity (nmol.min^{-1}.mg^{-1}prot) of the mature leaves of eight control tobacco plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene and eight transgenic tobacco plants with either low, medium or high invertase activity due to the functional expression of a *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to either the apoplast (Apo-Inv) or vacuole (Vac-Inv). The error bars indicate the standard error.

4.3.2 Tissue-specific soluble sugar content

The source leaves and roots of Apo-Inv, Vac-Inv and control lines were analysed for soluble sugar content. The levels of Suc, Glc and Fru were determined in these tissues with different sink/source status (Fig. 2). In comparison to the control lines, the average Suc content of the source leaves decreased by 1.3-fold and 1.7-fold (P=0.05) in the Apo-Inv and Vac-Inv lines, respectively. The Vac-Inv source leaves exhibited a fourfold decrease in average hexose content, whereas the Apo-Inv lines displayed a 2.7-fold (P=0.13) increase in average hexose levels. In comparison to the control plants, the Suc content in the roots decreased by approximately threefold (P=0.03) in the Vac-Inv lines, whereas a 10-fold (P=0.04) decrease was observed in the Apo-Inv lines. No significant differences in the average hexose levels were observed for the Vac-Inv roots, whereas the average total hexose content of the Apo-Inv roots decreased by approximately 1.4-fold.

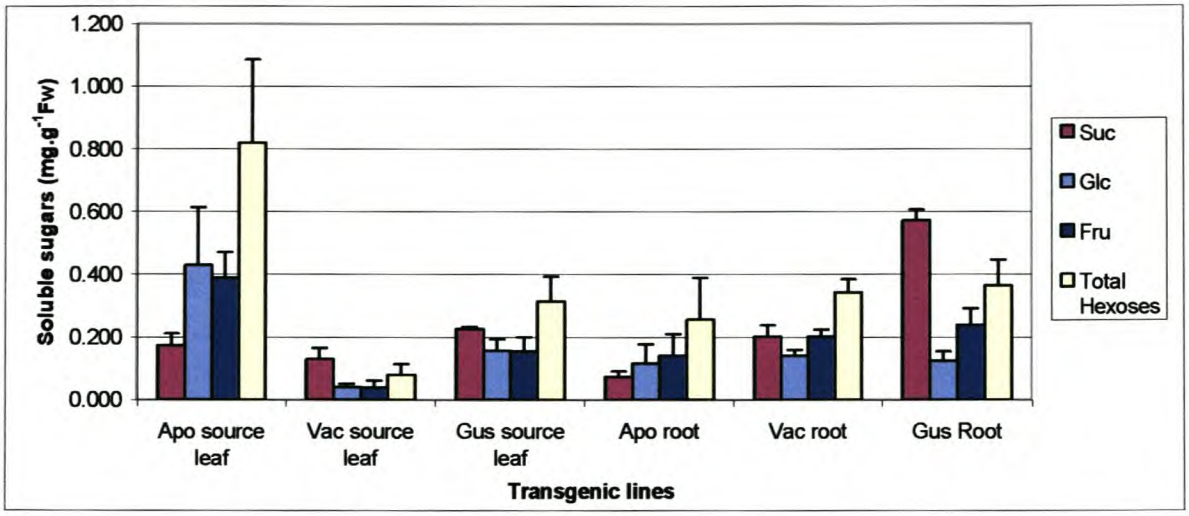

Figure 2. Sucrose (Suc), glucose (Glc), fructose (Fru) and total hexose levels in source leaves and roots of control tobacco lines (Gus) transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene and transgenic tobacco lines with medium activity levels of a *Saccharomyces cerevisiae* invertase (SUC2) targeted to the apoplast (Apo-Inv) or vacuole (Vac-Inv). The error bars indicate the standard error of the mean of four lines analysed.

4.3.3 Altered sugar transporter expression levels

To investigate the effect of the altered sugar levels in the source leaves and roots on sugar transporter expression, total RNA was isolated from these tissues of Apo-Inv and Vac-Inv lines, as well as control plants. Northern blots were probed with *NtSut1*, *NtMst1* and *NtGlcT* cDNA. The relative expression levels of the Suc transporter *NtSut3* gene were not analysed in this study since the expression of this transporter is restricted to late pollen development, pollen germination and pollen tube growth (Lemoine et al., 1999). In this study, the transgenic plants were harvested at the vegetative stage, prior to flowering. A Northern blot probed with *NtSut1* and expression levels of this transporter relative to rRNA levels is represented in Figure 3.

NtpGlcT mRNA levels of control and yeast-invertase expressing transformants varied greatly and thus no significant analysis could be made regarding the expression of this transporter in response to the altered sugar levels (results not shown). Expression levels of the sugar transporters NtSUT1- and NtMST1-encoding genes, relative to rRNA expression levels, however, revealed significant changes when compared to control plants (Fig. 4).

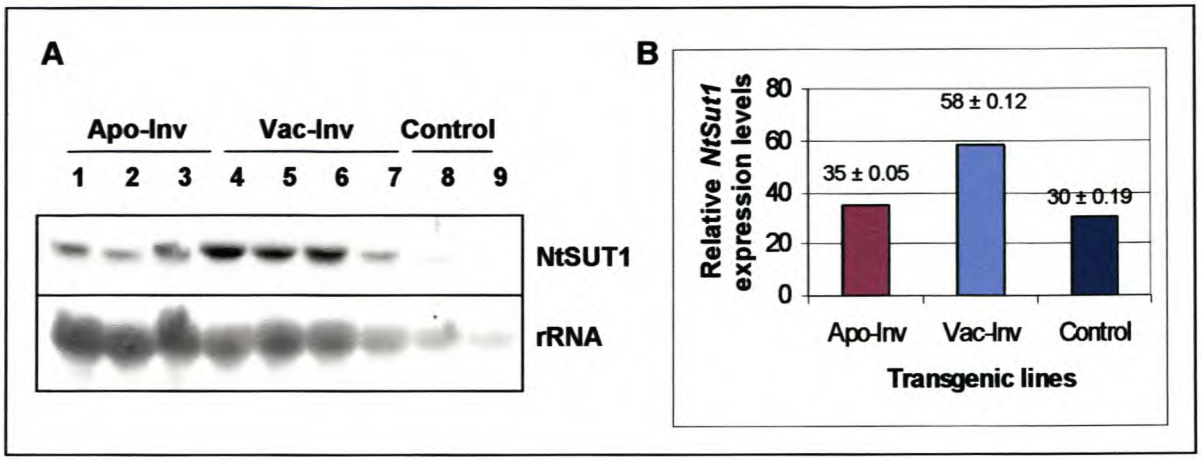

Figure 3. (A) Northern blot analysis of total RNA isolated from the roots of tobacco plants expressing the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the apoplast (Apo-Inv) (lanes 1-3) or vacuole (Vac-Inv) (lanes 4-7), as well as control plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene (lanes 8-9) probed for NtSUT1 and rRNA. (B) Expression levels of the NtSUT1 sucrose transporter, relative to rRNA expression levels, in the roots of control (GUS) plants, as well as Apo-Inv and Vac-Inv lines.

In the source leaves, the *NtSut1* and *NtMst1* mRNA levels remained constant in the Apo-Inv lines, whereas the average mRNA content of these transporters doubled in the Vac-Inv lines (Fig. 4). These values are not strictly statistically significant due to the variation in the Gus source leaf samples, however a trend of higher average expression levels is observable in the Vac-Inv lines.

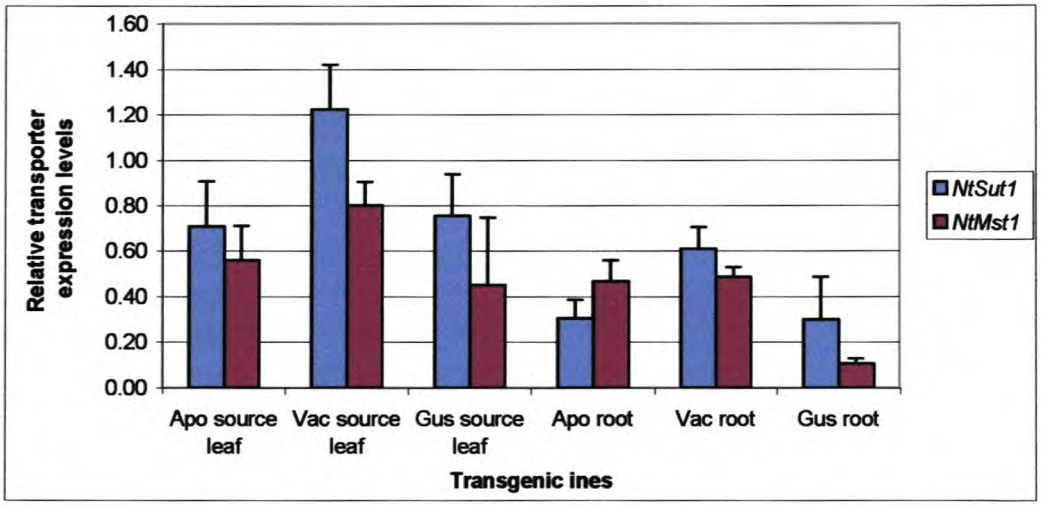

Figure 4. Relative expression levels of the sucrose transporter NtSUT1- and hexose transporter NtMST1-encoding genes, relative to rRNA expression levels, in source leaves and roots of medium invertase activity tobacco lines transformed with the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the apoplast (Apo-Inv) and vacuole (Vac-Inv), as well as control plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene. The error bars indicate the standard error of the mean of four lines analysed.

In the root tissues of the Apo-Inv lines, the *NtSut1* mRNA levels were similar to the control plant levels, whereas the *NtSut1* transcript levels doubled in the Vac-Inv lines (P=0.06). *NtMst1* expression levels increased approximately fivefold in the root tissues of both Apo-Inv (P=0.06) and Vac-Inv (P=0.03) lines in comparison to that in the control plant roots.

4.4 DISCUSSION

Although sugar transport has been the subject of much investigation in the past, it is only recently that Suc transporters were linked to the regulation of sugar partitioning. The aim of this work was to analyse the expression of the native sugar transporters of tobacco in plant lines that express a yeast-derived invertase in either the apoplast or vacuole. The data represented here indicate that the changed sugar levels in the various plant tissues, induced by the transgene, results in the altered expression of the NtMST1 and NtSUT1 sugar transporter-encoding genes.

Both sugar transporters analysed in this study, NtSUT1 and NtMST1, are H^+-sugar symporters associated with sugar movement along a proton gradient, and albeit at different levels, are expressed in most types of tissues. The sucrose/H^+-co-transporter NtSUT1 is most highly expressed in mature leaves, although lower levels of expression have also been observed in sink tissues, including floral organs (Bürkle et al., 1998). Antisense inhibition studies have shown that this Suc transporter is important for Suc loading into the phloem via an apoplastic route and possibly for intermesophyll transport. In contrast, the H^+–monosaccharide co-transporter NtMST1 has been shown to be expressed in most of the various tissues of mature tobacco plants, including roots, flowers, young leaves, as well as source leaves (Sauer and Stadler, 1993). Heterologous expression in yeast has confirmed that this transporter catalyses the uptake of hexoses, such as D-glucose and D-galactose, and pentoses such as D-xylose (Sauer and Stadler, 1993). These studies have also indicated that NtMST1 might play an important role in the import of hexoses, generated from Suc hydrolysis by cell-wall invertase following phloem unloading, into sink cells. It should be noted, however, that these sugar transporters are members of large gene families and thus not the only potential transporters for these sugars.

In this study, the expression levels of these sugar transporters were investigated in two types of plant tissue, namely a typical source (mature leaves) and a typical sink (root tissue) with altered sugar levels as induced by heterologous invertase activity. Transporter expression levels, as well as sugar levels, were measured in whole-plant tissues and therefore no differentiation is made between the different subcellular compartments. The flux of sugars between the various subcellular compartments was not determined. Thus, for the purpose of this discussion, it is presupposed that the changes in sugar levels occur in the various compartments in which the yeast-derived invertase is active, unless stated otherwise.

compartments in which the yeast-derived invertase is active, unless stated otherwise.

