# The Regulation and Role of Plant Invertases

A thesis submitted by

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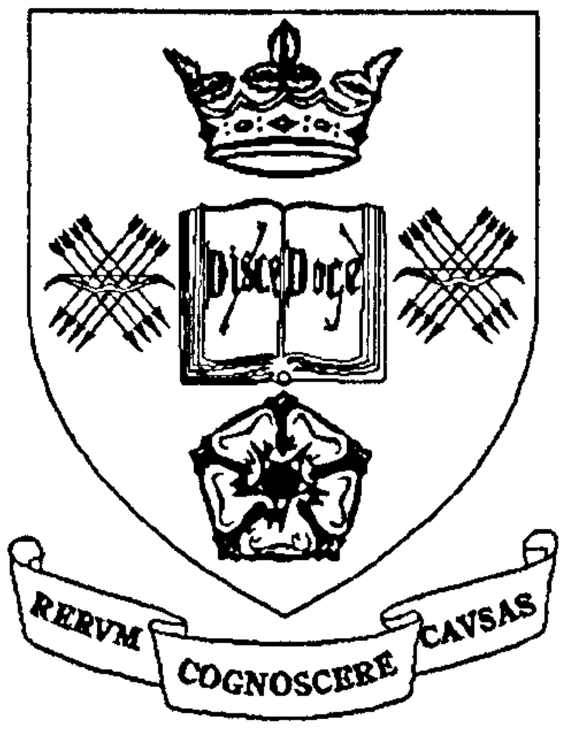

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# SUMMARY OF THESIS

## The Regulation and Role of Plant Invertases

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The aim of this thesis was to investigate the regulation and role of invertases in plant carbohydrate metabolism. In the first part of this thesis a molecular approach was adopted and the expression of five invertase genes were examined in different organs and in leaves of different ages in the model plant species *Arabidopsis thaliana* (L.). Of the five genes examined two encoded apoplastic invertases (ATβFRUCT 1 and ATβFRUCT 2), two encoded soluble invertases with a probable vacuolar localisation (ATβFRUCT 3 and 4) while the fifth encoded an invertase with an unknown sub-cellular localisation (ATβFRUCT 5). Generally, Northern hybridisation assays were sufficiently sensitive for the detection of invertase gene expression in *A. thaliana*, however, in order to examine the expression of rare ATβFRUCT 1 and ATβFRUCT 2 mRNA the reverse transcriptase polymerase chain reaction (RT-PCR) was the method of choice. The development of an RT-PCR internal standard enabled these data to be semi-quantitative in nature. Expression analysis revealed that each of the five invertase genes were differentially regulated in *A. thaliana*. However, high levels of invertase gene expression were associated with tissues typically considered sinks for carbohydrate. Examination of these tissues also revealed a relationship between invertase activity and the ratio of sucrose to hexoses.

Previous work has shown that certain environmental stimuli can influence invertase activity and gene expression. In this thesis the infection of *A. thaliana* leaves with the biotrophic pathogen *Albugo candida* resulted in the localised stimulation of cell-wall associated invertase activity. Examination revealed that the majority of this increase was attributable to the stimulation of the host apoplastic invertase gene, ATβFRUCT 1. Furthermore, expression of this gene was also elevated in response to mechanical leaf wounding. The high expression of ATβFRUCT 1 in sink tissues and in response to pathogenesis and wounding suggested that this gene plays an important role in establishing a supply of hexoses to tissues under a wide range of conditions.

In the second part of this thesis transgenic tomato plants (prepared by Zeneca Plant Science) with a range of leaf vacuolar invertase activities were examined. There was a linear relationship between vacuolar invertase activity and the amount of leaf hexose. In plants with no detectable leaf vacuolar invertase activity there was an accumulation of sucrose. This suggests that hexoses generated in the leaves of tomato plants are the product of sucrose cycling through the vacuole. The implications of such cycling are discussed in detail.

# ABBREVIATIONS

| | |
|---|---|
| **ABA** | abscisic acid |
| **ADP** | adenosine 5'-diphosphate |
| **ATβFRUCT 1** | *Arabidopsis thaliana* β-fructosidase gene 1 |
| **ATβFRUCT 2** | *Arabidopsis thaliana* β-fructosidase gene 2 |
| **ATβFRUCT 3** | *Arabidopsis thaliana* β-fructosidase gene 3 |
| **ATβFRUCT 4** | *Arabidopsis thaliana* β-fructosidase gene 4 |
| **ATβFRUCT 5** | *Arabidopsis thaliana* β-fructosidase gene 5 |
| **ATP** | adenosine 5'-triphosphate |
| **BA** | benzamidine |
| **BICINE** | (N,N-bis[2-hydroxyethyl]-glycine) |
| **CTAB** | hexadecyltrimethylammonium bromide |
| **DEPC** | diethylpyrocarbonate |
| **DNSA** | 3,5-dinitrosalicylic acid |
| **dNTP** | deoxynucleotide triphosphate |
| **DTT** | dithiothreitol |
| **EDTA** | ethylenediaminetetraacetic acid |
| **EST** | expressed sequence tag |
| **FW** | fresh weight |
| **$GA_3$** | gibberellic acid |
| **GUS** | β-glucuronidase |
| **Hepes** | N-2-hydroxyethylpoperazine-N'-2-ethane sulphonic acid |
| **IAA** | indol-3-yl acetic acid |
| **IEF** | isoelectric focusing |
| **IRGA** | infra-red gas analyser |
| **JA** | jasmonic acid |
| **LB** | Luria Broth |
| **MS** | Murashige and Skoog growth medium |
| **NAD** | nicotinamide adenine dinucleotide (oxidised) |
| **NADH** | nicotinamide adenine dinucleotide (reduced) |
| **NADP** | nicotinamide adenine dinucleotide phosphate (oxidised) |

| | |
|---|---|
| **NADPH** | nicotinamide adenine dinucleotide phosphate (reduced) |
| **NTP** | nucleotide triphosphate |
| **PCR** | polymerase chain reaction |
| **PGA** | polygalacturonic acid |
| **Pi** | inorganic phosphate |
| **pI** | isoelectric point |
| **PMSF** | phenylmethylsulphonyl fluoride |
| **RT-PCR** | reverse transcriptase polymerase chain reaction |
| **Rubisco** | ribulose-1,5-bisphosphate carboxylase/oxygenase |
| **SDS** | sodium dodecyl sulphate |
| **SE-CC** | sieve element companion cell complex |
| **TP** | triose phosphate |
| **Tris** | 2-amino-2-(hydroxymethyl)propane-1,3-diol |

# CONTENTS

# Chapter 1

# General Introduction

# 1.1 INTRODUCTION

Invertase or β-D-fructofuranosidase (EC 3.2.1.26) is an enzyme of plant carbohydrate metabolism that catalyses the hydrolysis of terminal α(1-2) linked fructose moieties. However, under normal physiological conditions the primary role of invertase is to catalyse the hydrolysis of sucrose yielding the hexoses glucose and fructose. In this thesis I will explore the role of invertases during the development of a plant and in response to external stimuli such as wounding and attack by pathogens.

## 1.2 The synthesis and utilisation of sucrose within the plant

Green plants are autotrophic, in other words they are able to extract their nutritional requirements from purely inorganic sources. The process of photosynthesis is the sequence of highly complex reactions that result in the fixation of atmospheric carbon into organic carbon compounds. During photosynthesis light energy is captured by light harvesting chlorophyll-protein complexes embedded in the thylakoid membrane of the chloroplast and funnelled through the electron transport chain. The final result of this process is the conservation of light energy in the chemical compounds ATP and NADPH. These compounds are used during the 'dark' reaction of photosynthesis or Benson-Calvin Cycle to provide energy for the enzymatic fixation of carbon dioxide into carbon compounds. An acceptor molecule, ribulose-1,5-bisphosphate (RuBP), combines with $CO_2$ in a carboxylation reaction in the presence of the enzyme RuBP carboxylase-oxygenase (Rubisco) giving two molecules of 3-phosphoglyceric acid (3PGA). The acceptor molecule is regenerated from 3PGA in reactions consuming NADPH and ATP. The reactions of the Benson-Calvin cycle are located within the stroma of the chloroplast. The first utilisable product of the Benson-Calvin Cycle is the three carbon compound triose-phosphate.

Triose-phosphates have one of three fates (Fig. 1.1). Firstly, they can be used for the regeneration of the carbon dioxide acceptor molecule RuBP. Secondly, they can be used for the synthesis of starch within the chloroplast (for review on starch synthesis see ap Rees, 1992) or finally they can be exported to the cytosol in exchange for Pi via the phosphate translocator. In the cytosol triose phosphates are utilised in biosynthesis

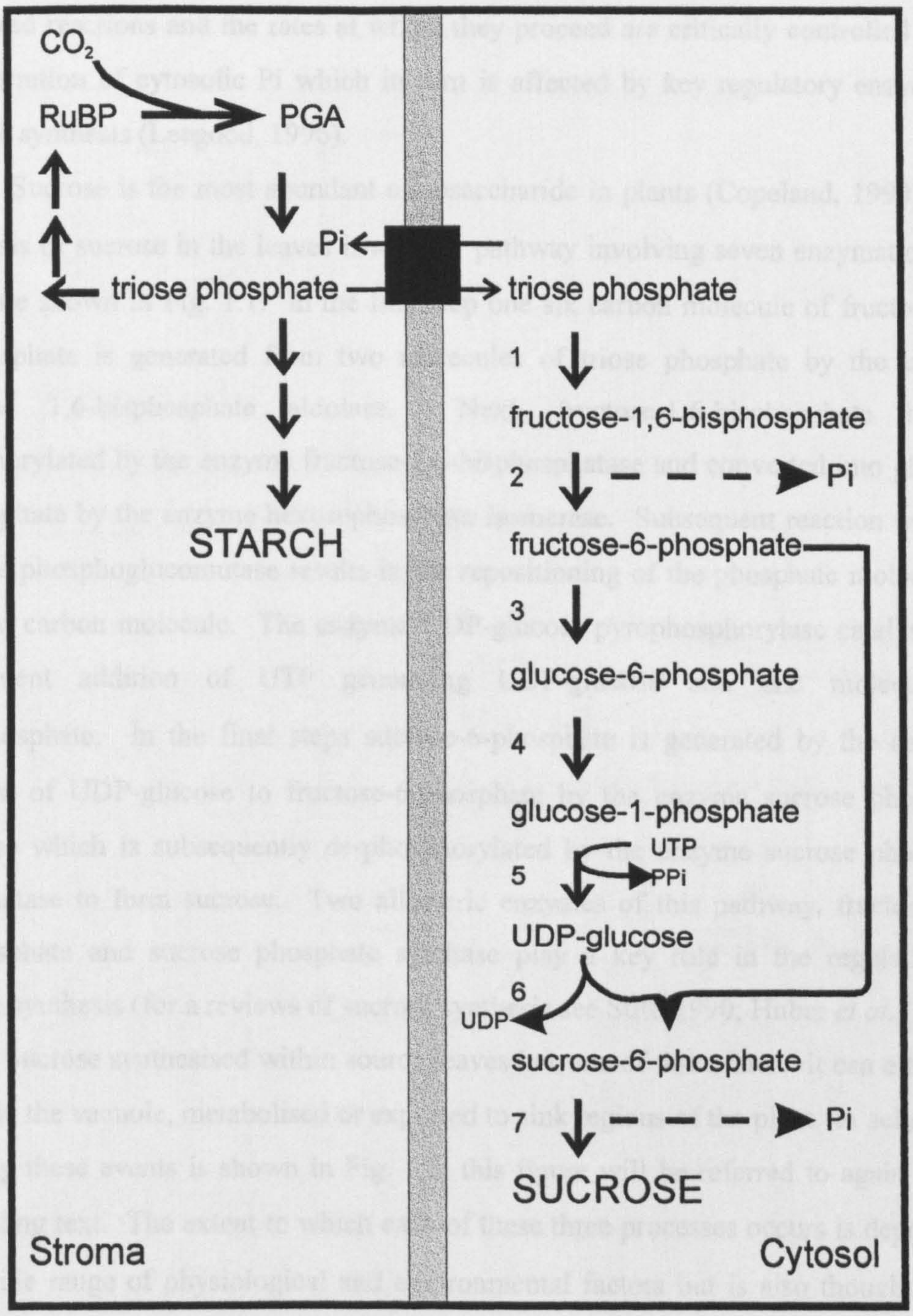



**Figure 1.1 The enzymatic synthesis of sucrose in the cytosol of photosynthetic cells.** 1: aldolase; 2: fru-1,6-bisphosphatase; 3: hexosephosphate isomerase; 4: phosphoglucomutase; 5: UDP-glucose pyrophosphorylase; 6: sucrose phosphate synthase; 7: sucrose phosphate phosphatase. Figure taken and adapted from Stitt (1990b).

pathways such as sucrose synthesis. Sucrose and starch synthesis are highly co-ordinated reactions and the rates at which they proceed are critically controlled by the concentration of cytosolic Pi which in turn is affected by key regulatory enzymes of sucrose synthesis (Leegood, 1996).

Sucrose is the most abundant oligosaccharide in plants (Copeland, 1990). The synthesis of sucrose in the leaves is a linear pathway involving seven enzymatic steps, these are shown in Fig. 1.1. In the first step one six carbon molecule of fructose-1,6-bisphosphate is generated from two molecules of triose phosphate by the enzyme fructose 1,6-bisphosphate aldolase. Next, fructose-1,6-bisphosphate is de-phosphorylated by the enzyme fructose-1,6-bisphosphatase and converted into glucose-6-phosphate by the enzyme hexosephosphate isomerase. Subsequent reaction with the enzyme phosphoglucomutase results in the repositioning of the phosphate molecule to the first carbon molecule. The enzyme UDP-glucose pyrophosphorylase catalyses the subsequent addition of UTP generating UDP-glucose and one molecule of pyrophosphate. In the final steps sucrose-6-phosphate is generated by the catalytic addition of UDP-glucose to fructose-6-phosphate by the enzyme sucrose phosphate synthase which is subsequently de-phosphorylated by the enzyme sucrose phosphate phosphatase to form sucrose. Two allosteric enzymes of this pathway, fructose-1,6-bisphosphate and sucrose phosphate synthase play a key role in the regulation of sucrose synthesis (for a reviews of sucrose synthesis see Stitt, 1990; Huber *et al.*, 1992).

Sucrose synthesised within source leaves has one of three fates. It can either be stored in the vacuole, metabolised or exported to sink regions of the plant. A schematic showing these events is shown in Fig. 1.2; this figure will be referred to again in the proceeding text. The extent to which each of these three processes occurs is dependent on a wide range of physiological and environmental factors but is also thought to be critically dependent on the activity of two sucrose hydrolysing enzymes, invertase and sucrose synthase.

Sucrose synthase (EC 2.4.1.13) catalyses the reversible conversion of sucrose and UDP into UDP-glucose and fructose (Fig. 1.3a). Under normal physiological conditions this reaction is thought to act in the direction of sucrose degradation (Cardini *et al.*, 1955; Avigad, 1982; Cobb and Hannah, 1988; Copeland, 1990; Kruger, 1990; Geigenberger *et al.*, 1993; Quick and Schaffer, 1996). In the direction of sucrose

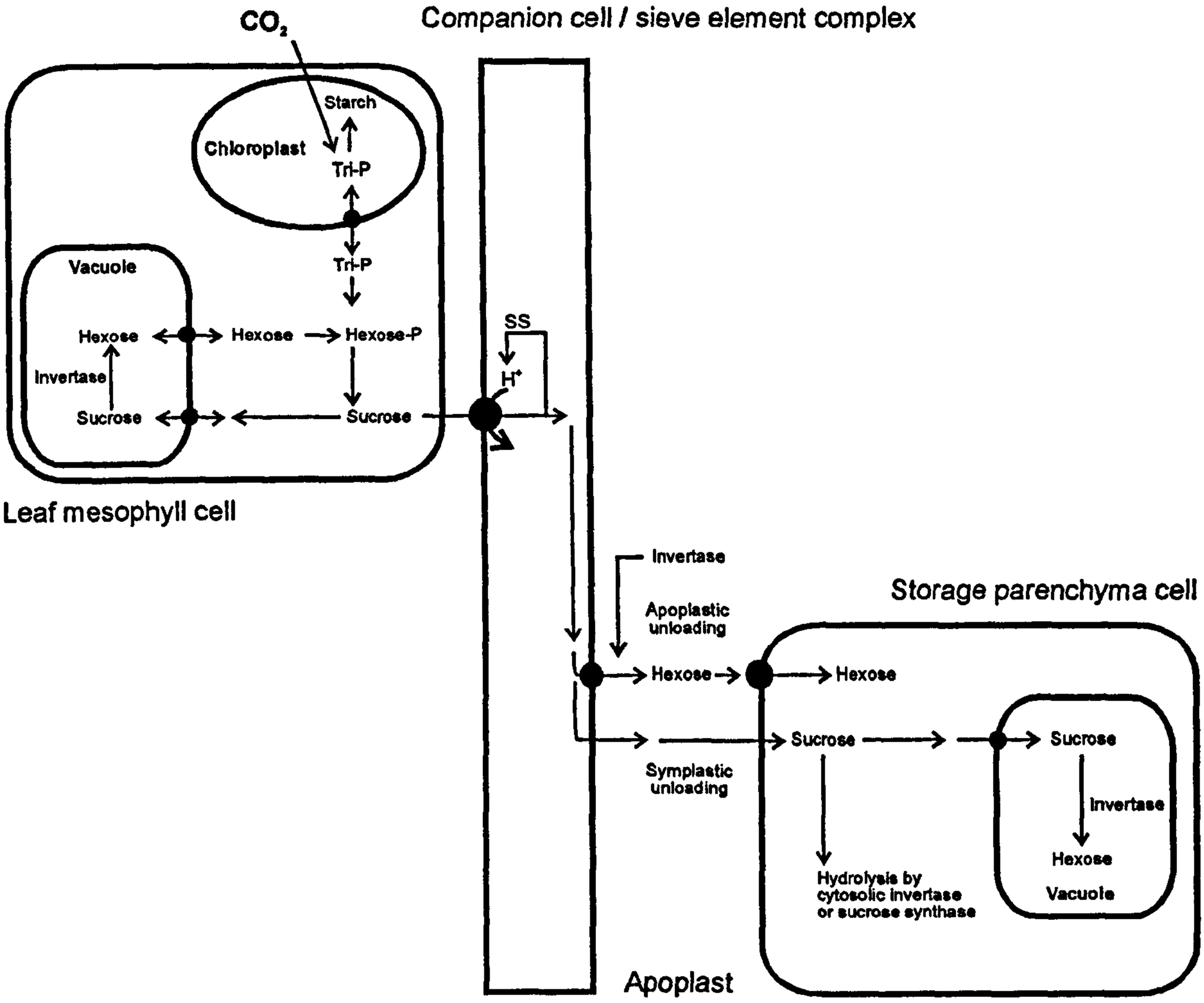
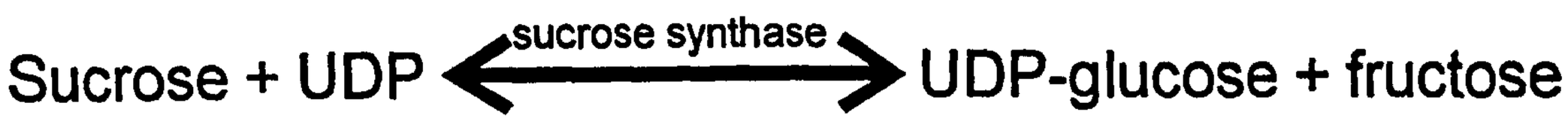



**Figure 1.2 A model of sucrose partitioning in plants with invertase activity indicated at key steps.** Sucrose is synthesised in the mesophyll cells and then either utilised or transported via the phloem to sink regions of the plant. Black circles represent membrane-located pumps while arrows traversing directly through membranes represent plasmodesmatal transport. *Tri-P*, triose-phosphate; *SS*, sucrose synthase. Diagram taken and adapted from Sturm *et al.* (1995).

A)




B)


**Figure 1.3 The sucrolytic activity of sucrose synthase and invertase.** Sucrose synthase (A) and invertase (B) are the two major enzymes of plant metabolism responsible for the catalysis of sucrose into its constitutive hexoses. The reaction with sucrose synthase is readily reversible and the energy of the reaction is preserved in the phosphorylated glucose moiety. The reaction with invertase is best described as a catalytic 'hydrolysis' and is essential irreversible.

cleavage sucrose synthase has a pH optimum of between pH 6 and pH 7. High sucrose synthase activity is commonly associated with sink organs (Avigad, 1982) where its activity has been linked with the maintenance of sink strength in some species (Chourey and Nelson, 1976; Chourey, 1981). Sucrose synthase activity is also present in mature leaves and is thought to reside within the phloem-companion cells (Chen and Chourey, 1989; Lowell *et al.*, 1989; Martin *et al.*, 1993, Nolte and Koch, 1993). Phloem loading in many species is thought to occur against a concentration gradient via a sucrose proton co-transporter (Slone *et al.*, 1991; Van Bel, 1993) and it has been suggested that sucrose synthase provides the energy required for this active process (Black *et al.*, 1987; Farrar and Williams, 1990, Martin *et al.*, 1993). The production of UDP-glucose by sucrose synthase is also thought to be used for the biosynthesis of cell wall and starch polymers in sink organs (Hendrix, 1990).

Invertases are a ubiquitous group of highly polymorphic plant enzymes that catalyse the irreversible hydrolysis of sucrose into the hexoses glucose and fructose (Fig. 1.3b). Investigations have revealed more than one activity of invertase on the basis of pH optima, solubility, isoelectric point and sub-cellular location. Invertases with acidic pH optima (4.5 to 5.5) are *N*-linked glycoproteins (Sturm, 1991; Unger *et al.*, 1992) and are either soluble proteins, thought to reside within the vacuole (Boller and Kende, 1979; Leigh *et al.*, 1979; Boudet and Canut, 1981; Vattuone *et al.*, 1983; Laurière *et al.*, 1988; Obenland *et al.*, 1993), or particulate proteins ionically bound to the cell apoplast (Unger *et al.*, 1994). Invertases with neutral or alkaline pH optima (6.8 to 8.0) are non-glycosylated and are located in the cytoplasm (ap Rees, 1988; Copeland, 1990; Chen and Black, 1992; Lee and Sturm, 1996).

Following synthesis in the mesophyll cells sucrose is thought, in many species, to move into the vacuole by a process of facilitated diffusion (Fig. 1.2) (Jones *et al.*, 1977; Fisher and Outlaw, 1979; Leigh *et al.*, 1979; Vattuone *et al.*, 1983; Huber *et al.*, 1989). However, species appear to differ in the extent to which they accumulate sucrose in the vacuole during the photoperiod. Huber (1989) examined ten species and found that in the mature leaves of some plants such as broad bean, pea and spinach the accumulation of sucrose was high (31.1, 23.0 and 22.6 mg $dm^{-2}$ respectively) while in cucumber, tobacco and soybean leaves accumulation was low (6.0, 5.7, 4.9 mg $dm^{-2}$ respectively). Analysis revealed that sucrose accumulation was negatively correlated

with the activity of soluble acid invertase. Huber (1989) proposed that in species with high soluble invertase activity sucrose which moved into the vacuole was rapidly hydrolysed to hexose sugars which subsequently moved back into the cytoplasm for phosphorylation (Fig. 1.2).

It must be remembered that in an actively photosynthesising leaf the net carbon flux is in the direction of sucrose synthesis and that in the model proposed by Huber (1989) only the sucrose entering the vacuole is hydrolysed. The majority of sucrose under these conditions is exported from the leaf to sink regions of the plant such as developing organs, regions with rapid metabolism or heterotrophic plant parts such as the roots. Export from source tissue begins with the symplastic movement of sucrose from mesophyll cell to mesophyll cell down a sucrose concentration gradient toward the phloem (Kursanov, 1984; Sivak *et al.*, 1989, Van Bel, 1993). The actual mechanism for the loading of sucrose into the sieve element companion cell complex (SE-CC) differs between species but falls into one of two groups; type 1 and type 2 phloem loaders (including sub-divisions thereof). In type 1 phloem loading species sucrose moves symplastically from the mesophyll cells to the SE-CC. However, as work in this thesis is going to examine the role of invertases in *Arabidopsis thaliana* and tomato (*Lycopersicon esculentum*), which are type 2 phloem loaders, the mechanisms involved in type 1 phloem loading will not be discussed further. In type 2 phloem loading species it has been suggested that specialised mesophyll cells may release photosynthate into the apoplast adjacent to their site of loading (Franceschi and Giaquinta, 1983; Williams *et al.*, 1989). Uptake of sucrose into the SE-CC is thought to be achieved by a carrier-mediated proton motive force driven sucrose/proton co-transporter (Delrot *et al.*, 1989) as discussed earlier.

Apoplastic invertase activity is often high in rapidly developing structures such as root tips and young leaves but residual activity can often be measured in the mature leaves of some species. The presence of apoplastic invertase in mature leaves would seem to be inconsistent with an apoplastic phloem loading step in type 2 loaders. The fact that apoplastic invertase activity in these tissues does not block the export of sucrose in these species may be due to the spatial separation of apoplastic sucrose hydrolysis and phloem loading. What is certain is that in plants where apoplast invertase activity increases, either as a result of wounding (Matsushita and Uritani,

1974) or due to over-expression in transgenic plants (Von Schaewen *et al.*, 1990), there is an accumulation of carbohydrate within the leaf.

Sucrose transport between source and sink organs appears to be driven by a turgor pressure gradient caused by a sucrose concentration gradient (Ho and Baker, 1982). In the roots a variety of mechanisms are thought to operate in order to achieve this gradient and all ultimately involve the breakdown of sucrose. One or more of these mechanisms may be in operation in any given species. In the first model sucrose leaves the SE-CC and is hydrolysed by apoplastic invertase prior to possible uptake by hexose transporters (Eschrich, 1980; Truernit, 1996). Alternatively, sucrose may be transported into the root parenchyma cells without prior hydrolysis either via the apoplast or symplastically through plasmodesmatal connections (Patrick, 1997). Where sucrose is unloaded from the phloem without prior hydrolysis in the apoplast the sucrose concentration gradient is thought to be maintained by intracellular hydrolysis either by sucrose synthase or by cytoplasmic or vacuolar invertase (Fig 1.2) (Sturm and Chrispeels, 1990).

## 1.3 The sub-cellular and tissue specific localisation of invertase

The role of plant invertases in carbohydrate metabolism is critically dependent on their cellular and sub-cellular localisation. This will now be explored in more detail.

### 1.3.1 Soluble acid (vacuolar) invertases

The presence of a soluble invertase with an acidic pH optimum has been measured in a wide range of plant organs from different species including seedling hypocotyls, roots and cotyledons, reproductive structures, developing and mature leaves, storage tissue and roots. The isolation of intact vacuoles from beetroot roots (*Beta vulgaris*) (Leigh *et al.*, 1979), tobacco pith (Boller and Kende, 1979), castor-oil plant leaves (*Ricinus communis*) (Vattuone *et al.*, 1983), *Melilotus alba* leaves (Boudet *et al.*, 1981), potato tubers (*Solanum tuberosum*) (Isla *et al.*, 1992), tomato fruit (*L. esculentum*) (Konno *et al.*, 1993), *Lolium temulentum* leaves (Walker *et al.*, 1997) and celery petioles (Keller and Matile, 1989) has revealed that this particular invertase isoenzyme has a vacuolar sub-cellular localisation. The evidence for this has been

further strengthened by the recent cloning and isolation of invertase genes from a number of species including carrot (Unger *et al.*, 1994) and *A. thaliana* (Haouazine-Takvorian *et al.*, 1997) which predict proteins with a short C-terminal amino acid extension thought to be involved in vacuolar targeting (Bednarek and Raikhel, 1992). Due to the large accumulation of data suggesting a vacuolar localisation for soluble acid invertase this enzyme will termed 'vacuolar invertase' for the duration of this thesis. Despite the relatively large amount of data on the sub-cellular localisation of vacuolar invertase and its activity in different plant organs relatively little is known about its tissue specific distribution.

Huber (1989) measured vacuolar invertase activity in the leaves of ten species. This work revealed that despite an age dependent decline in activity during leaf development many species retained considerable activity in the mature leaves. To date the exact location of this activity in the mature leaves is unknown and may reside throughout the leaf or within the vasculature, epidermal or mesophyll cells. The small amount of evidence that does exist is contradictory and suggests that the tissue localisation of vacuolar invertase in the leaf may be species dependent. For example vacuolar invertase activity was detected in intact vacuoles derived from the mesophyll cells of *Lolium temulentum* leaves (Walker *et al.*, 1997). While in contrast micro-assays of cells from barley leaves (Koroleva *et al.*, 1997) revealed little vacuolar invertase activity in either the epidermis, mesophyll or bundle sheath cells leaving the authors to speculate upon a vascular location to account for the high total leaf activities measured. Tissue prints of leaves from a wide range of species hybridised to an antibody raised to an 'acid invertase' did indeed suggest species variation in the distribution of leaf vacuolar invertase activity. Tissue prints of pea (*Pisum sativum*) and barley (*Hordeum vulgare*) leaves showed a vascular location for total acid invertase activity while those from fuchsia (*Fuchsia hybrida*) showed a widespread distribution throughout the leaf (Kingston-Smith and Pollock, 1996). These authors suggest that the variation in leaf vacuolar invertase activity may reflect differences in the phloem loading strategy between type 1 symplastic loaders and type 2 apoplastic loaders.

As in leaves very little is known about the tissue specific distribution of vacuolar invertase activity within roots. The presence of vacuolar invertase throughout the roots of some species has been shown by activity measurements such as those in

beetroot (Leigh *et al.*, 1979), carrot tap root (Sturm and Chrispeels, 1990) and the storage tissue of potato tubers (Isla *et al.*, 1992) but has also been inferred by the recent examination of gene expression in species such as maize (Xu *et al.*, 1996), *A. thaliana* (Haouazine-Takvorian *et al.*, 1997), fava bean (Weber *et al.*, 1995) and vine (Davies and Robinson, 1996). However, only in carrot (*Daucus carota*) has this gene expression analysis been extended to different root tissue types (Sturm *et al.*, 1995). These authors found equal expression of a vacuolar invertase gene throughout a cross section of mature tap roots including the periderm, phloem, cambium and xylem and have suggested a role for this enzyme in the maintenance of sink strength.

Gene expression studies have also revealed the presence of vacuolar invertase in reproductive structures such as the kernels, elongating silks and anthers of maize (Xu *et al.*, 1996), the gynoecia, stamens and petals of tomato (Godt and Roitsch, 1997) and the anthers, gynoecia and pods of fava bean (Weber *et al.*, 1995). Cheng *et al.* (1996) measured vacuolar invertase activity in developing maize endosperm. Miller and Ranwala (1994) purified three vacuolar invertase isoforms from the flower buds of *Lilium longiflorum* and found that isoform I was located within the anthers while isoforms II and III predominated in non-anther organs of the flower. These authors speculate that the presence of multiple isoforms within the flower provide greater control of sucrose metabolism during growth and under different environmental conditions.

Evidence indicates that in some species vacuolar invertase activity is important in determining the accumulation of hexoses during fruit ripening. For example, analysis of domestic tomato (*L. esculentum*) has revealed that during the latter stages of ripening vacuolar invertase activity increases concomitant with a decrease in sucrose and an increase in hexoses (Yelle *et al.*, 1991). In a closely related wild species (*L. chmielewskii*) lacking vacuolar invertase activity during the latter stages of fruit ripening sucrose not hexoses were the predominant soluble sugars (Yelle *et al.*, 1991). The role of vacuolar invertase in determining the hexose to sucrose ratio of ripening fruit was confirmed by analysis of transgenic tomato plants (*L. esculentum*) containing an antisense vacuolar invertase construct. In fruit with reduced vacuolar invertase activity there was an increase in the sucrose concentration (Klann *et al.*, 1996; Ohyama *et al.*, 1995).

The limited evidence available suggests that vacuolar invertase activity may be found in tissue throughout ripening fruit. For example measurements in muskmelon (*Cucumis melo*) revealed vacuolar invertase activity in the peduncle, ovule, pericarp, exocarp, mesocarp, endocarp, placenta and seeds. The amount of activity in each of these tissues altered during fruit development (Chrost and Schmitz, 1997). In grapefruit (*Citrus paradisi*) vacuolar invertase activity was measured in the dorsal vascular bundles, segment epidermis and juice sacs (Tomlinson *et al.*, 1991) while in grape berries vacuolar invertase gene expression was detected in both the grape berry skin and flesh (Davies and Robinson, 1996).

### 1.3.2 Apoplastic (cell-wall bound) invertases

Only a small number of studies have examined the tissue specific localisation of apoplastic invertases in different plant organs.

In the roots evidence for the distribution of apoplastic invertase is patchy and it appears to vary between species. In one of these studies Ramloch-Lorenz (1993) fused the promoter from the carrot apoplastic invertase gene (isolated by Sturm and Chrispeels, 1990) to the reporter gene β-glucuronidase (GUS) and introduced this construct into tobacco (*Nicotiana tabacum*) via *Agrobacterium tumefaciens* mediated transformation. These authors found that strong GUS activity was detectable histochemically in the root meristem of seedlings up to three weeks old. However in older plants GUS activity was not detectable either histochemically or by the more sensitive fluorometric assay. While this study localised expression of the apoplastic invertase gene to a specific region of the root tissue it gave no indication of the subsequent localisation of activity as the cells differentiated, expanded and matured behind the meristematic root tip.

However, microscopic examination of pea seedling roots by *in situ* hybridisation with an $^{35}$S-labelled RNA sense probe specific for the pea PcI-1 apoplastic invertase gene showed the presence of a weak signal in the root phloem (Zhang *et al.*, 1996). This indicated that in some species apoplastic invertase in the roots may reside within the vasculature.

In another study Benhamou *et al.* (1991) studied the distribution of apoplastic invertase in healthy and diseased roots of susceptible and resistant tomato. To achieve

this seedling tomato plants at the three leaf stage were infected with the fungal wilt pathogen *Fusarium oxysporum* and the roots collected daily for 5 d. Root sections were hybridised to an antibody raised against purified and deglycosylated apoplastic invertase from carrot (Laurière *et al.*, 1989) and visualised by electron microscopy using immuno-gold labelling of the antibodies. This work showed that in healthy roots apoplastic invertase was only sparsely distributed but that it was distributed throughout the root sections. This was in contrast to roots infected with *F. oxysporum* where apoplastic invertase accumulated in the cell walls of cells in contact with the hyphae.

In the leaves equally little is known about the tissue specific distribution of apoplastic invertase although several studies point to a vascular location.

In tobacco transformed with the carrot apoplastic invertase promoter fused to the GUS reporter gene (Ramloch-Lorenz, 1993) GUS activity was detected histochemically in 7 d old seedlings at the base of the cotyledons. However, three week old seedlings stained for GUS activity followed by chlorophyll bleaching showed blue staining of the major leaf veins. As previously discussed no GUS activity was detected in older transgenic tobacco. This work suggests that apoplastic invertase activity may be associated with the leaf vasculature in some species.

Kingston-Smith and Pollock (1997) showed that acid invertases (both soluble and apoplastic) were associated with the vasculature in the leaves of some plants. Those species in which acid invertase was distributed widely throughout the leaf were type 1 symplastic phloem loaders.

Leaves of two week old pea seedlings were examined for the expression of apoplastic invertase by *in situ* hybridisation with a portion of the pea apoplastic invertase cDNA (Zhang *et al.*, 1996). These authors could not detect the expression of the pea apoplastic invertase gene in any part of the leaf. However, upon wounding apoplastic invertase gene expression was seen associated with the vasculature. As expression in this study was induced by an environmental perturbation it does not necessarily follow that activity was present in this region under normal conditions.

In general a greater number of studies have examined the distribution of apoplastic invertases in reproductive structures than in leaves, roots or other vegetative plant organs. This work has revealed that within a species there may be expression and activity of different isozymes of apoplastic invertase in different floral structures and

fruits. This tissue specific localisation may enable subtle metabolic control over of hexose supply both spatially and temporally.

For example, apoplastic invertase in seeds is thought to supply carbohydrates to the developing embryo and endosperm in some species. This phenomenon was clearly shown in maize seeds (Cheng *et al.*, 1996). In normal maize seeds the maternal and filial cells are attached at a single point called the pedicel. Photosynthate and nutrients are unloaded into the pedicel through vascular elements prior to their uptake by the basal endosperm cells and translocation to the upper endosperm and embryo. As there are no plasmodesmatal connections between the pedicel and filial tissue all photosynthate must reach the endosperm apoplastically. The isolation of the *miniature1* (*mn*) seed mutant of maize (Lowe and Nelson, 1946) has shed light on the tissue specific localisation of apoplastic invertase in maize seeds and illuminated their possible function in the metabolism and development of the seed embryo. The *mn* mutant seed is only 20% of the weight of the normal seed and histological studies show that 9 d after pollination there is a gradual withdrawal of the maternal cells from the endosperm and a gap forms between the endosperm and the pedicel. Specifically, this gap is caused by the loss of a layer of pedicel cells known as the chalazal bridge and results in arrested endosperm development (Miller and Chourey, 1992). Invertase activity measurements and histological studies using an antibody raised against deglycosylated carrot apoplastic invertase have shown that the *mn* locus encodes an apoplastic invertase and it is the specific loss of this activity that results in the degeneration of the pedicel (Cheng *et al.*, 1996). A series of reciprocal crosses between wild type and a range of different *mn* mutant plants resulted in seeds showing a range of apoplastic invertase activities in the seeds. Interestingly, this work revealed that apoplastic invertase activity could decline to 6% of wild type activity before pedicel degeneration was observed. Cheng *et al.* (1996) propose that apoplastic invertase located in the pedicel plays a fundamental role in the supply of hexoses to the filial generation and that loss of activity causes a build-up of sucrose and osmotic imbalance that results in the degeneration of the ultra-thin placento-chalazal cells.

Apoplastic invertase is also located in specific regions of fava bean seeds (Weber *et al.*, 1995, 1996) and is thought to be important in controlling photosynthate partitioning to the developing embryo and in controlling the hexose:sucrose ratio during

the pre-storage phase of development (Weber *et al.*, 1995). Activity measurements and *in situ* hybridisation with apoplastic invertase gene probes revealed that apoplastic invertase was located in the seed coat of fava bean. This region is known to be a site of photoassimilate unloading. High apoplastic invertase activity in the seed coat correlated with a high hexose:sucrose ratio in the endosperm and led to speculation that apoplastic invertase was responsible for the establishment of sink strength in young seeds. Further evidence for this was provided by the observation that as the seed endosperm enlarged it crushed a layer of cells in the seed coat in which apoplastic invertase had been localised. The removal of activity from this region preceded a switch from the accumulation of hexoses in the endosperm to the accumulation of sucrose (Weber *et al.*, 1995). A further role for apoplastic invertase in fava bean seed development was suggested when studies of 'large' and 'small' seeded varieties showed that in the large seed varieties apoplastic invertase activity in the seed coat was present for longer. This led to speculation that the generation of hexoses favoured cell division over cell expansion. This was proven when sucrose added *in vitro* caused a decrease in cell division and initiated cell expansion. These results revealed that apoplastic invertase activity was a key enzyme in the regulation of seed development and seed size (Weber *et al.*, 1996).

In the flowering organs of tomato three apoplastic invertase genes have been shown to be expressed in a highly tissue specific manner (Godt and Roitsch, 1997). One apoplastic invertase gene, *Lin6*, was expressed in small and large flower buds and in the flowers and its expression was also associated with sink tissues throughout the plant. Two further apoplastic invertase genes *Lin5* and *Lin7* were shown to be expressed almost exclusively in the gynoecia and stamens respectively. The authors suggest that the highly specific nature of this expression may play a role in the supply of hexoses to these tissues during critical phases of development. Further evidence for this comes from a study of wheat flowers where it was shown that induction of male sterility by water-stress was preceded by a decline in invertase activity (Dorion *et al.*, 1996). Stamen/gynoecia specific apoplastic invertase gene expression was also observed in fava bean (Weber *et al.*, 1996) and a flower bud specific apoplastic invertase gene has also been isolated from carrot (Lorenz *et al.*, 1995).

## 1.4 The invertase gene family

Yeast (*Saccharomyces*) invertases have been studied for over a century (for review see Lampen, 1971) and the intracellular and excreted forms have been purified and characterised (Gascón and Lampen, 1968; Gascon *et al.*, 1968). The molecular characterisation of yeast invertase genes (SUC genes) has revealed the presence of a dispersed multigene family. Yeast carrying a SUC gene produce an excreted and intracellular invertase both arising from the same gene. The SUC 2 invertase gene has been extensively studied and has been shown to encode two mRNAs of 1.8 and 1.9 Kb in size. Sequence analysis has shown that the 1.9 Kb message encodes a signal peptide directing excretion from the cell. This signal peptide is absent from the 1.8 Kb message (Taussig and Carlson, 1983).

The first invertase gene characterised from a higher plant was an apoplastic invertase gene from carrot (Sturm and Chrispeels, 1990). The cDNA from this gene was isolated from a λgt11 expression library screened with an antibody raised against the purified and deglycosylated cell-wall enzyme. Sequence alignment analysis revealed only 28% homology with yeast invertase although short highly conserved motifs were identified, one of which was at the active site.

The second invertase gene from a higher plant to be identified was a vacuolar invertase from mung bean (Arai *et al.*, 1992). The overall similarity in terms of amino acid sequence between the mung bean vacuolar and carrot cell wall invertase was only 49.6% but there were many conserved sequences.

To date vacuolar and apoplastic invertase genes have been cloned from a wide range of species and this has revealed that in many species two or more different genes may encode each of the compartment specific invertases thus providing spatial and temporal flexibility over invertase activity. Table 1.1 shows the invertase genes and cDNAs cloned to date. At present only one invertase gene has been identified that encodes a cytosolic invertase (Gallagher and Pollock, 1998). However, a cDNA from tomato (Ehness and Roitsch, 1997) and one from *A. thaliana* have been cloned which predict invertases with unusual characteristics, these may also prove to be alkaline or neutral invertases.

Figure 1.4 shows a dendrogram based on the deduced amino acid sequence of a

## Table 1.1 Plant invertase genes and cDNAs identified to date.

| Plant species | Gene name | cDNA | Enzyme Location | Accession number of: Gene | cDNA | Reference |
|---|---|---|---|---|---|---|
| **Thale cress** *Arabidopsis thaliana* | ATβFRUCT 1 | Same as gene name | Apoplast | X74515 | X74514 | Schwebel-Dugué *et al.* (1994) |
| | ATβFRUCT 2 | | Apoplast | U11033 | | Mercier and Gogarten (1995) |
| | ATβFRUCT 3 | | Vacuole | X99111 | X95537 | Both vacuolar genes Haouazine-Takvorian *et al.* (1997) |
| | ATβFRUCT 4 | | Vacuole | X97749 | Y11559 | |
| | ATβFRUCT 5 | | ? | | T46086 | EST (Newmann *et al.*, 1994) |
| **Oat** *Avena sativa* | | As Inv | Apoplast | | X73601 | Wu *et al.* (1993) |
| **Red goosefoot** *Chenopodium rubrum* | | CIN1 | Apoplast | | X81792 | Roitsch *et al.* (1995) |
| | | CIN2 | Vacuole | | X81793 | Ehness and Roitsch (1997) |
| | | CIN3 | ? | | X81794 | Ehness and Roitsch (1997) |
| **Carrot** *Daucus carota* | Inv*Dc1 | Inv*Dc1 | Apoplast | X69321 | M58362 | Sturm and Chrispeels (1990) Ramloch-Lorenz *et al.* (1993) |
| | Inv*Dc2 | | Apoplast | X78423 | | Lorenz *et al.* (1995) |
| | Inv*Dc3 | | Apoplast | X78424 | | Lorenz *et al.* (1995) |
| | | sI | Vacuole | | X75352 | Unger *et al.* (1994) |
| | | sII | Vacuole | | X67163 | Unger *et al.* (1994) |
| **Pea** *Pisum sativum* | | Pc1 | Apoplast | | X85327 | Zhang *et al.* (1994) |
| **Tobacco** *Nicotiana tabacum* | | CW 1 | Apoplast | | X81834 | Grenier *et al.* (1995) |
| **Potato** *Solanum tuberosum* | | pCD 111 | Apoplast | | S37047 | Hedley *et al.* (1993) |
| | | pCD 141 | Apoplast | | Z22645 | Hedley *et al.* (1994) |
| | | pAI 11 | Vacuole | | L29099 | Zhou *et al.* (1994) |
| **Grapevine** *Vitis vinifera* | | GIN 1 | Vacuole | | G1839578 | Davies and Robinson (1996) |
| | | GIN 2 | Vacuole | | G1839579 | |
| **Maize** *Zea mays* | | CW-1 | Apoplast | | U17695 | Shanker *et al.* (1995) |
| | | CW-2 | Apoplast | | N/A | Cheng *et al.* (1996) |
| | Ivr 1 | | Vacuole | U16123 | | Xu *et al.* (1995) |
| | | Ivr 2 | Vacuole | | U31451 | Koch *et al.* (1995) |
| **Fava bean** *Vicia faba* | | VfCWINV 1 | Apoplast | | Z35164 | Weber *et al.* (1995) |
| | | VfCWINV 2 | Vacuole | | Z35162 | Weber *et al.* (1995) |
| | | VfVCINV | Vacuole | | Z35163 | Weber *et al.* (1995) |
| **Mung bean** *Vigna radiata* | | AI | Vacuole | | D10265 | Arai *et al.* (1992) |
| **Tomato** *Lycopersicon esculentum* | | TAI 1 | Apoplast | | S70040 | Sato *et al.* (1993) |
| | | Lin5 | Apoplast | | X91389 | Godt and Roitsch (1997) |
| | | Lin6 | Apoplast | | X91390 | Godt and Roitsch (1997) |
| | | Lin7 | Apoplast | | X91391 | Godt and Roitsch (1997) |
| | | Lin8 | Apoplast | | X91392 | Godt and Roitsch (1997) |
| | | TIV1 | Vacuole | | M81081 | Klann *et al.* (1992) |
| | InvLe 23g | InvLe 31 | Vacuole | Z12027 | Z12025 | Elliot *et al.* (1993) |
| *Lycopersicon pimpinellifolium* | InvLp 6g | InvLp 11 | Vacuole | Z12028 | Z12026 | Elliot *et al.* (1993) |

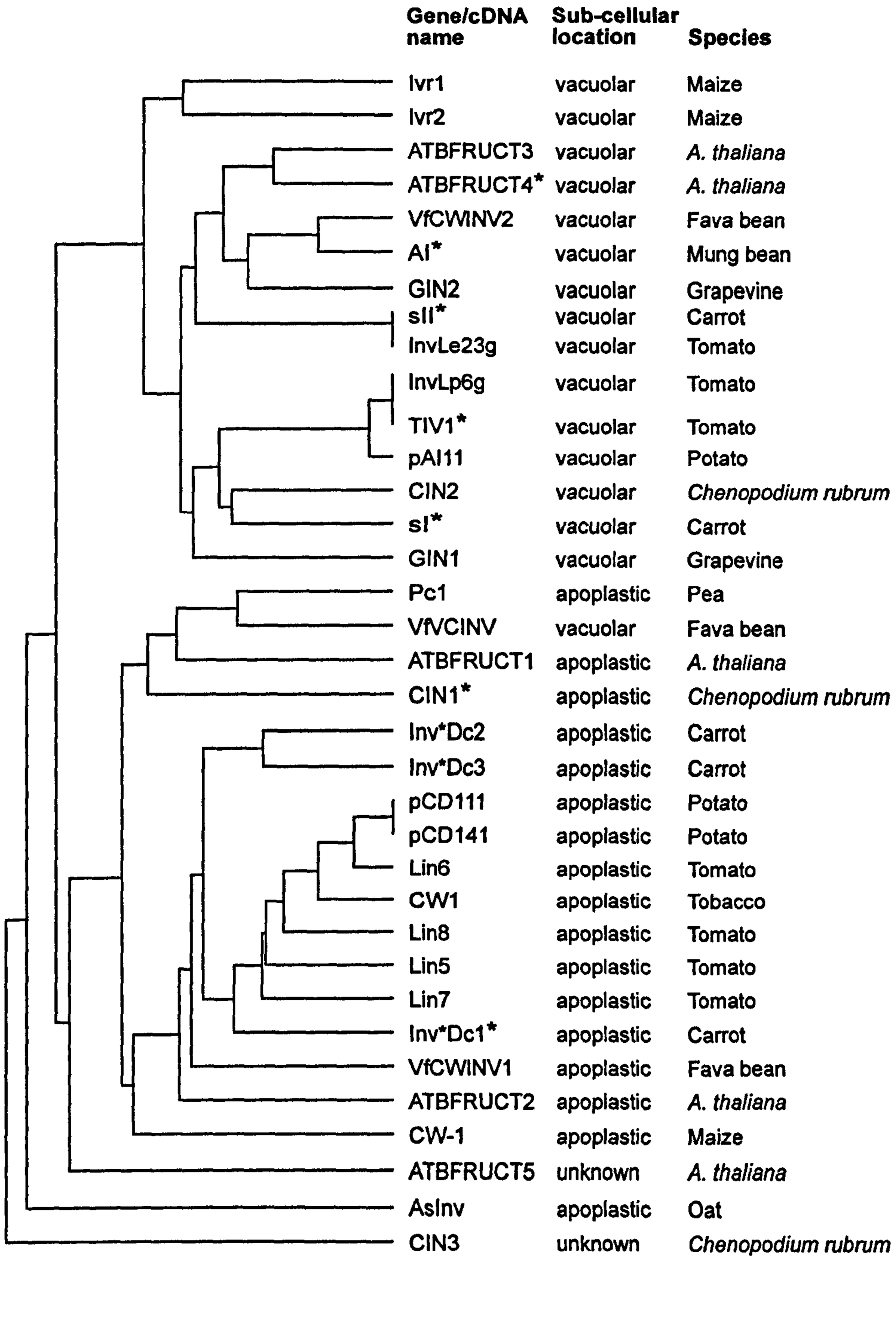



**Figure 1.4 Phylogenetic relationship of plant invertases.** The dendrogram was generated by the PILEUP programme (Dervereux *et al.*, 1984) by comparison of the deduced amino acid sequences of plant invertases. Sequences for which the identity has been proven by purification of the corresponding protein are marked with an asterisk (*). The references and accession numbers of the invertases are given in Table 1.1.

number of invertase cDNAs and shows clearly the existence of two separate groups representing apoplastic and soluble acid isoenzymes. The identity of both of these clusters has been fully verified by the purification of the corresponding proteins and by sequence analysis of the tryptic peptides.

## 1.5 The regulation of sucrose synthase and invertase activity and gene expression

As the major enzymes of sucrose degradation in plants, invertase and sucrose synthase, are essentially regulated in two ways; modulation of the activity of the mature protein by various mechanisms or alterations in the amount of *de novo* synthesis of the protein by induction or suppression of gene expression. Under normal conditions both of these mechanisms will be in operation and will enable the fine and coarse control of carbohydrate metabolism during development and under different environmental conditions.

### 1.5.1 Sucrose synthase

Although studies have shown that in maize, sucrose synthase gene expression is regulated by the cellular hexose concentration (Koch *et al.*, 1992), little is known about the mechanisms for the regulation of the mature protein. It has been shown in maize endosperm that sucrose synthase activity, in the cleavage direction, may be regulated by hexose mediated end-product inhibition (Doehlert, 1987). Recent studies indicate that sucrose synthase activity may also be regulated by protein phosphorylation. Evidence for the *in vitro* phosphorylation of sucrose synthase was provided when maize suspension cultured cells, provided with [$\gamma$-$^{32}$P]ATP in the growth medium, produced an extractable $^{32}$P labelled sucrose synthase (Shaw *et al.*, 1994). Similar results were obtained by Koch *et al.* (1995) who fed excised root tips [$^{32}$P]Pi. Huber *et al.* (1996) examined this mechanism in greater depth and found that two maize leaf sucrose synthase isoforms were $^{32}$P labelled on similar seryl residues when excised shoots were fed [$^{32}$P]orthophosphate. These authors also revealed that sucrose synthase was phosphorylated by an endogenous protein kinase in a strictly $Ca^{2+}$ dependent manner. Phosphorylation was found to increase the activity of the cleavage reaction by

increasing the affinity of the enzyme for sucrose and UDP.

Until recently the effect of soluble carbohydrate on sucrose synthase activity and gene expression appeared to be contradictory. Sucrose synthase activity was reportedly both repressed (Koch *et al.*, 1988) and enhanced (Salanoubat and Belliard, 1989) by high cellular concentrations of soluble carbohydrate. Recent work by Koch *et al.* (1992) showed that this may have been due to the presence of a sucrose synthase gene family differentially regulated by hexoses. These authors examined the expression of two sucrose synthase genes (*Sh1* and *Sh2*) in maize root tips under conditions of carbohydrate famine and abundance. This work revealed that the *Sh1* gene was up-regulated by carbohydrate famine while *Sh2* showed higher levels of expression when hexoses were abundant. This 'feast-famine' response may confer the ability to regulate carbohydrate partitioning under a wide range of physiological and environmental conditions.

## 1.5.2 Invertase

As invertases are the major focus of this thesis the regulation of their activity and gene expression will now be explored in detail.

### 1.5.2.1 The regulation of invertase activity by proteinaceous inhibitors

The first proteinaceous invertase inhibitor was identified in crude extracts of potato tubers (Schwimmer *et al.*, 1961). This was also the first tissue from which the protein was purified to homogeneity (Pressey, 1966, 1967). Since then invertase inhibitors have been identified or isolated from several plant species including red beet and sugar beet (Pressey, 1968), maize (Jaynes and Nelson, 1971), sweet potato (Matsushita and Uritani, 1976), tobacco (Weil and Rausch, 1994) and tomato (Pressey, 1994) although the universality of these inhibitors is as yet unknown. These proteinaceous inhibitors are able to bind effectively to both vacuolar and apoplastic invertases (Bracho and Whitaker, 1990; Sander *et al.*, 1996). However, until recently, the physiological role of these inhibitors, which were only operationally defined from their *in vitro* regulatory activity, remained unknown. Isla *et al.* (1992) showed that the

proteinaceous inhibitor was located in the apoplast of potato tuber cells and that therefore its role *in vivo* was probably the regulation of apoplastic rather than vacuolar invertase activity. Recently, cell fractionation studies have revealed that a proteinaceous inhibitor may also be present in the microsomal membranes, Golgi and/or endoplasmic reticulum of tobacco cell suspension cultures (Krausgill *et al.*, 1996).

The proteinaceous inhibitor from tobacco is a small 17 KDa acid stable non-glycosylated polypeptide (Weil *et al.*, 1994) and evidence suggests that inhibitory binding to apoplastic invertase is extremely tight, probably due to strong ionic forces (Krausgill *et al.*, 1996). Recently, it has been shown that low concentrations of sucrose (above 1 mM) protect apoplastic invertase against inhibition (Sander *et al.*, 1996). This has lead to speculation that *in vivo* the proteinaceous inhibitor may 'clamp' apoplastic invertase activity only when the concentration of sucrose in the cell-wall compartment falls below a certain level (Krausgill *et al.*, 1996).

### 1.5.2.2 The regulation of invertase activity by sucrose (substrate) and hexoses (products)

Analysis of purified invertases from a range of species have shown that sucrolytic activity is often modulated by the products of hydrolysis. The nature of this inhibition appears to vary between different invertase isozymes within and between species.

Walker *et al.* (1997) purified invertases with acid and alkaline pH optima from the leaves of *Lolium temulentum* and found that both forms were inhibited in a non-competitive manner by fructose. These authors found that vacuolar and apoplastic invertase activity showed 95% inhibition in the presence of 10 mM sucrose and 20 mM fructose while alkaline invertase activity was inhibited by 80% in the presence of 50 mM sucrose and 20 mM fructose. Lee and Sturm (1996) found that neutral and alkaline invertases purified from carrot suspension cell cultures were also inhibited by fructose ($K_i$ of 15 mM). However, in contrast to invertases from *L. temulentum* the nature of this inhibition was competitive. In addition, both of these enzymes were also inhibited by glucose in a non-competitive manner ($K_i$ 30 mM).

In a detailed study of invertases in potato, Burch *et al.* (1992) found that while a vacuolar invertase purified from the leaves and a vacuolar invertase purified from the tubers were both inhibited by fructose and glucose the nature of this inhibition differed between the isozymes. The kinetics of the glucose inhibition of the potato leaf enzyme indicated that glucose only bound to the enzyme after sucrose ($K_m$ for sucrose and $V_{max}$ both decreased to the same extent). In contrast both glucose and sucrose appeared to bind to the tuber invertase independently. The inhibition of the purified leaf invertase by fructose appeared to result from the altered affinity of the enzyme for sucrose. While in contrast the activity of the purified tuber invertase appeared to be modulated by the competitive binding of fructose to a second site on the enzyme (the $K_m$ for sucrose increased while the $V_{max}$ remained unchanged). Isla *et al.* (1992) also concluded that fructose inhibition of tuber vacuolar invertase operated through fructose binding to a second site on the enzyme as did Lopez *et al.* (1988) examining purified vacuolar invertase from *Carica papaya* fruits.

What are the possible physiological consequences of product inhibition of invertase activity? The presence of substantial acid invertase activity in the vacuoles is difficult to reconcile with the observation that in some species sucrose may be stored in the same compartment prior to export from the leaf. Although these kinetic studies were performed *in vitro* it is probable that invertase activity could be modulated by physiological concentrations of glucose or fructose found within the cell (Walker *et al.*, 1997). Under these conditions end-product inhibition could operate to prevent sucrose hydrolysis where sucrose accumulates in the same cell compartment as the enzyme that can hydrolyse it. However, in some species another strategy is thought to prevent the hydrolysis of sucrose prior to export from the leaf. Farrar (1989) demonstrated that in barley leaves there was a spatial separation of invertase and sucrose. There was an accumulation of sucrose in the mesophyll cells but no detectable vacuolar invertase activity.

### 1.5.2.3 The effects of soluble carbohydrates on invertase gene expression

It has been demonstrated in yeast that above certain concentrations the SUC2

structural gene for invertase is repressed by glucose (Fig. 1.5) This pathway functions to prevent the continued accumulation of a hexose already plentifully supplied within the cell (Sarokin and Carlson, 1984). Glucose repression is a major regulatory mechanism in yeast. However, under conditions where the supply of cell glucose is low SUC gene expression is re-induced (Fig. 1.5). The mechanism for this de-repression is thought to involve an enzyme called SNF1 protein kinase. The precise function of this kinase in the regulatory pathway is unclear but it is thought that it may interact with a protein showing DNA-binding activity (Estruch and Carlson, 1990).

In plants the effects of sucrose or hexoses on invertase gene expression is more complex. This is perhaps not surprising when considering that all plants develop from a purely heterotrophic embryo and that plant growth is accompanied by changes in the source:sink status of individual organs. It is also important to consider that each of the compartment-specific invertases plays a different role in metabolism within and between species.`

Cell suspension cultures of *Chenopodium rubrum* shifted from autotrophic to mixotrophic growth on 100 mM glucose, sucrose or fructose showed a large induction in CIN1 apoplastic invertase gene expression which was accompanied by an increase in enzyme activity (Roitsch *et al.*, 1995). This confirms work by Sturm and Chrispeels (1990) who noted high levels of apoplastic invertase gene expression when carrot cell suspension cultures were placed in a medium containing glucose, fructose or sucrose. The induction of the *C. rubrum* apoplastic invertase gene was also mimicked by a non-metabolisable glucose analog, 6-deoxyglucose. This indicates that hexose and not one of its metabolites is the signal that stimulates the putative transduction pathway (Roitsch *et al.*, 1995). As apoplastic invertase is thought to play a role in the maintenance or establishment of sink strength in some species Roitsch *et al.* (1995) suggest that sucrose and hexoses unloaded from the phloem may further up-regulate apoplastic invertase gene expression in these cells (feed-forward regulation) further contributing to the sucrose concentration gradient within the phloem. However, Sturm and Chrispeels (1995) did not find any evidence that apoplastic invertase activity in carrot was important in the maintenance of sink strength. These authors found that the expression of the Inv*Dc 1 apoplastic invertase gene was down-regulated early in root development and prior to tap root development, the stage of root development

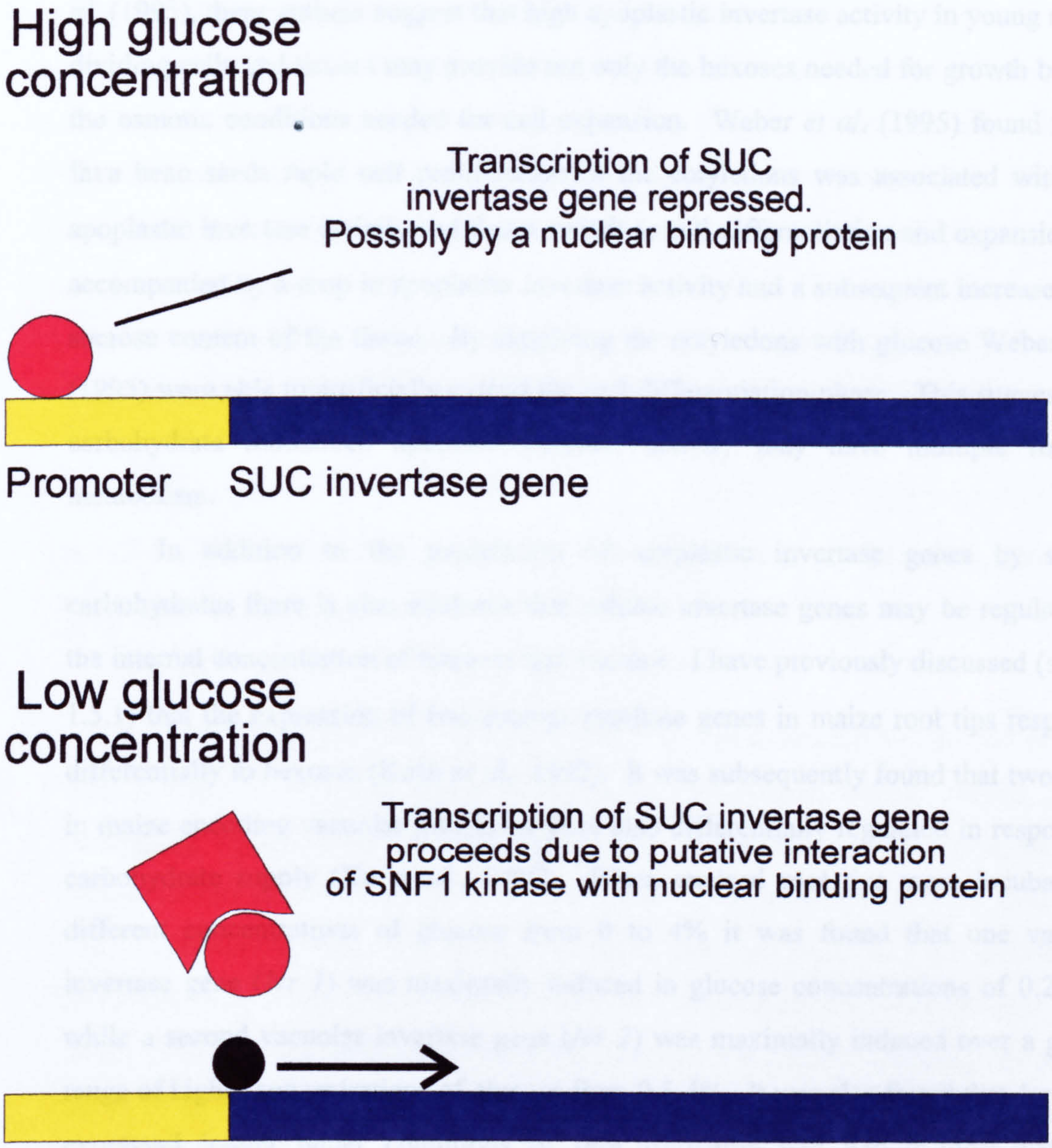



**Figure 1.5 Putative regulation of SUC invertase gene expression in yeast.** High concentrations of glucose have been shown to repress SUC invertase gene expression. When the supply of glucose is low SUC gene expression is re-induced. It is thought that this de-repression is mediated by SNF1 kinase. The precise mode of action of this enzyme is unclear but may involve interaction with a nuclear binding protein.

exhibiting the greatest carbohydrate demand. In contrast to the hypothesis of Roitsch *et al.* (1995), these authors suggest that high apoplastic invertase activity in young rapidly dividing cells and tissues may provide not only the hexoses needed for growth but also the osmotic conditions needed for cell expansion. Weber *et al.* (1995) found that in fava bean seeds rapid cell proliferation in the cotyledons was associated with high apoplastic invertase activity and that a switch to cell differentiation and expansion was accompanied by a drop in apoplastic invertase activity and a subsequent increase in the sucrose content of the tissue. By supplying the cotyledons with glucose Weber *et al.* (1995) were able to artificially extend the cell differentiation phase. This suggests that carbohydrate modulated apoplast invertase activity may have multiple roles in metabolism.

In addition to the modulation of apoplastic invertase genes by soluble carbohydrates there is also evidence that soluble invertase genes may be regulated by the internal concentration of hexoses and sucrose. I have previously discussed (section 1.5.1) that the expression of two sucrose synthase genes in maize root tips responded differentially to hexoses (Koch *et al.*, 1992). It was subsequently found that two genes in maize encoding vacuolar invertases were also differentially regulated in response to carbohydrate supply (Xu *et al.*, 1996). When excised root tips were incubated in different concentrations of glucose from 0 to 4% it was found that one vacuolar invertase gene (*Ivr 1*) was maximally induced in glucose concentrations of 0.2-0.5% while a second vacuolar invertase gene (*Ivr 2*) was maximally induced over a greater range of higher concentrations of glucose from 0.5-4%. It was also found that *Ivr 1* was expressed longer under conditions of root starvation and had a more narrowly distributed pattern of tissue expression indicating specialised roles during development. This gene may potentially maintain or aid import for areas of critical function for the plant during important phases of development. However, the differential modulation of these two genes in response to carbohydrate supply will provide enhanced metabolic flexibility under a wide range of environmental and developmental conditions.

The response of soluble invertase genes to changes in carbohydrate status may not be a universal phenomenon. For example, Ehness and Roitsch (1997) found that two soluble invertase genes, CIN2 and CIN3, in *C. rubrum* cell suspension cultures did not respond to transfer from autotrophic to mixotrophic growth on 100 mM sucrose,

glucose or fructose. This may indicate that different species have adopted different strategies in respect to the control of carbohydrate metabolism.

Recently, a gene (*Akin10*) has been isolated from *A. thaliana* that shows a deduced amino acid sequence homology of 65% to the SNF1 phosphate kinase in yeast (Le Guen *et al.*, 1992). Northern hybridisation assays showed that this gene was highly expressed in roots grown in liquid culture and in six week old leaves. Expression was also detected at a lower level in two week old shoots. This gene may be involved in the regulation of carbohydrate metabolism in a similar manner to the SNF1 kinase and possibly in the transduction of metabolic signals such as the availability of sugars (Le Guen *et al.*, 1992).

### 1.5.2.4 The effects of plant growth regulators on invertase activity and gene expression

It has commonly been reported that rates of cell expansion and growth correlate with changes in acid invertase activity (Morris and Arthur, 1984). Indeed, tissues undergoing rapid growth typically contain low concentrations of sucrose and high concentrations of hexose (Ricardo and ap Rees, 1970) indicating sucrose hydrolysis. This is perhaps not surprising when considering that high levels of plant growth must be supported by a supply of incoming nutrients, including soluble carbohydrates. High levels of the sucrose metabolising enzymes invertase and sucrose synthase may provide the hexoses required for rapid cell growth. As plant cell expansion and growth are known to be regulated by plant growth regulators such as auxin, gibberellic acid and cytokinin it may be expected that the application of these hormones may have a direct effect on invertase activity and/or gene expression.

The phytohormone gibberellic acid ($GA_3$) is responsible for cell elongation and is also thought to be important in the initiation of flowering. $GA_3$ has been reported to increase invertase activity in several plant organs including Jerusalem artichoke tubers (Sacher *et al.*, 1963) and lentil epicotyls (Seitz *et al.*, 1968). In oat stem segments the activities of both apoplastic and soluble invertase increased when pulsed with $GA_3$. This increase correlated with the active growth phase and then declined as the growth of the segments slowed (Kaufman *et al.*, 1973). However, it was found that a

continuous supply of $GA_3$ retarded this decline in activity. Interestingly, these authors found that a pulse of sucrose administered simultaneously with $GA_3$ resulted in a doubling of soluble invertase activity above that seen when $GA_3$ was administered alone. This suggests that sucrose and $GA_3$ may have a co-ordinated affect on invertase activity in oat (Kaufman *et al.*, 1973). It has not been fully established whether the effect of $GA_3$ on invertase activity results from the *de novo* synthesis of the enzyme via an increase in gene expression or whether this particular phytohormone exerts some regulatory effect over the mature protein. Several lines of evidence suggest that in oat this hormone acts at the level of gene expression. Firstly, *in vitro* enzyme assays have shown that soluble invertase activity is not stimulated by the presence of $GA_3$ (Kaufman *et al.*, 1968). Secondly, the apparent Km of the enzyme for sucrose is not altered during the growth of oat stem segments (Kaufman *et al.*, 1973). Thirdly, the administration of cycloheximide blocks the action of $GA_3$ when pulsed to oat stem segments (Kaufman *et al.*, 1973). Recent work with tomato suspension cultured cells showed that $GA_3$ had no effect on the expression of four apoplastic invertase genes or a vacuolar invertase gene when the hormone was exogenously supplied (Godt and Roitsch, 1997). This suggests that species may vary in their response to $GA_3$.

It has been shown that in the leaves and stems of bean (*Phaseolus vulgaris*) the highest activities of vacuolar invertase activity and the maximum rates of cell expansion occur at the time of peak free indol-3-yl-acetic acid (IAA) (Morris, 1982) and that exogenously supplied IAA resulted in a stimulation of vacuolar invertase activity (Morris and Arthur, 1984). Vacuolar invertase activity but not sucrose synthase activity also increased in the ovaries, fruit tissue and leaves of egg plant (*Solanum melongena*) in response to exogenously supplied IAA (Claussen *et al.*, 1986; Lee *et al.*, 1997). Weil and Rausch (1990) found that in an auxin deficient tobacco cell suspension culture the application of auxin to the growth medium resulted in a large stimulation of apoplastic invertase activity. Rudelshein *et al.* (1987) saw a correlation in various transformed tobacco cell suspension lines between apoplastic invertase activities and the level of endogenous auxin. However, species vary in the response of sucrose metabolising enzymes to the amount of IAA. For example, in pea exogenously supplied IAA did not alter invertase activity (Miyamoto *et al.*, 1993).

Auxins are widely believed to play an important role in the gravitrophic growth

response. Gravitropism has also been shown to act as a stimulus for changes in invertase activity and gene expression. Wu *et al.* (1993a, b) have shown that in oat-shoot pulvini gravistimulation results in the maximal induction of apoplastic invertase gene expression 1h after applying the stimulus. It is thought that gravistimulation results in the redistribution of auxin toward the lower side of the gravi-reacting organ and that this leads to the reorientation of the tissue (Philosoph-Hadas, 1996). It is possible that localised changes in the auxin level may lead to the stimulation of invertase gene expression required to supply the hexoses needed for reorientation growth.

To date there have been no reports on the effect of IAA on invertase gene expression although as with $GA_3$ it is widely believed that this phytohormone operates at the level of *de novo* synthesis (Miyamoto, 1993). In a further line of evidence Haouazine-Takvorian *et al.* (1997) found putative auxin responsive elements on the promoters of two soluble invertase genes (ATβFRUCT 3 and 4) in *A. thaliana*.

Cytokinins are a group of phytohormones that are known to promote cell division (Skoog and Armstrong, 1970). Godt and Roitsch (1997) found that a 2 μM application of zeatin, a naturally occurring cytokinin, was enough to trigger the induction of the LIN6 apoplastic invertase gene in tomato suspension-cultured cells. It has also been shown that cytokinin stimulates apoplastic invertase activity in suspension-cultured cells of *C. rubrum* and that this results from the direct stimulation of the CIN1 apoplastic invertase gene (Ehness and Roitsch, 1997). These authors suggest that in rapidly dividing meristematic cells apoplastic invertase activity may help to establish a supply of carbohydrate and that both rapid cell division and the supply of carbohydrate is co-ordinated by cytokinin.

### 1.5.2.5 The effect of developmental and environmental signals on invertase activity and gene expression

The activity of each of the compartment specific invertases is known to alter during plant development and within different plant tissues. High apoplastic invertase activity is commonly associated with rapidly growing tissues such as root tips and young leaves while high vacuolar invertase activity is commonly associated with certain

stages of fruit development, the storage tissues of some species and rapidly expanding structures. Neutral or alkaline invertases are less well characterised but activity is often high in storage tissue and within the leaves of certain grasses. The recent cloning of invertase genes and cDNAs has enabled these changes to be investigated at the molecular level. Interestingly, this has often revealed that genes belonging to the same sub-family, for example vacuolar invertases, are often regulated differentially by growth and development. A detailed discussion of the changes in invertase activity and gene expression during development and in different organs can be found in chapter 2.

Evidence has also accumulated which shows that apoplastic and soluble invertase gene expression and activity are up-regulated by events as diverse as pathogen attack by fungi or bacteria and physical damage resulting from herbivory or wind damage, all of which can be described as environmental stresses. These environmental stresses result in the establishment of a complex and energetically costly defence response which is mediated via a network of interconnected signal transduction pathways (Ehness *et al.*, 1997). The increase in invertase activity associated with these stresses may help to establish a localised sink for carbohydrate and thus provide hexoses for the stress induced increase in the respiratory rate (Sturm and Chrispeels, 1990; Benhamou *et al.*, 1991; Zhang *et al.*, 1996). As these topics are the focus of detailed investigation in chapter 3 they will not be discussed any further at this point.

## 1.6 Aims and objectives of this thesis

The first aim of this thesis is to perform a thorough characterisation of invertase gene expression in the cruciferous plant *A. thaliana*. One of the primary advantages of using this species is the relatively large number of invertase genes and cDNAs extracted from this species to date. These cloned sequences include two apoplastic invertase genes, two vacuolar invertase cDNAs and a fifth invertase cDNA with unusual characteristics and unknown subcellular localisation. In chapter 2 these probes are used to analyse invertase gene expression during leaf development and in different plant organs including the inflorescence, seedling roots and cotyledons. The examination of invertase gene expression during *A. thaliana* development will also be accompanied by measurements of invertase activity and isoform pattern and measurements of soluble

and storage carbohydrates. These analyses will allow us to determine how the different genes are regulated i.e. in a organ specific or developmental manner and provide new insights into the role of these isoenzymes.

Having completed an investigation of invertase gene expression and activity during the development of *A. thaliana*, chapter 3 will focus on the impact of environmental stimuli on invertase gene expression and activity in this species. As previously discussed, invertase gene expression and activity are stimulated by pathogenesis and wounding. In the first part of the chapter the impact of the biotrophic fungus *Albugo candida* on invertase gene expression, activity and on the carbohydrate content of infected leaves will be examined. Previous work has shown that *Albugo candida* infection of the leaves results in the stimulation of apoplastic invertase activity (Tang *et al.*, 1996; Chou, 1997) but the origin of this increase, host or fungal, is not known. I will examine whether the changes in apoplastic invertase activity result from changes in host gene expression. In addition, by correlating the timing, extent and origin of the increase in apoplastic invertase activity it may be possible to hypothesise about the nature of the signal responsible for these changes.

In the second part of the chapter the impact of wounding on invertase gene expression and activity will be examined. The majority of investigations on wounding and invertase to date have focussed on wounded roots and storage organs (Matsushita and Uritani, 1974, Sturm and Chrispeels, 1990). In this thesis the area of investigation will be limited to mature leaves. Previous studies have also tended to wound tissue severely. In this study tissue will be wounded in a way that is more representative of damage possible in the natural environment. Preliminary studies of wounded leaves will help to establish which invertase isoenzymes alter and this will be used for the basis of gene expression studies.

In the final experimental chapter (chapter 4), the role of vacuolar invertases in leaf metabolism will be investigated using transgenic tomato plants with reduced vacuolar invertase activity.

# Chapter 2

# The developmental regulation of invertase activity and gene expression in *Arabidopsis thaliana*

## 2.1 INTRODUCTION

Invertase is of ubiquitous occurrence among plant species and its activity (with that of sucrose synthase) represents the first stage in the utilisation and metabolism of sucrose for the processes of growth and biosynthesis. However, the role of invertases in metabolism is complex and critically depends upon their sub-cellular localisation.

One approach to understanding the role of the different compartment specific invertases is to examine changes in their activity during plant development. Numerous investigations have revealed that each of the compartment specific invertases alter in response to growth and development. For example during the expansion of soybean leaves vacuolar invertase activity declined from a peak of approximately 650 μmol sucrose hydrolysed $g^{1}$ (fresh weight) $h^{-1}$ to approximately 300 μmol sucrose hydrolysed $g^{1}$ $h^{1}$. This is in contrast to spinach leaves in which vacuolar invertase activity was low throughout development and disappeared almost completely as the leaves expanded (Huber, 1989). A decline in vacuolar and apoplastic invertase activity was also seen during the expansion of leaves of celery, *Lolium temulentum*, oat, wheat and grapevine (Roberts, 1953; Pollock and Lloyd, 1979; Greenland and Lewis, 1981; Keller and Matile, 1989, Ruffner *et al.*, 1990). In these studies there was a strong correlation between acid invertase activity and the sucrose to hexose ratio of the tissue.

Alterations in invertase activity have also been demonstrated during fruit and seed development. In certain species of wild tomato vacuolar invertase activity declines as the fruit ripens. This in contrast to domestic tomato where vacuolar invertase activity dramatically increases as the fruit ripens (Stommel, 1992; Klann *et al.*, 1993). Again these changes correlated strongly with the sucrose: hexose ratio of the tissue. Weber *et al.*, (1995, 1996) showed that apoplastic invertase activity in the seed coat of Fava bean altered dramatically during maturation. These changes occurred suddenly with a sharp decline in apoplastic invertase activity that marked the end of embryo development and the beginning of the final stages of seed maturation.

High levels of apoplastic invertase activity have also been measured in young roots (Ricardo and ap Rees, 1970). Typically the activity of this enzyme declines as the cells mature in the root cell elongation zone (Ramloch-Lorenz *et al.*, 1993).

Recently, invertase genes and cDNAs have been identified and cloned from a

number of plant species and this has frequently revealed that each of the compartment specific invertases are encoded for by more than one gene (Table 1.1 shows the invertase genes and cDNAs identified so far). For example in carrot two genes have been identified that encode for vacuolar invertases (Unger *et al.*, 1994) and three that encode for apoplastic invertases (Sturm and Chrispeels, 1990; Lorenz *et al.*, 1995). This is not unusual and indeed many plant species contain a small invertase multi-gene family although the specific number of genes encoding each isoenzyme is highly variable and species specific. The identification of these genes has provided a new tool with which to examine their developmental regulation.

Sturm *et al.* (1995) observed that upon carrot tap root development the expression of one vacuolar invertase gene (sI) declined while that of a second (sII) was induced. It was also observed that the expression of an apoplastic invertase gene (Inv*Dc1) ceased very early in root development. These data shed new light onto the role of each of these enzymes. Previously it had been hypothesised that apoplastic invertase was responsible for the maintenance of sink strength in the roots of many species (Eschrich, 1980). By analysing invertase gene expression, Sturm *et al.* (1995) revealed that this hypothesis was probably not true of carrot. In addition this work revealed that each of the vacuolar isoenzymes played a different role in metabolism.

In maize two vacuolar invertase genes, *Ivr1* and *Ivr2*, were found to be differentially regulated during development (Xu *et al.*, 1996). The expression of *Ivr1* was predominantly associated with reproductive structures while expression of *Ivr2* was associated with developing vegetative structures. Further analysis revealed that expression of the *Ivr1* gene was up-regulated during hexose famine while in contrast expression of *Ivr2* was up-regulated as the concentration of hexoses in the tissue reached the upper limit of their physiological range. This differential pattern of responses may enable tighter control of sucrose metabolism. In tissues with low concentrations of soluble carbohydrate the expression of *Ivr1* may enable critical supplies of hexoses to be maintained. In tissues with high concentrations of soluble carbohydrate the expression of *Ivr2* may enable these supplies to be maintained for the purposes of storage or rapid cell expansion.