In the source leaves of tobacco plants expressing a yeast-derived invertase in the apoplast (Fig. 5), a 1.3-fold decrease in Suc levels was observed with a concomitant 2.7-fold increase in average hexose content. These changes did not lead to any obvious alterations in either *NtSut1* or *NtMst1* expression levels. It would appear that in tissues with a predominant apoplastic loading capacity, neither a decrease in Suc levels nor an increase in hexose levels in the apoplastic compartment, can induce the up-regulation of either of these transporters. One possibility is that tobacco is not adapted to sense changes in sugar levels from this compartment in source tissues. These results might also be explained by the sink-

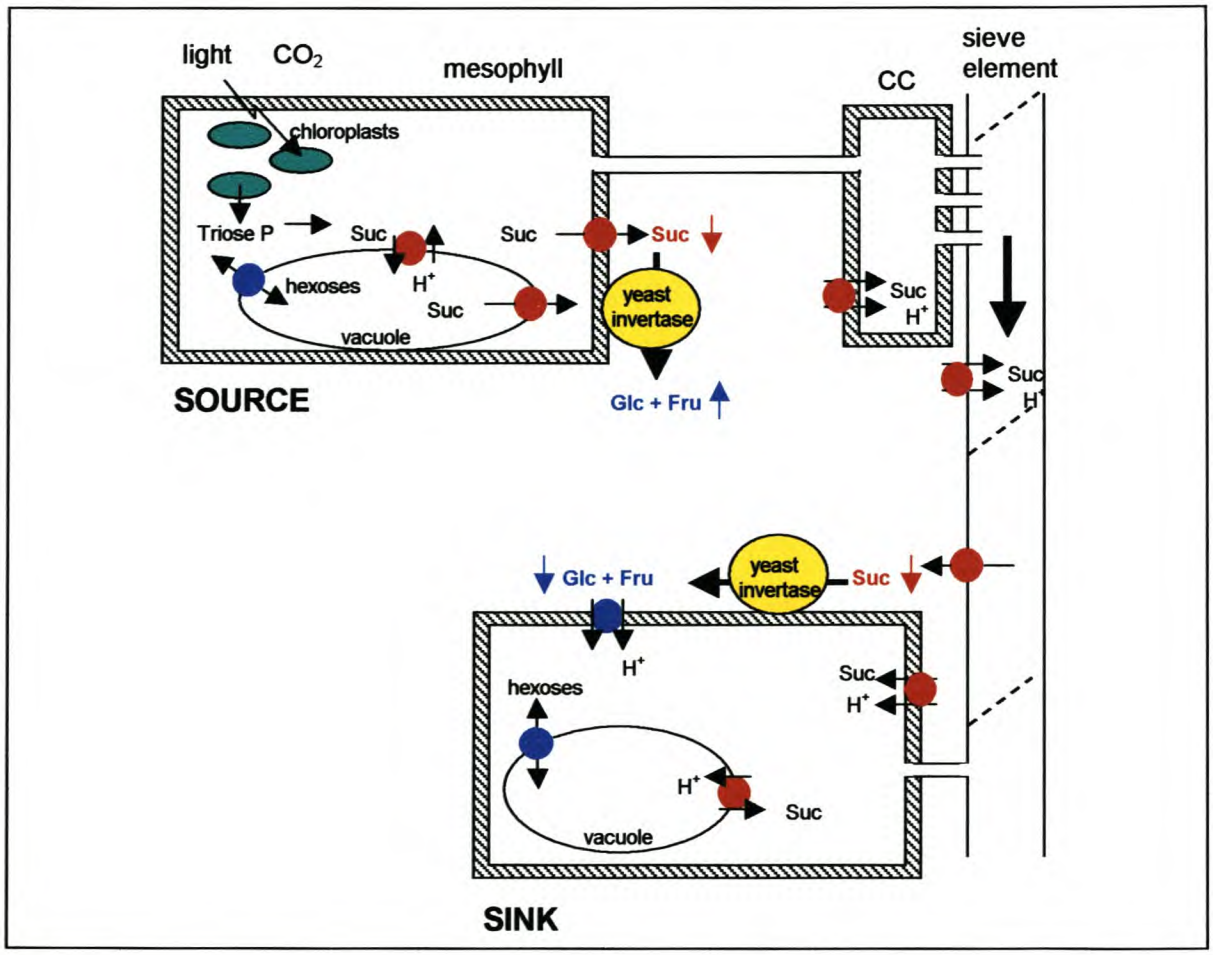

Figure 5. A diagrammatic representation of a transgenic tobacco plant expressing a yeast invertase in the apoplast with the resulting changes in sugar indicated. Blue circles represent the hexose transporter NtMST1 and red circles represent the sucrose transporter NtSUT1. Blue and red arrows correspond to sugar levels in comparison with that of the control lines. Abbreviations: CC, companion cell; Fru, fructose; Glc, glucose; P, phosphate; Suc, sucrose.

source interactions in these lines: *NtSut1* expression is not up-regulated in the roots of the Apo-Inv lines, which might lead to no subsequent decrease of Suc in the

In the root tissue of the Apo-Inv lines, a ca.10-fold reduction in Suc content was found, with a concomitant 1.4-fold decrease in average hexose levels. This significant decrease in Suc content had no influence on the expression of the Suc symporter *NtSut1* in these tissues. Again, it would appear that *NtSut1* does not respond to a decrease in Suc levels in the apoplast. This could be in concurrence with the fact that tobacco employs an apoplastic phloem unloading mechanism in developed roots (Imlau et al., 1999). This unloading pathway relies on the hydrolytic action of tobacco's native cell wall invertase, with a concomitant lowering in Suc levels, in this compartment. Expression of the hexose transporter *NtMst1*, however, increased by fivefold, most likely to increase the uptake of hexoses produced by the invertase activity. This predicted increase in hexose content is not reflected in the measured steady-state hexose levels, probably due to rapid uptake and consumption by the sink cells. It would therefore appear that hexose signalling occurred via the HK sensing system in that the carbon flux, related to the rate of hexose phosphorylation, is sensed as opposed to steady-state hexose-P levels (Jang and Sheen, 1994; Umemura et al., 1998).

In the source leaves of tobacco plants expressing a yeast-derived invertase in the vacuole (Fig. 6), Suc levels decreased by 1.7-fold, whereas the average hexose levels decreased by fourfold. The average transcript levels of both the Suc transporter *NtSut1* and hexose transporter *NtMst1* doubled in these tissues. The up-regulation of *NtSut1* in the source leaves is possibly due to sink-source interactions: a decrease in Suc content in the vacuoles of the sink tissues possibly resulted in the up-regulation of *NtSut1* in these tissues. This, in turn, might result in a decrease of Suc in the phloem that could be sensed in the source leaves by the Suc transporters, which is subsequently up-regulated (Chiou and Bush, 1998). The up-regulation of *NtMst1* (possibly located at the tonoplast) expression is possibly due to a decrease in hexose levels in the vacuoles of the source leaves. The transporter is probably up-regulated in order to facilitate the transport of hexoses to the cytosol for the resynthesis of Suc by fructo- and hexokinase. This remobilisation of hexoses would result in the measured decrease in hexose levels.

The root tissue of transgenic plants expressing a yeast-derived invertase in the vacuole exhibited a threefold reduction in Suc levels, with no significant change in the average hexose content. Expression levels of the *NtSut1* sucrose transporter was doubled in these tissues, probably due to the lowered Suc levels in the vacuole and potential subsequent decrease in the cytosol. This could lead to the up-regulation of this transporter in order to replenish Suc levels from the apoplast and phloem. A ca. fivefold increase in *NtMst1* hexose transporter transcript levels was observed in the roots to possibly, as in the case of the Apo-Inv lines, remobilise the hexoses that are produced during the catalytic action of yeast invertase in the vacuole. Again, no increase in hexose levels was measured, most likely due to remobilisation and utilisation of the hexoses in the cytosol.

vacuole. Again, no increase in hexose levels was measured, most likely due to remobilisation and utilisation of the hexoses in the cytosol.

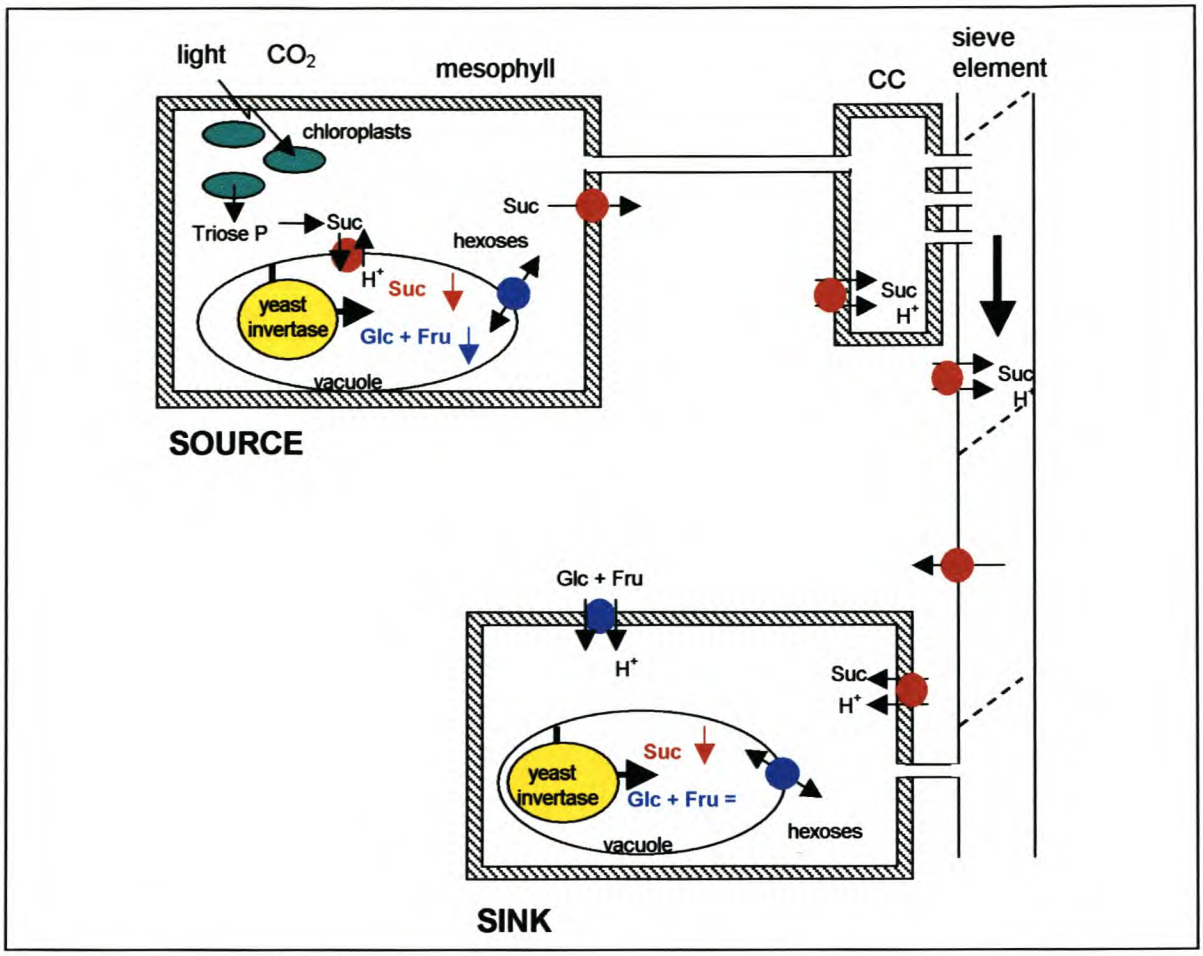

Figure 6. A diagrammatic representation of a transgenic tobacco plant expressing a yeast invertase in the vacuole with the resulting changes in sugar levels indicated. Blue circles represent the hexose transporter NtMST1 and red circles represent the sucrose transporter NtSUT1. Blue and red arrows correspond to sugar levels in comparison with that of the control lines. Abbreviations: CC, companion cell; Fru, fructose; Glc, glucose; P, phosphate; Suc, sucrose.

The up-regulation of the NtMST1 hexose transporter in the presence of altered sugar levels in this study is in contrast with the findings of Roitsch and Tanner (1994). They concluded that hexose transporter genes are not regulated by sugars, but are rather constitutively expressed and that differential expression of these transporters reflects on tissue-specific promoters. However, their data were obtained from suspension cells, which might not reflect the true responses in expression as obtained in the study of whole-plants.

The sugar H^+-symporters of most higher plants provide the driving force for the transport of Suc within the phloem and thus play an essential role in the systemic distribution thereof. As sugar partitioning is a major determinant of plant growth, development and yield, it is essential to comprehend its regulatory processes. The results represented here support the concept that this is a highly regulated process,

compartments of the various tissues analysed, might be sensed and manifested in the regulation of the genes essential to their distribution (Koch, 1996). From the data represented here it can be concluded that the sugar transporters NtSUT1 and NtMST1 are differentially regulated by Suc and/or hexose sugars at the transcriptional level. Furthermore, it would seem that the regulatory effect of the altered sugar levels on transporter expression depended on the subcellular compartment in which the yeast invertase was expressed.

4.5 ACKNOWLEDGEMENTS

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CHAPTER 5

RESEARCH RESULTS

The expression of the endogenous cell wall invertase and invertase inhibitor in transgenic tobacco overexpressing a yeast-derived invertase

Preliminary findings that will form part of a manuscript to be submitted for publication

The expression of the endogenous cell wall invertase and invertase inhibitor in transgenic tobacco overexpressing a yeast-derived invertase

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Transgenic tobacco plants expressing a yeast-derived invertase (*Suc2*) gene targeted to either the apoplast (Apo-Inv) or the vacuole (Vac-Inv) were utilised to study the expression of the tobacco endogenous cell wall invertase (CWI) and cell wall invertase inhibitor (CWI-Inh) encoding genes in the presence of the heterologous invertase. Northern blot analyses were employed to examine the expression of *Cwi* and *Cwi-Inh* in the different sink and source tissues at both the vegetative and flowering growth stages. In comparison with the control lines, the various tissues of the Apo-Inv and Vac-Inv lines displayed altered *Cwi* and *Cwi-Inh* expression levels, depending on the sink-source status and growth stage. However, no obvious correlation between the *Cwi* and *Cwi-Inh* expression levels and soluble sugar content of these tissues was found. Analysis of the *Cwi:Cwi-Inh* mRNA ratio and growth observations of the various tissues of control as well as Apo-inv and Vac-Inv lines indicated that this transcription ratio could be an accurate indicator of the sink strength of sink organs.

5.1 INTRODUCTION

Sucrose is central to most of the physiological activities and biochemical needs of higher plants by acting as a nutrient, an osmoticum and a signal molecule. By hydrolysing sucrose (Suc) into its monomeric constituents, glucose (Glc) and fructose (Fru), invertases (β-D-fructofuranosidases) maintain vital functions in the growth and developmental processes of plants (Tymowska-Lalanne and Kreis, 1998; Roitsch et al., 2003). The different invertase isoforms play essential roles in phloem unloading (Ruan and Patrick, 1995; Weber et al., 1995), sugar partitioning (Roitsch et al., 1995; Ehness and Roitsch, 1997; Roitsch, 1999), osmoregulation (Perry et al., 1987), stress signalling in plant defence responses (Herbers et al., 1996, 2000; Roitsch et al., 2003), as well as gravitropism (Wu et al., 1993).