Hedley *et al.* (1994) examined the expression of two apoplastic invertase genes in potato. These authors found that although both genes showed close sequence

homology they displayed very different expression profiles. The expression of one apoplastic invertase gene, pCD111, was associated with sink leaves, however, expression declined dramatically as the leaves developed. Expression of this gene was also low in stems. The second apoplastic invertase gene, pCD141, was also expressed in sink tissues but was up-regulated as the leaves matured. Expression of this gene was high in stems. Expression of neither gene was detected in the tubers or mature roots.

In tomato three apoplastic genes showed a highly tissue specific pattern of expression, particularly in the flowering organs. One apoplastic invertase gene, Lin5, was associated with the gynoecia while a second, LIN7, was associated with the stamens. The expression of the LIN6 apoplastic invertase gene was strongly associated with sink organs throughout the plant (Godt and Roitsch, 1996).

The data from all these species suggest that the provision of hexoses for tissues is a highly co-ordinated event and that the possession of an invertase multi-gene family enables each gene to be regulated in a specialised manner.

In this chapter I aim to examine invertase gene expression in different tissues of the model plant species, *Arabidopsis thaliana* (ecotype OY0). To date five invertase genes have been identified in this species. Two of these genes (ATβFRUCT 1 and ATβFRUCT 2) encode apoplastic invertases (Schwebel-Dugué *et al.*, 1994; Mercier and Gogarten, 1995) while two genes (ATβFRUCT 3 and ATβFRUCT 4) encode soluble invertases with a probable vacuolar localisation (Haouazine-Takvorian *et al.*, 1997). A fifth invertase gene (ATβFRUCT5) shows sequence homology to apoplastic invertase genes but is predicted to encode a protein with an acidic pI, which is more typical of vacuolar invertases. The sub-cellular localisation of this fifth invertase gene is unknown.

Some preliminary work has been carried out by Tymowska-Lalanne *et al.* (1996) and Haouazine-Takvorian *et al.* (1997) to characterise invertase gene expression in *A. thaliana*. Tymowska-Lalanne *et al.* (1996) found that the expression of ATβFRUCT 1 was high in roots but low in leaves and stems whilst Haouazine-Takvorian *et al.* (1997) found that the expression of ATβFRUCT 3 and 4 was low in mature leaves but high in flowers, flowering stems and young roots. To date there is no information on the expression of the second apoplastic invertase gene, ATβFRUCT 2, in *A. thaliana*.

The aim of the work presented in this chapter is to carry out a comprehensive analysis of the expression patterns of all five invertase genes in a wide range of tissues and to examine the relationship between patterns of expression, invertase activity and carbohydrate status of the tissue. Specifically I aim to: (1) Characterise the expression of all five invertase genes in seedlings, mature plants and during leaf development. (2) Set-up a semi-quantitative reverse transcriptase polymerase chain reaction system (RT-PCR) for the detection of low abundance invertase mRNAs. (3) Correlate changes in invertase gene expression, activity, isoform pattern and the amount soluble and storage carbohydrate within each tissue examined. (4) Use this information to speculate on the possible roles of invertase in different tissues and during leaf development.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Growth and harvesting of *Arabidopsis thaliana*

Seeds of *Arabidopsis thaliana* (ecotype OY0) were sown in Erin multi-purpose potting compost and were placed at 4 °C for 4 d to trigger even germination. Seeds were then transferred to a growth room with a day temperature of 22 ± 1 °C and a night temperature of 15 ± 1 °C with a 9 h photoperiod. Irradiance (130 μmol $m^{-2}$ $s^{-1}$) was provided by fluorescent lamps (Osram L 58W/77 Fluora, Germany). Seedlings were transplanted into pots (6 x 6 $cm^2$) 2 weeks after sowing and grown in the same growth room.

*Harvesting of leaf material and flowering stems*

When the plants were six weeks old, they were selected for uniformity and the leaf material harvested. Leaves of *A. thaliana* develop as a rosette. Each whorl of the rosette represents a different stage of leaf development. The innermost whorls contain young emergent leaves while whorls toward the outside of the rosette contain leaves that are fully expanded. For experimental purposes leaves were harvested at four developmental stages spanning the entire range of growth from emergence to full expansion. Emerging leaves less than 1.5 cm in length were harvested and termed 'L1'. Leaves 1.5-3.5 cm were harvested and termed 'L2'. Class 'L3' leaves were 3.5-4.5 cm in length but had not expanded fully while the youngest fully expanded leaves were harvested and termed 'L4'. The experimental sampling is shown in Fig. 2.1.

Flowering stems were harvested from intact plants when 8 cm in length. Under the conditions described above this occurred after 8 weeks of plant growth.

*Aseptic growth of A. thaliana seedlings*

Seeds of *A. thaliana* (ecotype OY0) were sterilised prior to germination on agar plates. Seeds were sterilised in an eppendorf tube by washing in 70% (v/v) ethanol for 5 min and then in 5% (v/v) bleach containing 0.1% (v/v) Tween 20 for 20 min and

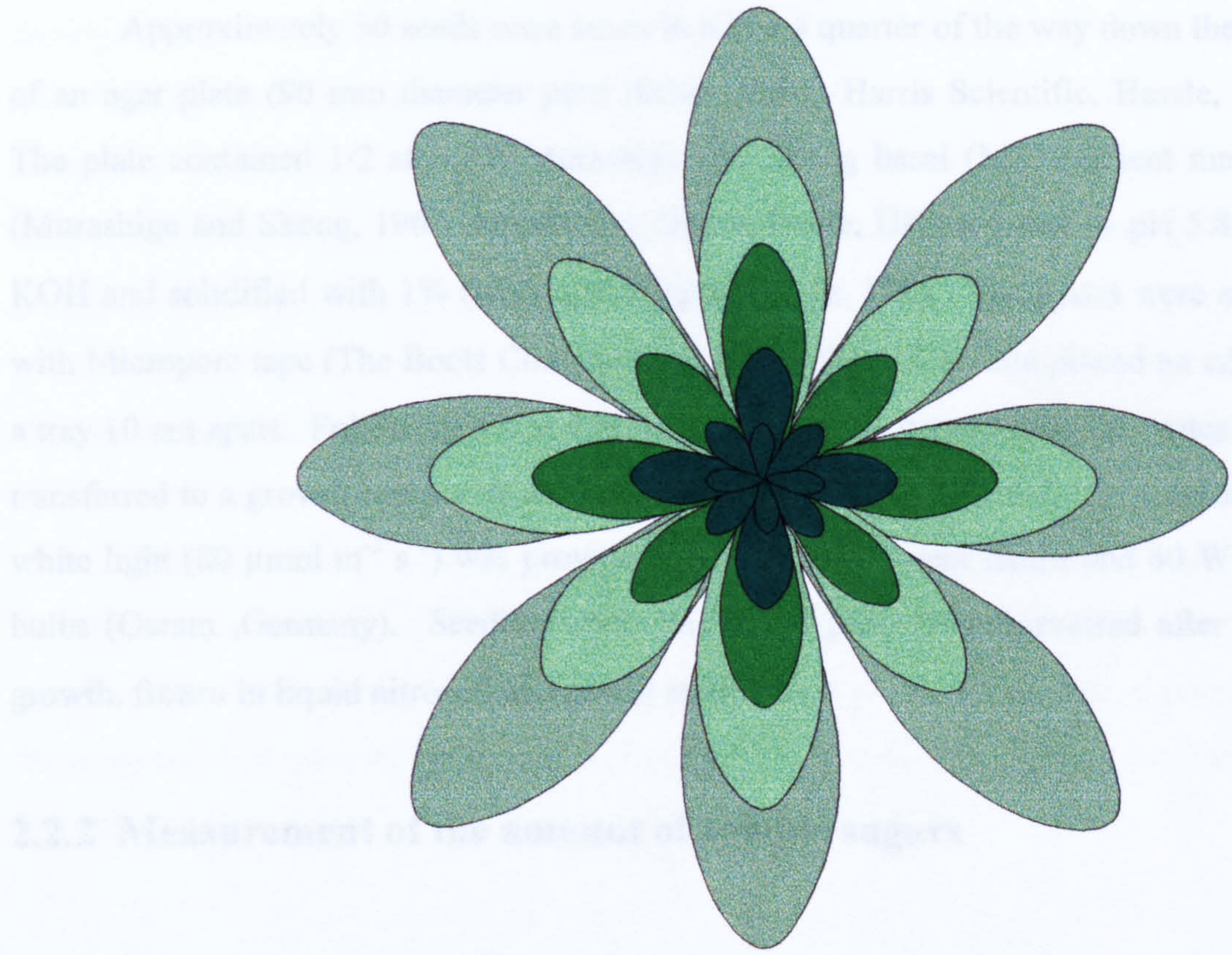



**Figure 2.1 Leaf sampling of soil-grown *A. thaliana*.** Leaf tissue from the rosette of 6 week old soil-grown *A. thaliana* (ecotype OY0) plants was harvested and separated according to length. Leaf length was used as an indicator of the developmental stage. Leaves in class L1 were less than 1.5 cm in length. Leaves in class L2 were between 1.5 and 3.5 cm in length. Leaves in class L3 were between 3.5 and 4.5 cm in length while leaves in class L4 constituted the youngest fully expanded leaves.

rocked continuously. The seeds were then washed five times in sterile distilled water.

Approximately 50 seeds were sown in a line a quarter of the way down the face of an agar plate (90 mm diameter petri dishes, Philip Harris Scientific, Hessle, UK). The plate contained 1/2 strength Murashige and Skoog basal (MS) nutrient medium (Murashige and Skoog, 1962, supplied by Sigma, Poole, UK) adjusted to pH 5.8 with KOH and solidified with 1% (w/v) agar (Sigma, Poole, UK). The plates were sealed with Micropore tape (The Boots Company plc, Nottingham, UK) and placed on edge in a tray 10 cm apart. Following 4 d at 4 °C to promote even germination the plates were transferred to a growth room with a constant temperature of 22 °C ± 1 °C. Continuous white light (80 μmol $m^{-2}$ $s^{-1}$) was provided by 80 W fluorescent lamps and 60 W light bulbs (Osram ,Germany). Seedling roots and aerial parts were harvested after 10 d growth, frozen in liquid nitrogen and stored at -80 °C.

## 2.2.2 Measurement of the amount of soluble sugars

The tissue specific and developmental pattern of soluble sugar accumulation in *A. thaliana* was examined by measuring the amounts of sucrose, glucose and fructose in the leaves (developmental stages 1-4) and flowering stems of soil grown plants and in the roots and cotyledons of aseptically grown seedlings.

Leaves (L1-L4) and flowering stems were harvested from soil grown plants toward the end of the photoperiod after 6 h in the light. Sixty mg of each tissue type was harvested from each of four replicate plants, placed into individually labelled foil packets and frozen immediately in liquid nitrogen. One 60 mg sample of seedling roots and one 60 mg sample of seedling cotyledons (consisting of many pooled roots or cotyledons) were harvested, placed into individually labelled foil packets and frozen at the same time. All samples were placed at –20 °C for storage.

*Extraction of soluble carbohydrates*

Soluble sugars were extracted from samples by incubation in 1 ml of 80% ethanol (v/v) at 70 °C for 20 min. The solvent was replaced and the procedure repeated until all the chlorophyll had been removed from the leaves or flowering stems;

comparable treatment times were used for roots. The ethanolic supernatants were pooled and dried in a centrifugal vacuum evaporator (Speedvac, Savant, France). Soluble sugars were resuspended in 1 ml of distilled water by vortexing vigorously for 30 s. The extract was transferred to a clean eppendorf tube and stored at –20 °C until analysed. Tissues from which soluble sugars had been extracted were frozen at -20 °C for later analysis of starch content.

***Enzyme linked assay for the measurement of the amount of soluble carbohydrates***

Soluble carbohydrates were measured using an enzyme linked assay as described in Scholes *et al.* (1994). Sucrose, glucose and fructose were selectively converted into glucose-6-phosphate which, upon the addition of glucose-6-phosphate dehydrogenase, caused the reduction of NAD to NADH with a change in absorbance at 340 nm as shown below:

$$\text{Glucose - 6 - phosphate} + \text{NAD} \xrightarrow[\text{dehydrogenase}]{\text{Glucose-6-phosphate}} \text{6 - phosphogluconate} + \text{NADH}$$

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{glucose - 6 - phosphate}$$

$$\text{Fructose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{fructose - 6 - phosphate}$$

$$\text{Fructose - 6 - phosphate} \xrightarrow{\text{Phosphogluco-isomerase}} \text{glucose - 6 - phoshate}$$

$$\text{Sucrose} + \text{H2O} \xrightarrow{\text{Invertase}} \text{glucose} + \text{fructose}$$

Multiple assays were carried out simultaneously using an ELISA plate reader (Anthos hT III, Labtec, Salzburg, Austria). Twenty µl of each extract were placed into wells of a micro-titre plate (NUNC MaxiSorp™ immuno plate, GibcoBRL, Paisley, UK) containing 125 µl of assay buffer (100 mM HEPES, 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD, pH 7.0) and the enzymes were then added in the order shown above.

To remove residual glucose-6-phosphate from the sample glucose-6-phosphate dehydrogenase from *Leuconostoc meserentoides* (2 units/assay) was added. Once this

reaction had gone to completion the absorbance of the wells was measured and this reading used as the starting point for the addition of further enzymes. The amount of glucose in the samples was measured by adding hexokinase (3 units/assay). This resulted in the conversion of glucose and fructose into their respective hexose-6-phosphates. The immediate conversion of glucose-6-phosphate into 6-phosphogluconate resulting from the pre-addition of glucose-6-phosphate dehydrogenase was accompanied by a change in absorbance proportional to the reduction of NAD to NADH. When this reaction had gone to completion phosphoglucoseisomerase (2 units/assay) was added to convert fructose-6-phosphate to glucose-6-phosphate. Invertase (85 units/assay) was added to the wells to catalyse the hydrolysis of sucrose into glucose and fructose respectively. These were then converted to hexose-6-phosphates and finally to 6-phosphogluconate with an accompanying absorbance change. All enzymes were obtained from Boerhinger Mannheim (Lewes, UK) with the exception of invertase which was obtained from Sigma Chemicals (Poole, UK).

The amount of soluble sugars in each extract was calculated by reference to a standard curve. Serial dilutions of glucose (87mM), fructose (87mM) and sucrose (43mM) were prepared and measured simultaneously on each micro-titre plate.

## 2.2.3 Measurement of the amount of starch

*Extraction of starch*

Samples were assayed for starch content after extraction of soluble sugars as described in section 2.2.2. Plant tissues were washed in distilled water to remove any ethanolic residues before being ground in 1.5 ml of 100 mM sodium acetate buffer, pH 4.7. Samples were placed in 1.5 ml screw capped eppendorf tubes (Scientific Laboratory Supplies, Nottingham, UK) and centrifuged for 10 min at 20 000 *g*. The supernatant was removed and the pellet resuspended in 400 µl of buffer prior to autoclaving for 30 min.

*Enzyme linked assay for starch*

Starch was broken down into glucose residues by overnight incubation of samples at 37 °C with 15 units of amyloglucosidase (Boerhinger Mannheim, Lewes, UK), which cleaves the α-1,6 linkage in starch, and 6 units of amylase (Sigma, Poole, UK), which cleaves the α-1,4 linkage. Following incubation the tubes were centrifuged at 20 000 *g* for 10 min. An aliquot of the supernatant (5 µl) was removed and analysed for glucose as described in section 2.2.2. except that only the enzymes glucose-6-phosphate dehydrogenase and hexokinase were added. The amount of starch was calculated with reference to a standard curve as described in section 2.2.2. and expressed as glucose equivalents.

## 2.2.4 Measurement of invertase activity

Invertase activity (apoplastic and soluble) in leaves of different ages and in different organs of *A. thaliana* were examined. Samples of leaves (L1-L4) and flowering stems from soil grown plants were harvested at noon, 3 h into the photoperiod. Two hundred mg of each tissue type were harvested from each of four replicate plants and placed into individually labelled foil packets and frozen immediately in liquid nitrogen. One 200 mg sample of seedling roots and one 200 mg sample of seedling cotyledons (consisting of many pooled roots or cotyledons) were harvested, placed into individually labelled foil packets and frozen at the same time. All samples were placed at –80 °C for long term storage.

*Extraction of invertases*

The activity of soluble and cell wall invertase was measured as described by Tang *et al.* (1996).

Samples were ground to a powder with a pestle and mortar chilled with liquid nitrogen. One ml of extraction buffer (50 mM sodium phosphate buffer, 1 mM EDTA, pH 6.5) containing 1 mM dithiothreitol (DTT), 1 mM benzamidine (BA) and 0.1 mM phenylmethyl sulphonyl fluoride (PMSF) was added to the powder and mixed

thoroughly. Once thawed the extract was transferred to an eppendorf tube and centrifuged at 20 000 *g* for 10 min at 4 °C. Following centrifugation the supernatant (containing soluble invertases) was divided and transferred into two clean tubes; one was kept on ice for measurement of soluble invertase activity while the second was frozen in liquid nitrogen and stored at –80 °C for protein measurement and isoelectric focussing (IEF).

The pellet, which contained apoplastic invertase activity, was resuspended in 1 ml of extraction buffer and then centrifuged at 20 000 *g* for 10 min at 4 °C. After centrifugation the supernatant was discarded. This was repeated three times but during the last wash 50 mM NaCl was added to the buffer to aid the complete removal of soluble invertases. Finally the pellet was used for the measurement of apoplastic invertase activity.

***Measurement of invertase activity; optimisation of assay conditions***

Invertase activity was measured according to a modified procedure of Farhendorf and Beck (1990). This assay involves the incubation of invertase extracts in an assay buffer containing sucrose. Invertase activity present within the extract hydrolyses the sucrose producing the reducing sugars glucose and fructose. These reducing sugars are quantified by reaction with a colourimetric agent resulting in an absorbance change when measured at 540 nm. The amount of invertase activity in each sample can be quantified by reference to a standard curve of known amounts of reducing sugar.

In order to optimise the assay conditions prior to use with experimental plants a pH profile was constructed. Extracts of soluble and apoplastic invertase were taken from the first fully-expanded leaves (L4) of six week old plants and assayed in different buffers chosen to cover a range of pH's from 3.5-9.0 at intervals of 0.5 pH units. The buffers used for the construction of the pH profile were citrate phosphate buffer (pH 3.0-5.5), sodium acetate buffer (pH 4.0-5.5), sodium phosphate buffer (pH 6.0-7.5) and BICINE buffer (N,N-bis[2-hydroxyethyl]-glycine) (pH 7.5-9.0). All the buffers were made to a concentration of 0.2 M and contained 0.1 M sucrose. The addition of the extract to the assay buffer resulted in a shift from the desired pH of 0.1-0.5 pH units.

This shift was measured and the actual pH was used to construct the pH profiles, these are shown in Fig. 2.2. The construction of the pH profiles revealed that soluble invertase activity was highest when assayed at pH 5.5 in 0.2 M sodium acetate buffer (Fig. 2.2a) and that apoplastic invertase activity was highest when assayed at pH 4.5 in 0.2 M sodium acetate buffer (Fig. 2.2b). The exact procedure used for the measurement of invertase activity is given in the sections below.

***Measurement of soluble invertase activity***

Fifty µl of soluble invertase extract was added to an eppendorf tube containing 450 µl of 0.2 M sodium acetate buffer, pH 5.5, containing 0.1 M sucrose and incubated for 30 min at 37 °C. After incubation the reaction was stopped by the addition of 500 µl of DNSA reagent (1% 3,5-dinitrosalicylic acid (w/v) in 0.5 M KOH, 1 M K/Na tartrate solution). Samples were vigorously boiled for 10 min to allow the DNSA to produce the characteristic orange/red reaction product and then placed rapidly on ice to cool. Comparable reactions were performed using 50 µl of pre-boiled extract (to denature the invertase) in order to measure pre-existing sugars in the sample.

The absorbance of each sample was measured at 540 nm in an ELISA plate reader (Anthos HT III, labtec, Salzburg, Austria). The absorbance of the sample containing the pre-boiled extract was subtracted from that of the active sample. Invertase activity was calculated with reference to a standard curve. Samples for the standard curve contained known amounts of glucose (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2 and 2.5 µmol glucose) and were treated in an identical manner to the active extracts. Invertase activity was expressed as the amount of sucrose hydrolysed per $cm^{-2}$ $min^{-1}$ or $g^{-1}$ $min^{-1}$.

***Measurement of apoplastic invertase activity***

The pellet was resuspended and incubated in 600 µl of assay buffer (0.2 M sodium acetate buffer, pH 4.5 containing 0.1 M sucrose) at 37 °C for 45 min. Samples were immediately placed on ice to slow the reaction and then centrifuged at 20 000 *g* for 10 min at 4 °C. Five hundred µl of supernatant was added to an eppendorf tube

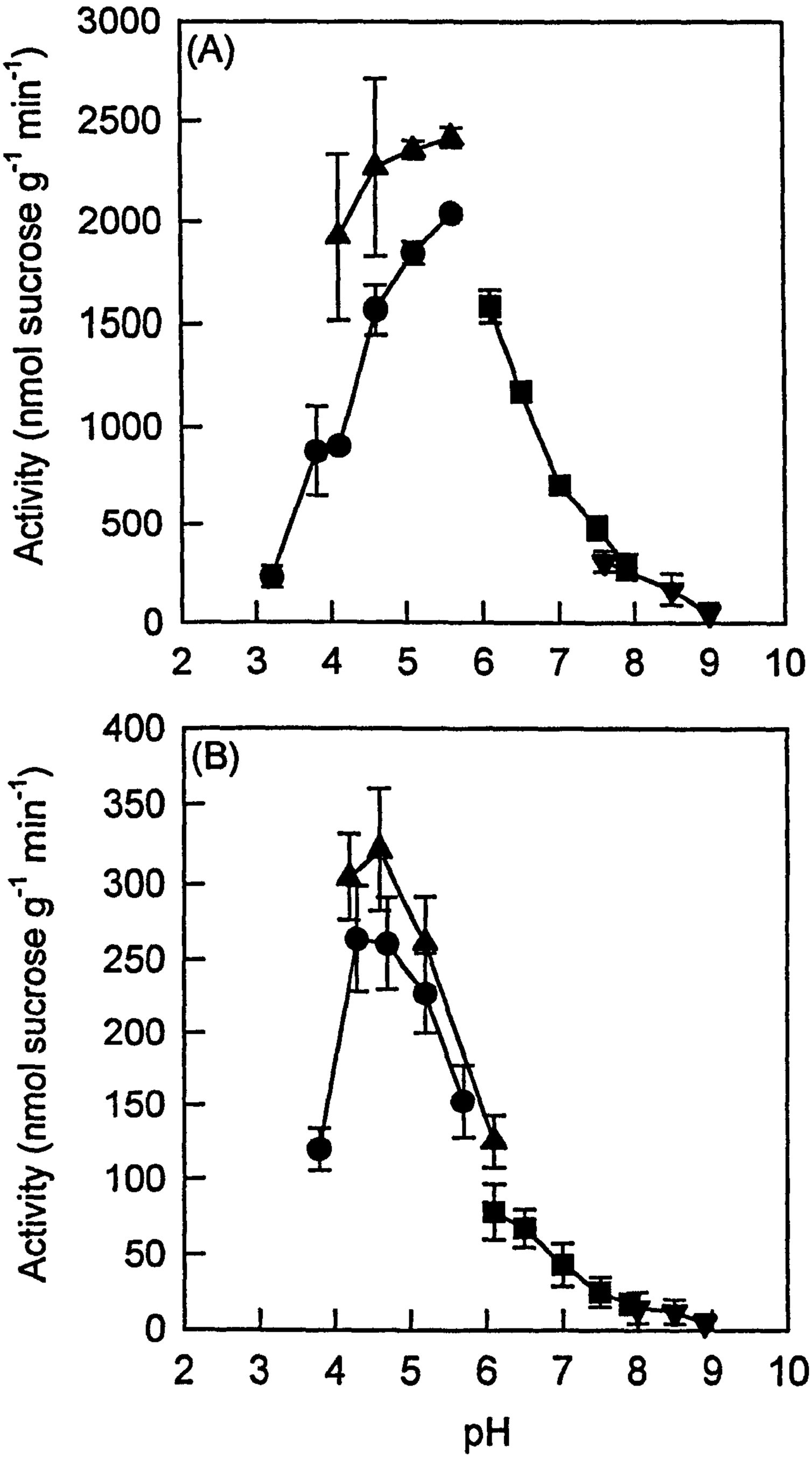



**Figure 2.2 Activity-pH profiles of soluble and apoplastic invertase from leaves of *A. thaliana* plants.** Soluble (A) and apoplastic (B) invertases were extracted from mature leaves of six week old *A. thaliana* plants and assayed in 0.2 M citrate phosphate buffer (-●-), sodium acetate buffer (-▲-), sodium phosphate buffer (-■-) and BICINE buffer (-▼-) containing 100 mM sucrose at 37 °C for 30 min. The results are the mean ± standard error of four replicate measurements.

containing 500 µl of DNSA reagent and treated as described above.

## 2.2.5 Isoelectric focusing (IEF) of soluble invertase proteins

The isoform pattern of soluble invertase protein was examined in the leaves (L1-L4) and flowering stems of soil grown plants and in the roots and cotyledons of aseptically grown seedlings. Extracts were made as described in section 2.2.4 and the activity measured prior to focusing.

Proteins were resolved by IEF using a Multiphor apparatus and pre-cast polyacrylamide gels (Ampholine PAGplate, pH 4.0-6.5) according to the manufacturer's instructions (Pharmacia Biotech, Uppsala, Sweden). The gel was maintained at 10 °C throughout the experiment by circulating water through the apparatus. Wicks were placed onto the gel surface soaked in electrode buffers (for the anode, 0.1 M glutamic acid in 0.5 M $H_3PO_4$ and for the cathode, 0.1 M β-alanine). The gel was pre-focused for 1 h with maximum power limits of 2000 V, 25 mA and 25 W. After pre-focussing, a strip of plastic wells (Pharmacia Biotech, Uppsala, Sweden) was applied to the gel surface into which the samples were loaded on an equal activity basis and focussed for a further 2.5 h.

### *Activity staining of invertase activity*

An activity stain was used to detect the presence of soluble invertase isoforms on the IEF gel. The gel was removed from the apparatus and incubated in 0.1 M sodium acetate buffer pH 5.5 containing 0.6 M sucrose at 37 °C for 30 min. Reducing sugars were detected by staining the gel with 0.2% (w/v) 2,3,5-tetrazolium chloride (Sigma, Poole, UK) dissolved in boiling 1 M NaOH for 2 min (Faye, 1981). The reaction was stopped by washing the gel in 10% acetic acid. Sharp red bands indicated soluble invertase activity.

## 2.2.6 Protein assay

Total soluble protein in extracts of plant tissue was measured using a modified

dye-binding procedure of Bradford (1976). Seven hundred and ninety µl of distilled water, 10 µl of sample and 200 µl of Bio-Rad protein assay solution (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) were placed into a 1 ml cuvette. The solutions were mixed and the colour left to develop for 5 min. The absorbance was measured at 595 nm in a spectrophotometer (UV-2101PC scanning spectrophotometer, Shimadzu, Kyoto, Japan).

The amount of soluble protein in the sample was calculated with reference to a standard curve. A standard curve was prepared from serial dilutions of bovine serum albumin (BSA Fraction V, Sigma, Poole, UK) ranging from 0-0.9 mg $ml^{-1}$ and dissolved in the same buffer as the sample. Protein in the standard was measured as described above.

### 2.2.7 Analysis of invertase gene expression in *A. thaliana*

The expression patterns of five invertase genes in *A. thaliana* were examined in response to organ specific and developmental stimuli. DNA probes for all five invertase genes were prepared and analysed for gene specificity using Southern hybridisation assays. The expression of three genes, encoding soluble invertases, were examined using Northern blotting and hybridisation techniques. However, the sensitivity of Northern blotting was insufficient for the detection of the two genes encoding apoplastic invertases and required the use of a more sensitive detection system. The expression of these genes was examined using the reverse transcriptase polymerase chain reaction (RT-PCR). This system was adapted to include an internal standard which allowed semi-quantitative measurements of apoplastic invertase gene expression to be made.

The following sections describe the preparation and labelling of DNA probes and their use for Southern and Northern hybridisation and the establishment of a semi-quantitative RT-PCR system for the detection of apoplastic invertase gene expression.

### 2.2.8 Growth of bacteria

Plasmid DNA was used as the vector for all the DNA probes used in the

Northern and Southern hybridisation analysis of invertase gene expression in developing *A. thaliana*. DNA probes cloned into bacterial plasmid vectors allowed for the easy replication, purification and manipulation of the inserts. Bacteria containing plasmid vectors were grown using standard molecular biological techniques as described in Sambrook *et al.* (1989).

For long term storage the bacteria (usually *E.coli* strain DH5α) were placed at -80 °C in 15% glycerol. Bacteria were grown by streaking out onto plates containing Luria Broth [LB, 1% (w/v) bactotryptone (Difco Laboratories, Detroit, USA), 0.5% (w/v) yeast extract (Difco Laboratories, Detroit, USA) and 1% (w/v) NaCl, pH 7.5] and solidified with 1% (w/v) agar (Sigma, Poole, UK). Where appropriate, antibiotics were added into the medium (usually 100 μg $ml^{-1}$ ampicillin) prior to the pouring of the plates. The plates were incubated overnight at 37 °C to encourage bacterial growth.

Single colonies were picked and used to inoculate 3 ml of liquid culture (LB containing antibiotic). These were grown overnight at 37 °C with shaking (800 cycles $min^{-1}$ on a KCH-VIBRAX-VXR shaker, Kinematica, Luzern, Switzerland). These liquid cultures were either used directly for small scale preparations of plasmid DNA or for inoculating larger volumes of liquid medium for large scale plasmid preparations.

### 2.2.9 Purification of plasmid DNA

Plasmid DNA, containing cloned DNA inserts used as probes for Northern and Southern hybridisation assays, was replicated in bacterial cultures and purified in a variety of ways depending upon the final use of the plasmid vector. Two methods were used for the small scale purification of plasmid DNA. The first method or 'boiling mini-prep' was used to screen large numbers of bacterial colonies for cloned inserts. This preparation yielded low quality plasmid DNA suitable for restriction enzyme digests. The second method used a commercially available kit (Wizard Minipreps™ DNA purification system, Promega, Southampton, UK) and yielded high quality plasmid suitable for restriction enzyme digests, DNA sequencing or further cloning and manipulation. Both the small scale plasmid preparations yielded a maximum of 30 μg of plasmid DNA per 3 ml overnight culture.

Larger scale preparations of plasmid DNA of up to 1 mg were achieved using an

alkaline lysis and caesium chloride density centrifugation technique that yielded high quality plasmid DNA from large bacterial cultures.

***Small scale purification - boiling mini-prep***

One ml of an overnight culture of bacterial cells was pelleted in an eppendorf tube by centrifugation at 12 000 *g* for 20 s at room temperature. Cells were resuspended in 70 µl of STET buffer (50 mM Tris-Cl, 8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM EDTA, pH 8.0) and 5 µl of fresh 10 mg $ml^{-1}$ lysozyme (Sigma, Poole, UK) and vortexed vigorously. The suspension was plunged into boiling water for 30 s and immediately placed on ice to cool before centrifugation at 20 000 *g* for 10 min at 4 °C. A sterile toothpick was used to remove the gelatinous precipitate from the bottom of the eppendorf tube and was discarded.

The plasmid DNA was precipitated by the addition of 1/4 volume of 10 M ammonium acetate and 2 volumes of ethanol, mixed, and incubated on ice for 30 min. The plasmid DNA was recovered by centrifugation at 20 000 *g* for 5 min at 4 °C. The supernatant was discarded and the pellet washed gently in 200 µl of ice-cold 70% (v/v) ethanol, centrifuged as above and the ethanol solution carefully removed. The pellets were dried in a centrifugal vacuum evaporator (Speedvac, Savant, France) and resuspended in 20 µl of distilled water. Ten µl of plasmid preparation were used for restriction enzyme digestion and treated with 1 µl of 10 mg $ml^{-1}$ RNAse (Ribonuclease A, Sigma, Poole, UK) prior to gel electrophoresis.

***Small scale purification - commercial kit***

Small quantities of plasmid DNA (up to 30 µg) were purified from 3 ml bacterial liquid cultures using a commercially available kit (Wizard™ Minipreps DNA Purification System, Promega, Southampton, UK). This kit utilises an alkaline lysis procedure to isolate plasmid DNA which is then purified on resin; all reagents were supplied as part of the kit. Plasmid DNA purified in this manner is free from RNA, genomic DNA contamination, salts, protein and is super-coiled. All steps were performed at room temperature.

Bacterial cells from a 3 ml overnight culture were pelleted in an eppendorf tube

by centrifugation at 10 000 *g* for 30 sec. The pellet was then gently resuspended in 200 µl of 'cell resuspension solution'. The cells were then lysed by adding 200 µl of 'cell lysis solution' and mixed by rocking the tube end over end six times. Two hundred µl of 'cell neutralisation solution' was added and mixed as above and resulted in the formation of a white precipitate. The sample was centrifuged at 10 000 *g* for 5 min and the supernatant carefully decanted into a fresh eppendorf tube to avoid transferring precipitate. One ml of 'DNA purification resin' was added to the supernatant, mixed by pipetting and left to stand for one minute to allow the DNA to bind to the resin. A syringe barrel (provided in the kit) was attached to a Wizard™ Minicolumn and placed on a vacuum manifold (Vac-Man®, Promega, Southampton, UK). The resin/DNA complex was pipetted into the barrel of the syringe and the vacuum was applied drawing the solution through the column and into the manifold while the resin was retained on the column. Once the solution had passed through the column the vacuum was broken. Two ml of 'column wash solution' was added to the syringe barrel and the vacuum reapplied. Once the wash solution had completely passed into the manifold the vacuum was applied for a further 30 sec to dry the resin. The column was then detached from the syringe barrel and manifold and placed on top of an eppendorf tube and centrifuged at 10 000 *g* for 2 min to remove any residual wash solution. The column was then transferred to a fresh eppendorf tube and 50 µl of distilled water, heated to 65 °C, was pipetted into the top of the column. After waiting one min the Minicolumn™ was centrifuged at 10 000 *g* for 20 sec to elute the plasmid DNA into the eppendorf tube. Plasmid DNA was quantified by UV spectroscopy prior to further manipulation or long term storage at –20 °C.

***Large scale preparation of plasmid DNA - alkaline lysis and density centrifugation***

The large scale purification was employed for commonly used plasmids and prevented the need for numerous small preparations to be made while yielding high quality DNA suitable for most purposes. The large scale purification was achieved using the method of Sambrook *et al.* (1989).

A 3 ml overnight culture was poured into a 2 L conical flask containing 500 ml of Luria broth (LB). The bacterial growth medium contained the appropriate antibiotic

for plasmid selection and was prepared as described in section 2.2.8. The bacteria were grown by overnight incubation on an orbital shaker (SO3 shaker, Stuart Scientific Company Ltd., Redhill, UK) at 37 °C.

Bacterial cells were pelleted by centrifugation at 5000 *g* for 5 min at room temperature and the supernatant discarded. The pellet was washed gently by resuspension in 20 ml of TE (10mM Tris-HCl, 1 mM EDTA, pH 8.0) and re-pelleted as described above. The pellet was then resuspended in 18 ml of 'cell resuspension buffer' (25 mM Tris-Cl, 50 mM glucose, 10 mM EDTA, pH 8.0), the bacterial cell wall being weakened by the addition of 2 ml of freshly prepared 10 mg $ml^{-1}$ lysozyme (Sigma, Poole, UK) suspension prepared in 10 mM Tris-Cl, pH 8.0. Forty ml of freshly prepared 'alkaline cell lysis solution' [0.2 M NaOH, 1% (w/v) SDS] was added immediately and the solutions mixed by turning the tube end over end several times. The solution was allowed to stand for 10 min at room temperature before addition of 20 ml of ice cold 'neutralisation buffer' (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml distilled water). The solution was placed on ice for 10 min during which a flocculent white precipitate formed containing RNA, chromosomal DNA and membrane complexes. The precipitate was pelleted by centrifugation at 5000 *g* for 10 min at 4 °C and allowed to stop gently without braking. The supernatant, containing plasmid DNA was carefully decanted into a fresh tube. The supernatant was filtered through four layers of cheesecloth and mixed with 0.6 volumes of isopropanol and allowed to stand for 10 min at room temperature to precipitate the plasmid DNA. The solution was centrifuged at 3000 x *g* for 15 min at room temperature and the supernatant carefully discarded. The pellet was rinsed with 5 ml of ice cold 70% (v/v) ethanol and the tube allowed to drain on layers of absorbent paper. The pellet was resuspended in 3 ml of TE buffer.

Caesium chloride was added to the plasmid solution at a concentration of 1 g $ml^{-1}$ and warmed to 30 °C to facilitate the dissolution of the salt. Eighty µl of ethidium bromide (10 mg $ml^{-1}$ in distilled water) was added per 1 ml of solution and immediately mixed before centrifugation at 8000 x *g* for 5 min at room temperature. Plasmid damage was minimised by reducing light exposure to a minimum from this point on. Centrifugation resulted in a furry scum that floated on the surface of the solution. A Pasteur pipette was used to transfer the red solution below the scum into a high speed

centrifugation tube (13 x 51 mm Quick-Seal™ tube, Beckman Instruments Inc., Palo Alto, USA). Full centrifuge tubes were heat sealed and placed in a Ti65 vertical centrifuge rotor (Beckman Instrumentation Inc., Palo Alto, USA) and spun at 178 000 *g* for 16 h at room temperature in a L7-65 ultracentrifuge (Beckman Instrumentation Inc., Palo Alto, USA).

Following centrifugation illumination of the tubes with long wave UV light (UVL unit, UVP Inc., San Gabriel, USA) revealed two fluorescing bands. The upper band consisted of linear bacterial DNA and nicked circular plasmid DNA. The lower band consisted of purified closed circular plasmid DNA. Syringe needles (18 gauge) were used to remove supercoiled plasmid DNA from the centrifuge tube. Ethidium bromide was removed from the solution with 1-butanol which had been vigorously shaken with an equal volume of distilled water saturated with NaCl. One volume of 1-butanol was added to the plasmid/ethidium bromide solution, vortexed and centrifuged briefly to separate the layers. The upper solvent layer was removed and the procedure repeated until all traces of the ethidium bromide had been removed (typically 5 x). Pure plasmid DNA was quantified in a spectrophotometer prior to further analysis or long term storage at –20 °C.

*Quantification of plasmid DNA*

Plasmid DNA was quantified using the method of Sambrook *et al.* (1989). Ten µl of plasmid DNA was placed in a 1 ml quartz cuvette containing 990 µl of distilled water and the absorbance measured between 350 nm to 250 nm in a spectrophotometer (UV-2101PC scanning spectrophotometer, Shimadzu, Kyoto, Japan). The amount of plasmid DNA in the cuvette was calculated by assuming that a 50 µg $ml^{-1}$ solution of DNA has an absorbance at 260 nm of 1.0. The purity of the isolated plasmid DNA was assessed by the ratio of the absorbances at 260 nm and 280 nm. Pure DNA has a ratio of approximately 1.8.

## 2.2.10 Preparation of competent cells

Some of the DNA probes used for Northern and Southern hybridisation assays

were occasionally acquired as plasmid DNA. This plasmid DNA was used to transform competent cells of *E. coli* (strain DH5α) prior to further manipulation and purification.

The preparation of competent cells was achieved using the method of Sambrook *et al.* (1989). One ml of a fresh 3 ml overnight culture (see section 2.2.8) of *E. coli* (DH5α with no plasmids or antibiotic resistance) was pipetted into a conical flask containing 50 ml of Luria Broth and incubated with shaking at 37 °C. At 45 min intervals the optical density of the culture was measured at 550 nm in a spectrophotometer (UV-2101PC scanning spectrophotometer, Shimadzu, Kyoto, Japan). Once an optical density of 0.25 had been reached the bacteria were incubated on ice for 10 min before pelleting by centrifugation at 5000 x *g* for 10 min at 4 °C. The pellet was gently resuspended in 20 ml of ice cold 100 mM $CaCl_2$ and incubated on ice for 20 min; the bacterial cells were then pelleted by centrifugation as before. The pellet was gently resuspended in 500 µl of ice cold 100 mM $CaCl_2$ containing 15% glycerol and 50 µl aliquots dispensed into eppendorf tubes. The cells were stored at –80 °C until use.

### 2.2.11 Transformation of E.coli with plasmid DNA

A 50 µl aliquot of *E. coli* competent cells was removed from storage at –80 °C and placed immediately on ice. The competent cells were gently mixed with a 10 µl plasmid ligation reaction (containing ~50 ng of plasmid) and incubated on ice for 10 min followed by incubation at 37 °C for 5 min and returned to ice. The cells were mixed with one ml of LB containing no antibiotic and incubated with gentle rocking at 37 °C for 1 h. The cells were plated onto LB 1% (w/v) agar plates containing antibiotic (usually 100 µg $ml^{-1}$ ampicillin in distilled water) onto which 50 µl of 50 mg $ml^{-1}$ X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, Sigma, Poole, UK) and 100 µl 0.1 M IPTG (Isopropyl β-D-thiogalactopyranosidase, Sigma, Poole, UK) had been spread and allowed to dry. The plates were incubated at 37 °C overnight. Following incubation white colonies were picked, grown in a 3 ml overnight culture and the plasmids examined using diagnostic restriction enzyme digests.

## 2.2.12 DNA sequencing of purified plasmids

Purified plasmids were sequenced to verify the identity of the DNA inserts used as probes for the analysis of invertase gene expression in developing *A. thaliana* or to gain sequence information about cloned RT-PCR products. Sequencing was achieved using a dye terminator cycle sequencing reaction supplied as a kit (ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin-Elmer, Warrington, UK), the manufacturer's instructions were followed throughout.

Between 0.2-0.5 μg of purified plasmid was placed in a 500 μl thin walled PCR tube (Radleys Laboratory Equipment, Saffron Walden, UK) containing 8 μl of sequencing mix (containing dye terminator nucleotides, nucleotides, $MgCl_2$, thermal stable pyrophosphatase and AmpliTaq DNA Polymerase), 3.2 pmol T7 primer (Promega, Poole, UK) and made up to 20 μl with distilled water. The reaction was overlaid with 40 μl of light mineral oil (Sigma, Poole, UK).

The reaction mix was placed into a thermal cycler (PHC-3 Thermal Cycler, Techne, Cambridge, UK) and the programme set to the following conditions:

- Rapid thermal ramp to 96 °C
- 96 °C for 30 s
- Rapid thermal ramp to 50 °C
- 50 °C for 15 s
- Rapid thermal ramp to 60 °C
- 60 °C for 4 min
- All steps repeated for 25 cycles, finish with 4 °C hold

Following the completion of thermal cycling the DNA was purified from the mineral oil overlay and reaction components. This was achieved by mixing with an equal volume of chloroform:isoamyl alcohol (24:1), vortexing for 20 s, and centrifuging at 12 000 *g* for 30 s at room temperature. The top layer was removed and placed in a 0.5 ml eppendorf tube and mixed with 3 μl of 2 M sodium acetate, pH 4.6 and 50 μl of absolute ethanol. The solution was placed on ice for 10 min to precipitate the nucleic acid. The DNA was recovered by centrifugation at 20 000 *g* for 10 min at 4

°C. The supernatant was discarded and the pellet washed with 100 µl of ice-cold 70% (v/v) ethanol and centrifuged as above. After centrifugation the ethanol solution was discarded and the pellet dried in a centrifugal vacuum evaporator (Speedvac, Savant, France).

Completed reactions were sequenced (Krebs Sequencing and Synthesis Unit, University of Sheffield, UK) using a ABI PRISM 373A DNA sequencer (Perkin-Elmer, Warrington, UK).

## 2.2.13 Preparation and purification of DNA fragments

*Restriction enzyme digests*

DNA probes for use in the Northern and Southern hybridisation analysis of invertase gene expression in *A. thaliana* were excised from plasmid vectors by restriction enzyme digestion. The DNA sequence was used to determine the appropriate restriction enzyme and the digests were performed according to the manufacturer's instructions (Promega, Southampton, UK). Typically, 1 µg of plasmid DNA was incubated in 5 units of restriction enzyme with the appropriate buffer supplied by the manufacturer (1x concentration) at 37 °C for 2 h. Up to 5 µg of plasmid were digested if the insert was to be purified.

*Non-denaturing gel electrophoresis*

Completed restriction enzyme digests were mixed with 1/5 volume of gel loading solution (Sigma, Poole, UK) containing bromophenol blue as a visible marker. 1 x TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) was solidified with 1% (w/v) agarose (Sigma, Poole, UK) and cast into a submarine, horizontal gel apparatus (Wide Mini-Subcell, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Samples and DNA markers (1 Kb ladders or λ Hind III fragments, GibcoBRL, Paisley, UK) were loaded and electrophoresed at 150 mA for 1 h using 1 x TAE as a running buffer. The gel was immersed in 1 x TAE containing 0.5 µg $ml^{-1}$ ethidium bromide for 20 min and the DNA visualised on a UV transilluminator (Genetic Research

Instrumentation Ltd., Felsted, UK).

*DNA fragment purification from agarose gels*

DNA fragments, revealed by trans-illumination, were cut from gels using a razor blade and placed in eppendorf tubes. The DNA was then purified from the agarose gel using a Wizard™ DNA Clean-Up System (Promega, Southampton, UK). The agarose was dissolved in 1 ml of a supplied buffer by gently shaking at room temperature. The suspension was drawn through a Wizard™ Minicolumn attached to a vacuum manifold and the bound DNA/resin complex cleaned with 2 ml 80% (v/v) *iso*-propyl-alcohol; similar to the procedure described in section 2.2.10. The Minicolumn was placed onto an eppendorf tube and centrifuged at 10 000 *g* for 30 sec at room temperature to remove all traces of *iso*-propyl-alcohol. The Minicolumn was placed onto a clean eppendorf tube and 50 µl of distilled water, heated to 65 °C, placed into the top of the column. After 1 min the column was centrifuged at 10 000 *g* for 20 sec to elute the DNA into the eppendorf tube. Gel electrophoresis was used to confirm the intactness of the purified fragment and to ensure the complete removal of vector DNA. Purified fragments were stored at -20 °C until use.

## 2.2.14 Preparation of radioactive DNA probes

DNA probes used for the Northern and Southern hybridisation analysis of invertase gene expression in *A. thaliana* were radiolabelled using a commercially available kit (Megaprime™, Amersham International PLC, Little Chalfont, UK). 25 ng of probe DNA was placed into an eppendorf tube containing 5 µl of random nonamer primers and distilled water added to a total volume of 50µl. The solution was boiled for 5 min to denature the DNA and then centrifuged at 10 000 *g* for 10 s at room temperature. To this was added 10 µl of labelling solution (containing buffer, dATP, dGTP and dTTP), 5 µl of [$\alpha$ $^{32}$P]-dCTP (370 MBq ml$^{-1}$ aqueous solution, Amersham International Plc., UK) and 1 unit of Klenow fragment of DNA polymerase (Amersham International PLC, Little Chalfont, UK). The solution was mixed by pipetting and incubated at 37 °C for 1 h to allow the synthesis of radiolabelled DNA probe fragments.

Following incubation the DNA was purified away from the unincorporated nucleotides using a Wizard™ DNA Clean-Up System (Promega, Southampton, UK) as in section 2.2.13 but with syringes being used rather than a vacuum manifold. The success of the incorporation of radio label was measured using a scintillation counter (1600TR Liquid Scintillation analyser, Canberra Company). Probes yielding a count of between 1-3 x $10^7$ cpm of $^{32}P$ (6700-20000 Bq $ng^{-1}$) were used for hybridisations.

Table 2.1 shows the source, preparation and identity of the probes used throughout this thesis.

## 2.2.15 Extraction of DNA from plant material

DNA was extracted from the leaves of *A. thaliana* (ecotype OY0) and used to generate Southern blots. These blots were used to examine the specificity of DNA probes to five invertase genes. The protocol for the extraction of DNA from plant material was adapted from the method of Shillito and Saul (1985) and typically yielded 1 mg of total DNA per 10 g of starting material. This method utilises hexadecyltrimethylammonium bromide (CTAB) to extract genomic DNA from plant sources.

To avoid the contamination of samples with foreign DNA all glassware, pestles, mortars and spatulas etc were baked at 180 °C prior to use and all solutions and plastic ware autoclaved (except chloroform:isoamylaclohol and phenol).

Ten g of *A. thaliana* (DNA from ecotypes OY0 and Columbia were prepared separately) leaf material was ground for 15 min with a pestle and mortar, pre-chilled with liquid nitrogen, until the sample appeared as a fine paste. The sample was quickly transferred to a polypropylene centrifuge tube (Nalgene, Rotherwas, UK) and 10 ml of 2 x CTAB buffer [100 mM Tris-Cl buffer, pH 8.0, 2% (w/v) CTAB, 1% (w/v) polyvinylpyrollidone (MW 360K), 1.4 M NaCl, 20 mM EDTA] heated to 65 °C added and mixed. Five µl of a 5 mg $ml^{-1}$ solution of transfer RNA (Sigma, Poole, UK) was added to aid the precipitation of the genomic DNA. Fifteen ml of chloroform:isoamyl alcohol (24:1) was added and the tube vortexed vigorously for 1 min. The sample was centrifuged at 12 000 *g* for 5 min at room temperature and the supernatant transferred to a fresh tube. To the supernatant 1/10 volume of 10% CTAB buffer [10% (w/v) CTAB,

**Table 2.1 DNA probes used for Southern and Northern hybridisation assays.** All probes were isolated from *A. thaliana*.

| Gene | Gene name | GenBank ID | Probe preparation | Reference |
|---|---|---|---|---|
| Apoplastic invertase 1 | ATβFRUCT 1 | X74515 | Genomic clone (~1000 bp EcoRI HindIII fragment) | Schwebel-Dugue *et al.*,(1994) |
| Apoplastic invertase 2 | ATβFRUCT 2 | U11033 | Genomic clone (~1000 bp EcoRI HindIII fragment) | Mercier and Gogarten, (1995) |
| Vacuolar invertase 1 | ATβFRUCT 3 | X95537 | cDNA clone (1373+479 bp EcoRI HindIII fragment) | Haouazine-Takvorian *et al.*, (1997) |
| Vacuolar invertase 2 | ATβFRUCT 4 | Y11559 | cDNA clone (~500 bp EcoRI SalI fragment) | Haouazine-Takvorian *et al.*, (1997) |
| Unknown invertase | ATβFRUCT 5 | T46086 | cDNA clone (502 bp EcoRI HindIII fragment) | Newmann *et al.*, (1994) |
| Small subunit of Rubisco | rbcS1A | X13611 | Genomic clone (1800 bp HindIII fragment) | Krebbers *et al.*, (1988) |
| Photoassimilate responsive gene 1c | PAR-1c | N97294 | cDNA clone (400+300 bp EcoRI BamH1 fragment) | Newmann *et al.*, (1994) |
| β-tubulin-4 | ABT4 | M21415 | Genomic clone (1998 bp HindIII fragment) | Oppenheimer *et al.*, (1988) |
| Chlorophyll a/b binding protein | AB180 | X03908 | cDNA clone (1800 bp EcoRI fragment) | Leutwiler *et al.*, (1986) |

0.7% (w/v) NaCl] was added and shaken gently. An equal volume of chloroform:isoamyl alcohol (24:1) was added and the sample vortexed vigorously for 1 min and centrifuged as above. Protein components of the sample could be clearly seen on the interface following centrifugation. The supernatant was twice re-extracted in an equal volume of chloroform:isoamyl alcohol (24:1) or until contaminants were no longer visible at the interface. An equal volume of CTAB precipitation buffer [50 mM Tris-Cl buffer, pH 8.0, 1% (w/v) CTAB, 10 mM EDTA] was added to the supernatant, mixed and chilled on ice for 20 min. Following centrifugation at 12 000 *g* for 10 min at 4 °C the supernatant was discarded and the pellet re-suspended in 1 ml of high-salt TE buffer (10 mM Tris-Cl buffer, pH 8.0, 1 mM EDTA, 1 M NaCl) heated to 65 °C to aid dissolution. The DNA was precipitated by incubation on ice for 20 min in 2 volumes of absolute ethanol. The DNA was recovered by centrifugation as above and the supernatant discarded. The pellet was washed gently in ice cold 70 % (v/v) ethanol and centrifuged for a further 5 min. The ethanol was discarded and the pellet air dried before resuspension in 1 ml of distilled water.

The purified genomic DNA was quantified spectrophotometrically as described in section 2.2.9 and stored at –20 °C until use.

## 2.2.16 Southern blotting

Five probes used to analyse the expression of invertase genes in developing *A. thaliana* were each examined for their gene specificity. Southern blots were generated from DNA extracted from the leaves of *A. thaliana* plants and hybridised to the radiolabelled invertase gene probes. The banding pattern of the resulting autoradiographs revealed if each probe was specific for a separate invertase gene.

***Restriction enzyme digest***

Total genomic DNA isolated from the leaves of *A. thaliana* (ecotype OY0) was digested with restriction enzymes prior to electrophoresis and Southern blotting. Five μg of DNA was incubated with 50 units of BamHI, EcoRI or HindIII restriction enzyme (Promega, Southampton, UK) at 37 °C for 2 h. The appropriate buffer (supplied by

manufacturer) was used for the digests in a total reaction volume of 50 µl.

*Non-denaturing gel electrophoresis*

Completed restriction enzyme digests were mixed with 1/5 volume of gel loading solution (Sigma, Poole, UK) containing bromophenol blue as a visible marker. Two hundred and forty ml of 1 x TAE was solidified with 1 % (w/v) agarose and cast into a large submarine, horizontal gel apparatus as described in section 2.2.13. Samples and 1 µg of λ Hind III DNA fragment markers (GibcoBRL, Paisley, UK) were loaded onto the gels and electrophoresed at 20 mA overnight using 1 x TAE as a running buffer.

After electrophoresis the DNA on the gel was stained to ensure that the restriction enzyme digestion was complete. The gel was carefully removed from the tank and immersed in 1 x TAE containing 0.5 µg $ml^{-1}$ ethidium bromide and rocked for 15 min. The DNA was examined using a UV transilluminator (Genetic Research Instrumentation Ltd, Felsted, UK).

Before blotting, the gel was incubated in 0.2 M HCl for 10 min at room temperature with rocking to depurinate the DNA. This procedure is known to enhance blotting of DNA fragments greater than 20 Kb in length. The gel was rinsed briefly in distilled water before immersing in a solution of 0.5 M NaOH, 1.5 M NaCl and rocked gently for 45 min. The gel was neutralised by immersion in 1 M Tris-Cl, 1.5 M NaCl, pH 7.4 which was replaced after 15 min and incubated with rocking for a further 30 min. At this point the gel was ready for Southern blot transfer of the DNA to a nylon membrane.

*Blotting*

The equipment for capillary transfer was set up as shown in Fig. 2.3. DNA was transferred from the gel to a nylon membrane (Zetaprobe GT, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) by capillary action as the blotting buffer was drawn from reservoirs, through the gel and into absorbent tissues. The gel was placed onto a wick comprised of two sheets of Whatman 3MM paper soaked in 10 x SSC buffer (1.5 M

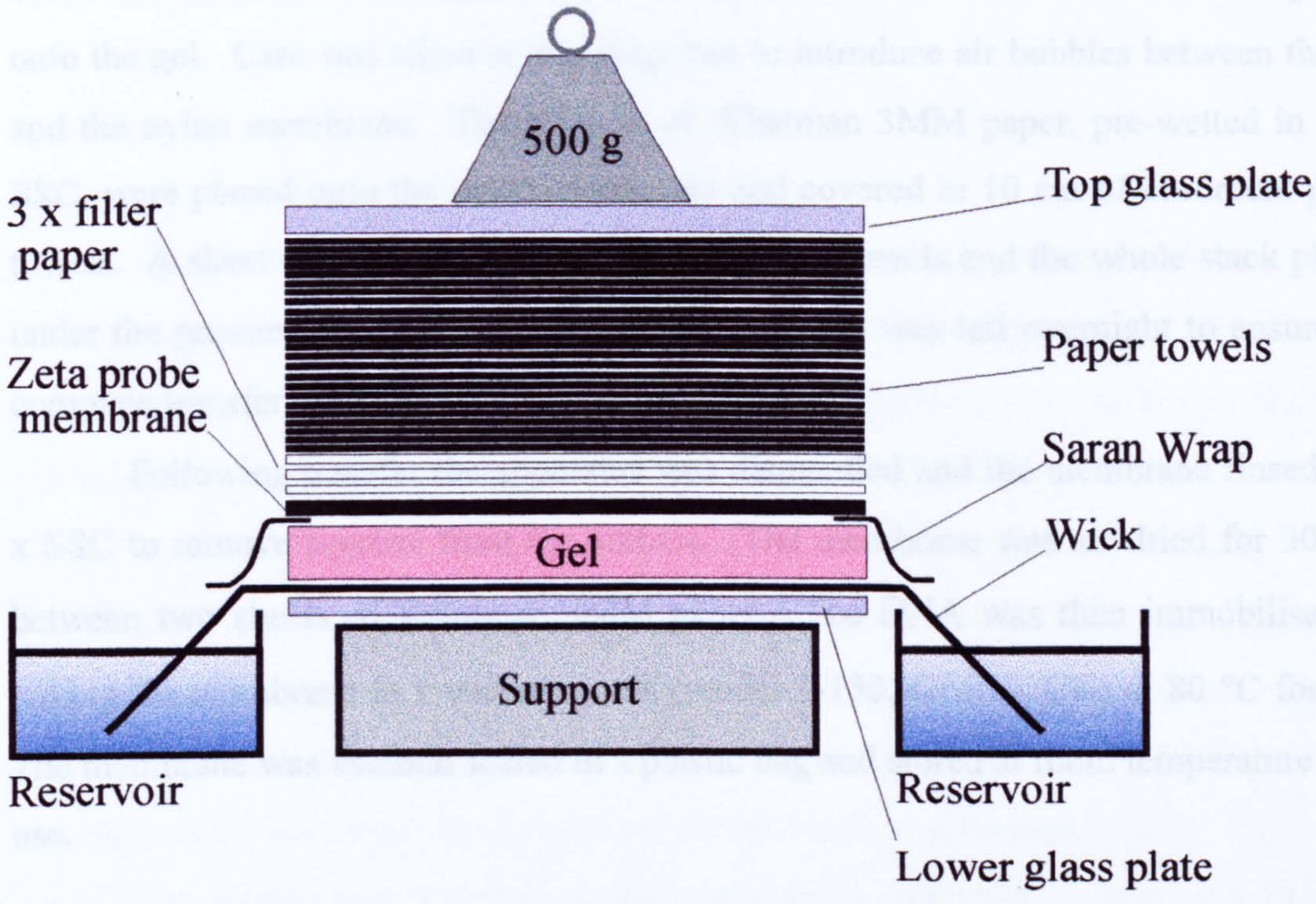



**Figure 2.3 Diagrammatic representation of Northern and Southern blotting apparatus.** Nucleic acids were transferred to a nylon membrane by capillary action as described in Materials and Methods.

NaCl, 0.15 M sodium citrate, pH 7.0) taking care not to trap any air bubbles under the gel. The wick was supported on a glass plate and the ends of the wick immersed in reservoirs containing 300 ml of 10 x SSC buffer. A rim of plastic film (Saran Wrap™, The Dow Chemical Company, UK) was placed around the edge of the gel to ensure that capillary action took place only through the gel. A piece of nylon blotting membrane cut to the same size as the gel and pre-wetted in distilled water for 5 min was placed onto the gel. Care was taken at this stage not to introduce air bubbles between the gel and the nylon membrane. Three layers of Whatman 3MM paper, pre-wetted in 10 x SSC, were placed onto the nylon membrane and covered in 10 cm of absorbent paper towels. A sheet of glass was placed onto the paper towels and the whole stack placed under the pressure of a 500 g weight. The apparatus was left overnight to ensure the complete transfer of the DNA to the nylon membrane.

Following transfer the apparatus was dismantled and the membrane rinsed in 3 x SSC to remove agarose from the surface. The membrane was air dried for 30 min between two sheets of Whatman 3MM paper. The DNA was then immobilised by baking the membrane in a vacuum oven (model 1-150, Griffin, UK) at 80 °C for 1 h. The membrane was vacuum sealed in a plastic bag and stored at room temperature until use.

## 2.2.17 Extraction of RNA from plant material

RNA was extracted from various organs of *A. thaliana* and from developing leaves and used to generate Northern blots for the examination of the expression of five invertase genes in those tissues. Samples of leaves (L1-L4) and flowering stems from soil grown plants were harvested at noon, three hours into the photoperiod. Samples were placed into foil packets and immediately frozen in liquid nitrogen. Samples of aseptically grown seedling roots and cotyledons were harvested and frozen at the same time. All tissue was placed at –80 °C for storage.

***Preparation of solutions and apparatus.***

To avoid the degradation of RNA by RNA nucleases (RNases) all glassware,

mortars, pestles and spatulas were baked at 180 °C for 8 h. In addition all disposable plasticware was used directly from unopened packs and autoclaved prior to use. Non-disposable plastics were soaked in distilled water containing 0.1% (v/v) diethylpyrocarbonate (DEPC) at 37 °C overnight to inactivate RNAses. The DEPC was subsequently removed by autoclaving. All solutions were treated with DEPC as above or used directly from high quality unopened sources.

*Extraction of RNA*

RNA was prepared from plant tissue using an adapted method of Loening (1969). Five g of sample was ground into a fine powder in mortar and pestle with liquid nitrogen. The tissue, while frozen, was transferred into a 30 ml Teflon Oakridge centrifuge tube (Nalgene, Rotherwas, UK). Ten ml of Tris-saturated phenol (liquid phenol equilibrated with 100 mM Tris-Cl, pH 8.0) and 10 ml of Kirby buffer [100 mM Tris-Cl buffer, pH 8.0, 1% (w/v) triisopropylnaphthalene sulphonic acid (TNS), 6% (w/v) paraaminosalicylic acid (PAS), 1% (w/v) NaCl, 6% (v/v) Tris-saturated phenol, pH 8.0] were added to the sample and any clumps of tissue broken-up with a spatula so that the powder was immediately exposed to the buffer as it thawed.

The sample was immediately homogenised 3 times for 15 s using a Polytron (Kinematica, Luzern, Switzerland) on power setting 8. The sample was kept on ice between each homogenisation to allow any foaming to recede. The sample was centrifuged at 12 000 *g* for 5 min at 4 °C and the upper aqueous layer transferred to a clean tube. The lower phase was retained, re-extracted with 5 ml of Kirby buffer, centrifuged (after vortexing) as before and the aqueous layer pooled with the first. The lower organic layer was discarded and the aqueous layer extracted with an equal volume of Tris-saturated phenol and centrifuged as above.

The aqueous layer was transferred to a clean tube and 0.1 volumes of NaCl and 2 volumes of absolute ethanol added. The sample was incubated for 4 h at -20 °C to precipitate the nucleic acids. Following centrifugation at 14 000 *g* for 30 min at 4 °C the supernatant was carefully discarded and the pellet washed with 5 ml of 70 % (v/v) of ice cold ethanol. The ethanol wash was discarded and the pellet allowed to air dry. The pellet was re-suspended in 1.5 ml of distilled water and transferred to an eppendorf

tube. The RNA was precipitated by the addition of 1/3 volume of 8 M LiCl and incubated overnight at 4 °C. The RNA was recovered by centrifugation at 20 000 *g* for 15 min at 4 °C and the pellet gently washed in 1 ml of 70 % (v/v) ice cold ethanol. The sample was centrifuged as before and the supernatant discarded. The pellet was dried in a centrifugal vacuum evaporator (Speedvac, Savant, France) before re-suspending in 1 ml of distilled water. Insoluble material was removed from the sample by centrifugation and the purified RNA stored at –80 °C.

*Quantification and quality of purified RNA*

The amount of RNA extracted from plant tissue was measured using UV spectroscopy as described in section 2.2.9. The amount of RNA in the cuvette was calculated by assuming that a 40 μg $ml^{-1}$ solution of RNA has an absorbance at 260 nm of 1.0. The purity of the isolated plasmid DNA was assessed by the ratio of the absorbances at 260 nm and 280 nm. Pure RNA has a ratio of approximately 1.8-2.0.

The integrity of the purified RNA extract was examined by gel electrophoresis. One μg of RNA was applied to a non-denaturing 1% (w/v) agarose gel as described in section 2.2.14 and electrophoresed at 150 mA for 1 h. The RNA was stained by immersion of the gel in 0.5 μg $ml^{-1}$ ethidium bromide. Intact RNA showed two distinct bands of 18S and 28S ribosomal RNAs and an indistinct smear of messenger RNA when viewed on a UV transilluminator (Genetic Research Instrumentation Ltd, Felsted, UK). Contamination of the RNA with genomic DNA could be seen as a sharp, high molecular weight, band on the stained gel.

## 2.2.18 Northern blotting

RNA extracted from the leaves (L1-L4) and flowering stems of soil grown plants and from the roots and cotyledons of aseptically grown seedlings were used to generate Northern blots. These blots were used to examine the expression of five invertase genes in *A. thaliana*.

*Sample preparation*

Sample RNA was mixed with an equal volume of RNA gel loading buffer (50% (v/v) deionised formamide, 6% (v/v) formaldehyde in 1 x MOPES buffer. MOPES buffer is 20 mM MOPS, pH 7.0 (3-[N-Morpholino]propanesulphonic acid), 5 mM sodium acetate, 1 mM EDTA). One-fifth volume of RNA gel loading buffer was added to the sample and heated to 65 °C for 5 min and left on ice until use.

*Denaturing gel electrophoresis*

RNA samples were separated by denaturing gel electrophoresis. Two g of agarose was dissolved in 148 ml of distilled water and 20 ml of 10 x MOPES buffer by heating in a microwave. The solution was cooled to 55 °C and 32.4 ml of formaldehyde (37% aqueous solution) added and mixed. The solution was poured into a gel casting tray of a horizontal gel electrophoresis system (Sub-cell, Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and left to solidify in a fume hood.

Equal amounts of RNA samples were loaded onto the gel (typically 5-20 µg of RNA per lane) and the electrophoresis performed at 75 V (constant voltage) for 3 h in 1 x MOPES running buffer. After electrophoresis the RNA was visualised by immersing the gel in 1 x MOPES buffer containing 0.5 µg $ml^{-1}$ ethidium bromide for 30 min with rocking. The gel was de-stained for 15 min in 1 x MOPES and the RNA examined on a UV transilluminator (Genetic Research Instrumentation Ltd, Felsted, UK). Two bands of ribosomal RNA could be seen following successful gel electrophoresis. The relative intensity of these bands was used to assess the equality of loading across the gel.

*Blotting*

The capillary transfer of RNA from a denaturing gel to a nylon membrane (Zetaprobe GT, Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) was achieved as described in section 2.2.16 and the equipment set-up as shown in Fig. 2.3 with one exception; the gel was blotted directly following nucleic acid visualisation and required no pre-treatment before blotting.

Following overnight blotting the membrane was gently washed in 3 x SSC to remove any agarose, air dried and the RNA immobilised as described in section 2.2.16.

## 2.2.19 Hybridisation of radioactive probes to Northern and Southern blots

*Hybridisation*

Baked nylon membranes (Zetaprobe GT, Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) with bound DNA (Southern blot) or RNA (Northern blot) were slowly immersed in 3 x SSC buffer to avoid trapping air bubbles within the structure of the membrane. The wetted membranes were placed between two nylon meshes (Biometra, UK). Up to four nylon membranes could be hybridised together provided they were separated by nylon meshes. The assembled blots and meshes were rolled up and placed in a glass hybridisation tube (Hybaid, Teddington, UK). The blots were incubated in 100 ml of hybridisation buffer (0.5 M Na-phosphate buffer, pH 7.2, 7% (w/v) SDS) at 65 °C for 30 min while rotating in a hybridisation oven (Mini Oven MK II, Hybaid, Teddington, UK). The hybridisation buffer was discarded and replaced with 50 ml of the same solution and rotated in the hybridisation oven at 65 °C while final preparations were made to the radioactive probe. The radioactive probe, prepared as described in section 2.2.14, was denatured by boiling for 5 min and then immediately chilled by placing on ice. The probe was added to the hybridisation tube containing the blots and rotated in the hybridisation oven at 65 °C overnight.

*Washing of blots*

The hybridisation buffer containing the unbound radioactive probe was discarded. The blots were washed 3 times in 50 ml of washing buffer (40 mM Na-phosphate buffer, pH 7.2, 5% (w/v) SDS) and 3 times in 50 ml of washing buffer with 1% (w/v) SDS with the washes being discarded each time. Each wash was performed at 65 °C for 30 min while rotating in a hybridisation oven. After washing the blots were removed from the glass hybridisation tube and prepared for autoradiography.

*Autoradiography*

The blot (Southern or Northern) was completely wrapped with a single layer of clear plastic film (Saran Wrap™, The Dow Chemical Company, UK) and placed on a piece of card for support. Onto the corners of the blot were placed phosphorescent stickers to mark the original orientation of the blot on the resulting autoradiograph. The blot was placed into a cassette (Hypercassette™, Amersham International plc, Little Chalfont, UK) between a layer of pre-flashed X-ray film (Biomax™ MS-1 Film, Eastman Kodak Company, Rochester, USA) and an intensifying screen and placed at –80 °C for between an hour and a week depending on the strength of the radioactive signal. The X-ray film was pre-flashed with a photographic flash gun to increase the sensitivity of the film and improve the linearity of the film response to radiation (Sambrook *et al.*, 1989). The effect of pre-flashing had been modified empirically to reduce the transmission of the X-ray film by 10%.

The X-ray film was developed using 1/4 strength PQ Universal Developer (Ilford, Mobberley, UK) for 2 min and fixed using 1/9 strength Hypam Rapid Fixer (Ilford, Mobberley, UK) for 2 min. The X-ray film was rinsed under running water for 30 min and allowed to air dry.

*Re-probing of Northern blots*

Northern blots to be used for further radioactive probing were not allowed to dry out between hybridisations. The blot was washed twice in 1 L of stripping solution (0.1 x SSC, 0.5% (w/v/) SDS) at 95 °C for 20 min to remove traces of the hybridised probe. The blot was stored moist at 4 °C until use.

## 2.2.20 Reverse transcriptase polymerase chain reaction

Northern hybridisation was used for the examination of five invertase genes during the development of *A. thaliana* but failed to detect the expression of ATβFRUCT 1 and 2 (apoplastic invertase genes). The low expression of these genes necessitated the use of more sensitive detection techniques and the reverse transcriptase

polymerase chain reaction (RT-PCR) was the method of choice.

RT-PCR offers a unique method for the detection of extremely rare messenger RNA (mRNA) transcripts. Traditional methods for the detection and analysis of RNA molecules including Northern, dot and slot blotting allow for the rapid processing of large numbers of samples but require 0.1 to 1.0 pg of target sequence or $10^5$-$10^6$ average mRNA molecules (Kawasaki, 1990). With the adaptation of the polymerase chain reaction (PCR) to amplify cDNA made from mRNA this level of detection has been decreased by several orders of magnitude.

PCR is a method for nucleic acid synthesis that can be targeted to amplify specific regions of DNA and allows the target sequence to be visualised as a distinct band/s on an agarose gel. During a PCR reaction DNA polymerase synthesises DNA between two oligonucleotide primers that flank opposite strands of the target DNA. Amplification is a three step process that begins with the high temperature denaturation of DNA hybrids followed by the annealing of the primers to their complementary sequences which is initiated by a decrease in temperature. In the final step DNA synthesis extends across the region between the two primers. As each of the newly synthesised DNA sequences themselves contain primer binding sites successive cycles of synthesis can theoretically result in the exponential increase of the target sequence. A DNA polymerase originally extracted from the thermophilic bacteria *Thermophilus aquaticus* (*Taq* DNA polymerase) that has a half-life at 95 °C of 40 min+ (Innis and Gelfand, 1990) enables many cycles of PCR to be carried out without need for further addition of enzyme activity.

In an extension of the principles of PCR specific mRNAs can be detected from a complex pool of total RNA. The enzyme reverse transcriptase is used to make an initial DNA copy (cDNA) from µg quantities of total RNA. The cDNA is then used in a normal PCR reaction. This approach gives valuable information about the presence or absence of specific sequences but due to tube to tube variability exaggerated during the exponential phase of PCR can offer no information about the relative abundance of messages between different samples. Even when the material to be examined has been pooled, copied into cDNA but amplified separately on the same run significant differences in yield still occur. This may be due to small temperatures variations across the thermal block (Gilliland *et al.*, 1990).

Several methods have been used to take account of the inherent variability of PCR and so produce quantitative data, these include competitive PCR (Gilliland *et al.*, 1990) and methods that include the use of primers specific for constitutively expressed genes (Tymowska-Lalanne *et al.*, 1996). Another method that obviates these problems is the use of an internal standard that utilises the same primer sites as the target sequence but can be distinguished from it by a change in the sequence length. When this internal standard is introduced as an RNA then even differences in the efficiency of reverse transcription between samples can be assessed. Described below is a quantitative method for the measurement of the expression of the apoplastic invertase gene ATβFRUCT 1 in *A. thaliana* using RT-PCR and an internal RNA standard. The expression of a second apoplastic invertase gene in *A. thaliana*, ATβFRUCT 2 was also examined using RT-PCR but in a non-quantitative manner.

***Design and synthesis of oligo-nucleotides***

Gene specific primers were designed for two apoplastic invertase genes present in *A. thaliana*, ATβFRUCT 1 and ATβFRUCT 2. Due to the close relationship between these two genes the nucleotide sequences (GenBank ID X74515 and U11033 respectively) were examined to find regions of low sequence homology. Primer sites were chosen to minimise the chance of cross hybridisation with similar regions of the other apoplastic invertase gene. In addition, primers were designed to hybridise to regions spanning an intron. This enabled amplified genomic DNA and amplified cDNA to be distinguished due to a difference in the size of the resulting product. A computer programme (Primer Designer Version 2.0, Scientific and Educational Software, UK) was used to design all the primers and checked for problems that might reduce the efficiency of PCR such as primer hairpin formation, primer dimer formation and high G/C content. The sequence and position of hybridisation of the oligonucleotides are shown in Table 2.2 and Fig. 2.7 respectively.

Oligonucleotides were synthesised at the Krebs Sequencing and Synthesis Unit (University of Sheffield, UK). Oligonucleotides were quantified in a spectrophotometer (UV-2101PC scanning spectrophotometer, Shimadzu, Kyoto, Japan) as described in section 2.2.9, diluted to a 10 μM stock and stored at –20 °C.

**Table 2.2 Primers used for the analysis of apoplastic invertase gene expression in *A. thaliana* using RT-PCR**

| Gene | Primer | Primer sequence | Position | Size of genomic product | Size of product from cDNA |
|---|---|---|---|---|---|
| ATβFRUCT 1 (apoplastic invertase 1) | ATβFRUCT 1A | 5′-CCCTTTCCACCGAAACTCTCCACT-3′ | 2211 | 782 bp | 349 bp |
| | ATβFRUCT 1B | 5′-GTGAGAGACTTGGAGAAAGCGGAT-3′ | 2992 | | |
| ATβFRUCT 2 (apoplastic invertase 2) | ATβFRUCT 2A | 5′-CCTTTTGCTCCAAAGCTCTCCACA-3′ | 1885 | 447 bp | 250 bp |
| | ATβFRUCT 2B | 5′-GTTTGATCACGTTGGCTACATCTG-3′ | 2331 | | |

*Preparation of RT-PCR standard*

To produce an internal standard of high quality several criteria must be fulfilled. The first of these is that the target sequence and the internal standard should be amplified with the same efficiency. One way to ensure this is to design an internal standard which utilises the same primer binding sites as the target sequence and also shares a similar DNA sequence. The second important criterion is that following amplification the internal standard and target sequence should be distinguishable from one another. To achieve this the standard is modified either by the addition or subtraction of a small amount of sequence or alternatively a novel restriction enzyme site can be introduced by point mutation allowing it to be modified in length after amplification. The conflict between these two important criteria is resolved by producing an internal standard with the minimum changes necessary to enable it to be distinguished from the target sequence following amplification. The steps in the production of the internal standard used for the quantification of ATβFRUCT 1 gene expression can be seen in the schematic diagram Fig. 2.4 and are described below.

To produce an internal standard genomic DNA isolated from *A. thaliana* (Section 2.2.15) was used as a template for the amplification of a 782 bp region of the apoplastic invertase gene ATβFRUCT 1 using the primers ATβFRUCT 1 'A' and 'B' as shown in Figure 2.4. Barrier pipette tips (Aeroseal™ Gold Filter, Fisher Scientific, Loughborough, UK) and clean sterile plasticware were used for all steps to avoid transfer of contaminating DNA. The following reaction mix was placed into a 500 µl thin-walled PCR tube (Thermo-tube, Radleys, Saffron Walden, UK) and made up to a total volume of 100 µl with distilled water:

| **PCR reaction stock solutions** | **Final concentration** |
|---|---|
| 10 µM Primer 'A' | 0.05 µM |
| 10 µM Primer 'B' | 0.05 µM |
| 10 x PCR buffer (Promega, Southampton, UK) | 1 x |
| 25 mM $MgCl_2$ (Promega, Southampton, UK) | 1.5 mM |
| 1 mM Mixed dNTP's (GibcoBRL, Paisley, UK) | 150 µM |
| Genomic DNA | 10 µg $ml^{-1}$ |

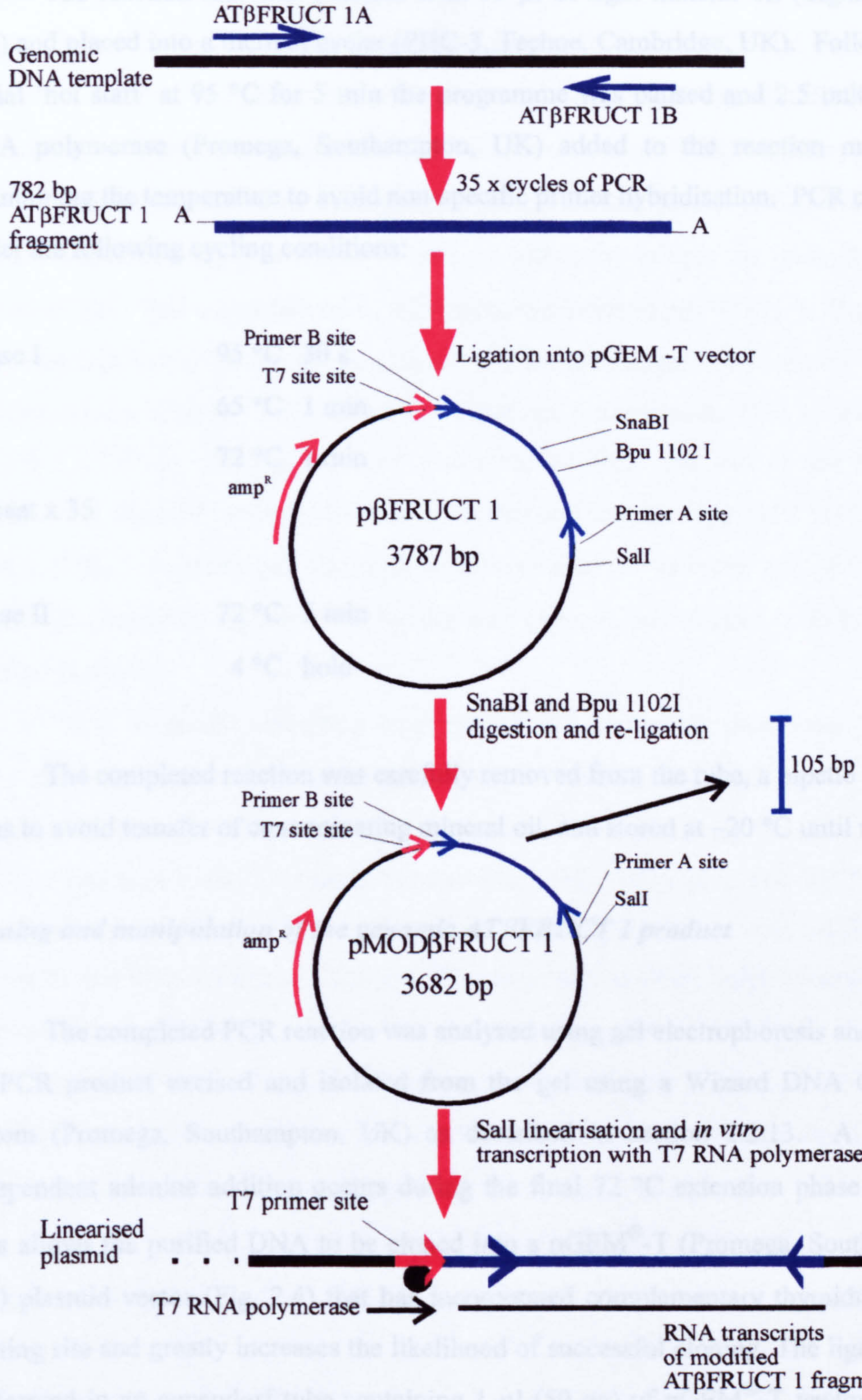



**Figure 2.4 Schematic diagram showing the development of an RNA internal standard.** An RNA internal standard was prepared for the semi-quantitative measurement of ATβFRUCT 1 gene expression in *A. thaliana*.