These invertase isoforms are distinct in their expression patterns and subcellular localisations. The soluble, acid invertases are typically found in the vacuole, whereas the insoluble neutral invertases are normally found in the cytosol and the insoluble acid invertases are ionically bound to the cell wall (Tymowska-Lalanne and Kreis, 1998). The cell wall invertases are of special importance in Suc partitioning (Sturm and Tang,

1999). Suc is the major form in which photosynthate is translocated in most plant species and cell wall invertases play an important part in establishing the differences in solute concentrations and osmotic potentials between sink and source organs: the effective cleavage of Suc to Glc and Fru in sink tissues assists in providing the driving force for Suc movement (Sturm and Tang, 1999).

In tobacco, a cell wall invertase (CWI) and a cell wall invertase inhibitor (CWI-Inh) have been identified and the corresponding genes cloned (Greiner et al., 1995; 1998). CWI is a glycosylated enzyme with a molecular mass of 63 kD, a pH optimum of 4.7 and a K_m of 0.6 mM for Suc (Weil and Rausch, 1990). The tobacco CWI-Inh is a 17-kD apoplastic protein (Weil et al., 1994), which operates as a regulatory switch of cell wall invertase (Krausgrill et al., 1998). The inactivational function of the invertase inhibitor is modulated by divalent cations at millimolar concentrations, whereas cell wall invertase activity can be protected by the presence of Suc (Sander et al., 1996). Furthermore, it was found that the expression of *Cwi* and *Cwi-Inh* is not always coordinated (Weil et al., 1994; Sander et al., 1996; Greiner et al., 1998). At the transcriptional level, both *Cwi* and *Cwi-Inh* show a tissue- and developmental specific pattern (Godt and Roitsch, 1997; Haouazine-Takvorian et al., 1997; Greiner et al., 1998). Cell wall invertases are regulated also on the transcriptional and posttranscriptional levels by sugars (Xu et al., 1995).

In the past decade, several plant species have been transformed with the *Saccharomyces cerevisiae* invertase *Suc2* gene (Von Schaewen et al., 1990; Dickinson et al., 1991; Sonnewald et al., 1991; Heineke et al., 1992; Büssis et al., 1997; Weber et al., 1998; Ma et al., 2000). Unlike plant invertases, yeast invertase has a broad pH optimum of 3.5 to 5.0, a lower affinity for Suc (K_m 25-26 mM) (Goldstein and Lampen, 1975) and is not inactivated by plant invertase inhibitors (Sander et al., 1996). Tobacco *Suc2*-transgenic lines accumulated large amounts of sugars in the source leaves and displayed modified Glc:Fru ratios in these organs (von Schaewen et al., 1990; Stitt et al., 1990; Sonnewald et al., 1991). These transgenic lines provided a valuable model system to study Suc metabolism and sink-source interactions.

In this study, the effect of a yeast-derived invertase activity on tobacco's endogenous invertase and inhibitor was examined. It was found that the expression of the plant's endogenous cell wall invertase and invertase inhibitor genes were modified by the presence of a heterologous invertase activity, depending on the sink-source status and growth stage of the tissues analysed.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

See Section 3.2.1 of Chapter 3.

5.2.2 Preparation and selection of transgenic plant material

Transgenic tobacco lines containing the *S. cerevisiae* invertase (*Suc2*) gene targeted to either the vacuole or apoplast, as well as control lines transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene, were prepared and selected as described in Chapter 3. Twelve plants from each of the Apo-Inv and Vac-Inv lines, as well as control plants, were selected for analysis. These lines were hardened off and grown in a green house with sunlight (ca. 1200 $\mu mol.m^{-2}.s^{-1}$ at midday) at 24°C.

Plant material was harvested at the vegetative growth stage (5 weeks of growth) as well as the flowering stage (8 weeks of growth). At the vegetative growth stage, young leaves (<5 cm in length), fully expanded leaves, old leaves, as well as roots were collected. At the flowering stage, young flower buds (<1.5 cm in length), fully expanded leaves, old leaves as well as roots were harvested. The plant material was frozen immediately in liquid nitrogen and stored at -80°C until required. Three individual plants of each transgenic line were sampled as replications for all subsequent analyses.

5.2.3 RNA extraction and Northern blot analysis

Total RNA was isolated from four leaf discs (40 mg) of each sample. The plant material was ground to a fine powder using liquid nitrogen and extracted with 1 mL TRIzol® Reagent according to the manufacturer's specifications (GIBCO/BRL, Life Technologies, California, USA). Total RNA was size fractionated by electrophoresis in 1.2% (v/v) formaldehyde containing agarose gels and blotted onto Hybond-N nylon membranes (Amersham Biosciences, Buckinghamshire, UK) using standard techniques (Sambrook et al., 1989). Hybridisation of DNA probes to immobilised RNA was performed at 50°C overnight in a standard DIG hybridisation buffer (Roche Molecular Biochemicals, Ingelheim, Germany) containing 50% (v/v) formamide. The non-specifically bound probe was removed by washing the membrane twice with 2 × tri-sodium citrate (SSC) and 0.1% (w/v) sodium dodecyl sulphate (SDS) for 15 min at room temperature and twice with 0.5 × SSC and 0.1% (w/v) SDS for 15 min at 65°C. Chemiluminescent signal detection proceeded according to the manufacturer's specifications (Roche Molecular Biochemicals). Normalisation of the Northern blots was done against the amount of 28S rRNA present, employing the Bandscan program (Glyko, California, USA). The DIG labelled probes utilised were polymerase chain reaction (PCR) labelled (Table 1) according to the manufacturer's specifications (Roche Molecular Biochemicals). The tobacco apoplastic invertase probe, *Cwi*, and the apoplastic invertase inhibitor probe, *Cwi-Inh*, consisted of a 1.7 kb and 0.3 kb PCR product, respectively. Tobacco genomic DNA was utilised as template for the amplification reactions. A 1.4-kb *Suc2* PCR product was utilised as the yeast invertase probe. The identity and integrity of the PCR products were confirmed by sequencing using an ABI Prism 377 automated DNA sequencer from PE Biosystems (California, USA) (results not shown).

Table 1. List of primers used in this study. Primers were designed according to published sequences.

Primer name	Primer sequence	Product and reference
CWI-forward	5'-TCC GAT CCA TAT CTC CGT AA-3'	Cell wall invertase (CWI)
CWI-reverse	5'-GGC CAT ACT CCA GGC ATT-3'	Greiner et al. 1995
CWI-Inh-forward	5'-ACA CAG GCA CAT CAT AAT AAA T-3'	Invertase inhibitor (CWI-Inh)
CWI-Inh-reverse	5'-ACC AAA GAG GCT ATA TAT TCT T-3'	Greiner et al. 1998
SUC2-Inv-forward	5'-AGC GAT AGA CCT TTG GTC CA-3'	Yeast invertase (SUC2)
SUC2-Inv-reverse	5'-CCG GTG GTC ATG AAG TAG GT-3'	Carlson and Botstein, 1982

5.2.4 Protein extraction and Western blot analysis

Total protein from 0.1 g of plant tissue was extracted with 200 μL extraction buffer (Ruffner et al., 1990). Subsequent to separation in standard 10% (w/v) SDS-polyacrylamide gels (Gabriel and Wang, 1969), proteins were transferred onto Hybond-C nitrocellulose membranes (Amersham) by means of the Bio-Rad wet system (Bio-Rad, California, USA). A rabbit anti-yeast invertase polyclonal antibody (generously provided by R. Schekman, UC Berkley, USA) was utilised to detect the yeast-derived invertase by means of an ECL detection kit (Amersham).

5.2.5 Invertase activity assays

CWI activity was assayed by discarding the supernatant of a crude protein extract and washing the remaining pellet four times with extraction buffer. A 5 μL aliquot of the extraction buffer was incubated with 25 mM Suc in 50 mM NaOAc buffer (pH 5.0) in a final reaction volume of 100 μL for 15 min at 37°C. Reactions were terminated by the addition of 50 mM Tris-HCl, pH 8.0. The liberated reducing sugars were measured using a D-glucose/D-fructose kit (Roche Molecular Biochemicals), an enzyme system coupled to nicotinamide adenine dinucleotide phosphate (NADP). The specific invertase activities were linear over time and proportional to the amount of protein being assayed. Protein was quantified using the Bradford assay according to the manufacturer's instructions (Bio-Rad). Invertase activity was expressed as nmol Suc hydrolysed per second (nkat).

5.2.6 Soluble sugars assay

Sugars were extracted and measured as described in Section 3.2.10 in Chapter 3.

5.3 RESULTS

5.3.1 Phenotype and growth rate of transgenic lines

No obvious differences in vegetative growth rate or phenotype were observed between the control, Apo-Inv and Vac-Inv lines when maintained under green house conditions with sunlight (ca. 1200 $\mu mol.m^{-2}.s^{-1}$ at midday).

At the flowering stage, however, the onset of flowering of the Apo-Inv transformants was delayed by approximately two weeks when compared to the Vac-Inv transformants and control plants. In addition, the total number of flowers observed for the apoplastic invertase lines was approximately 70% lower than that of the control plants, although no change in individual flower size was found (Fig. 1). In contrast, the vacuolar invertase lines showed an increase of 70% in flower number, but a 20% decrease in individual flower size.

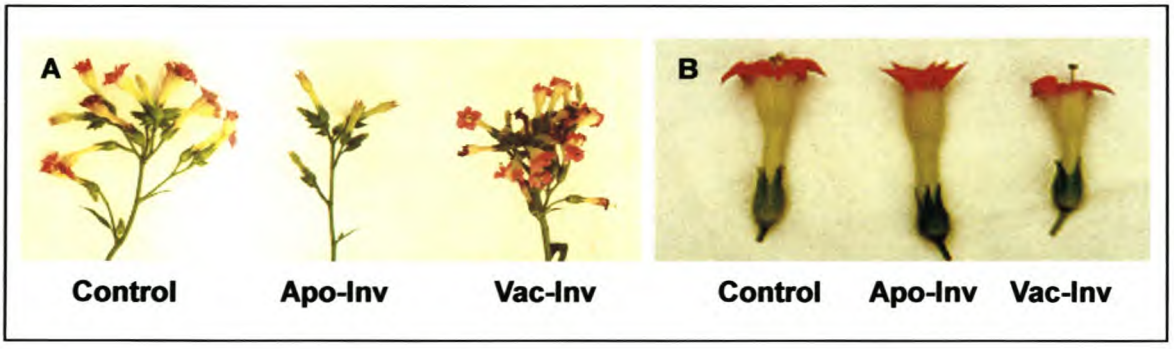

Figure 1. A comparison of (A) the number of flowers and (B) the size of the individual flowers between control tobacco plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene and plants expressing the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to either the apoplast (Apo-Inv) or vacuole (Vac-Inv).

5.3.2 Expression of the *Suc2, Cwi* and *Cwi-Inh* genes at the vegetative growth stage

The yeast invertase *Suc2* gene was expressed in all the analysed tissues of both the Apo-Inv and Vac-Inv lines, whereas no expression could be detected in the control (GUS) plants (Fig. 2A). The relative *Suc2* transcription levels were similar in the various tissues of the Apo-Inv lines (Fig. 2B). The same trend was observed in the Vac-Inv lines, which expressed double the amount of *Suc2* mRNA than that found in the corresponding tissues of the Apo-Inv lines.

A positive signal for the cell wall invertase (CWI) encoding gene was detected in

the control, Apo-Inv and Vac-Inv samples when hybridised with the tobacco *Cwi* probe (Fig. 2A). Except for the young leaves, the mRNA content of the CWI encoding gene was higher in the tissues of the control plants than in the corresponding tissues of the Vac-Inv or Apo-Inv lines (Fig. 2C). In the young leaves, the *Cwi* mRNA level was highest in the Apo-Inv lines. The highest overall *Cwi* transcription levels were found in the control (GUS) roots, which was approximately 1.5-fold higher than the levels found in the roots of the Vac-Inv and the Apo-Inv lines. In the old leaves, the *Cwi* mRNA levels were 1.7 and 1.2-fold higher in the control plants than in the Vac-Inv and Apo-Inv lines, respectively. High *Cwi* transcription levels were also detected in the fully expanded leaves of the control plants, which were approximately the same as in the Apo-Inv lines, but threefold higher than that in the fully expanded leaves of the Vac-Inv lines. In the young leaves of the Apo-Inv lines, the *Cwi* mRNA levels were 1.6 and 3.7-fold higher than the levels observed in the young leaves of the control and Vac-Inv lines, respectively.

The invertase inhibitor (CWI-Inh) encoding gene exhibited a much higher expression pattern in the source leaves of the control, Apo-Inv and Vac-Inv lines, than the levels measured in the sink tissues of these lines, with the lowest levels found in the young leaves (Fig. 2D). Similar *Cwi-Inh* transcript levels were displayed by the roots of the various lines, whereas in the old leaves of the Apo-Inv lines, the *Cwi-Inh* expression levels were approximately 1.2 and 1.5-fold higher than the levels found in the Vac-Inv and control lines, respectively. The *Cwi-Inh* mRNA levels were 1.2 and 1.7-fold higher in the fully expanded leaves of the Vac-Inv lines than the levels observed in the Apo-Inv and control lines, respectively. In the young leaves, these levels were 2.3-fold higher in the Vac-Inv lines than in the Apo-Inv and control lines.