The reaction mix was overlaid with 50 µl of light mineral oil (Sigma, Poole, UK) and placed into a thermal cycler (PHC-3, Techne, Cambridge, UK). Following an initial 'hot start' at 95 °C for 5 min the programme was paused and 2.5 units of Taq DNA polymerase (Promega, Southampton, UK) added to the reaction mix while maintaining the temperature to avoid non-specific primer hybridisation. PCR continued under the following cycling conditions:

| | | |
|---|---|---|
| Phase I | 95 °C | 30 s |
| | 65 °C | 1 min |
| | 72 °C | 1 min |
| Repeat x 35 | | |
| Phase II | 72 °C | 5 min |
| | 4 °C | hold |

The completed reaction was carefully removed from the tube, a pipette was used so as to avoid transfer of contaminating mineral oil, and stored at –20 °C until use.

***Cloning and manipulation of the genomic ATβFRUCT 1 product***

The completed PCR reaction was analysed using gel electrophoresis and the 782 bp PCR product excised and isolated from the gel using a Wizard DNA Clean-Up system (Promega, Southampton, UK) as described in section 2.2.13. A template independent adenine addition occurs during the final 72 °C extension phase of PCR. This allows the purified DNA to be cloned into a pGEM®-T (Promega, Southampton, UK) plasmid vector (Fig. 2.4) that has incorporated complementary thymidine at the cloning site and greatly increases the likelihood of successful cloning. The ligation was performed in an eppendorf tube containing 1 µl (50 ng) of pGEM®-T vector, 7 µl of PCR insert (with a 3:1 molar ratio of PCR insert to vector), 1 µl of 10 x ligation buffer and 1 µl (3 Weiss units) of T4 DNA ligase. The ligation reaction was performed at 16 °C for 24 h. The ligation reaction was heated to 65 °C for 15 min to destroy the activity of the T4 DNA ligase before transformation into *E. coli* (strain DH5α) competent cells

as described in sections 2.2.10 and 2.2.11.

Figure 2.4 shows the 782 bp genomic fragment of ATβFRUCT 1 cloned into a pGEM™-T vector. The plasmid was sequenced to ensure the correct identity of the cloned insert, as described in section 2.2.12, and renamed pβFRUCT 1. To produce an internal standard that could be distinguished from both amplified ATβFRUCT 1 cDNA and any contaminating genomic DNA present within the sample the internal standard was modified. This was achieved by shortening the insert in pβFRUCT 1 The plasmid was linearised using the restriction enzyme Sna B1 (Promega, Southampton, UK) and purified using a Wizard™ DNA Clean-Up (Promega, Southampton, UK) as described in section 2.2.13. A 105 bp fragment was removed from the end of the linearised pβFRUCT 1 plasmid by digestion with the restriction enzyme Bpu 1102 I (GibcoBRL, Paisley, UK). Agarose gel electrophoresis was used to examine the pβFRUCT 1 plasmid for complete digestion and the digested plasmid was isolated from the gel and purified as above.

The 5' overhangs resulting from restriction enzyme digestion were 'filled-in' using the large fragment of DNA polymerase I (Klenow fragment) prior to the re-ligation of the modified plasmid. A total reaction mix of 2.5 μg linearised plasmid DNA, 1 x Klenow buffer (Promega, Southampton, UK), 100 μM mixed dNTPs and 0.1 mg $ml^{1}$ acetylated Bovine Serum Albumin was made up to 100 μl with distilled water and incubated with 2.5 units of Klenow large fragment of DNA polymerase (Promega, Southampton, UK) for 15 min at room temperature. The reaction was stopped by incubation at 75 °C for 10 min and the plasmid purified using a Wizard™ DNA Clean-Up system as described above. The modified pβFRUCT 1 plasmid was ligated as described above and renamed pMODβFRUCT 1 as shown in Fig. 2.4.

***In vitro transcription***

The enzyme T7 RNA polymerase (Promega, Southampton, UK) was used to generate RNA transcripts using the modified insert in pMODβFRUCT 1 as a template. T7 RNA polymerase initiates transcription at a specific nucleotide sequence in the presence of appropriate salts and nucleotides. Figure 2.4 shows the location of this site upstream of the cloned ATβFRUCT 1 insert. High quality plasmid DNA is required

and was prepared using a Wizard™*Plus* Minipreps DNA Purification System (Promega, Southampton, UK), as described in section 2.2.9. In order to avoid the production of RNA transcripts from vector DNA and to ensure the transcripts were of equal length the plasmid was linearised using the restriction enzyme Sal I (Promega, Southampton, UK). The linearised plasmid was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), vortexed for 30 s, and centrifuged at 20 000 x *g* for 1 min. The aqueous phase was removed and mixed with 1 volume of chloroform:isoamyl alcohol (24:1), vortexed for 30 s and centrifuged as before. The aqueous phase was transferred to a clean eppendorf tube and the DNA precipitated on ice for 15 min with 0.1 volume of 3 M sodium acetate, pH 5.2 and 2 volumes of absolute ethanol. The plasmid DNA was recovered by centrifugation at 20 000 x *g* for 10 min at 4 °C. The supernatant was removed and the pellet washed in 1 ml of ice cold 70 % (v/v) ethanol and centrifuged as above. The supernatant was discarded and the DNA pellet dried in a centrifugal vacuum evaporator (Speedvac, Savant, France) the DNA was resuspended in RNase-free distilled water.

RNA transcripts were made by adding 5 µg of linearised plasmid into a clean eppendorf tube containing 5 x Transcriptase Optimised Buffer (Promega, Southampton, UK), 10 mM DTT, 100 units of Recombinant RNasin® Ribonuclease Inhibitor (Promega, Southampton, UK), 10 mM mixed NTPs, 100 units T7 RNA polymerase (Promega, Southampton, UK) and made up to 100 µl with RNase-free distilled water. The reaction was incubated at 37 °C for 1 h. Following transcription the DNA template was digested by incubation with 5 units of RQ1 RNase-Free DNase (Promega, Southampton, UK) for 30 min at 37 °C. The RNA was extracted with 1 volume of acid (pH 4.5) phenol:chloroform:isoamyl alcohol (25:24:1), vortexed for 30 s and centrifuged at 20 000 x *g* for 1 min. The aqueous supernatant was removed and vortexed for 30 s in 1 volume of chloroform:isoamyl alcohol (24:1), centrifuged as before and the aqueous supernatant transferred to a fresh tube. The RNA was precipitated on ice for 30 min in 0.5 volumes of 7.5 M ammonium acetate (RNase free) and 2 volumes of absolute ethanol and recovered by centrifugation at 20 000 x *g* for 10 min at 4 °C. The supernatant was discarded and the pellet washed in 1 ml of ice cold 70% (v/v) ethanol, centrifuged as above and the pellet dried in a centrifugal vacuum evaporator (Speedvac, Savant, France) and resuspended in 200 µl of RNase free

distilled water.

The amount of RNA was measured in a spectrophotometer (UV-2101PC scanning spectrophotometer, Shimadzu, Kyoto, Japan) as described in section 2.2.9 and diluted to a 100 ng $ml^{-1}$ stock and frozen at –80 °C until use.

### *Removal of contaminating DNA from RNA samples*

Samples of *A. thaliana* RNA extracted from developing leaves of soil grown plants (L1-L4) and from the roots and cotyledons of aseptically grown seedlings were treated with RNase-free DNase before use as a template for cDNA production; the RNA internal standard was also added at this stage. This enzyme removed small quantities of DNA carried over during the extraction of sample RNA and plasmid DNA used as the template for the RNA internal standard.

Five µg of sample RNA was placed into an eppendorf tube containing 1 unit of RQ1 RNase-free DNase (Promega, Southampton, UK), 150-200 pg of standard RNA and made up to 8 µl with RNAse free distilled water; the reaction was incubated at 37 °C for 30 min. The enzyme was inactivated by incubation at 75 °C for 5 min and the sample placed on ice for immediate use in cDNA synthesis.

### *cDNA synthesis*

cDNA was generated from 8 µl samples of DNAse-treated RNA using a commercially available kit (First-Strand cDNA Synthesis Kit, Pharmacia Biotech, Uppsala, Sweden). Five µl of Bulk First-Strand Reaction Mix was placed into an eppendorf tube containing 1 µl of DTT and 1 µl of random hexamer primers (all reagents supplied as part of kit). The 8 µl DNAse-treated sample containing the RNA standard was added to the reaction mix and pipetted gently to mix. The reaction was incubated at 37 °C for 1 h. Following incubation the sample was ready for immediate use in PCR.

*PCR of cDNA*

PCR has two distinct phases of amplification. During the first phase reagents are not limiting and amplification is exponential. During the second phase one or more of the reagents (primer, nucleotides, polymerase etc.) becomes limiting and amplification plateaus. Quantitative PCR is only possible during the exponential phase of PCR when differences in the amount of amplified product reflect differences in the amount of starting template and have not been influenced by limited reagents. An initial experiment was conducted to gauge the parameters of quantitative amplification. Internal standard RNA was used as a template to generate cDNA (as described above) and 1, 10, 20 , 50, 100 and 200 pg were amplified by PCR. Twenty µl aliquots were removed from the PCR reactions after 25, 30, 35 and 40 cycles. The aliquots were electrophoresed on 1.5% (w/v) agarose gels, the DNA stained using ethidium bromide and visualised by UV transilumination (see section 2.2.13). Following 25 cycles of PCR the amount of amplification was not sufficient to visualise the PCR products regardless of the amount of internal standard cDNA added to the reaction (data not shown). After 30 cycles of PCR the amplified internal standard was clearly visible with the fluorescence of the fragment proportional to the amount of cDNA added to the reaction (Fig 2.5). Following 35 or 40 cycles of amplification the fluorescence of the product was no longer an indication of the amount of cDNA added to the reaction as reagents had become limiting (Fig 2.5). From this initial experiment it was concluded that quantitative PCR was possible if the amplification products were analysed following 30 cycles of amplification with 10-100 pg of starting template.

RNA extracted from the developing leaves (L1-L4) and flowering stems of soil grown *A. thaliana* and from the cotyledons and roots of aseptically grown seedlings were used to generate cDNA as described above. For each cDNA sample three PCR reactions were conducted. Each reaction contained a different amount of cDNA and was amplified using primers specific for ATβFRUCT 1. This resulted in a range in the amount of amplified ATβFRUCT 1 product and internal standard following 30 cycles of PCR. Experimentation revealed that 0.05-5 µg of cDNA was required to visualise amplified ATβFRUCT 1 product on ethidium bromide stained gels after 30 cycles of PCR and this depended upon the tissue the RNA had been extracted from.

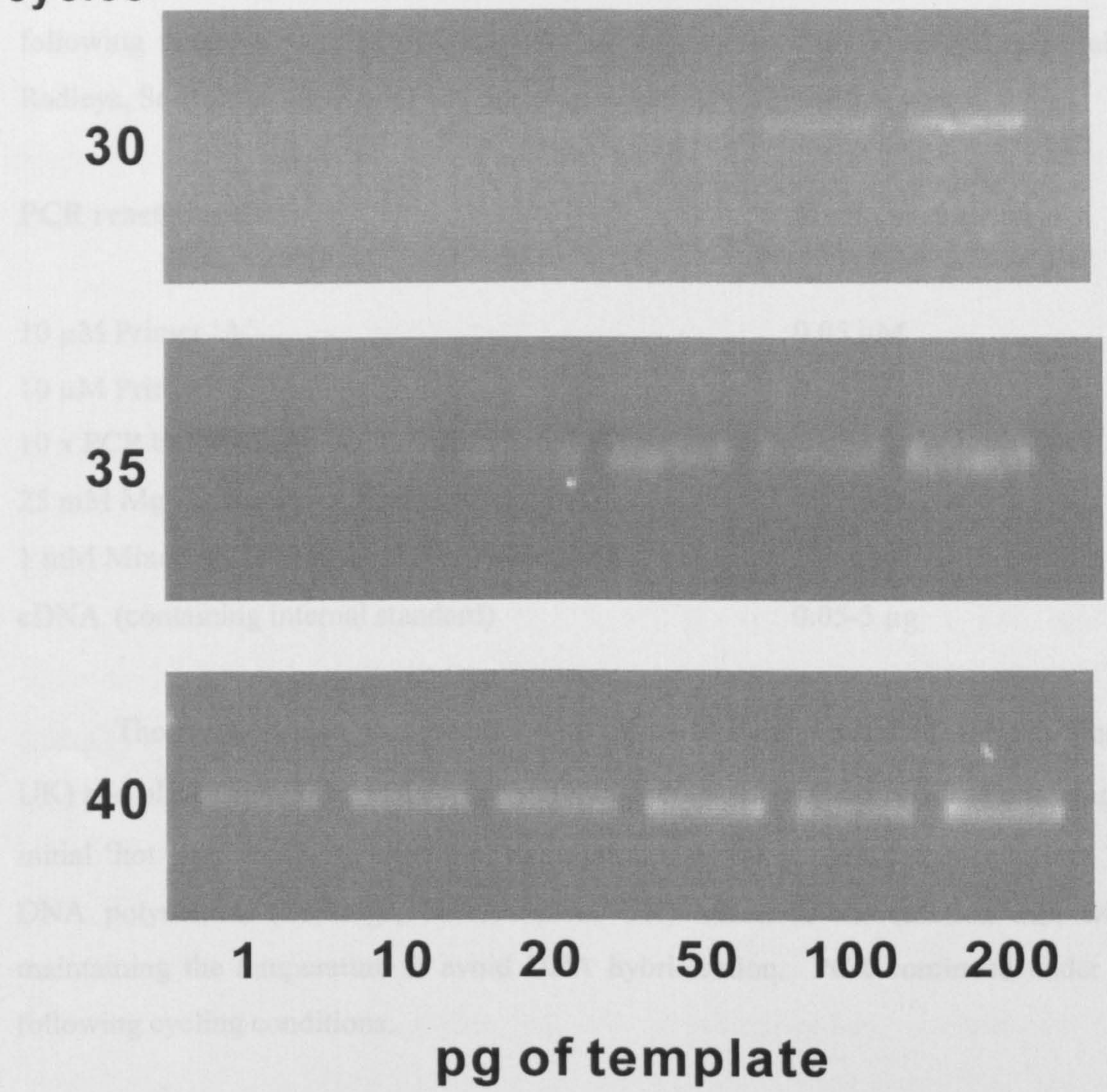



**Figure 2.5 A linear increase in the amount of PCR product is dependant on the number of cycles of amplification and the amount of starting template added to the reaction.** Different amounts of internal standard cDNA were added to PCR reactions and amplified for 25, 30, 35 and 40 cycles under standard conditions (see Materials and Methods). Twenty five cycles of PCR amplification was not sufficient for the visualisation of PCR product regardless of the amount of starting template (data not shown). Following 35 or 40 cycles of PCR the reagents had become limiting and the amount of product was not proportional to the amount of starting template. Following 30 cycles of amplification the amount of PCR product was proportional to the amount of starting template and fluorescence of the samples was visible over a wide range of initial starting template concentrations.

The PCR reaction mix was made as described below and barrier pipette tips and clean autoclaved plasticware were used to avoid DNA cross contamination. The following reagents were placed into 500 µl thin-walled PCR tubes (Thermo-tubes, Radleys, Saffron Walden, UK) and made up to 100 µl with distilled water:

| **PCR reaction mix** | **Final concentration** |
|---|---|
| 10 µM Primer 'A' | 0.05 µM |
| 10 µM Primer 'B' | 0.05 µM |
| 10 x PCR buffer (Promega, Southampton, UK) | 1 x |
| 25 mM $MgCl_2$ (Promega, Southampton, UK) | 1.5 mM |
| 1 mM Mixed dNTP's (GibcoBRL, Paisley, UK) | 150 µM |
| cDNA (containing internal standard) | 0.05-5 µg |

The reaction mix was overlaid with 50 µl of light mineral oil (Sigma, Poole, UK) and placed into a thermal cycler (PHC-3, Techne, Cambridge, UK). Following an initial 'hot start' at 95 °C for 5 min the programme was paused and 2.5 units of *Taq* DNA polymerase (Promega, Southampton, UK) added to the reaction mix while maintaining the temperature to avoid DNA hybridisation. PCR continued under the following cycling conditions:

| | | |
|---|---|---|
| Phase I | 95 °C | 30 s |
| | 65 °C | 1 min |
| | 72 °C | 1 min |
| Repeat x 30 | | |
| Phase II | 72 °C | 5 min |
| | 4 °C | hold |

The completed reaction was carefully removed from the tube, a pipette was used so as to avoid transfer of contaminating mineral oil, and stored at –20 °C until use.

The expression of the apoplastic invertase gene ATβFRUCT 2 was examined in

developing *A. thaliana*. This examination was performed in a non-quantitative way and was used to detect the presence or absence of expression, not the amount of expression. Samples of RNA from developing leaves and flowering stems of soil grown plants and from the cotyledons and roots of aseptically grown seedlings were examined. The reaction mix was prepared as above but contained more cDNA (5 μg), no internal standard and was amplified for 40 cycles.

***Imaging of the amplified ATβFRUCT 1 fragment***

Twenty μl of PCR reaction was mixed with 1/6$^{th}$ volume of gel loading solution [30% (w/v) glycerol, 0.25% (w/v) xylene cyanol FF (Sigma, Poole, UK), 0.1 M EDTA (pH 8.0)]. The samples were loaded onto a 1 x TAE agarose gel solidified with 1.5% (w/v) agarose (Sigma, Poole, UK) and cast into a submarine, horizontal gel apparatus (Wide Mini-Subcell, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Samples and DNA markers (1 Kb ladders, GibcoBRL, Paisley, UK) were loaded and electrophoresed at 150 mA for 1 h using 1 x TAE as a running buffer.

The DNA was stained by immersion of the gel in 1 x TAE containing 0.5 μg ml$^{-1}$ ethidium bromide for 20 min. Excess ethidium bromide was removed by immersion of the gel in 1 x TAE for 20 min.

The DNA was visualised by UV transillumination .(Genetic Research Instrumentation Ltd., Felsted, UK) and the image captured using a chilled CCD camera (C5985, Hamamatsu, Japan) with a Tiffin orange no. 15 filter (Tiffin, USA) and a Schott BG 18 filter (Schott, Mainz, Germany). Optimas image processing software (BioScan Inc., Edmonds, USA) was used to analyse the intensity of the fluorescing DNA fragments.

***Quantification of ATβFRUCT 1 gene expression***

Quantification of ATβFRUCT 1 gene expression was made possible by the inclusion of an internal standard. Because the amount of internal standard added to each RT-PCR reaction was known precisely, the fluorescence of this fragment could be related directly to the fluorescence of the ATβFRUCT 1 fragment. This enabled the

amount of ATβFRUCT 1 mRNA in the sample to be calculated. In the first step of this calculation a ratio of the fluorescence of the ATβFRUCT 1 fragment ($f^{inv}$) to the fluorescence of the standard ($f^{st}$) was made ($f^{inv}/f^{st}$). In the second step of the calculation this ratio was multiplied by the amount (g) of internal standard added to the reaction to give the amount (g) of ATβFRUCT 1 mRNA in the sample.

However, this calculation assumed that there was a linear relationship between the amount of DNA in the gel and the amount of DNA fluorescence. To test this assumption the fluorescence of known amounts of DNA, of approximately the same size as the internal standard and ATβFRUCT 1 fragments (electrophoresed, stained and visualised as described above) was examined. This examination revealed that from 0 to 0.4 μg of DNA fluorescence was linearly related to the amount of DNA in the gel (Fig. 2.6). Under the PCR conditions described above internal standard and ATβFRUCT 1 production was well within this range.

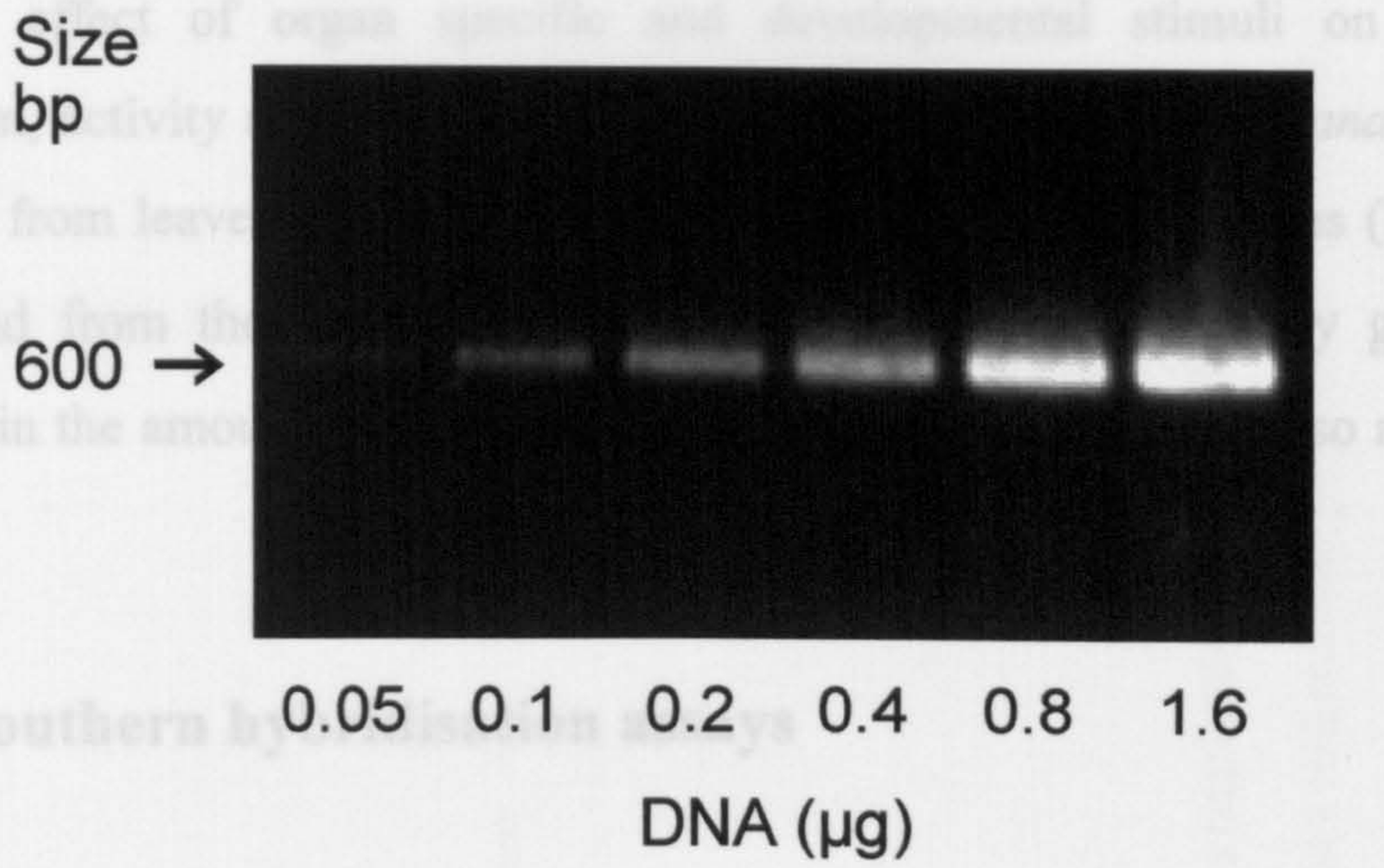



**Figure 2.6 The relationship between the amount of DNA and its fluorescence under UV light.** Different amounts of DNA (of similar size to that produced by the PCR amplification of *A. thaliana* DNA using ATβFRUCT 1 primers) were electrophoresed through agarose gels, stained with ethidium bromide and imaged following UV transillumination.

## 2.3 RESULTS

The effect of organ specific and developmental stimuli on invertase gene expression, activity and isoform pattern were examined in *A. thaliana*. Samples were collected from leaves of different ages (L1-L4) and flowering stems (F) of soil grown plants and from the cotyledons (C) and roots (R) of aseptically grown seedlings. Changes in the amount of soluble carbohydrates and starch were also analysed in these tissues.

### 2.3.1 Southern hybridisation assays

Total genomic DNA from *A. thaliana* was digested with the restriction enzymes BamHI, HindIII and EcoRI and used to construct a series of Southern blots. These blots were used to examine the gene specificity of five DNA probes which were constructed from portions of the cloned genes (ATβFRUCT 1 and 2 probes) or from portions of Expressed Sequence Tags (ATβFRUCT 3, 4 and 5 probes). The invertase genes ATβFRUCT 1-4 have been cloned and sequenced and are shown diagramatically in Fig 2.7 (Schwebel-Dugue *et al.*, 1994; Mercier and Gogarten, 1995; Tymowska-Lalanne *et al.*, 1997).

Southern hybridisation assays using the five invertase gene probes yielded a pattern of hybridising fragments (Fig 2.8) consistent with the published gene sequences and with the Southern blots of Haouazine-Takvorian *et al.* (1996) and Tymowska-Lalanne *et al.* (1997) (Fig. 2.7).

Southern hybridisation of the ATβFRUCT 1 probe yielded a 4.2 kb EcoRI fragment (Fig. 2.8) which was consistent with the published gene sequence and that observed by Haouazine-Takvorian *et al.* (1996) (Fig. 2.7). As the HindIII site downstream of the cloned ATβFRUCT 1 gene has not been sequenced the 4.6 kb hybridising fragment observed cannot be verified (Fig. 2.7 and 2.8). The same is true of the ATβFRUCT 2 gene for which none of the restriction enzyme sites downstream of the cloned fragment have been sequenced.

However, hybridisation of the ATβFRUCT 3 probe did yield the expected 2.5 kb EcoRI fragment (Fig. 2.8) as was also seen by Tymowska-Lalanne *et al.* (1997) (Fig.

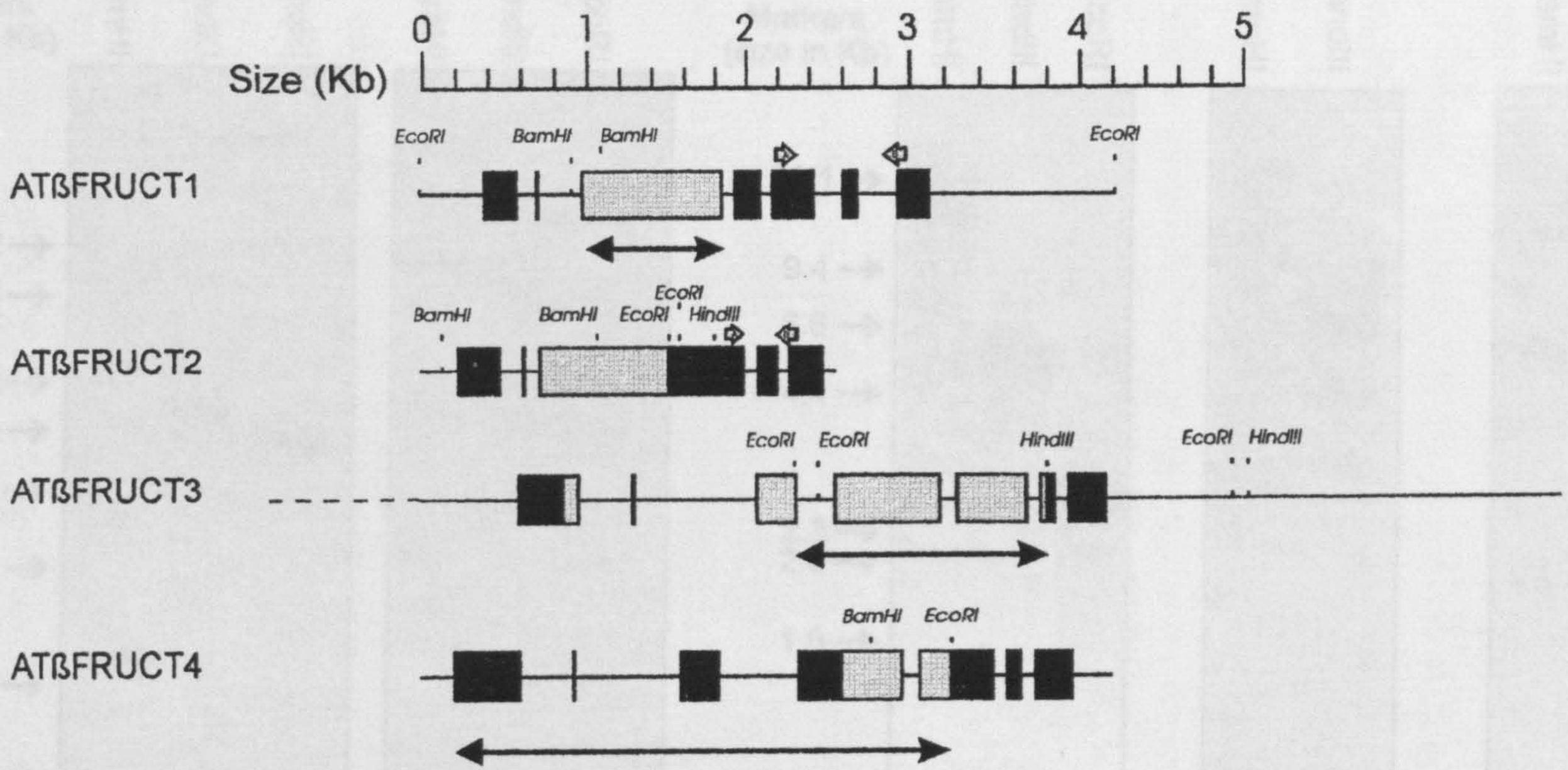



**Figure 2.7 Schematic illustration of the organisation of the *A. thaliana* genes encoding apoplastic (ATβFRUCT 1 and 2) and vacuolar (ATβFRUCT 3 and 4) invertase genes showing major restriction enzyme sites.** Closed boxes indicate the position of exons while grey boxes indicate the position of the probes used for this thesis. The position of primers used for RT-PCR gene expression work are indicated by short grey arrows. The position of probes used by Haouazine-Takvorian *et al.* (1996) for the examination of ATβFRUCT 1 and Tymowska-Lalanne *et al.* (1997) for the examination of ATβFRUCT 3 and 4 are shown by long black arrows. ATβFRUCT 1 was cloned and sequenced by Schwebel-Dugue *et al.* (1994), ATβFRUCT 2 was cloned and sequenced by Mercier and Gogarten (1995) and ATβFRUCT 3 and 4 were cloned and sequenced by Tymowska-Lalanne *et al.* (1997).

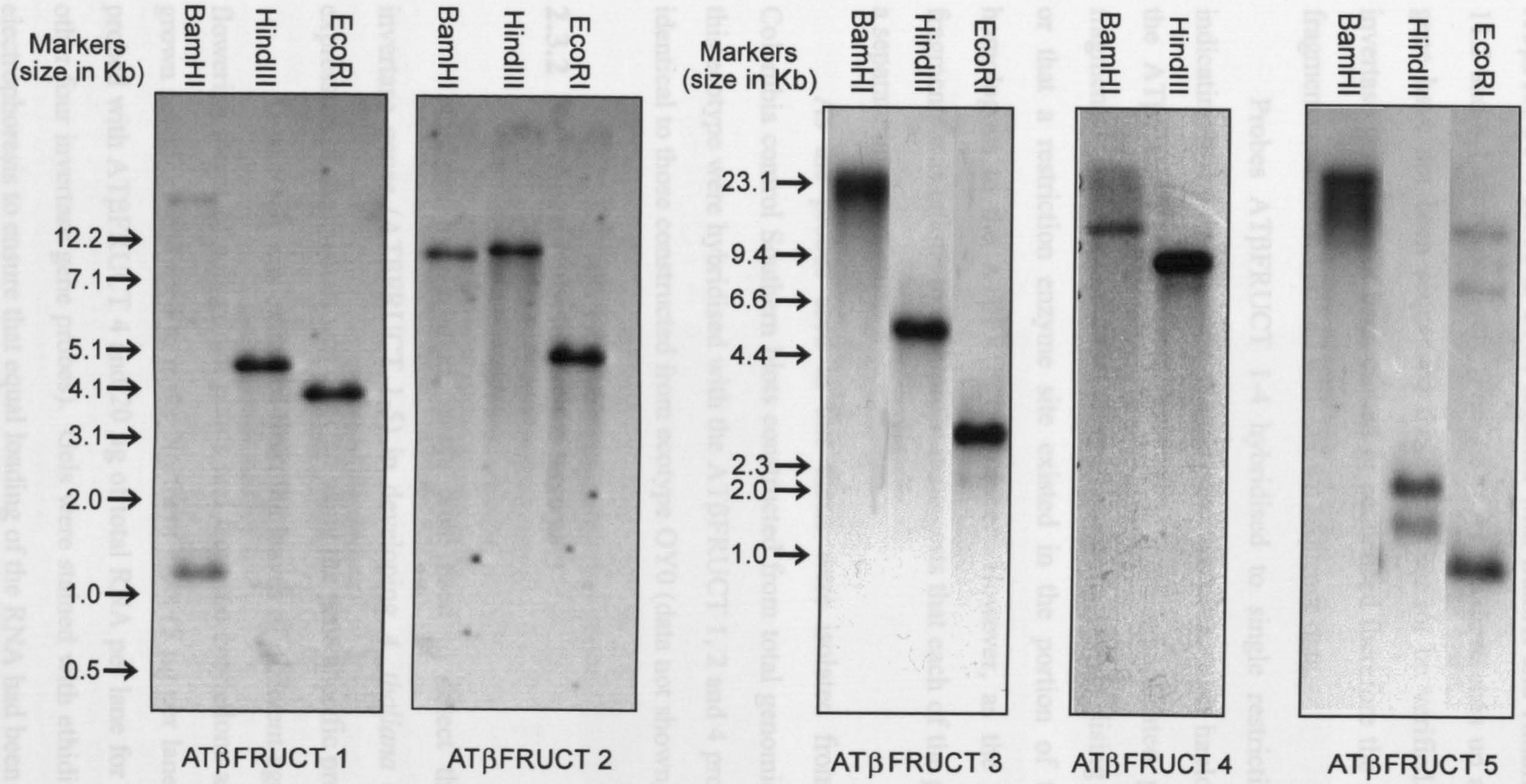



**Figure 2.8 Southern hybridisation assay of five invertase genes in *A. thaliana*.** DNA extracted from *A. thaliana* (ecotype OY0) was digested with restriction enzymes and used to prepare a series of Southern blots (5 µg of DNA per lane). Blots were then hybridised to radiolabelled DNA probes with sequence homology to five invertase genes.

2.7). As the HindIII and BamHI sites up and downstream of the cloned gene have not been sequenced the large hybridising fragments cannot be verified. Hybridisation of the ATβFRUCT 4 probe to DNA digested with BamHI and HindIII yielded fragments of 11.5 and 8.1 kb respectively (Fig. 2.8). As the these sites up and downstream of the gene have not been sequenced these sizes cannot be verified. The ATβFRUCT 5 invertase gene has not been cloned or sequenced therefore the size of the hybridising fragments cannot be verified with known sequence data.

Probes ATβFRUCT 1-4 hybridised to single restriction enzyme fragments indicating that only one copy of each gene was present per haploid genome. However, the ATβFRUCT 5 probe resulted in a more complicated pattern of hybridising fragments. This suggested that the probe was either hybridising to more than one gene or that a restriction enzyme site existed in the portion of the *A. thaliana* DNA homologous to the ATβFRUCT 5 probe. However, as the pattern of hybridising fragments was unique to each probe it suggests that each of the probes was specific for a separate invertase gene.

As the probes used in this thesis were isolated from *A. thaliana* ecotype Colombia control Southern blots constructed from total genomic DNA extracted from this ecotype were hybridised with the ATβFRUCT 1, 2 and 4 probes. These blots were identical to those constructed from ecotype OY0 (data not shown).

### 2.3.2 Northern hybridisation assays

Northern hybridisation assays were used to detect the expression of five invertase genes (ATβFRUCT 1-5) in developing *A. thaliana* (ecotype OYO). The expression of these genes was detected using the gene specific probes described above.

Total RNA was extracted from the leaves of different ages (classes L1-L4) and flowering stems of soil grown plants and from the cotyledons and roots of aseptically grown seedlings and used to make Northern blots (5 μg per lane of total RNA for blot probed with ATβFRUCT 4 and 20 μg of total RNA per lane for blots hybridised to the other four invertase gene probes). Gels were stained with ethidium bromide following electrophoresis to ensure that equal loading of the RNA had been achieved (Fig. 2.9a).

Northern blots, hybridised to probes specific for the invertase genes

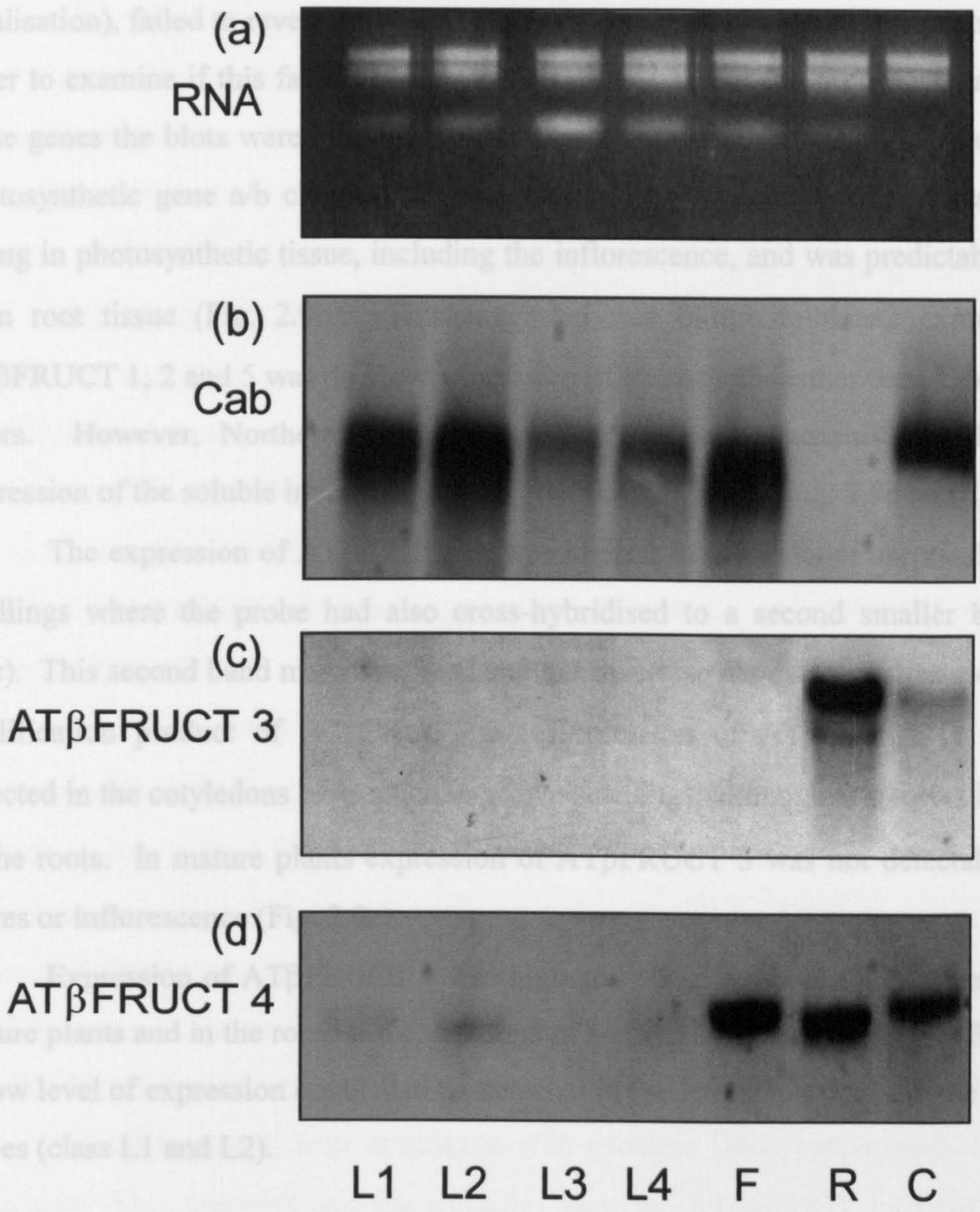



**Figure 2.9 Northern blot analysis of soluble invertase gene expression in *A. thaliana* (ecotype OY0).** Following electrophoresis and prior to Northern blotting agarose gels were stained with ethidium bromide and viewed with UV light to ensure equal loading of the RNA (a). A control blot was hybridised to a probe specific for the highly expressed photosynthetic gene chlorophyll a/b binding protein (b). Northern blots were then hybridised to probes specific for the soluble invertase genes ATβFRUCT 3 (c) and ATβFRUCT 4 (d). Total RNA was isolated from developing leaves (L1-L4); inflorescence (F); seedling roots (R) and seedling cotyledons (C). Twenty μg of total RNA was loaded per lane except blot (d) where 5 μg per lane was loaded.

ATβFRUCT 1 and 2 (apoplastic invertases) and ATβFRUCT 5 (invertase of unknown localisation), failed to reveal any bands following autoradiography (data not shown). In order to examine if this failure was due to technical errors or to the low expression of these genes the blots were re-hybridised to a probe specific for the strongly expressed photosynthetic gene a/b chlorophyll binding protein. As expected hybridisation was strong in photosynthetic tissue, including the inflorescence, and was predictably absent from root tissue (Fig. 2.9b). This suggested that failure to detect expression of ATβFRUCT 1, 2 and 5 was due low expression of these genes rather than experimental errors. However, Northern hybridisation was sufficiently sensitive to detect the expression of the soluble invertase genes ATβFRUCT 3 and 4 (Fig. 2.9c and d).

The expression of ATβFRUCT 3 was highest in the roots of aseptically grown seedlings where the probe had also cross-hybridised to a second smaller band (Fig 2.9c). This second band may have been another invertase gene or a post-transcriptional modification product of ATβFRUCT 3. Expression of ATβFRUCT 3 was also detected in the cotyledons of aseptically grown seedlings, although at a lower level than in the roots. In mature plants expression of ATβFRUCT 3 was not detectable in the leaves or inflorescence (Fig. 2.9c).

Expression of ATβFRUCT 4 was high and about equal in the inflorescence of mature plants and in the roots and cotyledons of aseptically grown seedlings (Fig. 2.9d). A low level of expression could also be detected in the leaves, particularly the youngest leaves (class L1 and L2).

### 2.3.3 ATβFRUCT 1 and 2 gene expression in *A. thaliana*

Northern hybridisation assays were insufficiently sensitive to detect the expression of the apoplastic invertase genes ATβFRUCT 1 and 2 in *A. thaliana*. The extra sensitivity was provided by the reverse transcriptase polymerase chain reaction (RT-PCR). Semi-quantitative RT-PCR (ATβFRUCT 1 only) was made possible by the inclusion of an internal standard.

### *ATβFRUCT 1 gene expression; analysis of RT-PCR products*

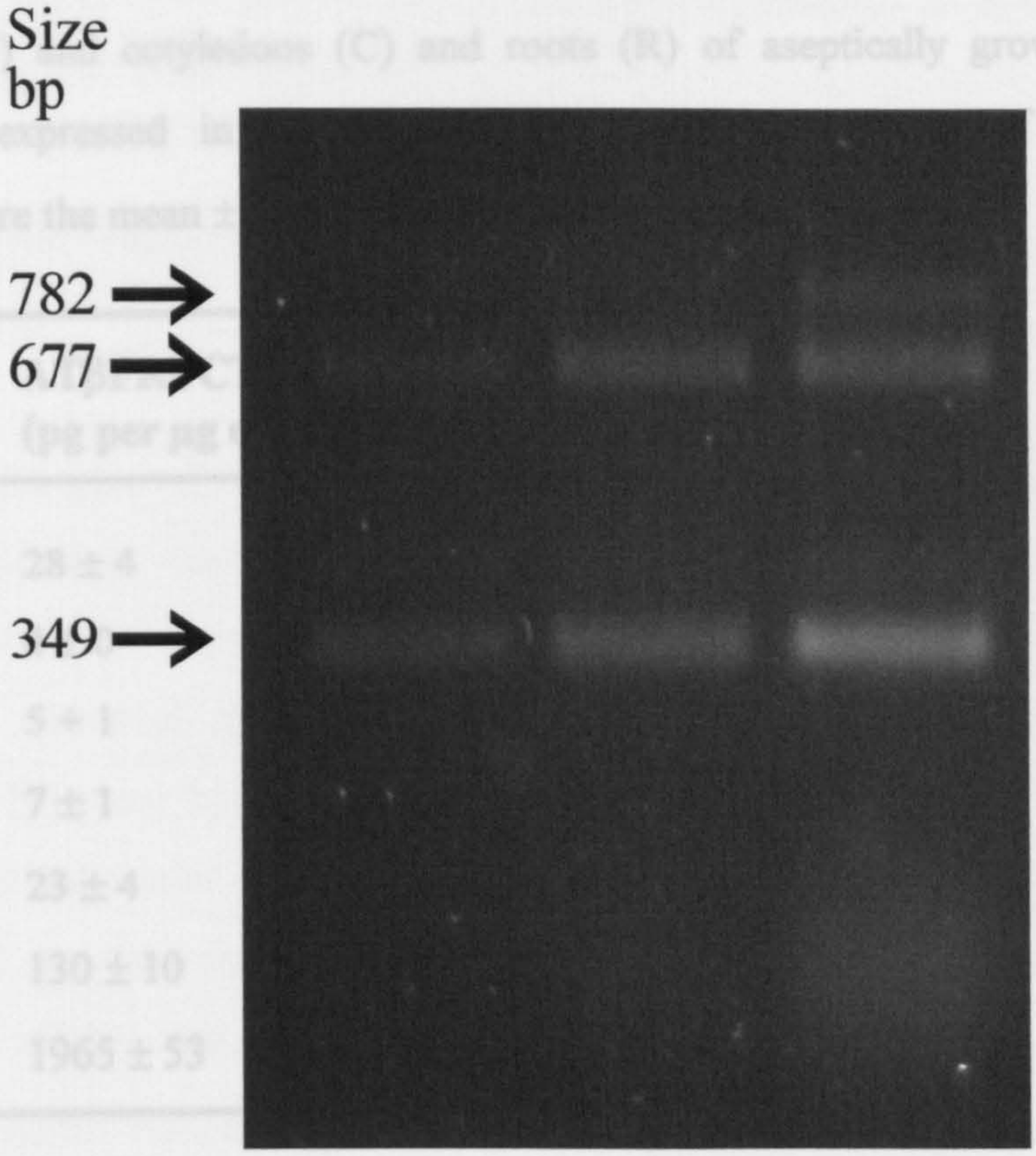
In order to perform an RT-PCR reaction total RNA, extracted from *A. thaliana*, and internal standard (also RNA) were incubated together with the enzyme reverse transcriptase to produce cDNA. This cDNA was used to perform three separate PCR reactions each containing primers specific for the ATβFRUCT 1 apoplastic invertase gene. Each of these PCR reactions were identical except that a different amount of cDNA was added to each one. Following PCR 20µl of each reaction mix was electrophoresed through a 1.5% (w/v) agarose gel, stained with ethidium bromide and viewed using UV transillumination as described in section 2.2.20.

In samples where both ATβFRUCT 1 and internal standard cDNA had amplified successfully UV transillumination of agarose gels revealed two DNA fragments (Fig. 2.10). Amplified ATβFRUCT 1 cDNA was visible as a 349 bp fragment and the amount of this fragment (as revealed by the fluorescence intensity) was proportional to the amount of ATβFRUCT 1 mRNA transcript present in the original sample. This fragment was cloned and sequenced to confirm its identity. Amplified internal standard was visible as a second 677 bp DNA fragment. As the amount of internal standard added to each PCR reaction was known precisely it was possible to use its fluorescence under UV transillumination as a measure of the amount of ATβFRUCT 1 gene expression in each sample.

A third fragment, seen in samples with genomic DNA contamination was 782 bp in size. This fragment was the amplified genomic ATβFRUCT 1 sequence. This fragment was also cloned and sequenced to verify its identity.

### *ATβFRUCT 1 gene expression in leaves of different ages and inflorescence of A. thaliana*

ATβFRUCT 1 gene expression was relatively high in the youngest leaves (L1) (28 pg of RNA per µg of total RNA) but declined rapidly as the leaves matured such that in class L2 expression represented only 4.1 pg of RNA per µg of total RNA, a six fold decrease (Table 2.3). Expression remained at about this level in the other classes examined.




| | | | |
|---|---|---|---|
| Standard (pg) | 12.5 | 25 | 50 |
| cDNA (μg) | 0.67 | 1.33 | 2.67 |

**Figure 2.10 RT-PCR amplification products.** Total RNA extracted from class L1 *A. thaliana* leaves (5 μg) and a known amount of RNA internal standard were incubated with the enzyme reverse transcriptase to form cDNA. Different amounts of this cDNA were used in three separate PCR reactions each containing ATβFRUCT 1 primers. Following 30 cycles of PCR 20 μl of each completed reaction was electrophoresed through an agarose gel, stained with ethidium bromide and viewed by UV transillumination. The three DNA fragments present on this gel resulted from the PCR amplification of ATβFRUCT 1 cDNA (349 bp), internal standard cDNA (677 bp) and amplified *A. thaliana* genomic DNA contamination (782 bp). Altering the amount of internal standard and sample cDNA added prior to thermal-cycling has a direct affect on the amount of DNA amplified.

**Table 2.3 Expression of the ATβFRUCT 1 apoplastic invertase gene in developing *A. thaliana*.** The amount of expression was measured in the developing leaves (L1-L4), inflorescence (F) and cotyledons (C) and roots (R) of aseptically grown seedlings. Expression is expressed in pg ATβFRUCT 1 mRNA per μg of total RNA. Measurements are the mean ± S.E. of three replicate samples.

| Sample | ATβFRUCT 1 gene expression (pg per μg of total RNA ± S.E.) |
|---|---|
| L1 | 28 ± 4 |
| L2 | 4 ± 0 |
| L3 | 5 + 1 |
| L4 | 7 ± 1 |
| F | 23 ± 4 |
| C | 130 ± 10 |
| R | 1965 ± 53 |

ATβFRUCT 1 gene expression in the inflorescence was comparable to that seen in the youngest leaves (L1). Expression in this organ represented an average of 23 pg of RNA per µg of total RNA (Table 2.3)

***ATβFRUCT 1 gene expression in the roots and cotyledons of aseptically grown A. thaliana seedlings***

High expression of ATβFRUCT 1 was seen in the roots of aseptically grown seedlings compared to that measured in the other tissues examined (Table 2.3). Expression in this tissue represented an average of 2000 pg of message per µg of total RNA or 0.2%. This high level of ATβFRUCT 1 gene expression should have been detected using Northern hybridisation assays and tends to suggest a quantification problem with this particular sample. Examination of the original gels revealed that the 782 bp internal standard fragment was faint compared with the 349 bp amplified ATβFRUCT 1 cDNA. It is possible that during PCR the high amounts of the ATβFRUCT 1 cDNA out competed the amplification of the internal standard. If true this phenomenon would predict that ATβFRUCT 1 gene expression was high in the seedling roots but competitive effects led to an over estimation of ATβFRUCT 1 gene expression in this tissue

Expression of the ATβFRUCT 1 apoplastic invertase gene was also high in seedling cotyledons representing 130 pg of message per µg of total RNA (Table 2.3).

***ATβFRUCT 2 gene expression in A. thaliana using non-quantitative RT-PCR***

The expression of the apoplastic invertase gene ATβFRUCT 2 was also examined but in a non-quantitative manner. Samples of RNA from developing *A. thaliana* were used to generate cDNA which was amplified by PCR for 40 cycles containing primers specific for the ATβFRUCT 2 gene as described in section 2.2.20. Gel electrophoresis and staining of the DNA with ethidium bromide resulted in the visualisation of a number of DNA fragments. A DNA fragment of ~250 bp representing the expression of the ATβFRUCT 2 gene could only be detected in samples extracted from the inflorescence (Fig. 2.11). The expression of this gene was

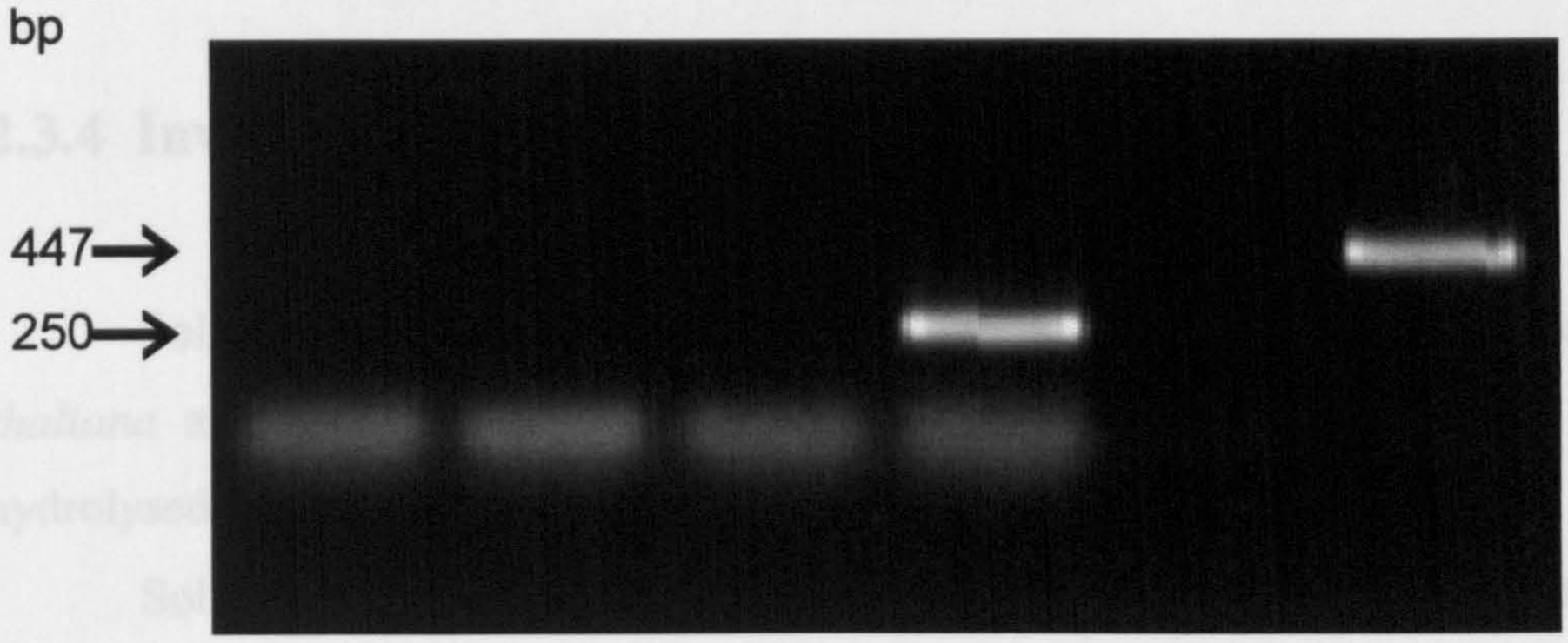



**Figure 2.11 ATβFRUCT 2 gene expression in developing *A. thaliana* plants**. Total RNA was extracted from leaves of different ages (L1 and L4), aseptically grown seedling roots (R) and the inflorescence of *A. thaliana* plants and used for the non-quantitative examination of ATβFRUCT 2 gene expression by RT-PCR. Sample cDNA was amplified in a non-quantitative manner for 40 cycles using ATβFRUCT 2 primers. Successful amplification of ATβFRUCT 2 mRNA yielded a DNA fragment of ~250 bp. Genomic DNA contamination amplified to yield a 447 bp fragment. A control PCR reaction with no cDNA added (-) was used to ensure that there was no external contamination of the sample. One reaction (+) was spiked with *A. thaliana* genomic DNA as a positive control to check for correct amplification.

not detected in either the developing leaves or roots and cotyledons of aseptically grown seedlings. The amplification of genomic DNA could be seen in all the samples examined and resulted in a fragment of 447 bp which is consistent with the known DNA sequence of this gene.

### 2.3.4 Invertase activity in *A. thaliana* plants

Soluble and apoplastic invertase activity was measured in different organs of *A. thaliana* and in leaves of different ages and expressed as the amount of sucrose hydrolysed per g fresh weight (FW) or per mg of protein.

Soluble invertase activity measured per g FW was highest in the inflorescence (Fig. 2.12a). High activity (2000 nmol sucrose hydrolysed $g^{-1}$ FW $min^{-1}$) was seen in the youngest leaves but declined by 50% as they aged. Soluble invertase activity in the roots of aseptically grown seedlings was high and comparable to that seen in the youngest leaves (L1). However, soluble invertase activity was considerably lower (approximately 3 fold) in the seedling cotyledons.

When soluble invertase activity was expressed per mg of total soluble protein a different profile of activity was observed (Fig. 2.12b). In the leaves soluble invertase activity remained constant as they developed. Activity in the inflorescence was approximately 2 fold higher than in the leaves but the highest activity was observed in the roots of aseptically grown seedlings. Soluble invertase activity in the seedling cotyledons was approximately three fold lower than in the roots but was higher than that observed in the leaves of mature plants (Fig. 2.12b).

Apoplastic invertase activity was considerably lower than soluble invertase activity in the tissues examined, typically one tenth of the activity in the aerial plant organs and one quarter the activity in the roots (Fig. 2.12c and d).

When apoplastic invertase activity was expressed per g FW (Fig. 2.12c) there was a small overall decline in activity as the leaves expanded although a considerable proportion of the activity remained in the oldest leaf class (L4). Apoplastic invertase activity in the inflorescence and seedling cotyledons was comparable to that observed in the leaves. The highest apoplastic invertase activity observed was in the roots of aseptically grown seedlings and was 2.5 to 5 fold higher than that measured in the aerial

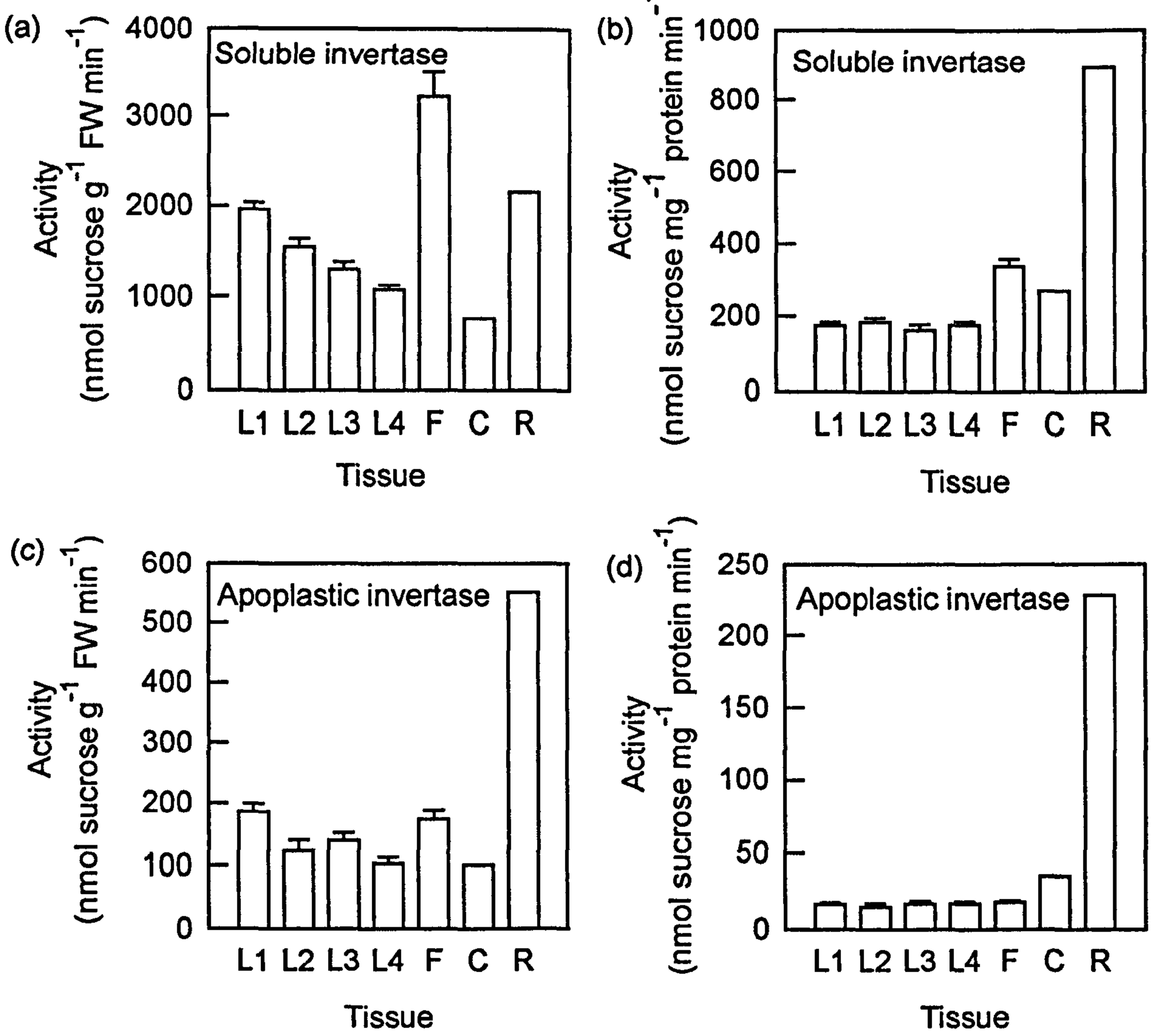



**Figure 2.12 Soluble and apoplastic invertase activity in *A. thaliana*.** Soluble (a and b) and apoplastic (c and d) invertase activity was measured in leaves of different ages (L1-L4), inflorescence (F), cotyledons (C), and seedling roots (R). Activity is expressed per g fresh weight (FW) (a and c) and per mg of total soluble protein (b and d). Soluble invertase activity was assayed at pH 5.5 and apoplastic invertase activity at pH 4.5. Measurements are the mean ± S.E. of four replicate measurements except seedling roots (R) and cotyledons (C) which were pooled prior to extraction of invertase activity.

parts of the plant (Fig.2.12c).

When apoplastic invertase activity was expressed per mg of total soluble protein no overall decline was observed during leaf expansion (Fig. 2.12d). Activity in the inflorescence was comparable to that measured in the leaves. Apoplastic invertase activity in the seedling cotyledons was nearly double that measured in the leaves and inflorescence of mature plants but again the highest activity observed was in the roots of aseptically grown seedlings which was approximately 10 fold higher than that observed in the aerial tissues examined (Fig. 2.12d).

### 2.3.5 The isoform pattern of soluble invertases in developing *A. thaliana* plants

Figure 2.13 shows the changes in the soluble invertase isoform pattern in samples extracted from different tissues of *A. thaliana*. All samples were loaded on an equal activity basis.

The soluble invertase isoform pattern did not alter as the leaves expanded and matured; four major and two minor isoforms were visible with pIs between 4.65 and 4.85. The same isoforms could also be seen in samples extracted from the inflorescence and the roots and cotyledons of aseptically grown seedlings although there was greater variation in the relative intensity of each of the isoforms.

### 2.3.6 Soluble and storage carbohydrates in developing *A. thaliana*

The amount of glucose in the youngest leaves (L1 and L2) remained constant at approximately 10 μmol $g^{-1}$ fresh weight (FW) (Fig 2.14a). However, as the leaves matured the amount of glucose declined markedly, by ~50 % in class L3 and by ~80% in class L4. The amount of fructose in the leaves was considerably lower than the amount of glucose throughout development. However, fructose in the leaves also showed an age dependent decline mirroring that of glucose (Fig 2.14a). The amount of sucrose in the leaves was comparable to that of fructose but in contrast to glucose and fructose did not decline as the leaves matured thus in the oldest leaves examined (L4) it was the predominant soluble carbohydrate (Fig. 2.14a).

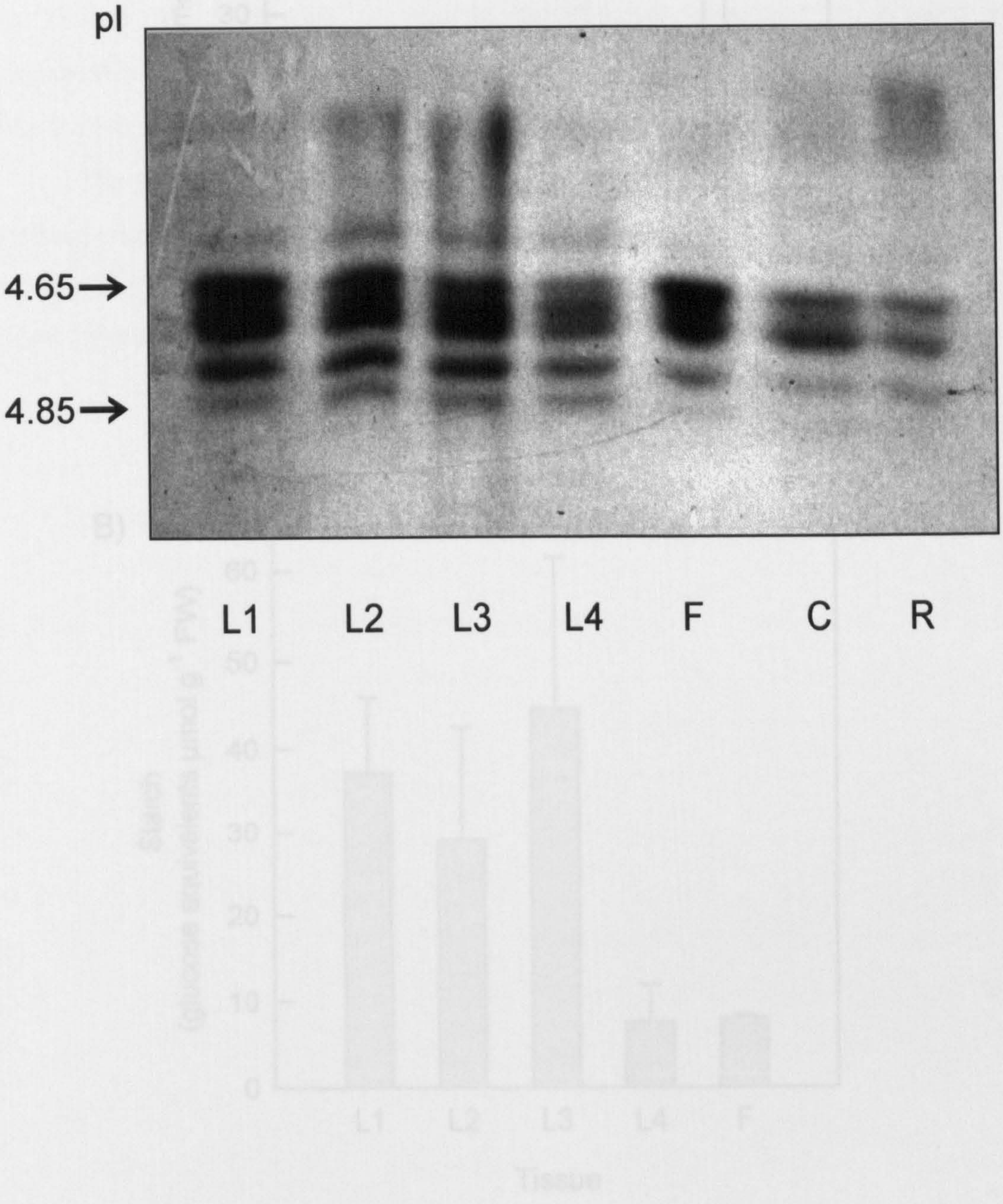



**Figure 2.13 Soluble invertase isoform pattern in *A. thaliana* plants.** Soluble invertase isoforms were separated by isoelectric focusing over a pH range of 4.0-6.5. Invertase activity was visualised by staining reducing sugars produced by the enzyme following incubation in sucrose with 2,3,5 tetrazolium chloride. Samples were prepared from developing leaves (L1-L4), inflorescence (F) and the cotyledons (C) and roots (R) of aseptically grown seedlings. Samples were loaded on an equal activity basis.

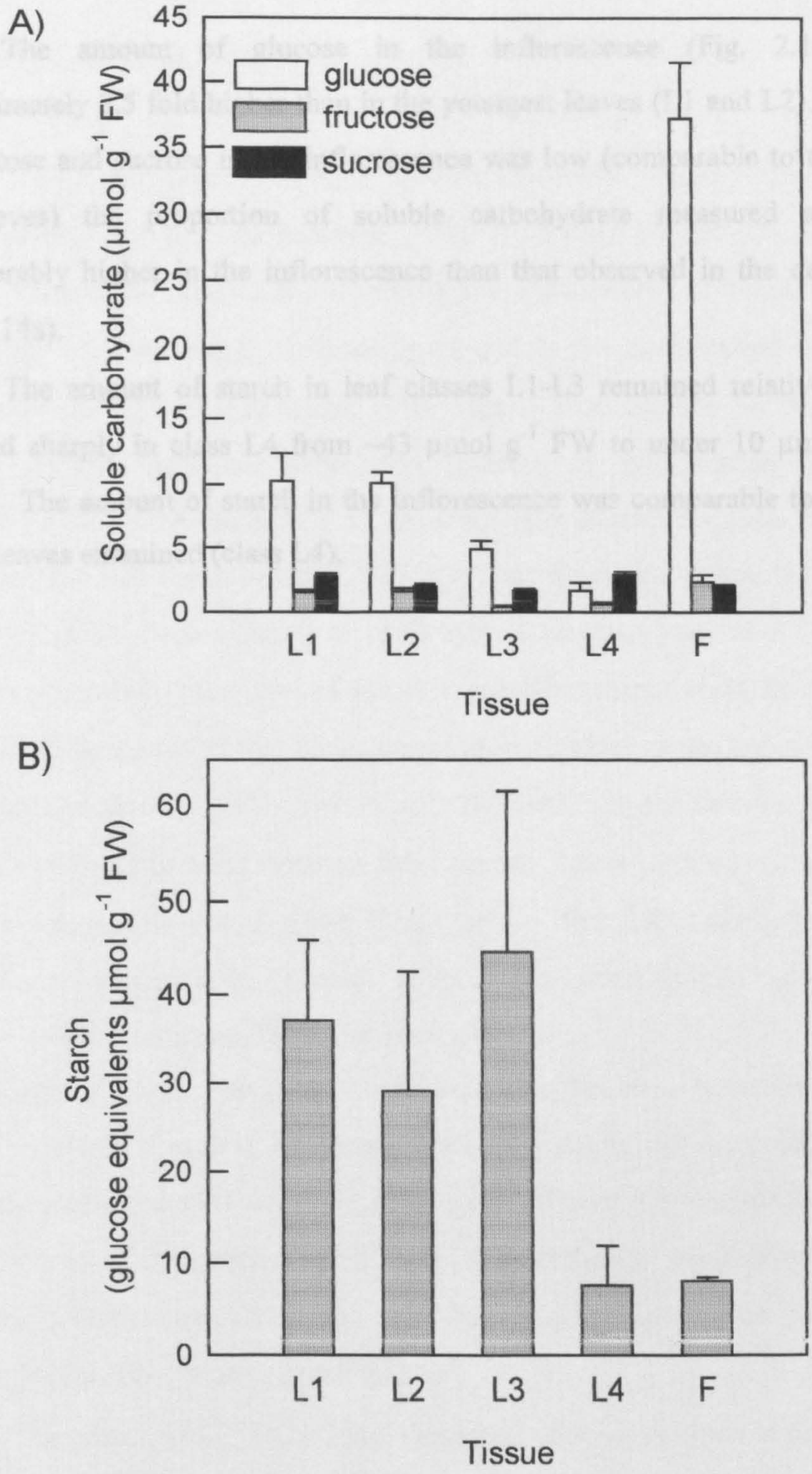



**Figure 2.14 Soluble and storage carbohydrate in *A. thaliana* plants.** Soluble carbohydrates (A) and starch (B) were extracted from developing leaves (L1-L4), inflorescence (F) and seedling roots (R) and cotyledons (C). Samples were harvested at mid-afternoon. Results are the mean ± S.E. of four replicate measurements except seedling roots and cotyledons which were pooled at harvest.

The amount of glucose in the inflorescence (Fig. 2.14a) was high, approximately 3.5 fold higher than in the youngest leaves (L1 and L2). As the amount of fructose and sucrose in the inflorescence was low (comparable to that measured in the leaves) the proportion of soluble carbohydrate measured as glucose was considerably higher in the inflorescence than that observed in the developing leaves (Fig. 2.14a).

The amount of starch in leaf classes L1-L3 remained relatively constant but declined sharply in class L4 from ~43 μmol $g^{-1}$ FW to under 10 μmol $g^{-1}$ FW (Fig. 2.14b). The amount of starch in the inflorescence was comparable to that seen in the oldest leaves examined (class L4).

## 2.4 DISCUSSION

The developmental and organ specific expression pattern of five invertase genes were analysed in *A. thaliana* (ecotype OY0). This work adds to that previously carried out by Tymowska-Lalanne *et al.* (1996) and Haouazine-Takvorian *et al.* (1997) by examining a greater range of tissue types and in the examination of invertase gene expression during leaf expansion. This thesis also reports for the first time the developmentally regulated expression patterns of two further invertase genes, ATβFRUCT 2 and ATβFRUCT 5. In addition to these new areas of investigation I have designed and developed an RT-PCR system for the semi-quantitative examination of ATβFRUCT 1 gene expression. This system was necessary as ATβFRUCT 1 gene expression was below the level of detection using Northern hybridisation assays.

Data presented in this thesis reveal an individual expression pattern for each of the genes examined possibly indicating a different role for each enzyme. This work adds to a growing body of evidence that suggests that invertase genes are under spatio-temporal control (Arai *et al.*, 1992; Hedley *et al.*, 1993; Elliot *et al.*, 1993; Sturm *et al.*, 1995; Lorenz *et al.*, 1995; Xu *et al.*, 1996; Tymowska-Lalanne *et al.*, 1996; Godt and Roitsch, 1997; Haouazine-Takvorian *et al.*, 1997).

During the first stage of the investigation Southern hybridisation assays were used to ascertain if each of the gene probes was specific for a separate invertase gene. This was confirmed and the simple pattern of hybridising fragments also suggested that only one copy of the genes ATβFRUCT 1-4 were present per haploid genome. These Southern blots were consistent with those produced by Tymowska-Lalanne *et al.* (1996) and Haouazine-Takvorian *et al.* (1997).

The probe ATβFRUCT 5 hybridised to multiple fragments on a Southern blot but none of these correlated to those seen with the other four invertase probes indicating that this probe was also gene specific.

### 2.4.1 Apoplastic invertase gene expression in developing *A. thaliana* plants

Northern hybridisation assays failed to detect the expression of the ATβFRUCT 1 apoplastic invertase gene in *A. thaliana* tissue. However, it was apparent from measurements of activity that one or more apoplastic invertase genes had been expressed, at least in young tissue, and so it was decided that a more sensitive detection technique would be needed to analyse their expression. The reverse transcriptase polymerase chain reaction (RT-PCR) was the method of choice. The use of RT-PCR to analyse invertase gene expression in *A. thaliana* has been reported previously (Tymowska-Lalanne *et al.*, 1996). However, the inclusion of an internal RNA standard makes this, to our knowledge, the first semi-quantitative assessment of apoplastic invertase gene expression in any species.

The apoplastic invertase gene ATβFRUCT 1 showed a developmentally regulated pattern of expression. Expression of this gene was detected in all the tissues examined and did not appear to show organ specificity confirming the analysis by Tymowska-Lalanne *et al.* (1996). However the semi-quantitative nature of these measurements also revealed that the expression of the ATβFRUCT 1 gene varied between different organs and as the leaves developed.

ATβFRUCT 1 gene expression was highest in the seedling roots; 15 times higher than in any other tissue examined. Expression was also elevated in regions of high metabolic activity such as the seedling cotyledons and the rapidly expanding inflorescence. Expression of this gene was also elevated in the youngest leaves but declined suddenly as they matured.

Similar patterns of apoplastic invertase gene expression have been detected in both carrot and tomato (Sturm *et al.*, 1996; Godt and Roitsch, 1997). In carrot apoplastic invertase gene expression declined as the leaves developed and was below the level of detection in mature leaves. Expression was high in young roots but ceased at the onset of tap root development. In tomato apoplastic invertase gene expression is complicated by the presence of four genes (*Lin5-8*). One of the genes *Lin6* shows a similar expression profile to ATβFRUCT 1, being developmentally regulated. This gene was not detected in mature leaves or stems but was detected in seedling roots. As

the roots matured expression dropped below the level of detection.

In this thesis ATβFRUCT 1 gene expression was not measured in mature roots. However, Tymowska-Lalanne *et al.* (1996) using non-quantitative RT-PCR demonstrated some level of expression in the root tissue of mature plants. This is in contrast to studies of carrot and tomato where apoplastic invertase gene expression was not detected in these tissues (Sturm *et al.*, 1995; Godt and Roitsch, 1997). There could be several reason for these apparent species differences. (i) Root development occurs behind an apical meristem and therefore the definition of sample root tissue as 'young' or 'mature' may depends more upon the ratio of root tips to old roots than upon the age of the plant when the roots were sampled. (ii) Tymowska-Lalanne *et al.* (1997) examined ATβFRUCT 1 gene expression using the highly sensitive RT-PCR method whereas the studies of carrot and tomato relied on less sensitive Northern hybridisation assays. Apoplastic invertase gene expression in the roots of carrot and tomato might also have been detected using RT-PCR. (iii) There may be real species differences in the level of apoplastic invertase gene expression in roots from mature plants. However, the physiological relevance of these differences will depend more upon residual apoplastic invertase activity in these tissues than the amount of detectable gene expression.

Generally apoplastic invertase activity and ATβFRUCT 1 gene expression correlated closely in all the tissues examined, particularly when activity was measured per mg of soluble protein. For example, in the seedling roots high levels of gene expression correlated closely to high activity. This correlation was also consistent between different organs for example in the youngest leaves and inflorescence where similar levels of ATβFRUCT 1 gene expression and activity were detected. However, in the leaves apoplastic invertase activity remained largely unchanged as the leaves developed. There was a small decrease in activity when measured on a fresh weight basis and no change when measured per mg of soluble protein. In contrast ATβFRUCT 1 gene expression virtually ceased as the leaves matured (from L1 to L2). These data argue for the high stability of the mature protein *in vivo* with activity remaining in the leaf long after the cessation of ATβFRUCT 1 gene expression.