5.3.3 Expression of the *Suc2, Cwi* and *Cwi-Inh* genes at the flowering growth stage

As at the vegetative stage, no *Suc2* expression was detected in the control plants (Fig. 3A and B). In the Vac-Inv lines, the *Suc2* mRNA levels in the roots were 10-fold higher than those observed in the corresponding tissue of the Apo-Inv lines, whereas the old leaves, fully expanded leaves and flowers exhibited 7, 9 and 65-fold higher expression levels than the corresponding tissues of the Apo-Inv lines.

At the flowering stage, a high level of variation was observed in the expression of the *Cwi* gene between the various tissues of the lines analysed (Fig. 3C). In the control and Vac-Inv lines, the levels of *Cwi* mRNA were highest in the flowers and roots, whereas the opposite expression pattern was exhibited by the Apo-Inv lines where the *Cwi* transcription levels were low in the flowers and roots, but high in the fully expanded and old leaves. The roots of the Vac-Inv lines showed 1.6 and 5.7-fold higher expression levels than the roots of the control and Apo-Inv lines, respectively. No detectable *Cwi* expression levels could be observed in the old leaves of either the control or Vac-Inv lines.

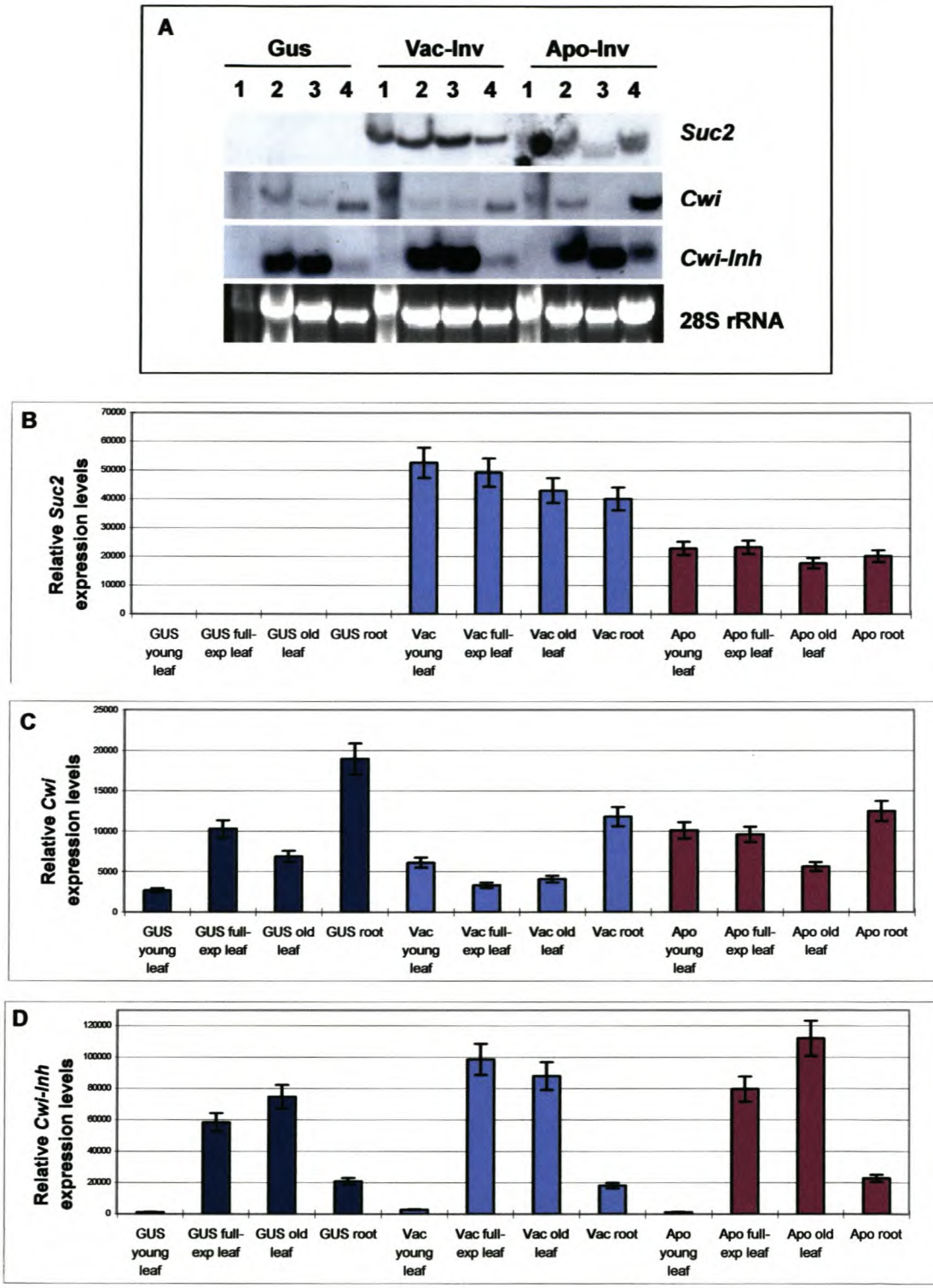

Figure 2. (A) Northern blot analysis of total RNA isolated from the (i) young leaves, (ii) fully expanded leaves, (iii) old leaves and (iv) roots of tobacco control plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene, as well as tobacco lines expressing the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the vacuole (Vac-Inv) or apoplast (Apo-Inv) at the vegetative growth stage. The blots were probed with tobacco cell wall invertase (*Cwi*), tobacco cell wall invertase inhibitor (*Cwi-Inh*) and yeast invertase (*Suc2*) probes. The corresponding 28S rRNA levels are indicated. Expression levels, relative to the 28S rRNA levels, of (B) the *Suc2*, (C) *Cwi* and (D) *Cwi-Inh* genes in the GUS control, Vac-Inv and Apo-Inv lines.

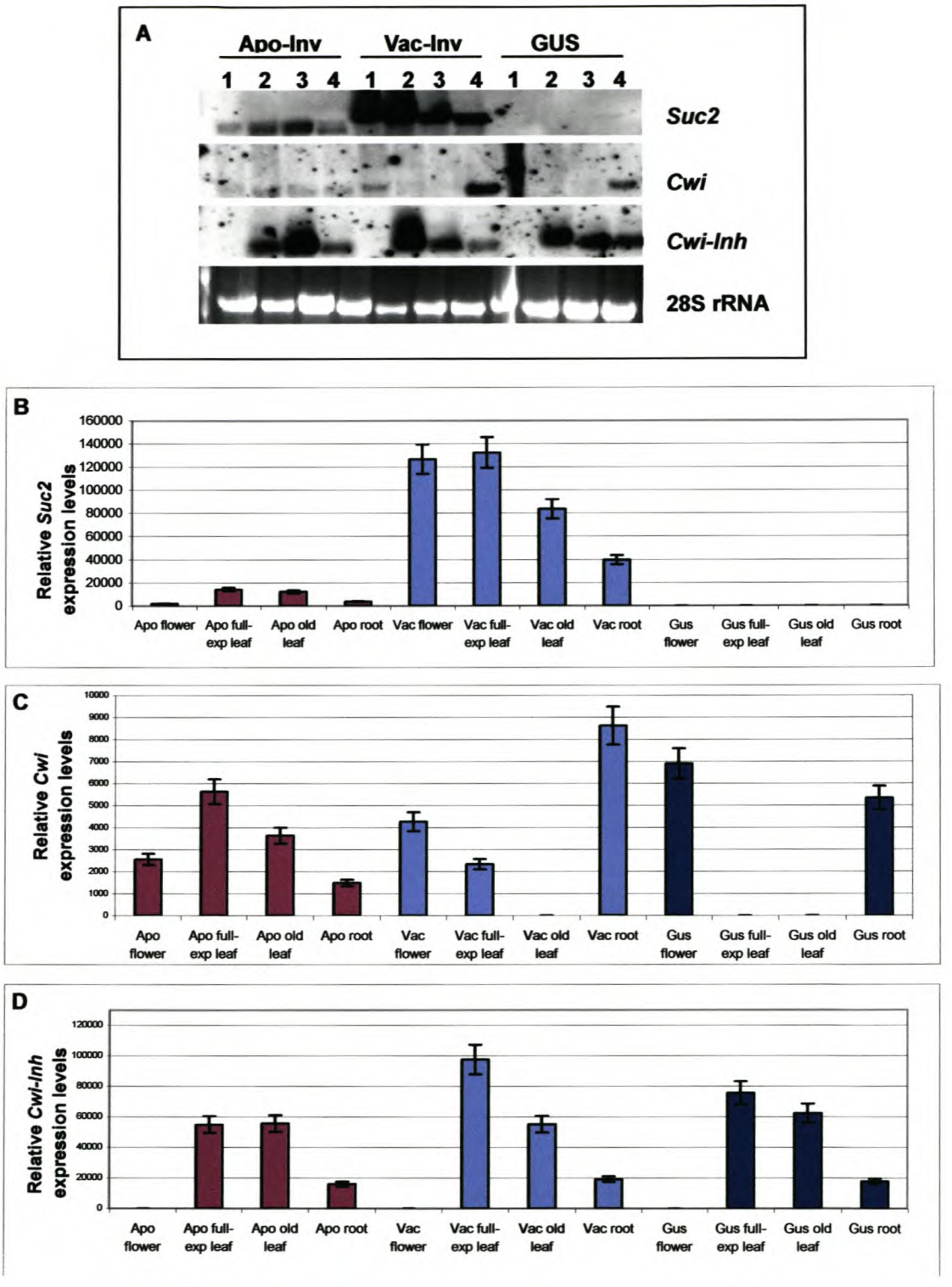

Figure 3. (A) Northern blot analysis of total RNA isolated from the (i) flowers, (ii) fully expanded leaves, (iii) old leaves and (iv) roots of tobacco control plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene, as well as tobacco lines expressing the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the vacuole (Vac-Inv) or apoplast (Apo-Inv) at the flowering growth stage. The blots were probed for the tobacco cell wall invertase (CWI)-, the tobacco cell wall invertase inhibitor (CWI-Inh) and the yeast invertase (SUC2) encoding genes. The corresponding 28S rRNA levels are indicated. Expression levels, relative to the 28S rRNA levels, of (B) the *Suc2*, (C) *Cwi* and (D) *Cwi-Inh* genes in the GUS control, Vac-Inv and Apo-Inv lines.

The fully expanded leaves of the Apo-Inv lines displayed a 2.4-fold higher expression level than the Vac-Inv lines, whereas no expression could be detected in the control lines. In the flowers of the control lines, however, 1.4 and 2.7-fold higher transcription levels were observed than found in the corresponding tissues of the Vac-Inv and Apo-Inv lines, respectively.

No Cwi-Inh signal was detected in the flowers of the control, Vac-Inv and Apo-Inv lines (Fig. 3D). The *Cwi-Inh* transcription levels furthermore displayed a decreasing trend from the fully expanded leaves to the roots in the Vac-Inv and control plants. In the Apo-Inv lines, the *Cwi-Inh* mRNA levels of the fully expanded and old leaves were similar, but 3.5-fold higher than in the roots. The fully expanded leaves of the Vac-Inv lines exhibited a 1.7 and 1.3-fold higher *Cwi-Inh* transcription than Apo-Inv and control lines, respectively. Similar *Cwi-Inh* expression levels were observed in the old leaves of the Vac-Inv, Apo-Inv and control lines. In the roots, however, the *Cwi-Inh* mRNA levels were similar in the Vac-Inv and control lines, but 1.2-fold higher than the levels found in the roots of the Apo-Inv lines.

5.3.4. Relative *Cwi:Cwi-Inh* mRNA ratios

In order to investigate the correlation in expression levels between the CWI and CWI-Inh encoding genes in the Apo-Inv, Vac-Inv and control lines, the relative *Cwi:Cwi-Inh* mRNA ratios were calculated at both the vegetative and flowering growth stages. At the vegetative stage, the sink tissues displayed a higher *Cwi:Cwi-Inh* mRNA ratio than found in the source organs (Fig. 4A). The *Cwi:Cwi-Inh* mRNA ratio was highest in the young leaves of the Apo-Inv lines, which was approximately fourfold higher than the ratio found in the young leaves of the Vac-Inv and control lines. In the control young leaves, this ratio was approximately 17-fold higher than in the source leaves, whereas this ratio was 43 and 100-fold higher in the corresponding tissues of the Vac-Inv and Apo-Inv lines, respectively.

At the flowering stage, the *Cwi:Cwi-Inh* mRNA ratio of the flowers far exceeded that observed in the other tissues analysed (Fig. 4B). The highest *Cwi:Cwi-Inh* mRNA ratio was found in the control flowers, which was 1.6 and threefold higher than the ratio found in the flowers of the Vac-Inv and Apo-Inv lines, respectively. In the Apo-Inv flowers, the *Cwi:Cwi-Inh* mRNA ratio was 50-fold higher than in the other tissues analysed, whereas this ratio was 85 and 138-fold higher in the corresponding tissues of the Vac-Inv and control lines, respectively.