What are the possible roles for apoplastic invertase in the different organs of *A. thaliana*? It is immediately evident from data presented here that

ATβFRUCT 1 gene expression and apoplastic invertase activity were considerably higher in the roots of aseptically grown seedlings than in any other tissue examined. High root apoplastic invertase activity has also been measured in a wide range of other species including pea (Lyne and ap Rees, 1971), bean (Robinson and Brown, 1952), tomato (Chin and Weston, 1973), *Ricinus* (Chapleo and Hall, 1989), maize (Hellebust and Forward, 1962) and oat (Pressey and Avants, 1980). One possible role of apoplastic invertase in the roots could be in the establishment and maintenance of sink strength. Sucrose transport through the phloem is thought to be generated by a sucrose concentration gradient (Ho and Baker, 1982). It has been proposed that the hydrolysis of sucrose in the apoplast of sink organs could help to maintain this concentration gradient (Eschrich, 1982, 1989). In this model sucrose leaving the sieve element/companion cell complex would be hydrolysed in the apoplast by apoplastic invertase with the resulting hexoses taken up into the root parenchyma by active transport (Patrick, 1997).

However, to date the experimental basis for such a role is largely speculative and correlative in nature. Indeed several studies have not only called into question the importance of apoplastic invertase activity in maintaining sink strength in the roots but whether it occurs at all in some species. In the first of these studies Duke *et al.* (1991) examined inbred maize plants (Oh 43) with an apoplastic invertase deficiency in the primary root system. These authors found that under the growth conditions examined the growth of Oh 43 plants was completely unaffected by the loss of apoplastic invertase activity in the primary root system. In a second study Sturm *et al.* (1995) examined apoplastic invertase gene expression in developing carrot. These authors found that apoplastic invertase gene expression was high in young filamentous roots but that expression of this gene had dropped below the level of detection by the onset of tap root development. Indeed tap root development was marked by the massive induction of a vacuolar invertase gene (sII). These authors suggest that vacuolar not apoplastic invertase activity may be important for maintaining sink strength in this species.

Recent work has shown that apoplastic invertase activity may have as yet unrecognised roles in rapidly growing tissues such as root extension zones and the inflorescence. In these regions the plant hormone cytokinin is thought to induce rapid cell division (Skoog and Armstrong, 1970). Work by Ehness and Roitsch (1997) has

shown that cytokinins induce the CIN 1 apoplastic invertase gene in *Chenopodium rubrum*. This has lead to speculation that apoplastic invertase not only establishes a supply of carbohydrates for these actively growing regions but also produces the necessary osmotic conditions needed for cell expansion. This work has been supported by evidence of glucose induction of the CIN 1 invertase gene resulting in a positive feedback loop (Ehness and Roitsch, 1997).

The role of apoplastic invertase activity in the leaves is equally complex. Data presented in this thesis reveal that ATβFRUCT 1 gene expression declines suddenly as the leaves mature (the decline occurs between class L1 and L2). Similar apoplastic invertase gene expression patterns were seen in developing carrot and tomato leaves (Sturm *et al.* 1995, Godt and Roitsch, 1997). These changes in gene expression appeared to coincide with the transition of the leaf from a sink to a source organ which generally occurs when the leaf is between 30 and 60% of its mature size (Turgeon, 1989). Whether apoplastic invertase gene expression in young leaf tissue may help to establish the organ as a sink for carbohydrates as has been suggested for root tissue is not known. However, results presented in this thesis show that considerable apoplastic invertase activity remained in mature leaves. The role of this remaining activity is unknown but may critically depend upon its cellular location. Several studies indicate that apoplastic invertase may be located in the leaf vasculature (Kingston-Smith and Pollock, 1996; Zhang *et al.*, 1996; Ramloch-Lorenz *et al.*, 1993). It is possible that under normal physiological conditions activity remaining in the mature leaf may help to retrieve sucrose exuded into the apoplast.

***The ATβFRUCT 2 gene is expressed in an organ specific manner***

Expression of the ATβFRUCT 2 apoplastic invertase gene was limited to the flowering organs of *A. thaliana* plants. This is the first time that the expression of this gene has been reported. Although the exact cell types in which the ATβFRUCT 2 gene were expressed are not known its expression profile is in clear contrast to that of ATβFRUCT 1. The expression of apoplastic invertase genes in flowering organs has also been reported in tomato (Godt and Roitsch, 1997). These authors reported that one apoplastic invertase gene *Lin7* was exclusively expressed in flower buds and flowers

and pinpointed the majority of this expression to the stamens. The expression of a second invertase gene, *Lin5*, in the gynoecia led these authors to speculate on the importance of these two genes in the supply of hexoses to the male and female organs. Indeed, this is supported by Dorion *et al.* (1996) who found that the induction of male sterility in wheat by water stress was preceded by a decline in invertase activity. The expression of a flower bud specific apoplastic invertase has also been reported in carrot (Lorenz *et al.*, 1995).

The importance of co-ordinated apoplastic invertase gene expression during critical phases of reproduction has also been demonstrated during seed development. Apoplastic invertase activity in seeds is thought to supply carbohydrates to the developing embryo. This was clearly shown using mutant maize seeds (Miller and Chourey, 1992, Cheng *et al.*, 1996). In normal maize seeds the maternal and filial cells are connected at a single point, the pedicel. In this region photosynthate and nutrients are unloaded prior to uptake by the basal endosperm. This step occurs apoplastically as there are no plasmodesmatal connections between the pedicel and filial tissue. The isolation of the fatal *miniature1* (*mn*) maize seed mutant in which apoplastic invertase activity is absent has shown how crucial apoplastic invertase activity is for the supply of hexoses to the developing embryo. Cheng *et al.* (1996) propose that apoplastic invertase located in the pedicel plays a fundamental role in the supply of hexoses to the filial generation and that loss of activity causes a build-up of sucrose and osmotic imbalance that results in the degeneration of the ultra-thin placento-chalazal cells.

Examination of Fava bean seed development has also revealed a critical role for apoplastic invertase activity for supplying hexoses to the developing embryo. Activity measurements and *in situ* hybridisation with apoplastic invertase gene probes revealed that apoplastic invertase was located in the seed coat of fava bean. This region is known to be a site of photoassimilate unloading. High apoplastic invertase activity in the seed coat correlated with a high hexose:sucrose ratio in the endosperm and lead to speculation that apoplastic invertase was responsible for the establishment of sink strength in young seeds (Weber *et al.*, 1995, Weber *et al.*, 1996).

Future work would aim to examine the expression and activity of ATβFRUCT 2 in the inflorescence in greater depth. This would begin by localising expression more precisely and examining expression during seed development. This may uncover a

similar role for ATβFRUCT 2 in *A. thaliana* seeds as was demonstrated for maize and fava bean.

### 2.4.2 Soluble invertase gene expression in *A. thaliana*

In *A. thaliana* two genes have been identified that are thought to encode soluble acid invertases, ATβFRUCT 3 and ATβFRUCT 4. It is widely believed that these enzymes are located within the vacuole (Leigh *et al.*, 1979).

The role of vacuolar invertase activity in plant metabolism is not fully understood although it has been implicated in the maintenance of sink strength in the roots of some species (Sturm *et al.*, 1995). In other species vacuolar invertase activity is thought to be important in determining the final sucrose to hexose ratio in ripening fruit (Yelle *et al.*, 1991; Elliot *et al.*, 1993; Klann *et al.*, 1996). In the leaves it has been suggested that vacuolar invertase activity may supply the hexoses needed for growth. It has also been suggested that in the mature leaves of some species vacuolar invertase activity may lead to the futile cycling of sucrose through the vacuole (Huber *et al.* 1989, Geigenberger and Stitt, 1991) a phenomenon which may regulate the amount of sucrose available for export, alter carbon partitioning between sucrose and starch or influence photosynthetic gene expression via a sugar sensing mechanism under certain conditions (Sheen, 1990; Jang and Sheen, 1994). The role of vacuolar invertases in the regulation of leaf carbohydrate metabolism will be examined in chapter 4.

In this study it was found that the ATβFRUCT 3 and ATβFRUCT 4 vacuolar invertase genes were differentially regulated in *A. thaliana*. Northern hybridisation assays revealed that the ATβFRUCT 3 gene was regulated in an organ specific manner while the ATβFRUCT 4 gene was developmentally regulated.

Expression of the ATβFRUCT 3 gene was limited to the roots and cotyledons of aseptically grown seedlings where there was strong hybridisation of the probe to RNA isolated from roots and weak hybridisation to RNA isolated from cotyledons. In addition, a second smaller and more weakly hybridising band was seen in the root tissue. The identity of this second band is unknown although it is possible that it resulted from a form of alternative gene splicing. Alternative splicing has been observed in an apoplastic invertase gene (CD 111) from potato following cold stress

(Bournay *et al.*, 1996). These authors suggest that this event could regulate the activity of the mature protein by the removal of important catalytic sequences. However, alternative splicing is unlikely to have given rise to the second hybridising fragment reported in this thesis as these events usually occur only with exons smaller than 51 bp in length and this would have given rise to second hybridising fragment indistinguishable in size on a Northern blot from the complete ATβFRUCT 3 mRNA. It is also possible that the second hybridising fragment was the result of hybridisation of the probe to another invertase mRNA. However, again this is unlikely as Southern hybridisation assays showed that each of the probes used were specific for a different invertase gene.

Expression of the ATβFRUCT 4 gene was detected in all the tissues examined. Expression was high and about equal in the inflorescence, seedling roots and cotyledons. Expression was low in the youngest leaves (L1 and L2) and declined below the level of detection as they matured.

These data add to those already published by Haouazine-Takvorian *et al.* (1997) who also examined the expression of soluble invertase genes in *A. thaliana*. These authors examined the expression of ATβFRUCT 3 and 4 in different organs but not during organ development. Interestingly, these authors found that ATβFRUCT 3 was also expressed in the roots of mature plants revealing that the expression in this organ is not limited to the seedling stage. However, some discrepancies are revealed when data published by these authors are compared to that presented here. Firstly, these authors report that expression of the ATβFRUCT 3 gene was detected in the flowering stem, flowers and leaves. This is in contrast to data presented here in which expression was not seen in any tissue from mature plants. There may be several explanations for these apparent differences. (i) A low level of ATβFRUCT 3 gene expression may have been present in the floral organs and leaves examined in this thesis. However, the sensitivity of Northern hybridisation assays is dependent on a range of non-biological factors and this may have influenced the visibility of the hybridisation. (ii) The plants used in the two studies were grown under different light conditions and the inflorescence harvested at different developmental stages. (iii) Examination of the ATβFRUCT 3 probes used in the two studies reveal that this is unlikely to be the source of the differences as they are both very similar in size and sequence.

The two studies concur on the expression profile of the ATβFRUCT 4 gene although, in addition to data reported here, Haouazine-Takvorian *et al.* (1997) report that the ATβFRUCT 4 gene was not expressed in the roots of mature plants. This suggest that at some point during root development the expression of this gene ceased and that new activity was the product of ATβFRUCT 3 gene expression alone. No second smaller hybridising fragment was reported by Haouazine-Takvorian *et al.* (1997).

The differential expression of vacuolar invertase genes has also been reported in other species. In carrot two vacuolar invertase genes (sI and sII) show a developmentally regulated pattern of expression (Sturm *et al.*, 1995). These authors found that the expression of sI mainly predominated in the primary roots while sII predominated in the developing tap root. Expression of both genes was also observed during early leaf development. When the leaves were removed from mature carrot plants sII gene expression in the roots declined markedly until leaf re-growth was well advanced. This suggested that the sII vacuolar invertase gene was important in regulating the partitioning of sucrose to the tap root and that changes in expression occurred in response to changes in the availability of photosynthate from source tissue.

The differential expression of two vacuolar invertase genes, *Ivr1* and *Ivr2* was also examined during development of maize (Xu *et al.*, 1996). These authors showed that *Ivr2* gene expression was prevalent in all sucrose importing structures whereas *Ivr1* expression was markedly higher in reproductive structures than vegetative tissues. The incubation of maize root tips in different concentrations of glucose also revealed that *Ivr1* and *Ivr2* are differentially regulated by hexose concentration. *Ivr1* gene expression was higher in low concentrations of glucose (0.2-0.5%) whereas *Ivr2* gene expression was elevated in higher concentrations of glucose (0.5-4.0%). This 'feast and famine' response may explain the differential expression of the genes in different tissues. For example, the more limited distribution of *Ivr1* mRNAs could enhance their potential to aid local expansion and import in organs such as root tips and reproductive structures whereas the sugar enhancement of *Ivr2* has the potential to aid import into carbohydrate storing structures. This work has shown for the first time that invertase genes may be both modulated by and effectors of carbohydrate metabolism in plants. These findings pose interesting questions as to the potential differential regulation of ATβFRUCT 3

and 4 by soluble carbohydrates.

In general it is difficult to correlate measurements of enzyme activity with those of gene expression as Northern hybridisation assays are semi-quantitative in nature. However, there was a broad correlation between the pattern of ATβFRUCT 3 and 4 gene expression and measurements of vacuolar invertase activity in the different organs of *A. thaliana*, particularly when activity was expressed per mg of total soluble protein. When activity was expressed per g fresh weight the correlation was not so precise.

Vacuolar invertase activity was approximately 10 fold higher than apoplastic invertase in each tissue examined. It was noticeable that despite a variation in vacuolar invertase activity between different organs activity was not low in any of the tissues examined.

Vacuolar invertase activity was particularly high in the roots of aseptically grown seedlings. The role of vacuolar invertase in the roots is not known although, as previously mentioned, it may help to maintain the roots as a sink for carbohydrate (Sturm *et al.*, 1995). Alternatively it may help to provide the osmotic conditions necessary for cell expansion behind the root tip (Ehness and Roitsch, 1997).

In the leaves vacuolar invertase activity was present long after a cessation in gene expression. This argues for the high stability of the mature protein *in vivo*.

Isoelectric focusing of soluble invertase proteins extracted from *A. thaliana* tissues revealed the presence of multiple isoforms. Up to six isoforms were detected in each tissue, four of which were stronger and had pIs of between 4.65 to 4.95. It is not clear whether these isoforms arose from post-translational modification of ATβFRUCT 3 and 4 products or whether they arose due to proteolysis during the extraction procedure. What is clear, however, is that the isoforms with pIs of 4.75, 4.70 and 4.65 (extracted from the leaves) all contain a 52 KDa protein with an N-terminal amino acid sequence homologous to that predicted from the ATβFRUCT 4 gene (Tang *et al.*, 1996). Although it is possible that the different isoforms arose due to proteolysis during the extraction procedure (Fahrendorf and Beck, 1990) it is perhaps more likely that the expression of ATβFRUCT 4 and/or ATβFRUCT 3 gave rise to two or more different isoforms. Multiple soluble invertase isoforms have also been observed in tissues from a number of other species including tomato fruit (Yelle *et al.*, 1991) and radish cotyledons (Faye *et al.*, 1986). The authors in both of these studies suggested

that the different isoforms arose from post-translational processing of invertase gene products rather than from proteolysis. The role of the different soluble invertase isoforms in *A. thaliana* is as yet unknown although they may have a role in the regulation of sucrose metabolism.

The role of soluble invertase activity in the leaves is a matter of debate and depends critically on the developmental stage in question. In young rapidly developing leaves soluble invertase activity is thought to supply the developing tissue with the hexoses needed for growth (Sturm *et al.*, 1996). Data presented here showed that high soluble invertase activity and high amounts of hexoses were indeed found in the young leaves of *A. thaliana* plants. However, in addition to supplying hexoses for the high metabolic requirements of the growing leaves soluble invertase activity may also provide the necessary osmotic conditions needed for cell expansion (Ehness and Roitsch, 1997).

Soluble invertase activity in the leaves of *A. thaliana* plants declined as they matured. Similar declines have been reported in a number of other species including *Phaseolus vulgaris* (Morris and Arthur, 1984), *Lolium temulentum* (Pollock and Lloyd, 1977) and *Citrus sinensis* (Schaffer, 1986). However, despite this decline a considerable proportion of the activity still remained in the mature leaves of *A. thaliana*. Huber *et al.* (1989) showed that different species varied in the extent to which soluble invertase activity was retained in the mature leaves. Plants retaining activity did not accumulate sucrose within the leaf. This led to speculation that in these species a futile cycle of sucrose synthesis and degradation exists (Foyer, 1988; Huber 1989; Goldschmidt and Huber, 1992). The possible role of a futile cycle may include the regulation of the amount of sucrose available for export or the regulation of photosynthetic gene expression via the sugar sensing mechanism under certain conditions (Sheen 1990; Jang and Sheen, 1994).

In this thesis the expression of a fifth invertase gene, ATβFRUCT 5, was examined for the first time. However, expression of this gene was not detected in any of the tissues examined using Northern hybridisation assays. Failure to detect expression could have been due to. (i) The low abundance of the mRNA as was the case for ATβFRUCT 1 and 2. (ii) Expression in an extremely localised cell type as was the case for ATβFRUCT 2 and as is common for invertases (Lorenz *et al.*, 1995; Godt

and Roitsch, 1997). (iii) Expression of the gene only under certain physiological and environmental conditions as has been reported for apoplastic invertase genes (Matsushita and Uritani, 1974; Sturm and Chrispeels, 1990; Benhamou *et al.*, 1991; Zhang *et al.*, 1997). However, one fact is clear, ATβFRUCT 5 is not a silent gene as the probe used to detect its expression was constructed from an Expressed Sequence Tag.

Analysis of the ATβFRUCT 5 EST sequence revealed some unusual characteristics. ATβFRUCT 5 shows higher sequence homology to apoplastic invertases than to vacuolar invertases but its low predicted pI is unusual for apoplastic invertases for which a high isoelectric point is required for ionic interaction with the negatively charged cell wall. One possibility is that the ATβFRUCT 5 gene may encode an intracellular invertase with a neutral or alkaline pH optimum. An invertase gene with similar characteristics has also been identified in *Chenopodium rubrum* (Ehness and Roitsch, 1997). Neutral and alkaline invertases are thought to be located in the cytoplasm and differ from soluble acid (vacuolar) invertases in being non-glycosylated (Copeland, 1990; Chen and Black, 1992). At present little is known about the role of neutral and alkaline invertases in metabolism although activity is generally confined to mature tissues. Ricardo and ap Rees (1970) suggested that the role of this enzyme was the catalysis of sucrose hydrolysis in the cytoplasm of cells lacking significant amounts of acid invertase activity.

### 2.4.3 Conclusion and future work

In this chapter the expression of five invertase genes in *A. thaliana* have been examined, two apoplastic, two vacuolar and fifth invertase of unknown sub-cellular localisation. The low expression of the ATβFRUCT 1 apoplastic invertase gene necessitated the development of a semi-quantitative RT-PCR system. Using this system it was revealed that the expression of this gene was regulated developmentally and was detected in all the tissues examined. Expression of this gene and corresponding activity was particularly high in seedling roots where I hypothesise that it may help in the maintenance of sink strength and where it may also provide the osmotic conditions necessary for rapid cell enlargement behind the root tip. Future work would

aim to produce transgenic *A. thaliana* plants with reduced apoplastic invertase activity. This approach may help to further clarify the role of this enzyme in metabolism.

The expression of a second apoplastic invertase gene, ATβFRUCT 2, was also examined using RT-PCR but in a non-quantitative manner. This gene was expressed in a highly localised manner being found only in the inflorescence. Future work would aim to localise expression further possibly by utilising *in situ* hybridisation techniques. Such localisation may help to clarify the role of this enzyme in reproduction as was achieved by Weber *et al.* (1996, 1997) in fava bean seeds.

The expression of an invertase gene encoding a protein with an unknown sub-cellular localisation, ATβFRUCT 5, was also examined. Expression of this gene was not detected in any of the tissues examined. This gene may encode a neutral or alkaline invertase.

The expression of two vacuolar invertase genes, ATβFRUCT 3 and ATβFRUCT 4, were differentially regulated during development. Such differential regulation has previously been shown for vacuolar invertases in carrot (Sturm *et al.*, 1995) and in maize (Xu *et al.*, 1996) where a different role for each invertase has also been suggested. Measurements of vacuolar invertase activity in different organs of *A. thaliana* correlated with changes in the ratio of sucrose to hexoses also measured in those tissues. Vacuolar invertase activity was found to be highest in rapidly expanding structures such as roots, inflorescence and young leaves where it may help to supply active tissues with the hexoses needed for growth. Future work would aim to examine the regulation of ATβFRUCT 3 and 4 in more detail by examining expression in response to changes in the concentration of soluble carbohydrate.

In this chapter invertase gene expression in developing *A. thaliana* has been characterised. In the next chapter the impact of different environmental stimuli on invertase gene expression will be examined in detail.

# Chapter 3

# The impact of pathogenesis, wounding and soluble carbohydrates on invertase activity and gene expression in *Arabidopsis thaliana*

## 3.1 INTRODUCTION

In the previous chapter we saw how the processes of growth and development influenced the expression of five invertase genes in *A. thaliana*. We saw that in a developing plant, changes in invertase gene expression and activity may have helped to regulate the supply of carbon to different organs within the plant. Generally, high overall levels of invertase gene expression tended to occur in tissues typically considered as sinks for carbohydrate. This was particularly true of the seedling roots in which high expression of both vacuolar (ATβFRUCT 3 and 4) and apoplastic (ATβFRUCT 1) invertase genes were observed. The association of invertase gene expression with sink tissues was clearly demonstrated in the developing leaves. Here the expression of both apoplastic and soluble invertase genes declined as the leaves matured and reached full expansion, thus mirroring the transition of the leaves from sink to source tissue. In the oldest leaf class examined (L4), expression of the ATβFRUCT 4 vacuolar invertase gene was barely detectable while expression of the ATβFRUCT 1 apoplastic invertase gene was low and only detectable using one of the most highly sensitive detection techniques, RT-PCR.

The low expression of invertase genes in mature leaves is not restricted to *A. thaliana* but has been observed in a wide range of plant species. For example, in carrot leaves the expression of one apoplastic invertase gene (Inv*Dc1) and two soluble invertase genes (sI and sII) declined below the level of detection within five weeks of emergence (Sturm *et al.*, 1995). Similarly, apoplastic invertase gene expression was below the level of detection in the mature leaves of tomato, pea and *Chenopodium rubrum* (Godt and Roitsch, 1997; Zhang *et al.*, 1996; Ehness *et al.*, 1997). However, despite the low levels of invertase gene expression often observed in leaf tissue two environmental stimuli, wounding and pathogenesis, have been shown capable of stimulating activity in these tissues.

The impact of wounding on invertase activity tends to vary between species and also depends on the organ affected (Zhang *et al.*, 1996). Generally, wounding results in a localised and transient increase in soluble acid and apoplastic invertase activity in the affected organ. In one such study Matsushita and Uritani (1974) wounded sweet potato roots by slicing, and measured acid invertase activity over the proceeding 48 h period.

These authors found that soluble acid and apoplastic invertase activity increased rapidly after a lag of 3-6 h and reached a maximum after 18 h; thereafter activity decreased over the next 30 h. Concomitant with the increase in invertase activity there was a decline in the sucrose concentration of the wounded root tissue suggesting that wounding had resulted in the induction of sucrose metabolism within the root possibly in order to provide hexoses for mounting an energetically costly wound response.

The recent cloning and identification of invertase genes from a number of plant species has enabled wound-induced changes in invertase activity to be examined at the level of gene expression for the first time. This has helped to clarify the role of specific invertase genes in a number of species. In one such study Godt and Roitsch (1997) examined the expression of one vacuolar and four apoplastic invertase genes in wounded tomato leaves. This study revealed that only one apoplastic invertase gene, LIN6, was induced by wounding. Previously, these authors had found that the expression of LIN6 was associated with sink tissues and was developmentally regulated. This work suggested that LIN6 played an important role in establishing a supply of hexoses to tissues with high metabolic activity. Apoplastic invertase genes showing elevated expression in sink tissues and in response to wounding have been identified in a number of other species including, carrot (Sturm and Chrispeels, 1990) and *Chenopodium rubrum* (Ehness *et al.*, 1997).

In the previous chapter I identified ATβFRUCT 1 as a sink associated apoplastic invertase gene, similar to LIN6 in tomato. In this chapter I aim to examine the impact of wounding on invertase activity and gene expression in *A. thaliana* and in so doing I hope to clarify the role of specific invertase genes. To achieve this I will wound mature leaves and measure subsequent changes in soluble acid and apoplastic invertase activity. I will then use this as a basis for gene expression studies. I have chosen to wound mature leaves as, in the natural environment, these organs are not only subject to herbivory by grazing animals and insect pests but are also exposed to damage by abiotic forces such as wind and rain. Furthermore, mature leaves represent the primary source tissue in plants and therefore induction of invertase gene expression and activity in this region represents the clearest induction of sink metabolism. In previous studies wounding has tended to be severe, non-standardised and destructive. In this study I aim to wound leaves in manner more representative of normal environmental

damage therefore I will develop a non-destructive wounding technique but also one which is standardised.

Another stimulus shown to induce invertase activity in plants is infection by pathogens. Such induction has been observed during infection by a wide spectrum of pathogens including bacteria, necrotrophic and biotrophic fungi (Sturm and Chrispeels, 1990; Benhamou *et al.*, 1991).

A stimulation of apoplastic and/or soluble invertase activity is commonly observed during infection of leaves by biotrophic fungi (for reviews see Whipps and Lewis, 1981; Farrar and Lewis, 1987; Scholes, 1992). For example, in barley leaves infected with powdery mildew (*Erysiphe graminis*) there was a three fold stimulation of acid invertase in the infected leaf 8 d after inoculation (Scholes *et al.*, 1994). Similarly, in flax leaves inoculated with the rust fungus *Melampsora lini* soluble acid and cell-wall associated invertase activity increased during infection such that 14 d after inoculation total acid invertase activity in rusted leaves was 24 times higher than in control leaves (Clancy and Coffey, 1980). The increases in invertase activity observed during leaf biotrophic infections are thought to result in an accumulation of soluble carbohydrate within the infected leaf. This accumulation of carbohydrate is thought to lead to a down regulation of photosynthetic gene expression (Scholes *et al.*, 1994).

However, in order to understand the role that invertases play in the changes in host carbohydrate and photosynthetic metabolism, and in carbon acquisition by the fungus we need to know the origin and location of the increase in invertase activity. Fungi also contain both particulate and soluble invertase (Geissmann *et al.*, 1991; Vainstein and Peberdy, 1991) which may be released during the extraction procedure. It is possible that the increases in invertase activity observed during infection are the result of invertases produced by the pathogen; this has been a source of much debate and research (Billet *et al.*, 1977; Callow *et al.*, 1980).

Infection of *A. thaliana* with the fungal biotrophic pathogen *A. candida* (white blister rust) results in the stimulation of cell-wall associated and soluble acid invertase activity in the infected leaf. These changes are also accompanied by the appearance of a new soluble invertase isoform late in infection (Tang *et al.*, 1996). Further investigation by Chou (1997) revealed that the increase in cell-wall associated and soluble acid invertase activity and the appearance of the new soluble isoform were only

observed in regions of the leaf directly colonised by the fungal mycelium. In this chapter I aim to examine this host-pathogen interaction and identify the origin of the increase in cell-wall associated invertase activity.

A number of approaches are possible in order to identify the origin of the increases in invertase activity observed during pathogenesis. One of these is the use of immuno-gold labelled antibodies raised against a purified host or fungal invertase. Using this approach Benhamou *et al.* (1991) found that the infection of tomato roots by the necrotrophic pathogen *Fusarium oxysporum* led to the accumulation of host apoplastic invertase in root cells 48-72 h after inoculation.

A second approach is to examine the changes at the level of gene expression. By virtue of their specificity, invertase gene probes offer a unique method for distinguishing the origin of the increases commonly observed. Using this method Sturm and Chrispeels (1990) found that infection of carrot tap roots by the bacterial pathogen *Erwinia carotovora* resulted in the induction of host apoplastic invertase gene expression within 1 h. However, to date there are no reports where this approach has been used in a host-biotrophic pathogen system.

The recent cloning and identification of invertase genes in *A. thaliana* makes this second approach ideal for examining the origin of the increase in cell-wall associated invertase activity during infection of *A. thaliana* leaves by *A. candida*. To achieve this I will inoculate *A. thaliana* leaves in such a manner that a portion of the leaf remains free of fungal mycelium throughout infection (using the method of Chou, 1997). This will enable changes in invertase gene expression to be localised more precisely.

In the final part of this chapter I will investigate the impact of the soluble carbohydrates sucrose and glucose on invertase activity in *A. thaliana* leaf tissue. To date a number of reports have shown that invertase gene expression and/or activity are influenced by the internal concentration of hexoses and/or sucrose (this was discussed in detail in chapter 1). Here I report the effect of glucose and sucrose feeding on invertase activity and the soluble invertase isoform pattern in *A. thaliana* leaf discs.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Impact of *A. candida* infection on invertases in the leaves of *A. thaliana*

***Growth and inoculation of plants***

Seeds of *A. thaliana* (ecotype OY0) were grown as described in section 2.2.1. When the plants were six weeks old they were selected for uniformity and inoculated with a zoospore suspension of *A. candida*.

***Preparation of zoospore suspension***

Sporangia were scraped from the surface of twenty leaves heavily infected with *A. candida* into a petri dish containing 20 ml of distilled water. The suspension was transferred into eppendorf tubes and centrifuged at 1500 *g* at 14 °C for 10 min. The sporangia were resuspended in 10 ml of distilled water and incubated at 15 °C for 2 h after which time the spore suspension was examined microscopically every 30 min until zoospore release had occurred. When 10-20 motile zoospores were visible in the field of view under 25 x magnification (inoculum concentration of approximately $10^6$ zoospores $ml^{-1}$) the spore suspension was used to inoculate plants.

***Inoculation and harvesting of plant material***

Six to eight of the youngest fully expanded leaves were inoculated on the adaxial leaf surface with four 10 µl droplets of zoospore suspension. Each of the four droplets was placed toward the tip of the spatulate leaves as shown in Fig. 3.1. Following inoculation the plants were kept under high humidity in a propagating chamber for 24 h (Dang *et al.*, 1992). Control plants were inoculated with distilled water and treated in an identical manner. Following the high humidity treatment the plants were grown with a 9 h photoperiod at an irradiance of 130 µmol $m^{-2}$ $s^{-1}$ as described in section 2.2.1. For experimental purposes the regions of the infected leaves

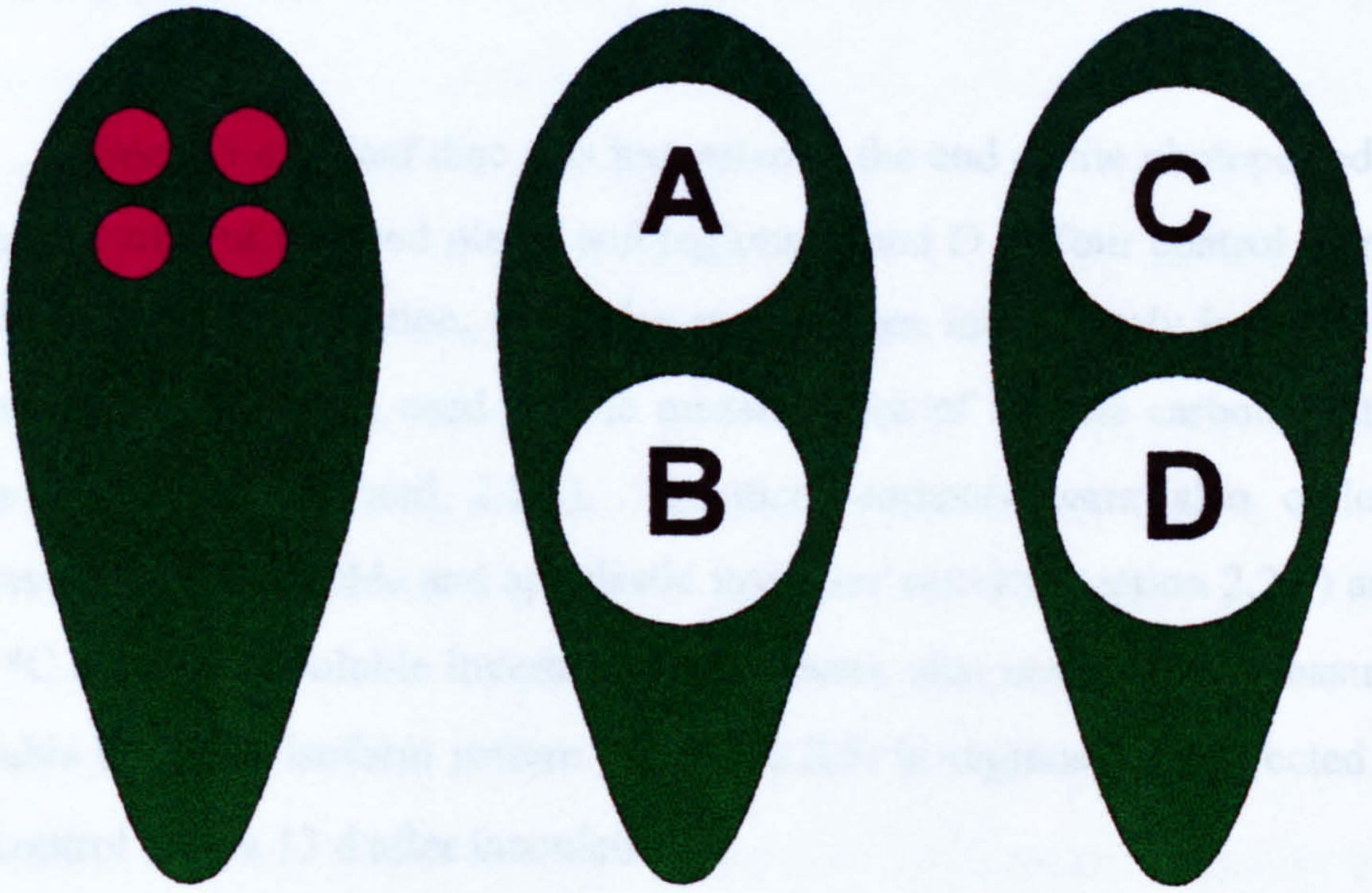



**Figure 3.1 Diagrammatic representation of the inoculation procedure and sampling of *A. thaliana* leaves infected with *A. candida*.** Leaves were inoculated with two 10 µl droplets of zoospore suspension placed on each side of the midrib at the tip of the adaxial leaf surface. Leaf discs were harvested 4, 6, 8, 10 and 13 days after inoculation. (A) Infected region of an infected leaf. (B) Uninfected region of an infected leaf. (C) Region of control leaf equivalent to infected region A. (D) Region of control leaf equivalent to uninfected region B.

inoculated with zoospores were termed region A, the uninoculated region of the same leaf, region B and identical regions on control leaves regions C and D respectively (Fig. 3.1).

***Analysis of invertase activity and soluble and storage carbohydrates in infected and control plants***

One 2.5 $cm^2$ leaf disc was harvested at the end of the photoperiod from regions A and B of four infected plants and regions C and D of four control plants 4, 6, 8, 10 and 13 d after inoculation. Samples were frozen immediately in liquid nitrogen and stored at –20 °C until used for the measurement of soluble carbohydrates and starch (see sections 2.2.2 and 2.2.3). Identical samples were also collected for the measurement of soluble and apoplastic invertase activity (section 2.2.4) and stored at –80 °C until use. Soluble invertase extracts were also used for the measurement of the soluble invertase isoform pattern (section 2.2.5) in regions A of infected leaves and C of control leaves 13 d after inoculation.

***RT-PCR analysis of ATβFRUCT 1 gene expression***

The reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect the expression of the ATβFRUCT 1 apoplastic invertase gene in regions A and B of infected leaves and regions C and D of control leaves 4, 6, 8, 10 and 13 d after inoculation. In total 60 – 100 $cm^2$ of leaf discs were harvested from each region at each time point and placed into individually labelled foil packets and frozen immediately in liquid nitrogen prior to storage at –80 °C. The leaf discs were harvested 5 h after the beginning of the photoperiod. Total RNA was prepared using the method of Loening (1969) and is described in section 2.2.17.

Semi-quantitative RT-PCR was used to analyse the expression of ATβFRUCT 1, the procedure for which is discussed in detail in section 2.2.20.

### 3.2.2 Effect of leaf wounding on invertases in *A. thaliana*

***Experimental design***

*A. thaliana* plants (ecotype OY0) were grown as described in section 2.2.1. Leaf wounding was achieved using a device that enabled the amount and severity of wounding to be carefully replicated each time it was performed. This device was made from a modified 1 ml plastic syringe (Fig. 3.2). This device was assembled by removing the plunger from the syringe barrel and shortening the plunger by ~2 cm. A spring (with a diameter equal to that of the syringe barrel) was placed onto the plunger and the plunger was reinserted into the barrel of the syringe. The external end of the plunger consisted of a plastic plate with a ridged surface. Leaves were wounded by placing the external end of the modified syringe onto the upper surface of a leaf and by pressing down onto a second plunger (held between the fingers) on the lower surface of the leaf. Pressure was applied quickly and ceased when the spring was fully compressed. The plunger ends were juxtaposed such that the ridges were at 90° to one another. Where the ridges crossed a series of small punctures were made in the leaf.

The eight largest leaves on each plant were wounded with the wound being made across the mid-rib toward the tip of the leaf. The wounded area (and comparable regions of control plants) was sampled using a 1.54 $cm^2$ cork borer 0, 8, 24, 48, 72 h after wounding.

***Analysis of invertase activity and ATβFRUCT 1 gene expression***

For the measurement of ATβFRUCT 1 gene expression and soluble and apoplastic invertase activity 60 $cm^2$ and 12 $cm^2$ respectively of leaf discs were harvested from control and wounded leaves at each time point. For the measurement of invertase activity replicate samples were taken from three plants. Samples were placed into labelled foil packets and frozen immediately in liquid nitrogen prior to storage at –80 °C.

RNA was extracted from the tissue as described in section 2.2.17 and ATβFRUCT 1 gene expression analysed using semi-quantitative RT-PCR as described

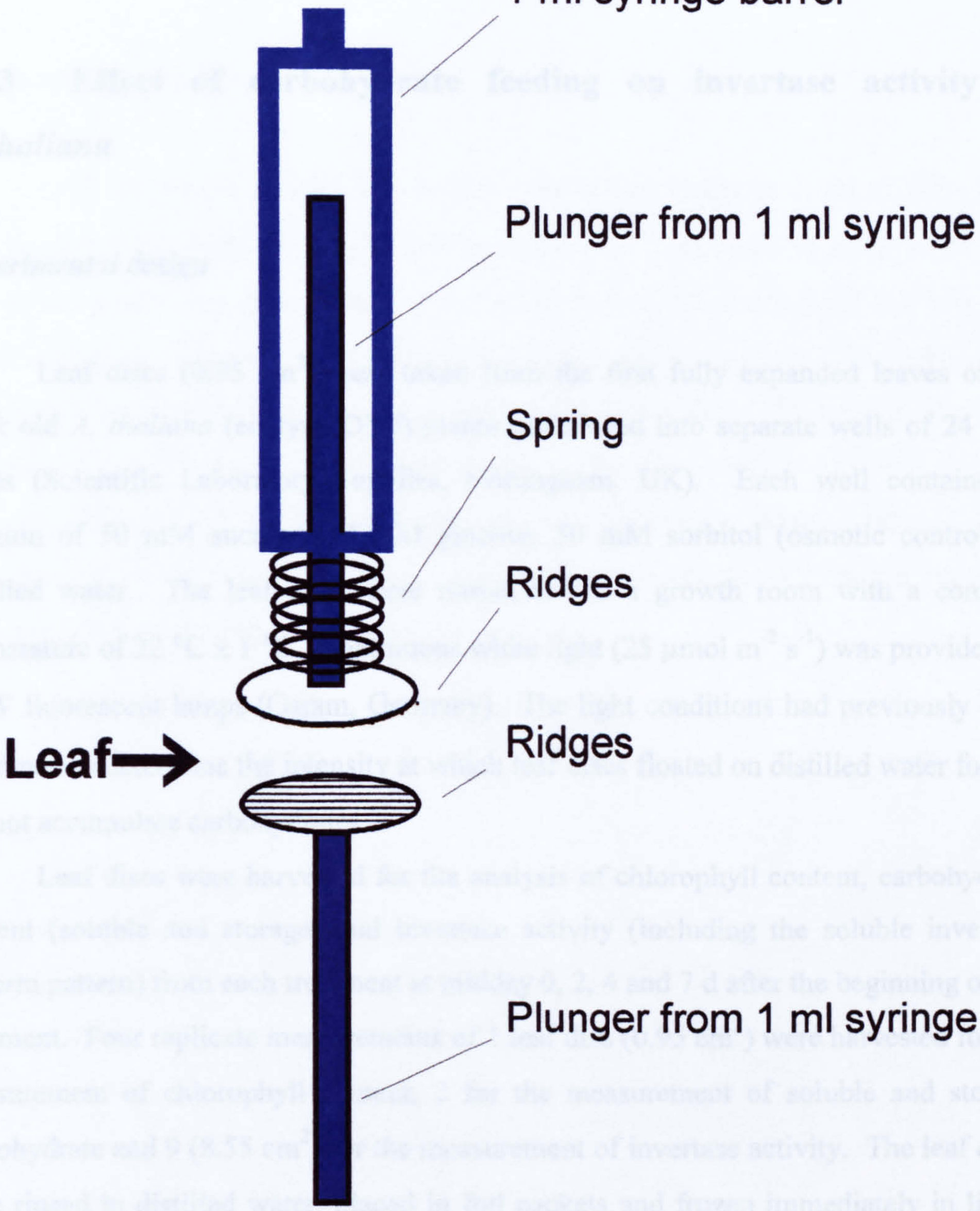



**Figure 3.2 Diagrammatic representation of the device used to wound the leaves of *A. thaliana* plants.**

in section 2.2.20. Soluble and apoplastic invertase activity was measured as described in section 2.2.3.