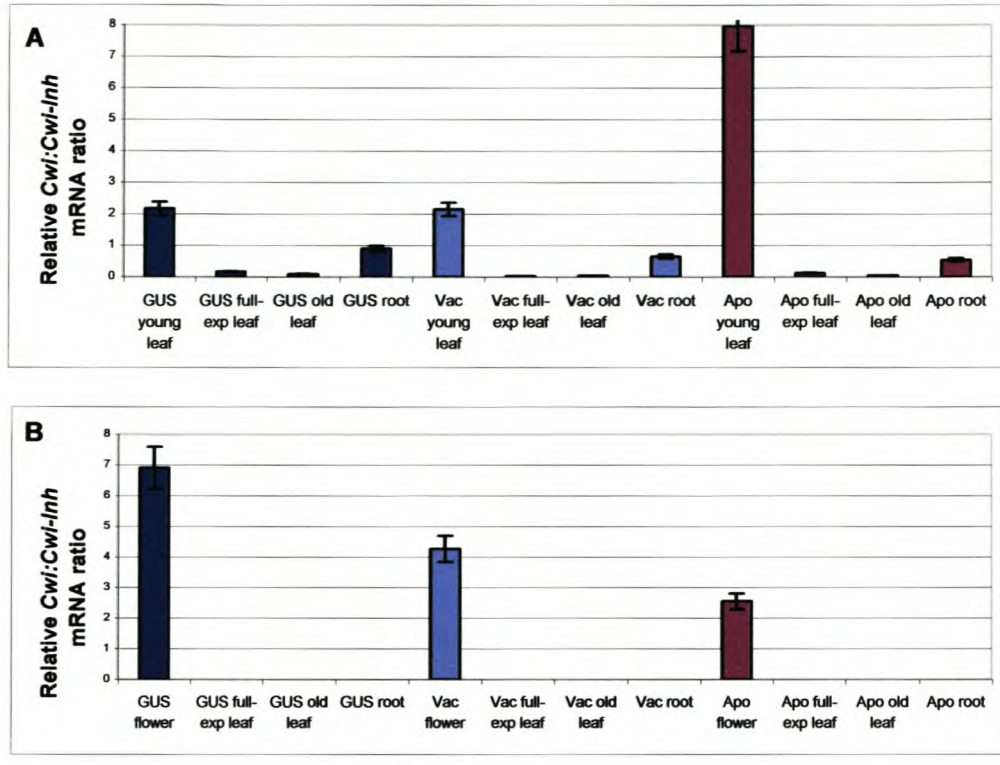

Figure 4. Relative mRNA transcript ratios of the tobacco cell wall invertase (CWI) and the tobacco cell wall invertase inhibitor (CWI-Inh) encoding genes in the young, fully expanded and old leaves, as well as roots at (A) the vegetative growth stage and the flowers, fully expanded and old leaves, as well as roots at (B) the flowering stage of tobacco control plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene, as well as tobacco lines expressing the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the vacuole (Vac-Inv) or apoplast (Apo-Inv).

5.3.5 Western blot analysis

Even though the Vac-Inv lines displayed a higher *Suc2* transcription level than the Apo-Inv lines, the Western blot analyses demonstrated a contrasting pattern of protein levels found at the vegetative stage when hybridised with a yeast invertase antibody (Fig. 5A). The yeast invertase signal was very feint in the Vac-Inv tissues, whereas the Apo-Inv lines exhibited a strong yeast invertase signal. A similar trend was observed at the flowering stage where a weak SUC2 signal was exhibited by the Vac-Inv lines (Fig. 5B). The Apo-Inv samples displayed even stronger SUC2 signals than in the vegetative stage. No yeast invertase signal was detected in the GUS control lines at either growth stage.

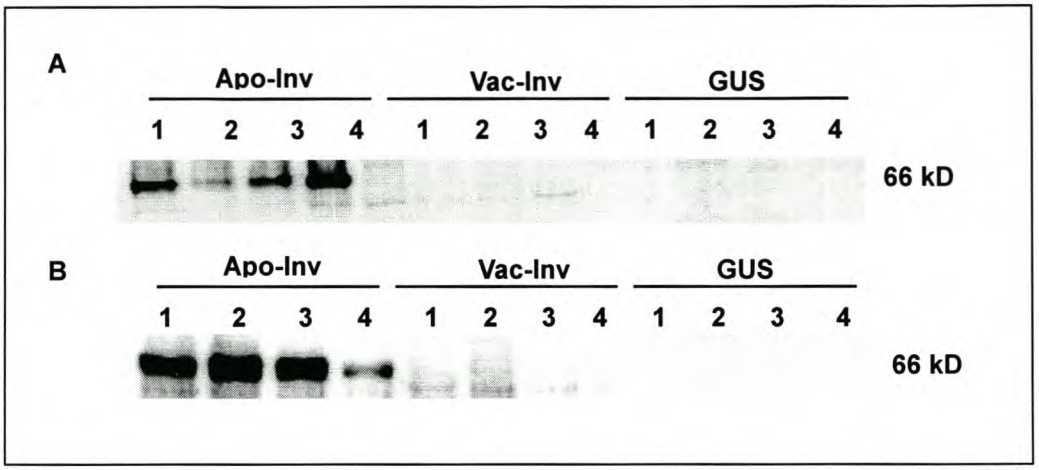

Figure 5. Western blot analysis, hybridised with a yeast invertase antibody, of total proteins extracted at (A) the vegetative growth stage from (1) young, (2) fully expanded and (3) old leaves, as well as (4) roots and at (B) the flowering stage from (1) flowers, (2) fully expanded and (3) old leaves, as well as (4) roots of tobacco control plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene, as well as tobacco lines expressing the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the vacuole (Vac-Inv) or apoplast (Apo-Inv).

5.3.6 Cell wall invertase activity

The Apo-Inv lines exhibited the highest cell wall invertase activity. A decreasing invertase activity pattern was displayed from the roots > old leaves > fully expanded leaves > young leaves at the vegetative growth stage (Fig. 6A). The cell wall invertase activity was 67 to 203-fold higher in these lines than in the corresponding tissues of the Vac-Inv and control lines. No significant differences in invertase activity pattern or levels were observed between the Vac-Inv and control lines.

At the flowering stage, the distribution pattern in invertase activity changed to an increasing pattern from the roots < old leaves < fully expanded leaves and then a decrease in the flowers of the Apo-Inv lines (Fig. 6B). At this growth stage the cell wall invertase activity was 37 to 793-fold higher in the Apo-Inv lines than in the corresponding tissues of the Vac-Inv and control lines. Despite a 10-fold higher invertase activity in the flowers of the Vac-Inv and control lines, no significant differences in invertase activity pattern or levels were observed between the remaining tissues of these lines.

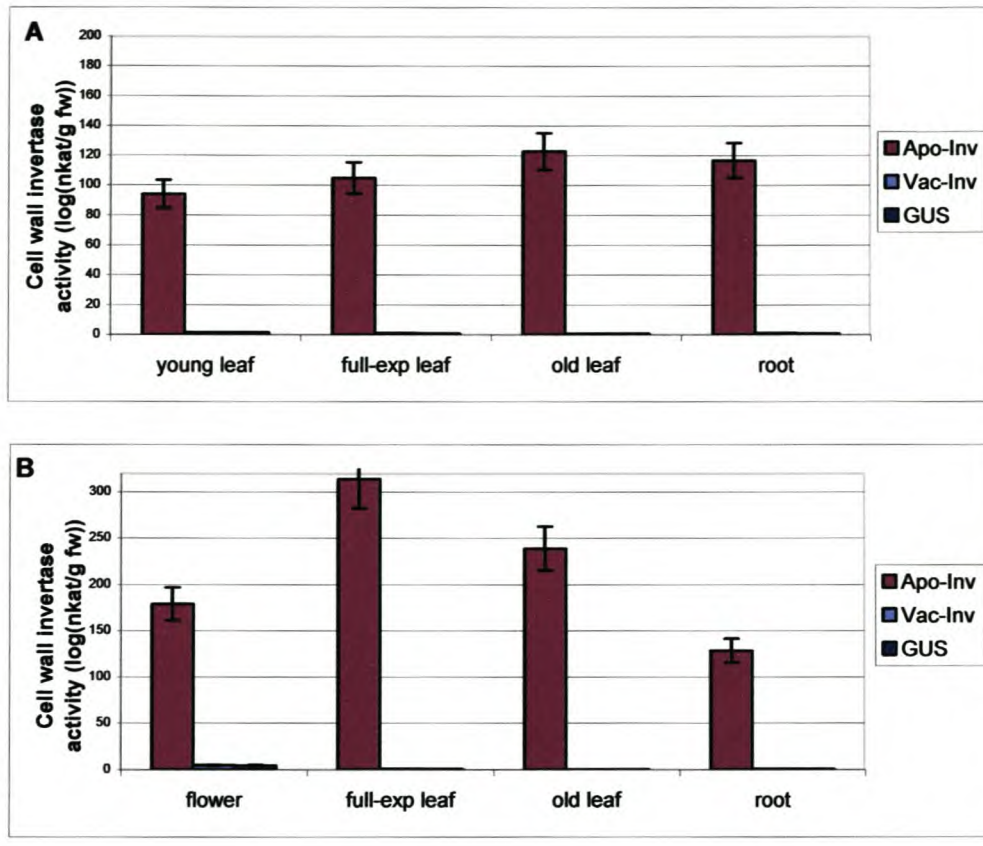

Figure 6. Cell wall invertase activity (log(nkat/g fw)) at (A) the vegetative growth stage in young, fully expanded and old leaves, as well as roots and at (B) the flowering growth stage in flowers, fully expanded and old leaves, as well as roots of tobacco control plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene, as well as tobacco lines expressing the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the vacuole (Vac-Inv) or apoplast (Apo-Inv).

5.3.7 Tissue-specific soluble sugar content

In order to investigate a possible correlation between the expression of *Cwi* and *Cwi-Inh* and soluble sugar levels, the various tissues of the Apo-Inv, Vac-Inv and control lines were analysed for their Suc, Glc and Fru content at the vegetative and flowering growth stages (Fig. 7). At the vegetative growth stage, the young leaves of the Apo-Inv plants displayed a ca. 1.7-fold lower Suc content and a 1.4-fold lower total soluble sugar content than the corresponding tissues of the Vac-Inv and control lines (Fig. 7A). No significant differences in hexose levels were observed between the young leaves of these lines. The fully expanded leaves of the Vac-Inv lines contained 1.8-fold less Suc and six-, eight- and fourfold less Glc, Fru and total soluble sugars, respectively, than the Apo-Inv and control lines. In the old leaves, the Vac-Inv lines displayed a 1.5, 6, 15 and fourfold decrease in Suc, Glc, Fru and total soluble sugar content in comparison with the control lines. In contrast, the old leaves of the Apo-Inv lines showed a 1.7, 3.6, 2.5 and 2.6-fold higher Suc, Glc, Fru and total soluble sugar

content than found in the control lines. In comparison with the control plants, the Suc and total soluble sugar levels in the roots of the Apo-Inv lines were decreased by ca. four and 1.5-fold, respectively, whereas the Glc and Fru content were unaltered. The roots of the Vac-Inv lines were characterised by a 1.6-fold decrease in Suc levels and 1.4-fold increase in both Glc and Fru when compared to the control lines. The total soluble sugar content of the roots was equal in these two lines.

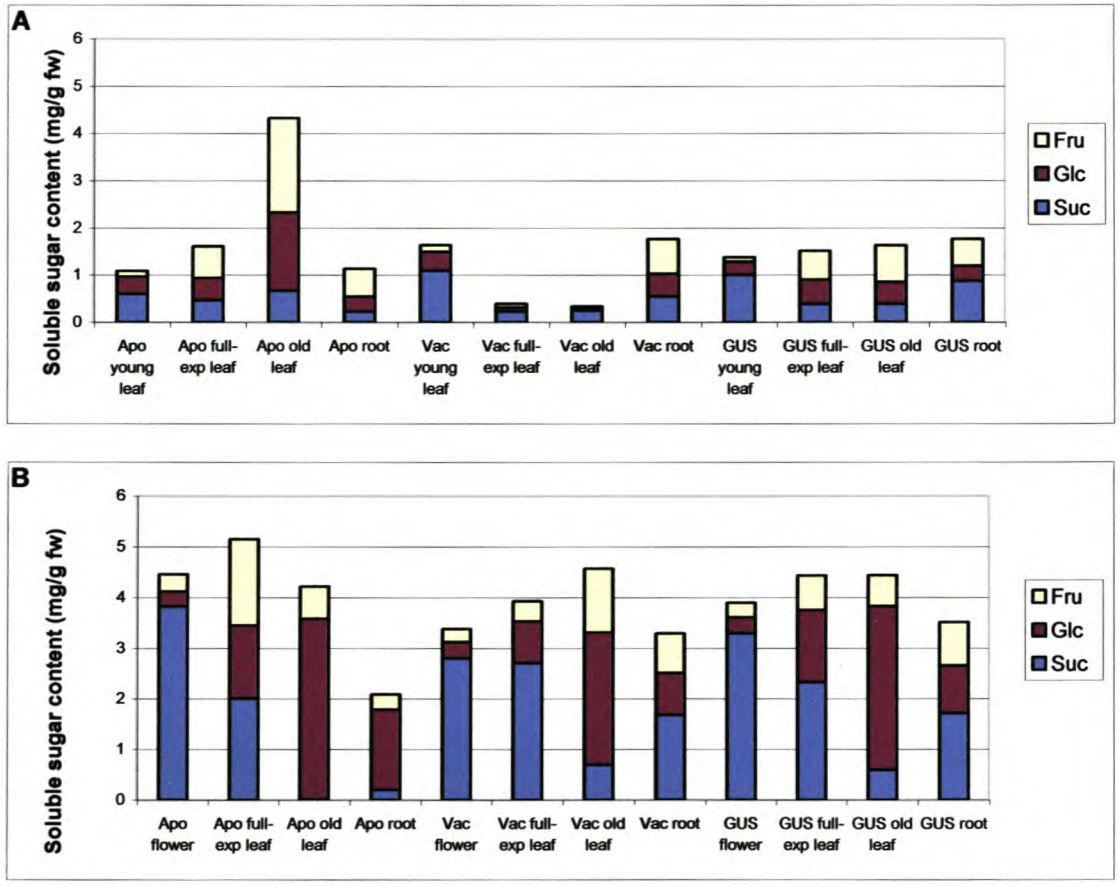

Figure 7. Sucrose (Suc), glucose (Glc) and fructose (Fru) levels in the young, fully expanded and old leaves, as well as roots flowers at (A) the vegetative growth stage and the flowers, fully expanded and old leaves, as well as roots at (B) the flowering stage of tobacco control plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene, as well as tobacco lines expressing the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the vacuole (Vac-Inv) or apoplast (Apo-Inv).

At the flowering growth stage, the total soluble sugar content of the various tissues analysed were, on average, fourfold higher than the levels measured at the vegetative stage (Fig. 7B). No significant differences in hexose content were measured between the flowers of the control, Apo-Inv and Vac-Inv lines. However, a 1.2-fold decrease in Suc and total soluble sugars in the Vac-Inv flowers and a 1.2-fold increase in these sugars in the Apo-Inv flowers, were observed in comparison with the control plants. In the fully expanded leaves, the Apo-Inv lines displayed a 1.2-fold decrease in Suc,

equal Glc levels, and a 2.5 and 1.2-fold increase in Fru and total soluble sugar content, respectively, when compared to the control lines. The Vac-Inv fully expanded leaves were characterised by a 1.2-fold increase in Suc levels, a 1.7-fold decrease in hexose content and unaltered total soluble sugar content in comparison to the control plants. The Apo-Inv old leaves displayed a 60-fold decrease in Suc amounts in comparison to the control lines, whereas an unaltered hexose and total soluble sugar content was measured. The old leaves of the Vac-Inv lines exhibited a 1.2-fold increase in Suc levels, a 1.2-fold decrease in Glc levels, a twofold increase in Fru levels and an unaltered total soluble sugar content when compared to the control lines. In the roots, similar soluble sugar levels were observed in the Vac-Inv and control lines, whereas the Apo-Inv lines displayed a 8.6-fold decrease in Suc, a 1.7-fold increase in Glc, a 2.8-fold decrease in Fru and a 1.7-fold decrease in total soluble sugar content in comparison to the control plants.

5.4 DISCUSSION

This study was aimed at examining the expression of the endogenous cell wall invertase (*Cwi*) and cell wall invertase inhibitor (*Cwi-Inh*) genes of tobacco in the presence of a yeast-derived invertase (SUC2). The transcription patterns of the CWI and CWI-Inh encoding genes, in Apo-Inv and Vac-inv lines, were compared in different tissues and developmental stages to those of the control (GUS) plants. A possible correlation between these transcription patterns and soluble sugar levels was also investigated.

The presence of the yeast invertase in the apoplastic space impairs photosynthate export by the hydrolysis of Suc to Glc and Fru (Von Schaewen et al., 1990; Sonnewald et al., 1991). The free hexoses cannot be taken up into the phloem as efficiently as Suc and are retrieved by the mesophyll cells, rephosphorylated by hexokinase and fructokinase and reconverted to Suc (Maynard and Lucas, 1982). This inhibition of Suc export from the source leaves of Apo-Inv lines has previously been shown to lead to severe changes in the growth rate and phenotype of these lines, including the development of bleached areas in mature leaves, when grown in a growth room under artificial light (50 $\mu mol.m^{-2}.s^{-1}$; 16-h light:8-h dark cycle) (section 3.3.3 of Chapter 3). However, when these lines were maintained in a green house under sunlight in this study (ca. 1200 $\mu mol.m^{-2}.s^{-1}$), no obvious differences in the vegetative growth rate or phenotype was observed in comparison with the control and Vac-Inv lines. It is thought that the increased light intensity results in an increased photosynthetic capacity together with an increased demand from sink tissues, which results in an increased growth rate. It is proposed that the increased translocation stream and utilisation of hexoses would exceed the low capacity of SUC2 to hydrolyse Suc (K_m=25-26 mM) (Goldstein and Lampen, 1975). The soluble sugars will therefore not accumulate to the threshold concentrations needed to cause vegetative phenotypic alterations.

At both the vegetative and flowering stages, the *Suc2* mRNA levels were between

two and 65-fold higher, depending on the tissue-type, in the Vac-Inv lines than the levels observed in the same tissues of the Apo-Inv lines. The results of the Western blot analysis, however, revealed a contrasting pattern in protein levels with much stronger yeast invertase signals observed in the tissues of the Apo-Inv lines than in the Vac-inv lines. Accordingly, cell wall invertase activity was 67 to 203-fold higher in the Apo-Inv lines than in the corresponding tissues of the Vac-Inv lines. Although no distinction could be made between the yeast invertase and the endogenous tobacco apoplastic invertase activities in the cell wall fraction of isolated plant samples, it is accepted that the cell wall invertase activities measured represent the heterologous yeast invertase activity as deduced by comparison to the control lines. The contrasts between the mRNA transcript levels and the invertase activities were also observed in previous experiments where a yeast-derived invertase was expressed in either the vacuole or apoplast of tobacco plants (Von Schaewen et al., 1990; Sonnewald et al., 1991). This is probably due to a lower stability of the fusion protein in the vacuole than in the apoplast (Sonnewald et al., 1991).

CWI has an important function in establishing and maintaining sink strength and metabolism by means of the apoplastic cleavage of Suc (Godt and Roitsch, 1997; Cheng and Chourey, 1999). Accordingly, high *Cwi* transcription levels were observed in the sink tissues of the control, Apo-inv and Vac-Inv lines at the vegetative stage. At the flowering stage, *Cwi* expression was especially high in the flowers and roots of the control and Vac-Inv lines, where high Suc levels (or Suc:hexose ratio) were measured. During the sink-to-source transition, *Cwi* transcription levels decreased in the source tissues of control and Vac-Inv lines, as have been observed in other plants (Ruffner et al., 1990; Lorenz et al., 1995, Tymowska-Lalanne and Kreis, 1998; Hirose et al., 2002). Low *Cwi* expression levels were observed in the source leaves of the control and yeast-invertase expressing lines at the vegetative stage. CWI activity in source tissues is thought to supply the energy needed for respiration and the synthesis of growth-related compounds (Roitsch et al., 2003). In contrast to the control and Vac-Inv lines, the *Cwi* mRNA levels increased in the fully expanded and old leaves of the Apo-Inv lines at the flowering stage. No obvious correlation between these *Cwi* transcription levels and soluble sugar content could be found. This is surprising since Suc, Glc and Fru have been shown to induce both the steady-state transcript levels and corresponding activity of cell wall invertase (Roitsch et al., 1995; Godt and Roitsch, 1997; Tymowska-Lalanne and Kreis, 1998; Sinha et al., 2002). It has also been shown that the strong effects of Suc on gene expression level and enzyme activity resulted from Suc hydrolysis and transcriptional activation by the resulting Glc (Roitsch et al., 1995).

Although only one CWI was purified from tobacco leaves, and the corresponding gene cloned (Greiner et al., 1995), many different isoforms could exist in sink and source tissues. These isoforms could display variations in gene expression and regulation by sugar concentrations or ratios. For example, a small CWI gene family, which have a different organ- and developmental stage-specific expression pattern

was reported in carrot, *Arabidopsis* and maize (Unger et al., 1994; Tymowska-Lalanne and Kreis, 1998; Sturm, 1999). Different isoforms of cell wall invertase could provide great flexibility to control sugar metabolism, storage and translocation (Tymowska-Lalanne and Kreis, 1998). In our Northern blot analysis, a single signal was detected with the *Cwi* probe, but cross–hybridisation with different cell wall invertase mRNAs cannot be excluded. Isoform-specific probes are needed to further investigate *Cwi* expression.

The expression of the *Cwi-Inh* gene was approximately three to fivefold higher in the source tissues than in the sink tissues of the control, Apo-Inv and Vac-Inv lines at both the vegetative and flowering stages. High *Cwi-Inh* expression in the source leaves could possibly ensure low CWI activity for optimal Suc loading into the phloem. At the vegetative stage, the *Cwi-Inh* transcription levels in the mature leaves of the Vac-Inv and Apo-Inv lines were 1.3-fold higher than in control lines, whereas at the flowering stage, lower *Cwi-Inh* mRNA levels were found in the Apo-Inv lines than in the control or Vac-Inv lines. No obvious correlation between these *Cwi-Inh* expression patterns and soluble sugar content could be found. Moreover, no specific pattern in *Cwi-Inh* expression levels was observed when detached tobacco leaves were treated with 0.4 M Suc, 0.4 M Glc and 0.4 M Fru, respectively (data not shown). The CWI-Inh is suggested to function as a regulatory switch of CWI when the Suc concentration is lower than a specific threshold (Weil et al., 1994; Sander et al., 1996; Krausgrill et al., 1998). Sucrose binding at the catalytic site of CWI prohibits binding of the inhibitor and it is therefore suggested that CWI should become susceptible to inhibition only after *in situ* substrate depletion (Anderson et al., 1980; Weil et al., 1994). It would seem that the post-transcriptional and post-translational control of CWI and CWI-Inh by sugars might play a more important role in their regulation than transcriptional control alone.

The *Cwi:Cwi-Inh* mRNA ratio was analysed to investigate the correlation between the expression levels of the CWI and CWI-Inh encoding genes. At the vegetative stage, the young leaves exhibited the highest *Cwi:Cwi-Inh* mRNA ratio of the various tissues analysed. These results seem to indicate that young leaves have priority over roots in sugar partitioning at this growth stage. Plant growth observations confirmed that the development and increase in biomass of young leaves were faster than that of the roots (results not shown). The young leaves of the Apo-Inv lines displayed a fourfold higher *Cwi:Cwi-Inh* transcript ratio than the ratios observed in control and Vac-Inv young leaves. At the flowering stage, the *Cwi:Cwi-Inh* mRNA ratio of the flowers far exceeded that observed in the other tissues analysed, which seem to indicate that these sink tissues became the predominant sink organs of the plant with regards to sugar partitioning. The highest *Cwi:Cwi-Inh* mRNA ratio was found in the flowers of the control lines, which was 1.6 and threefold higher than the ratio found in the flowers of the Vac-Inv and Apo-Inv lines, respectively. These results are consistent with the observed phenotypes of the flowers, i.e. the control plants displayed normal flowers and seed set, whereas the number of flowers of the Apo-Inv lines was decreased by approximately 70%. The individual flower size of the Vac-Inv lines was decreased by

20%, whereas the number of flowers was increased by 70%.

In conclusion, the presence of a yeast-derived invertase in the apoplast or vacuole of transgenic tobacco plants altered the expression of the tobacco endogenous *Cwi* and *Cwi-Inh* in the various tissues, depending on their sink-source status and growth stage. However, no obvious correlation between the *Cwi* and *Cwi-Inh* expression levels and soluble sugar content was found. The results obtained in this study also suggest that the transcript ratio between *Cwi* and *Cwi-Inh* could be an accurate indicator of the real invertase activity in plants tissues and therefore sink strength.

5.5 ACKNOWLEDGEMENTS

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CHAPTER 6

RESEARCH RESULTS

Sugar partitioning in transgenic tobacco overexpressing a yeast-derived invertase in either the sink or source organs

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Sugar partitioning in transgenic tobacco overexpressing a yeast-derived invertase in either the sink or source organs

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Sugar partitioning forms an integral part of sugar metabolism in higher plants and plays an important role in their growth, development and yield. However, little is known about the regulation of this multifaceted process. In order to gain further insight into the role of sink-source interactions in this process, a reciprocal-type grafting was performed between control plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) reporter gene and lines transformed with the *Saccharomyces cerevisiae* invertase *Suc2* gene targeted to the apoplast (Apo). The resulting Apo/GUS (Apo on GUS) scions did not exhibit a reduction in carbon export from the above-ground tissues as expected, whereas the GUS/Apo (GUS on Apo) scions displayed a significant shift in sugar partitioning towards the roots when compared to the GUS/GUS (GUS on GUS) control scions. Interestingly, these shifts in sugar partitioning had no impact on plant biomass or morphology, possibly due to an increase in respiration as reflected by sugar content. These results confirm the importance of invertases in the regulation of sugar partitioning and also highlight the intricate nature of the relationship between sink-source interactions and sugar partitioning.

6.1 INTRODUCTION

The partitioning of photoassimilates between autotrophic source tissues and a variable number of sink tissues enables higher plants to survive as multicellular organisms and accompanies all stages of plant growth and development (Gifford et al., 1984). This complex and integrated process is affected by exogenous factors that include temperature (Farrar, 1988), salinity (Gao et al., 1998), wounding (Roitsch, 1999), as well as virus and phytopathogenic fungal infection (Ehness et al., 1997; Lucas and Wolf, 1999). Although the regulation of assimilate partitioning is not well understood, it has been shown that some control is mediated by phytohormones (Ehness and Roitsch, 1997; Goetz et al., 2000), competition between the various storage and metabolic sinks, as well as transmembrane transport events (Truernit et al., 1996).

Sink-source interactions form an integral part of sugar partitioning and have been studied by means of various approaches. Of these methods, studies that involved the heterologous expression of a yeast-derived invertase (Stitt et al., 1990;

Von Schaewen et al., 1990; Sonnewald et al., 1991; Hajirezaei et al., 2000, Neubohn et al., 2000), as well as the analysis of plants expressing antisense sucrose transporters (Riesmeier et al., 1994; Bürkle et al., 1998; Schulz et al., 1998), revealed that sucrose signalling can regulate phloem loading, and thus sugar partitioning, at the level of phloem translocation with profound effects on growth and development (Chiou and Bush, 1998). These effects, including stunted growth, reduced root formation and bleached leaves, were attributed to a reduced photoassimilate loading capacity and thus starvation of the sink organs (Von Schaewen et al., 1990). However, in the previous experiments involving a yeast-derived invertase, the overexpression of this heterologous enzyme was on a whole-plant level (Von Schaewen et al., 1990; Sonnewald et al., 1991). The implications of this homogenous expression of the yeast invertase is that the interpretations made regarding the influence of altered sink or source organ capacity on sugar partitioning, disregarded the integrated nature of sink-source interactions.

The aim of this study was to determine the influence of altered sucrose metabolism, due to invertase activity, in either the above-ground tissues or roots. To this end, transgenic tobacco plants that overexpressed a yeast-derived invertase in the apoplast were used in reciprocal grafting with control plants. These scions were analysed to verify the influence of the heterologous invertase activity in either the above-ground tissues or roots on plant morphology and sugar partitioning.

6.2 MATERIALS AND METHODS

6.2.1 Reagents

See Section 3.2.1 of Chapter 3. ^{14}C was purchased from Amersham Biosciences (Buckinghamshire, UK).

6.2.2 Selection and preparation of transgenic plant material

Transgenic tobacco lines expressing the *Escherichia coli* β-glucuronidase (*Gus*) gene and lines expressing the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the apoplast, were derived as described in Sections 3.2.3 and 3.2.4 of Chapter 3 of this dissertation. Approximately 30 independent putative transgenic tobacco plants expressing a yeast-derived invertase in the apoplast (Apo-Inv) were regenerated and analysed as described in Chapter 3. A range of invertase activities was found among the transformants, with the highest invertase activities up to 25-fold higher than that of the control (GUS) plants (Fig. 8, Chapter 3). Transgenic lines displaying high invertase activities (ranging from 20 to 37 $nmol.min^{-1}.mg^{-1}$ prot) were selected for grafting with clonal copies of a control (GUS) line. These transformants were hardened off in sand (1:1 mixture of no.1 and no.12) (Consol Glass, Cape Town, RSA) in a growth chamber (50 $\mu mol.m^{-2}.sec^{-1}$) under a 16 h-light: 8 h-dark cycle,

at 24°C. Plants were watered daily and fed every two weeks with a 1:300 dilution of organic plant food (8% N, 2% P and 5.8% K). Following 10 days of growth (approximately 5 leaves per plant), reciprocal-type grafting was performed between the GUS and Apo-Inv lines, along with control grafts consisting of GUS/GUS (GUS on GUS) and Apo/Apo (Apo on Apo) (Fig. 1). Each graft combination was repeated between four to eight times and the resulting scions used as replications in subsequent analyses. Seven weeks of growth was allowed before the scions were subjected to analysis. The Apo/Apo grafting unions did not survive the grafting procedures, whereas the GUS/Apo scions exhibited a lower survival rate than that of the Apo/GUS and GUS/GUS scions.

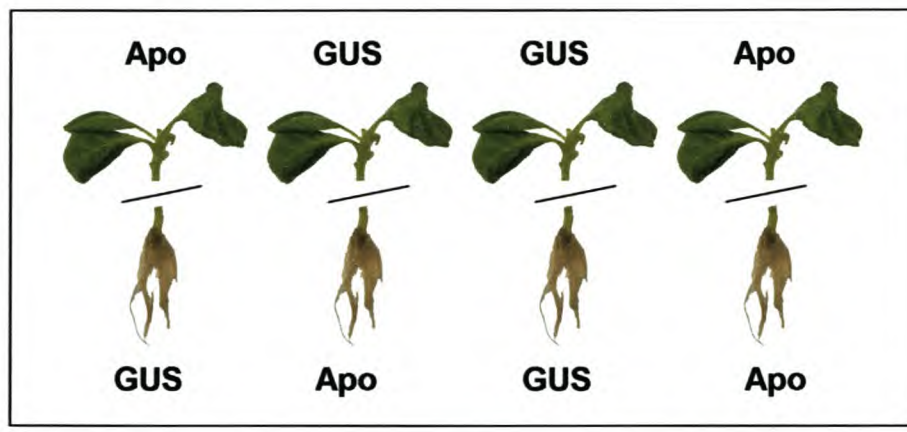

Figure 1. A representation of the respective reciprocal-type grafting unions between control plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene and lines transformed with the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the apoplast (Apo): Apo on GUS (Apo/GUS), GUS on Apo (GUS/Apo), GUS on GUS (GUS/GUS) and Apo on Apo (Apo/Apo).

6.2.3 [^{14}C]-radiolabelling of transgenic plants

$^{14}CO_2$ feeding of the scions was performed by exposure to 2 mCi $^{14}CO_2$ in a sealed glass chamber of 30 L. To prevent any $^{14}CO_2$ fixation in the rhizosphere the plant pots were individually sealed with plastic bags and tightened around the base of the stem with an elastic rubber band. The total volume occupied by the containers was 12 L (3 L per plant) and the estimated plant volume was 10 L. Air within the chamber was circulated with the aid of a fan mounted on the sidewall of the chamber. After the plants (four per treatment) were placed in the chamber and an airtight seal was achieved, the $^{14}CO_2$ was introduced into the chamber by syringe injection through a rubber septum. Preliminary experiments revealed that more than 90% of the $^{14}CO_2$ was fixed within 60 min of incubation. All labelling experiments were subsequently done with a total incubation time of 90 min. After incubation, any residual $^{14}CO_2$ in the atmosphere was removed by absorption with 12% (w/v) KOH for 15 min. The plants were then removed from the chamber and separated into above-ground tissues and roots. The separated tissues were ground to a fine powder in liquid nitrogen after

which 70% (v/v) ethanol was added in a 2:1 ratio. A 1 ml aliquot of the suspension was then mixed with 5 ml Ultima Flo™ M scintillation cocktail and total radioactivity in the sample was determined with a Beckman LS 1801 scintillation counter. Disintegration per minute (DPM) counts was corrected for quenching using the internal standard method of the apparatus.

6.2.4 Determination of plant biomass

The scions were harvested and separated into above-ground tissues and roots. The roots were washed from the potting medium under slow-running water to minimise loss of biomass before determining fresh weight.

6.2.5 Soluble sugars assay

Sugars were extracted from above-ground and root tissue and measured as described in Section 3.2.10 (Chapter 3).

6.2.6 Statistical analysis

See Section 3.2.12 of Chapter 3.

6.3 RESULTS AND DISCUSSION

It has been shown that the abundance or the depletion of sugars such as Suc and Glc play a role to co-ordinately and differentially regulate plant growth through various control mechanisms and complex signal transduction pathways (Koch, 1996; Chiou and Bush, 1998; Sheen et al., 1999; Ho et al., 2001). This is achieved by altering gene expression and/or enzyme activities, thereby controlling photosynthate production and utilisation and therefore sugar partitioning (Koch, 1996). In this study, transgenic tobacco plants overexpressing a yeast-derived invertase in the apoplast were used in reciprocal grafting with control plants to examine the effects of increased invertase activity, with concomitant altered sugar content and sink-source interactions in either the sink or source organs, on sugar partitioning.

6.3.1 Effect of grafting procedures on phenotype and growth

In previous studies, the expression of a yeast-derived invertase in the apoplast of transgenic tobacco plants resulted in severe changes in phenotype such as stunted growth, reduced root formation and bleached areas in old leaves (Von Schaewen et al., 1990; Sonnewald et al., 1991; Chapter 3 of this dissertation). The first two phenomena were linked to a lower photoassimilate supply to the sink organs, due to impaired Suc export from the source leaves (Von Schaewen et al., 1990; Sonnewald

et al., 1991). However, the yeast invertase was expressed on a whole-plant basis and therefore the influence of altered of sink or source capacity *per se* on sugar partitioning could not be distinguished.

In this study, opposite phenotypic changes could initially be observed in the GUS/Apo and Apo/GUS scions. Within the first four weeks of growth following the grafting procedures, the Apo/GUS scions displayed an approximate 10% reduction in growth of the above-ground tissues, whereas the Apo/GUS scions exhibited a 60% reduction in growth in comparison to GUS/GUS control grafts (Fig. 2). As time progressed, however, the differences in growth between the various scions became less pronounced until they were minimal at the time of analysis (seven weeks post-grafting). This is due to the slower-growing scions (expressing the yeast invertase gene in either the above-ground tissues or roots) gaining on the GUS/GUS control scions as the growth of the control scions slowed down prior to flowering.

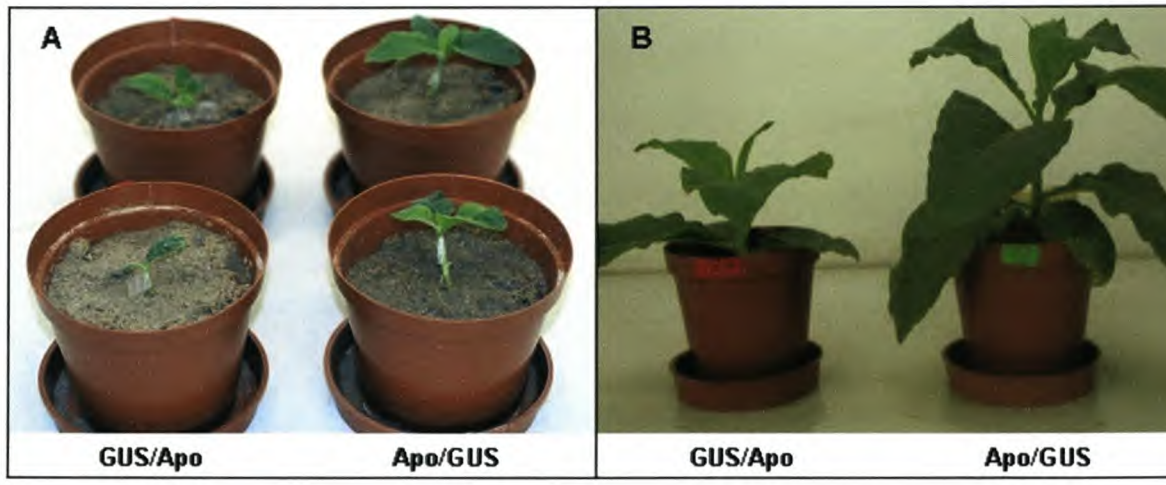

Figure 2. Examples of the phenotypical appearance of soil-grown tobacco scions subsequent to reciprocal-type grafting procedures between control plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene and lines transformed with the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the apoplast (Apo). The resulting GUS/Apo and Apo/GUS scions at (A) one week and (B) four weeks post-grafting.

6.3.2 Plant biomass distribution

The total biomass of above-ground tissues and roots was determined in Apo/GUS, GUS/Apo and GUS/GUS scions and the distribution of the biomass expressed as a percentage of total biomass (Table 1). The biomass distribution of above-ground tissues of the Apo/GUS scions were higher (P=0.04) than those of the GUS/Apo scions, whereas the Apo/GUS roots had a lower (P=0.02) biomass than the GUS/Apo roots. No significant differences in final biomass distribution were measured between the rest of the scion-types at seven weeks subsequent to the grafting procedures. These results were unexpected since the proposed reduced loading capacity of the Apo/GUS scion-source leaves did not manifest

morphologically as in the previous experiments with a yeast-invertase targeted to the apoplast (Von Schaewen et al., 1990; Sonnewald et al., 1991; Chapter 3 of this dissertation). Also, the GUS/Apo scions were expected to exhibit large increases in root biomass due to increased sink strength as a result of invertase activity in the apoplast of the roots. However, this was not reflected in the phenotype or biomass distribution of these plants.

Table 1. Biomass distribution, expressed as a percentage of total biomass, of above-ground tissues and roots of reciprocal grafting unions between control tobacco plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene and transgenic tobacco lines transformed with the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the apoplast (Apo), Apo/GUS, GUS/Apo and GUS/GUS, seven weeks post-grafting. The standard error of the mean is indicated.

	Apo/GUS[a]	GUS/Apo[a]	GUS/GUS[b]
Above-ground tissues	81 ± 1	75 ± 1	78 ± 1
Roots	19 ± 1	25 ± 1	22 ± 1

[a] The values for the Apo/GUS and GUS/Apo scions represent the mean of eight lines analysed per scion-type.

[b] The values for the GUS/GUS scions represent the mean of four lines analysed.

6.3.3 Sugar partitioning in grafted transgenic plants

To evaluate the actual ability of the above-ground tissues to export photosynthate, as well as the capacity of the roots to import the translocated carbon, the grafted scions were subjected to a $^{14}CO_2$ feeding experiment seven weeks subsequent to the grafting procedures. Results of the distribution of the radiolabel (Table 2) revealed no significant reduction in carbon export from the above-ground tissues by the Apo/GUS scions in comparison to GUS/GUS control scions, whereas the GUS/Apo scions displayed an approximate 18% shift in partitioning towards the roots (P=0.05).

Table 2. Distribution of radioactivity, expressed as a percentage of total disintegration per minute (DPM) counts, between the above-ground tissues and roots of reciprocal grafting unions between control tobacco plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene and transgenic tobacco lines transformed with the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the apoplast (Apo), Apo/GUS, GUS/Apo and GUS/GUS, seven weeks post-grafting. The standard error of the mean is indicated.

	Apo/GUS[a]	GUS/Apo[a]	GUS/GUS[a]
Above-ground tissues	94 ± 2	74 ± 5	92 ± 2
Roots	6 ± 3	26 ± 5	8 ± 1

[a] Each value represents the mean of four lines analysed.

This shift in sugar partitioning towards the roots in the GUS/Apo scions confirms increased sink strength in these tissues due to the invertase activity in the apoplast: Suc is removed by cell wall-bound invertase in the apoplastic space, the Suc gradient is steepened and sink activity is increased, resulting in increased sink strength and in turn increased sugar partitioning towards the sink (Eschrich, 1980; Ho, 1988; Sonnewald et al., 1997).

6.3.4 Tissue-specific soluble sugar content

The above-ground tissues and roots of Apo/GUS, GUS/Apo and GUS/GUS scions, differing in their sink/source status, were analysed for soluble sugar content seven weeks post-grafting. The levels of sucrose (Suc), glucose (Glc) and fructose (Fru) were determined in these lines (Fig. 3). The above-ground tissues of the Apo/GUS scions displayed a 1.2-fold (P=0.05) decrease in Suc, which was probably due to the yeast invertase activity in these tissues. Approximately equal hexose levels in comparison to the GUS/GUS control lines were also observed in the above-ground tissues of the Apo/GUS scions. In the roots, the Suc content of the Apo/GUS lines was characterised by a 1.5-fold (P=0.03) decrease, whereas the hexose levels was unaltered when compared to the control scions. This decrease in Suc levels in the roots is most likely the result of decreased Suc loading into the phloem by the above-ground tissues due to heterologous invertase activity.

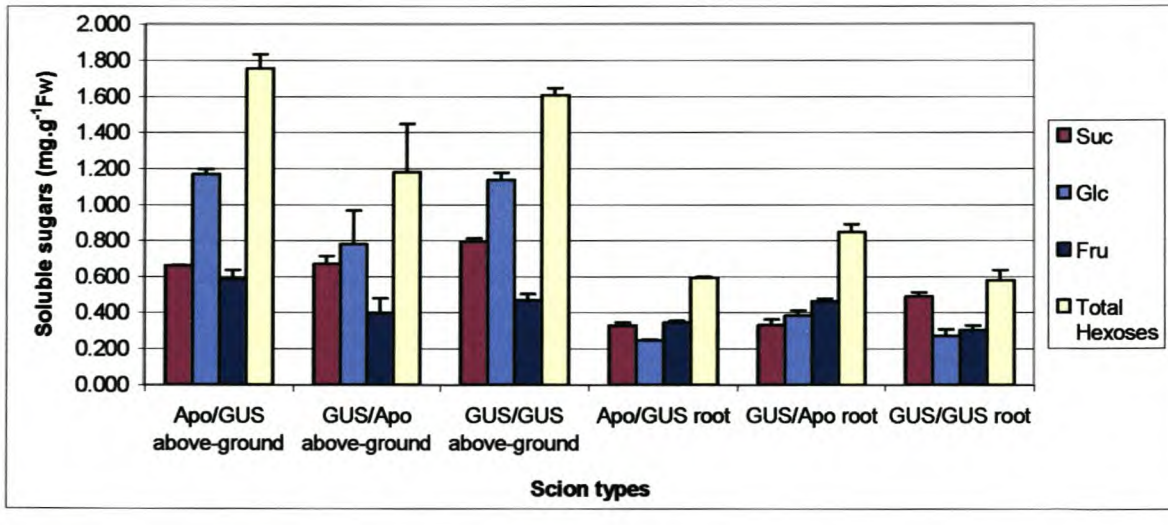

Figure 3. Sucrose (Suc), glucose (Glc), fructose (Fru) and total hexose levels of the above-ground tissues and roots of reciprocal grafting unions between control tobacco plants transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene and transgenic tobacco lines transformed with the *Saccharomyces cerevisiae* invertase (*Suc2*) gene targeted to the apoplast (Apo), Apo/GUS, GUS/Apo and GUS/GUS, seven weeks post-grafting. The error bars indicate the standard error of the mean of four lines analysed.

In comparison to the GUS/GUS control scions, the Suc levels in the above-ground tissues of the GUS/Apo-scions were decreased by 1.2-fold (P=0.06), whereas the averages in Glc and Fru content, although no statistically significantly, were decreased by 1.5-fold and 1.2-fold, respectively. The average total hexose content was decreased by approximately 1.3-fold. This decrease in soluble sugar content in the above-ground tissues might be due to the increased sink strength and therefore increased translocation towards the roots of these scions. The roots of the GUS/Apo scions exhibited a 1.5-fold (P=0.09) decrease in Suc content, whereas the average Glc and Fru levels were increased by 1.4-fold and 1.5-fold (P=0.05), respectively in comparison to the control lines. Thus the average total hexose content was increased by approximately 1.5-fold (P=0.07). This decrease in the amount of Suc, rather than an increase due to increased import of Suc into the roots, as well as the increased hexose levels, was measured as a possible result of the yeast invertase activity. A higher accumulation of hexose sugars was expected due to the significant increase in sugar partitioning towards these tissues. This might be a result of increased respiration in the tissues expressing a yeast-derived invertase in the apoplast (Stitt et al., 1990; Von Schaewen et al., 1990). In previous experiments involving the overexpression of a yeast-derived invertase in the apoplast, the accumulation of soluble sugars in the source leaves resulted in an increase in glycolytic enzymes and a concomitant increase in respiration (Stitt et al., 1990; Von Schaewen et al., 1990).

Thus in summary, the genetic and physical manipulations performed in this study resulted in scions with shifts in sugar partitioning in both above-ground tissues and roots, but no impact on final biomass or plant morphology. It is postulated that the increased carbon content in the above-ground tissues of the Apo/GUS scions and in the roots of the GUS/Apo scions is oxidised and released in the form of CO_2 due to an increased respiration rate. Although this postulation is supported by the fact that there is no considerable accumulation of hexoses in the transgenic lines, further investigation is required. Also, analysis of the scions at an earlier growth stage, e.g. four weeks post-grafting, instead of the seven weeks of growth allowed in this study might, might be more insightful regarding the correlation between growth phenomena and sugar partitioning.

In conclusion, the data presented here highlight the complexity and flexibility of Suc metabolism and sugar partitioning. One implication of these results is that the alteration of production levels of the sucrose cleaving enzymes, and those of other proteins intricately involved in Suc metabolism and transport in plants, are not promising targets for the manipulation of crop productivity (to increase the partitioning towards edible sink tissues) as thought previously (Riesmeier et al., 1993; Zrenner et al., 1996; Sonnewald et al., 1997; Veramendi et al., 1999; Leggewie et al., 2003). Due to the complex nature of sink-source interactions, more sophisticated strategies are required to succeed in the biotechnological improvement of crop yield. This was further illustrated by the work of Leggewie et al. (2003) who overexpressed the

spinach SoSUT1 sucrose transporter in potato in order to improve tuber yield. They observed an increase in sucrose uptake capacity by the transformed tubers that resulted in elevated sugar content, however, no increase in starch content or tuber yield. In this case, the shift in sugar partitioning was induced by an increase in sucrose import into the tubers as a result of increased sucrose transporter activity, although this was not reflected in tuber morphology.

The results obtained in this study confirm, however, that sugar partitioning can be modulated by sink-source interactions and emphasise the importance of invertases in the regulation of sugar partitioning through its ability to alter sink capacity.

6.4 ACKNOWLEDGEMENTS

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 GENERAL DISCUSSION AND CONCLUSIONS

The ability to disturb the sugar metabolism of plants at a particular step through recombinant DNA technology enables scientists to gain insight into the processes of sugar translocation and partitioning. For instance, valuable information was acquired with experiments involving the heterologous expression of the *Saccharomyces cerevisiae* invertase (*Suc2*) gene in various species (Von Schaewen et al., 1990; Dickinson et al., 1991; Sonnewald et al., 1991; Heineke et al., 1992; Büssis et al., 1997; Weber et al., 1998; Ma et al., 2000). Since the first descriptions of this model system and the valuable information derived from it in various plant species, several new insights regarding the regulation of this process and the role of sugar as a signal molecule have become available. This, together with the fact that certain initial observations were never further investigated or quantitatively analysed, left significant scope to further exploit this system with regards to sugar metabolism and partitioning.

This model system of transgenic plants expressing a yeast-derived invertase in different subcellular compartments was thus utilised to test the following research hypothesis: in higher plants, sugar signals are sensed by proteins integrally linked to sugar transport and/or partitioning to ultimately result in physiological responses. To this end, tobacco was successfully transformed with the targeted yeast-invertase *Suc2* gene. The overexpression of the yeast invertase in either the vacuole (Vac-Inv) or apoplast (Apo-Inv) of tobacco plants resulted in increased invertase activities in comparison with control lines transformed with the *Escherichia coli* β-glucuronidase (*Gus*) gene. The hydrolysis of sucrose (Suc) to glucose (Glc) and fructose (Fru) by invertase activity resulted in the severe disturbance of soluble sugar content in the source leaves and roots of the Vac-Inv and Apo-Inv lines. As a consequence, stunted growth and bleached regions in the interveinal leaf tissue were observed in the Apo-Inv lines. A causal relation was confirmed between the accumulation of hexoses, the decrease in chlorophyll (Chl) content and the visible phenotype in the source leaves of the Apo-Inv lines: an increase in hexose content lowers the rate of photosynthesis, which will decrease the demand for ATP and NADPH that in turn will result in the loss of Chl (Stitt et al., 1990; Von Schaewen et al., 1990; Stitt et al., 1995). The changes in growth and development were clearly linked to disturbed sink-source relationships as caused by the invertase activity and resulting increase in hexose levels. These results are consistent with previous experiments involving the overexpression of a yeast-derived invertase in tobacco (Von Schaewen et al., 1990; Sonnewald et al., 1991).

These transgenic lines, affected in their sink-source interactions, were utilised to examine the effect of the altered sugar levels, as a result of the heterologous invertase activity, in sink and source organs on gene expression and sugar partitioning. This was accomplished by investigating the expression of proteins integrally linked to sugar translocation and partitioning, such as plant sugar

transporters, as well as endogenous invertases and invertase inhibitors. The invertase-expressing lines were analysed for Suc transporter *NtSut1* and hexose transporter *NtMst1* mRNA levels in the source leaves and roots. Transcript levels of *NtSut1* and *NtMst1* increased significantly in the source leaves and roots of Vac-Inv transformants, whereas increased *NtMst1* transcript levels were also detected in the roots of Apo-Inv transformants. These increased mRNA levels could be correlated to the invertase activities and altered sugar levels in these tissues. It is concluded that *NtSut1* and *NtMst1* expression is differentially regulated by Suc and/or hexose content. In addition, the regulatory effect of the altered sugar levels on transporter expression depended on the subcellular compartment in which the yeast invertase was expressed. It would seem that the subcellular compartmentation of sugar metabolism is important for the dynamic regulation of sugar partitioning. The effect of this is evident when comparing the phenotypes of the Apo-Inv and Vac-Inv transformants. It is postulated that the up-regulation of sugar transporters in both source leaves and roots of Vac-Inv lines enabled these lines to efficiently sense and manage the disturbance in Suc metabolism, as brought about by the introduced yeast invertase activity. This is, to our knowledge, the first report that shows that *NtSut1* and *NtMst1* expression is regulated by sugars. The results obtained supports the research hypothesis that sugar signals are indeed sensed by certain proteins integrally linked to sugar transport.

The expression of tobacco's endogenous cell wall invertase (*Cwi*) and cell wall invertase inhibitor (*Cwi-Inh*), in the presence of the yeast-derived invertase, was examined in various tissues of Apo-Inv and Vac-Inv lines at both the vegetative and flowering growth stages. In comparison with the control lines, the various tissues of the Apo-Inv and Vac-Inv lines displayed altered *Cwi* and *Cwi-Inh* expression levels, depending on the sink-source status and growth stage. However, no obvious correlation between the *Cwi* and *Cwi-Inh* expression levels and soluble sugar content of these tissues was found. It is suggested that the post-transcriptional and post-translation control of these proteins by sugars might play an important part in their regulation. Our results indicate that certain proteins integrally linked to sugar partitioning, specifically CWI and CWI-Inh are not involved in the sensing of sugar signals on the transcriptional level, thereby not conforming to the research hypothesis. Furthermore, analysis of the *Cwi:Cwi-Inh* mRNA ratio and growth observations of the various tissues of control as well as Apo-inv and Vac-Inv lines indicated that this transcription ratio could be an accurate indicator of the sink strength of sink organs.

Apo-Inv transformants with high invertase activity and disturbed sugar metabolism and phenotype were used in reciprocal grafting procedures with control plants. The resulting scions were analysed to determine the influence of altered Suc metabolism in either the sink or source organs on phenotype, biomass, soluble sugar content and sugar partitioning. Contrary to expectations, the Apo/GUS scions did not exhibit a significant reduction in carbon export from the above-ground tissues,

whereas the GUS/Apo scions displayed a significant 18% shift in sugar partitioning towards the roots. However, no significant differences in the final phenotype or biomass could be observed between the Apo/GUS, GUS/Apo and GUS/GUS control scions at seven weeks post-grafting. This might be explained by an increase in the respiration rate of the tissues expressing the yeast invertase in the apoplast (Stitt et al., 1990; Von Schaewen et al., 1990). Furthermore, the phenomena of impaired root formation and stunted growth was not observed in the tissues expressing the yeast invertase. In previous studies the altered growth phenomena was linked directly to impaired sucrose export from the source leaves, which would result in the "starvation" of sink tissues (Von Schaewen et al., 1990; Sonnewald et al., 1991), but from our results it is clear that the sink-source interactions play a more fundamental role than previously thought. In addition, our results confirm the importance of invertases in the regulation of sugar partitioning through its ability to alter sink strength and emphasise the complex relationship between sugar partitioning and plant phenotype. The research hypothesis is thus met in that it has been shown through these results that in higher plants physiological responses do occur as a result of sink/source interactions through the sensing of sugar signals.

In conclusion, the introduction and expression of a targeted yeast-invertase gene in transgenic plants altered the sugar content and sink-source interactions in these lines to provide significant insights into the regulation of sugar partitioning in response to internal signals. It has been shown here that sugar transporters are intricately involved in the sensing and translation of sugar signals by means of their transcriptional regulation. Moreover, the results confirm that sugar partitioning is a highly integrated and adaptable process, which can be modulated by sink-source interactions. The data presented in this study also demonstrated the intricate and flexible nature of the relationship that exists between sugar metabolism, partitioning and growth phenomena.

7.2 LITERATURE CITED

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