### 3.2.3 Effect of carbohydrate feeding on invertase activity in *A. thaliana*

***Experimental design***

Leaf discs (0.95 $cm^2$) were taken from the first fully expanded leaves of six week old *A. thaliana* (ecotype OY0) plants and placed into separate wells of 24 well plates (Scientific Laboratory Supplies, Nottingham, UK). Each well contained a solution of 50 mM sucrose, 50 mM glucose, 50 mM sorbitol (osmotic control) or distilled water. The leaf discs were transferred to a growth room with a constant temperature of 22 °C ± 1 °C. Continuous white light (25 µmol $m^{-2}$ $s^{-1}$) was provided by 80 W fluorescent lamps (Osram, Germany). The light conditions had previously been examined to determine the intensity at which leaf discs floated on distilled water for 7 d did not accumulate carbohydrates.

Leaf discs were harvested for the analysis of chlorophyll content, carbohydrate content (soluble and storage) and invertase activity (including the soluble invertase isoform pattern) from each treatment at midday 0, 2, 4 and 7 d after the beginning of the treatment. Four replicate measurements of 1 leaf disc (0.95 $cm^2$) were harvested for the measurement of chlorophyll content, 2 for the measurement of soluble and storage carbohydrate and 9 (8.55 $cm^2$) for the measurement of invertase activity. The leaf discs were rinsed in distilled water, placed in foil packets and frozen immediately in liquid nitrogen prior to storage at –80 °C.

***Measurement of carbohydrates (soluble and storage), invertase activity and the soluble invertase isoform pattern***

Soluble and storage carbohydrates were extracted and measured as described in section 2.2.2 and section 2.2.3. Invertase activity (soluble and apoplastic) was extracted and measured as described in section 2.2.4. A portion of the soluble invertase extract

was retained for the analysis of the soluble invertase isoform pattern by isoelectric focussing as described in section 2.2.5.

*Measurement of chlorophyll content*

Leaf discs were ground in a chilled mortar and pestle in 1 ml of 80% (v/v) acetone under low light conditions and the suspension transferred to a test tube. The mortar and pestle was rinsed twice with 1 ml of 80% (v/v) acetone and this was also transferred to the test tube which was made up to a known volume. The tubes were centrifuged for 5 min at 4000 x *g* to remove the leaf debris. One ml of the clear supernatant was transferred into a glass cuvette and the absorbance read at 663, 646 and 470 nm (UV-1201 UV-VIS spectrophotometer, Shimadzu, Kyoto, Japan). The concentration of chlorophylls a, b and carotenoids were calculated from the equations of Lichtenthaler and Wellburn (1983).

## 3.3 RESULTS

### 3.3.1 Impact of *A. candida* infection on invertases in the leaves of *A. thaliana*.

*Symptom development*

Figure 3.3 shows the development of visible symptoms of *A. candida* infection on the leaves of *A. thaliana* plants. Four days after inoculation no visible symptoms of infection could be seen on either the upper or lower surfaces of the leaf. Eight days after inoculation fungal spores were visible on the lower surface of the leaf directly below the region of inoculation. These sporulating regions formed discrete patches which spread and merged to cover region A of the lower leaf surface by 14 days after inoculation. Late in the infection cycle (8-14 d) chlorotic regions were visible on the upper leaf surface. These regions were closely associated with the developing spore patches on the lower leaf surface. The edge of the chlorotic regions were often surrounded by a red border; probably regions of anthocyanin accumulation.

*ATβFRUCT 1 apoplastic invertase gene expression during disease development*

Table 3.1 shows the changes in ATβFRUCT 1 apoplastic invertase gene expression in regions A and B of infected leaves and regions C and D of control leaves 4, 6, 8, 10 and 13 days after inoculation with *A. candida*.

Four and 6 d after inoculation the amount of ATβFRUCT 1 gene expression in both regions of control and infected leaves were similar and low. Thereafter, the amount of expression in region A of infected leaves increased dramatically while that in region B of infected leaves and in regions C and D of control leaves remained stable and low. Eight days after inoculation the amount of ATβFRUCT 1 gene expression in region A had increased 8 fold and represented an average of 57 pg of message per μg of total RNA, 9.5 times higher than in region C (comparable region of control leaf). By 10 d after inoculation the amount of expression in region A increased slightly to represent an average of 69 pg of message per μg of total RNA. Thirteen d after inoculation

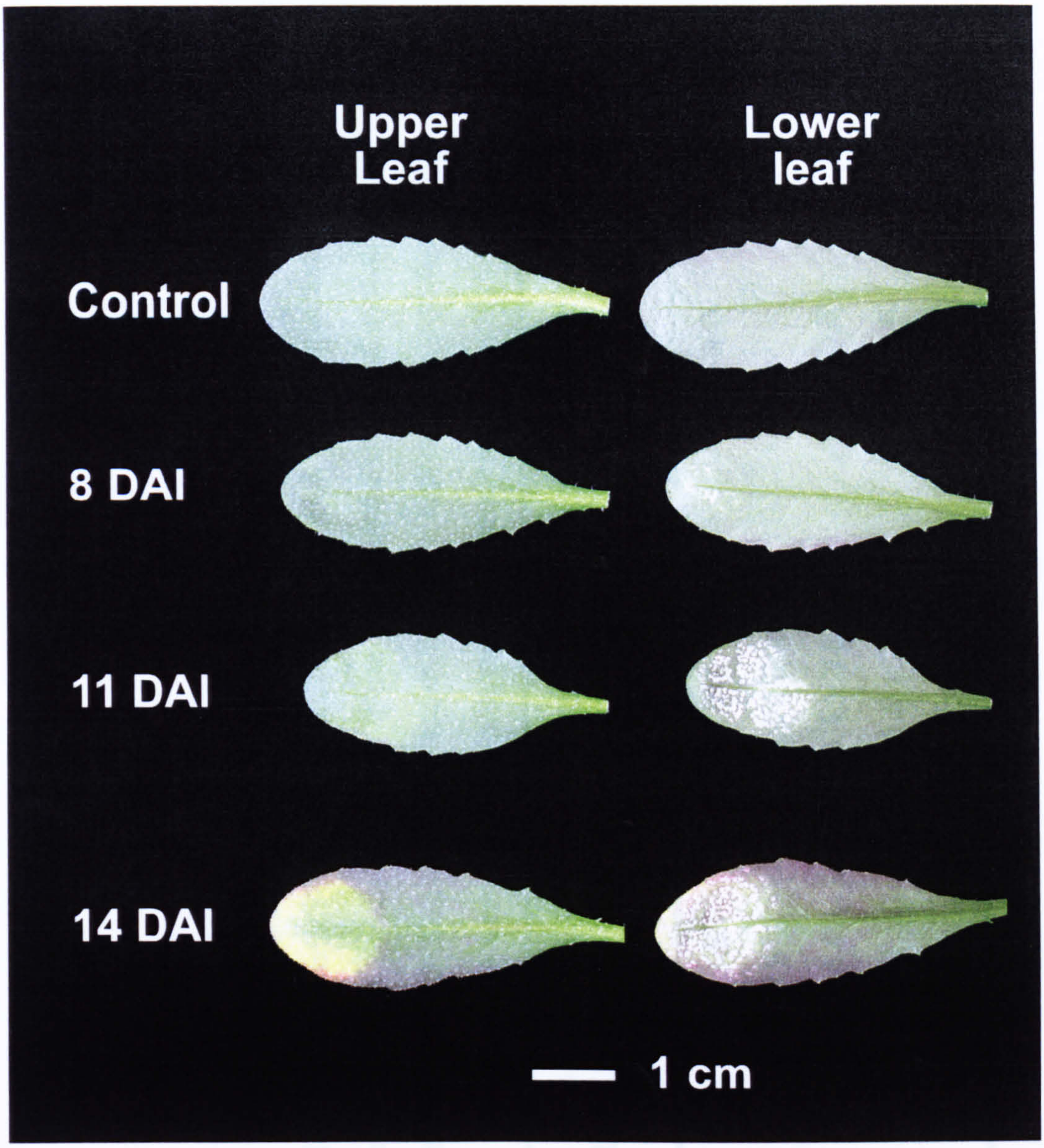



**Figure 3.3 Development of visible symptoms of *Albugo candida* infection on leaves of *A. thaliana* plants.** The upper and lower surfaces of *A. thaliana* leaves infected with *A. candida* are shown 8, 11 and 14 days after inoculation (DAI) with *A. candida*. The upper and lower surfaces of a healthy leaf are also shown for comparison.

**Table 3.1 Expression of the ATβFRUCT 1 apoplastic invertase gene in the leaves of *A. thaliana* infected with *A. candida*.** The amount of expression in regions A and B of infected leaves and regions C and D of control leaves were analysed during the infection cycle and measured using semi-quantitative RT-PCR. Expression is expressed in pg of ATβFRUCT 1 per μg of total RNA. Results are the mean ± S.E. of three replicate measurements.

| Day | **Region A** pg per μg total RNA ± S.E. | **Region B** pg per μg total RNA ± S.E. | **Region C** pg per μg total RNA ± S.E. | **Region D** pg per μg total RNA ± S.E. |
|---|---|---|---|---|
| **4** | 4.5 ± 0.4 | 10.1 ± 3.8 | <0.5 | 15.7 ± 2.0 |
| **6** | 6.6 ± 1.7 | 5.6 ± 0.9 | 0.8 ± 0.4 | <0.5 |
| **8** | 56.6 ± 3.7 | 17.6 ± 4.4 | 6.4 ± 0.5 | 14.7 ± 0.8 |
| **10** | 68.7 ± 8.9 | 2.3 ± 2.0 | 11.9 ± 2.1 | 11.9 ± 0.4 |
| **13** | 436.1 ± 105.7 | 15.1 ± 1.5 | <0.5 | <0.5 |

ATβFRUCT 1 gene expression had increased dramatically in region A to represent an average of 436 pg of message per μg of total RNA. At this point the amount of ATβFRUCT 1 gene expression was too low to detect in the control leaves.

***Soluble and apoplastic invertase activity in infected leaves***

Figure 3.4a shows the amount of soluble invertase activity in regions A, B, C and D of leaves infected with *A. candida* 4, 6, 8, 10 and 13 d after inoculation. Activity in regions A and B of infected leaves and C and D of control leaves declined sharply until 8 d after inoculation. Thereafter soluble invertase activity in regions A, C and D recovered. Thirteen d after inoculation soluble invertase activity in region A was significantly higher than in region B ($P < 0.05$).

Figure 3.4b shows the amount of apoplastic invertase activity in regions A, B, C and D after inoculation. Apoplastic invertase activity in the leaves was approximately 15 fold lower than soluble invertase activity. At all times the amount of apoplastic invertase activity in region A was higher than in regions B, C and D. Activity in region A doubled between 10 and 13 d after inoculation from 5.8 to 12 nmol of sucrose hydrolysed $cm^{-2}$ $min^{-1}$ and was significantly higher than in all other regions ($P < 0.05$). Apoplastic invertase activity in regions B, C and D remained constant throughout infection at approximately 3 nmol of sucrose hydrolysed $cm^{-2}$ $min^{-1}$.

***Changes in the soluble invertase isoform pattern during infection***

The soluble invertase isoform pattern was analysed in region A of infected leaves and region C of control leaves 13 d after inoculation (Fig. 3.5). In both control and infected leaves 4 major isoforms were detected with pIs between 4.65 and 4.85. The activity of these isoforms were stronger in region C than in region A. In region A an additional strongly staining isoform was detected with a pI of 5.1.

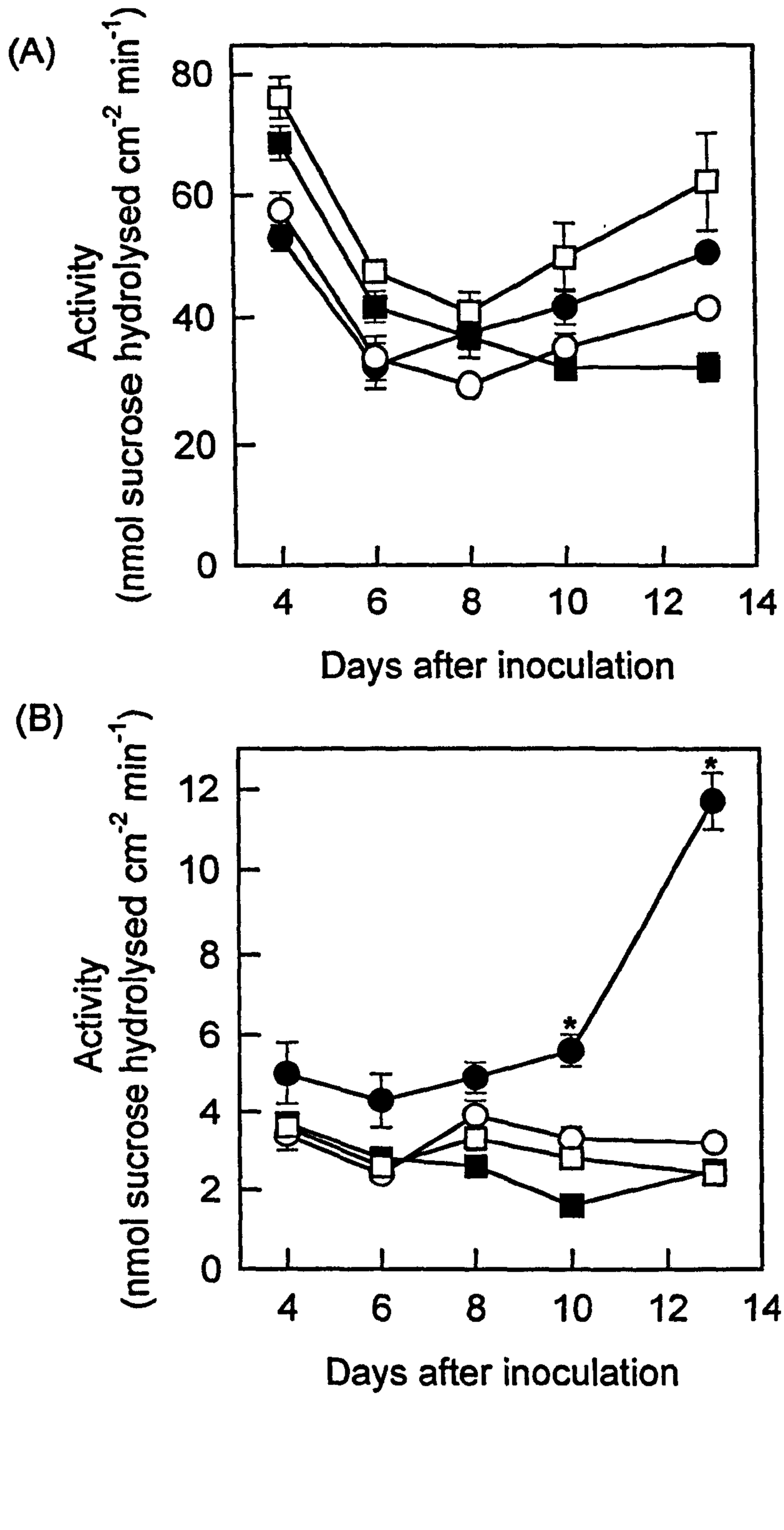



**Figure 3.4 The effect of *A. candida* infection on soluble and apoplastic invertase activity in the leaves of *A. thaliana* plants throughout infection.** Soluble acid (A) and apoplastic (B) invertase activity was measured in region A of infected leaves (-●-), region B of infected leaves (-■-) and equivalent control regions C (-○-) and D (-□-). These results are the means ± the standard error of four replicate measurements. * indicate that data are significantly different from the comparable control ($P < 0.05$).

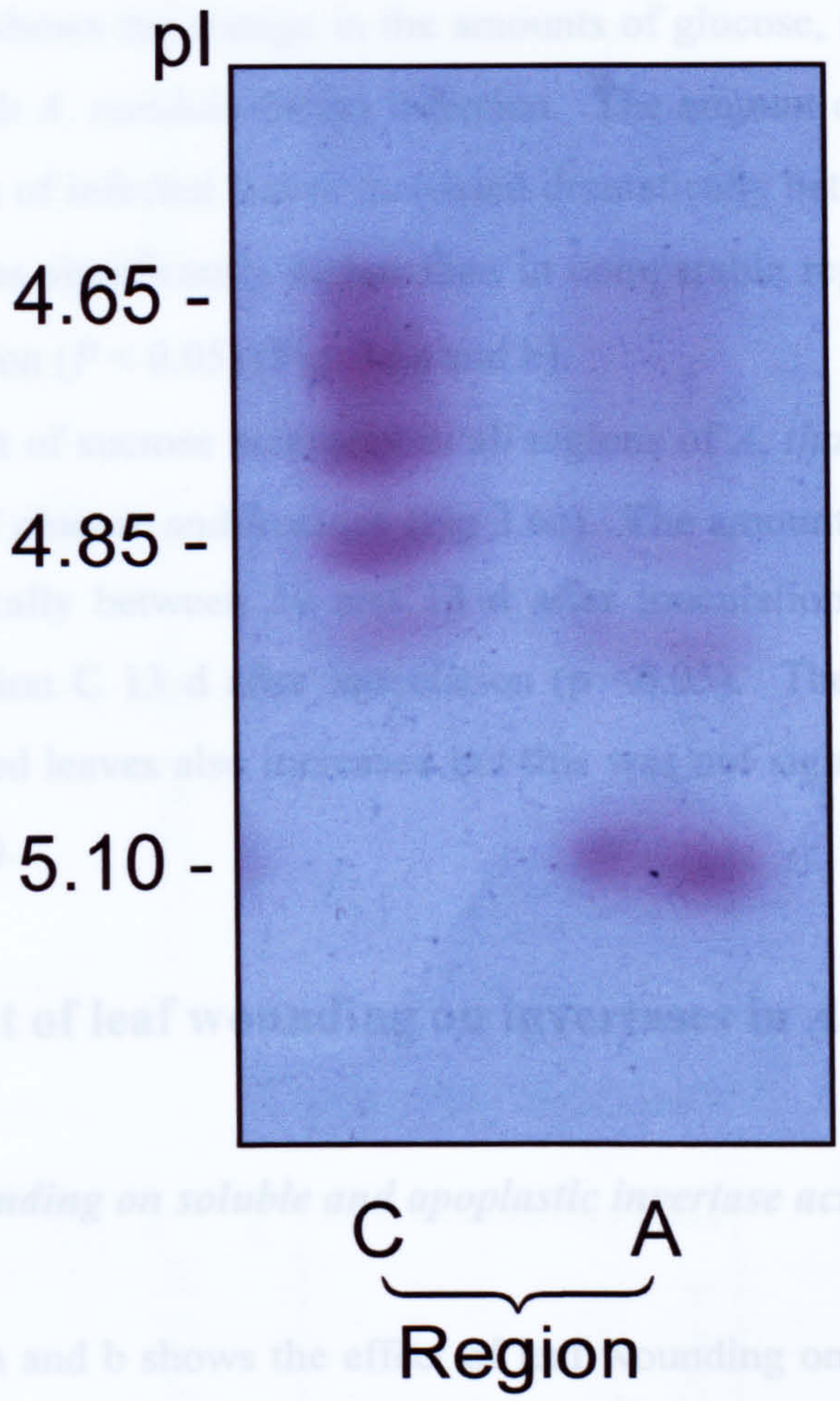



**Figure 3.5 Soluble invertase isoforms in leaves heavily infected with *A. candida* and comparable non-infected leaves.** Soluble invertase isoforms were separated by isoelectric focussing over a pH range of 4.0 – 6.5. Invertase activity was visualised by staining reducing sugars with 2, 3, 5 tetrazolium chloride. Samples were prepared from region A of infected leaves 13 d after inoculation (A) and from comparable regions of control leaves (C). Samples were loaded on an equal activity basis.

*Changes in leaf soluble carbohydrate content during disease development*

Figure 3.6 shows the change in the amounts of glucose, fructose and sucrose in leaves infected with *A. candida* during infection. The amount of glucose and fructose in regions A and B of infected leaves increased dramatically between 10 and 13 d after inoculation and was significantly higher than in comparable regions of control leaves 13 d after inoculation ($P < 0.05$) (Fig. 3.6a and b).

The amount of sucrose measured in all regions of *A. thaliana* leaves was lower than the amount of glucose and fructose (Fig 3.6c). The amount of sucrose in region A increased dramatically between 10 and 13 d after inoculation and was significantly higher than in region C 13 d after inoculation ($p < 0.05$). The amount of sucrose in region B of infected leaves also increased but this was not significantly higher than in region D (Fig 3.6c).

## 3.3.2 The effect of leaf wounding on invertases in *A. thaliana*

*Effect of leaf wounding on soluble and apoplastic invertase activity*

Figure 3.7a and b shows the effect of leaf wounding on soluble and apoplastic invertase activity 0, 8, 24, 48 and 72 h after wounding. Leaves were wounded in a localised area and this area was used for subsequent measurements of invertase activity. The same localised region of non-wounded leaves were analysed as a control.

There was a significant increase in apoplastic invertase activity following leaf wounding (Fig. 3.7b). This increase in activity was first observed 24 h after the wound was inflicted and was approximately 2 fold higher than in control leaves ($P < 0.05$). Activity remained significantly higher ($P < 0.05$) than in control leaves until 72 h (the last time point measured).

In contrast, there was no increase in soluble acid invertase activity following leaf wounding (Fig. 3.7a).

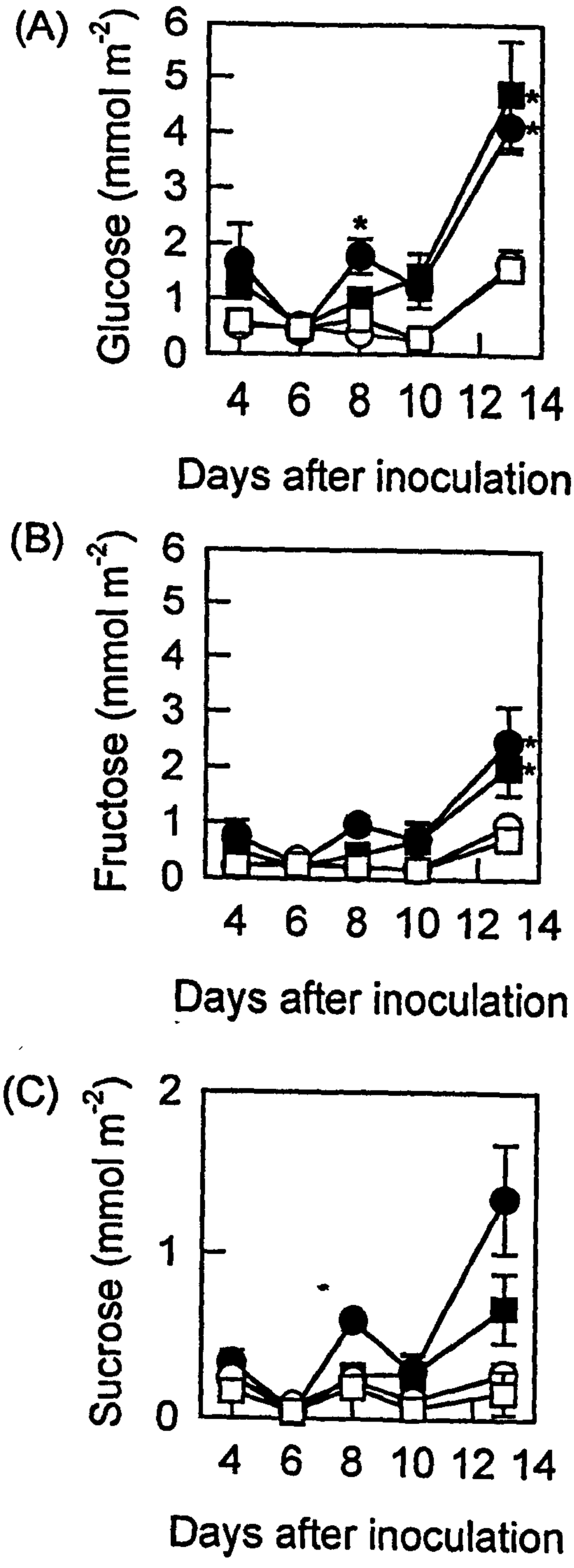



**Figure 3.6 The impact of *A. candida* infection on the amount of soluble carbohydrates in the leaves of *A. thaliana* plants throughout infection.** Leaf glucose (A), fructose (B) and sucrose (C) were measured in region A (-●-) and B (-■-) of infected leaves and regions C (-○-) and D (-□-) of control leaves 4, 6, 8, 10 and 13 d after inoculation of leaves with *A. candida*. These results are the means ± the standard error of four replicate measurements. * indicate that data are significantly different from the comparable control ($P < 0.05$).

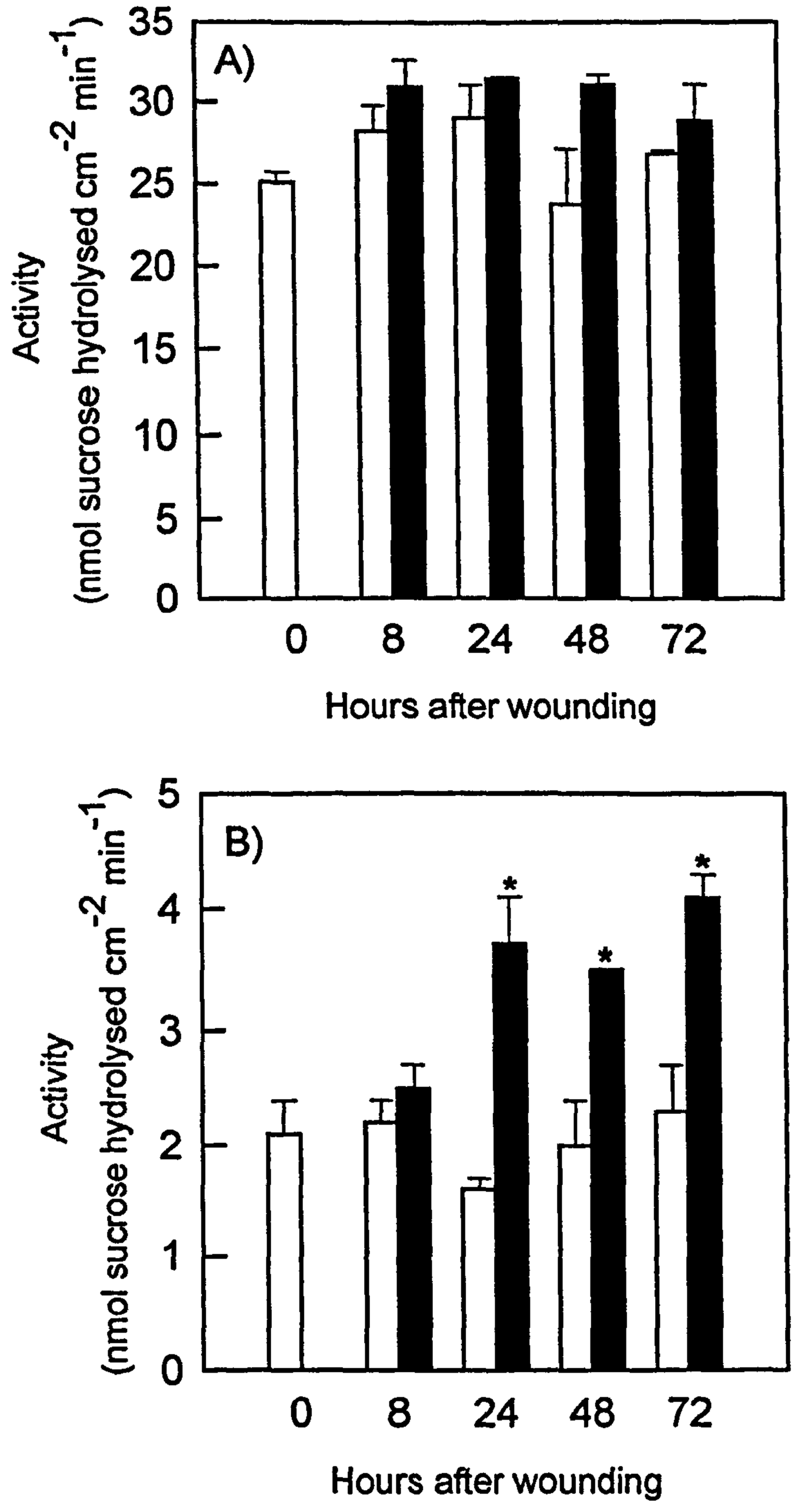



**Figure 3.7 Wound-induced changes in invertase activity.** Mature leaves of *A. thaliana* plants were wounded and measurements of soluble (A) and apoplastic (B) invertase activity were made 0, 8, 24, 48 and 72 hours after the wound was inflicted. Samples were taken from the wounded region of the leaf (filled bars) and from control leaves (open bars). The results are the mean ± the standard error of three replicate measurements. * indicate that the data are significantly different ($p<0.05$).

### *ATβFRUCT 1 gene expression in wounded leaves*

Semi-quantitative RT-PCR was used to analyse the expression of the ATβFRUCT 1 apoplastic invertase gene in wounded regions, and equivalent control regions, of *A. thaliana* leaves 0, 8, 24, 48 and 72 h after wounding. The RT-PCR system used in this thesis included an internal standard that co-amplified with the ATβFRUCT 1 cDNA; this was used as the basis of quantification (see section 2.2.20). However, for this particular analysis of ATβFRUCT 1 gene expression it was not possible to consistently amplify the internal standard. The reason for this failure is not known although previously it has been observed that contamination of sample RNA can lead to internal standard amplification problems.

Despite the failure of internal standard amplification it was still possible to observe broad trends in ATβFRUCT 1 gene expression in wounded and control leaves (Fig. 3.8b). This was possible, despite slight sample to sample variations in amplification efficiency, because large differences in gene expression still resulted in comparable differences in the amount of amplification product. These differences could be distinguished visually on an agarose gel in a similar manner to the hybridising fragments on a Northern blot.

Several parameters were examined in order to ensure that differences in amplification were due to differences in gene expression and not experimental inaccuracies. Firstly, the RNA used for this experiment was successfully and equally quantified (Fig. 3.8a). Secondly, when 8 µl and 4 µl of sample cDNA were amplified the amount of ATβFRUCT 1 amplification product in the 4 µl sample was approximately half that seen when 8 µl was amplified. This demonstrated that PCR was amplifying consistently and evenly between samples such that differences in the amount of cDNA added were reflected by differences in the amount of ATβFRUCT 1 amplification produced.

There was no amplification of the ATβFRUCT 1 fragment in RNA from pre-wounded leaves indicating extremely low expression of this gene (Fig. 3.8b). Eight h after wounding a 349 bp ATβFRUCT 1 amplification product could be observed but was of similar intensity in both wounded and control leaves. This indicated that 8 h after wounding there was no measurable increase in ATβFRUCT 1 gene expression

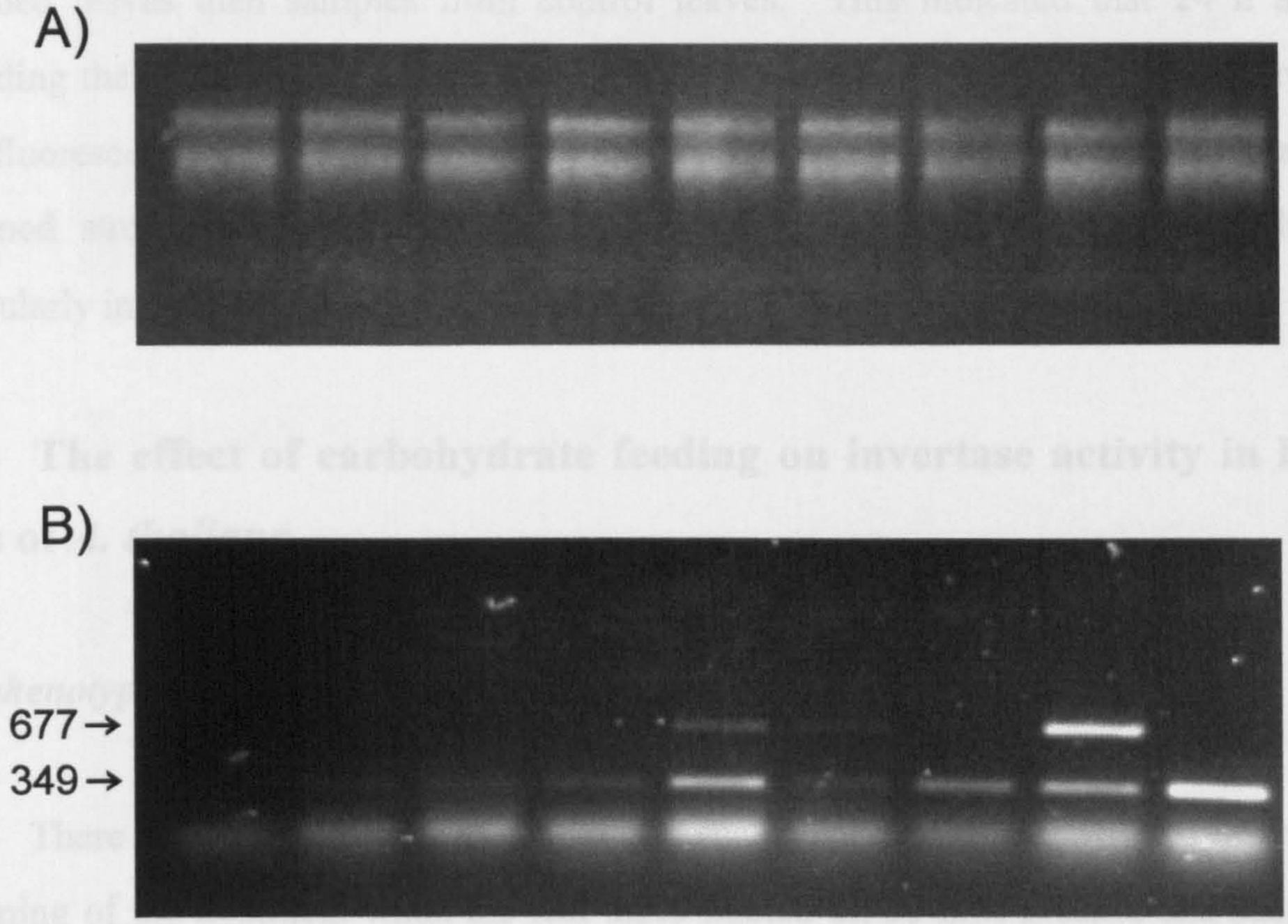



**Figure 3.8 ATβFRUCT 1 apoplastic invertase gene expression in wounded and control *A. thaliana* leaves.** Samples from the wounded region of wounded leaves (W) and the equivalent regions of control leaves (C) were harvested 0, 8, 24, 48 and 72 h after wounding and used to prepare total RNA. The RNA was quantified and 1 µg electrophoresed on an agarose gel to ensure correct quantification (A). Sample RNA was used for the examination of ATβFRUCT 1 gene expression using semi-quantitative RT-PCR (B). Following amplification 20 µl of sample was electrophoresed on an agarose gel, stained with ethidium bromide and visualised using UV light. Amplified ATβFRUCT 1 was visible as a 349 bp fragment while amplified internal standard was visible as a 677 bp fragment.

(Fig 3.8b). However, 24 h after wounding the fluorescence intensity of the 349 bp ATβFRUCT 1 amplification product was significantly stronger in samples from wounded leaves than samples from control leaves. This indicated that 24 h after wounding there was an increase in ATβFRUCT 1 gene expression in wounded leaves. The fluorescence of ATβFRUCT 1 amplification products from wounded leaves remained stronger than those from control leaves 48 and 72 h after wounding; particularly in the 72 h sample (Fig 3.8b).

### 3.3.3 The effect of carbohydrate feeding on invertase activity in leaf discs of *A. thaliana*

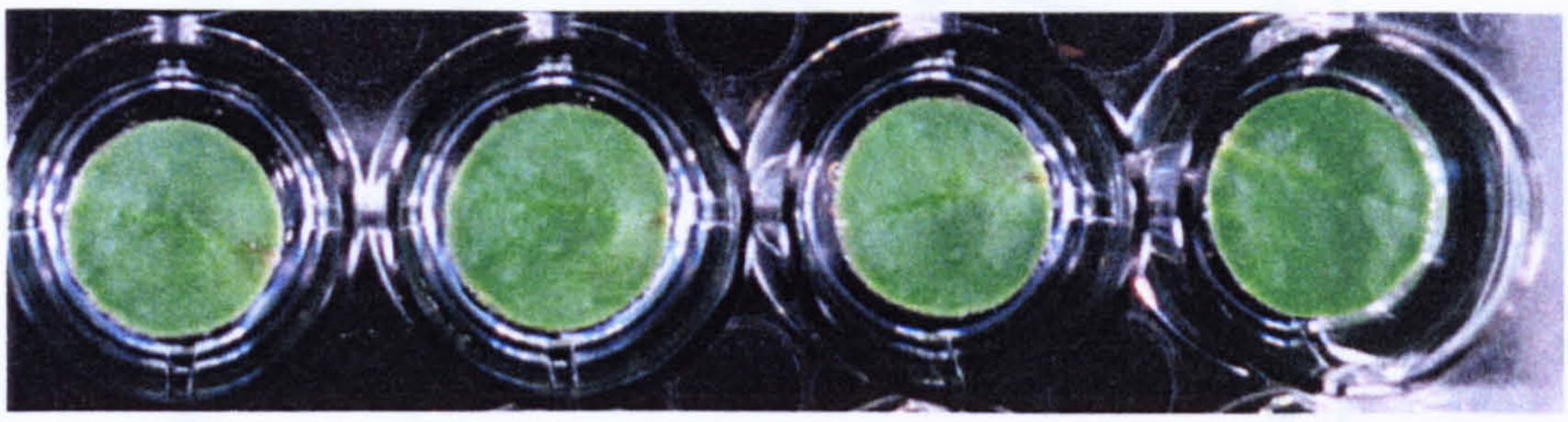
*The phenotype of carbohydrate-fed leaf discs*

There was no difference in the appearance of the leaf discs until 4 d after the beginning of the treatment when the leaf discs floated on 50 mM glucose and sucrose appeared paler in colour compared with the controls. Around the edge of the leaf discs a yellow border could be seen extending 1-2 mm into the centre of the discs. Figure 3.9 shows the appearance of the discs 7 d after the beginning of the treatment. The leaf discs floated on 50 mM glucose and sucrose were considerably paler than those floated on distilled water and showed zones of chlorosis around the edge of the discs. In contrast those discs floated on distilled water were uniformly green 7 d after the beginning of the treatment. Leaf discs floated on 50 mM sorbitol were similar in appearance to those floated on distilled water (data not shown).

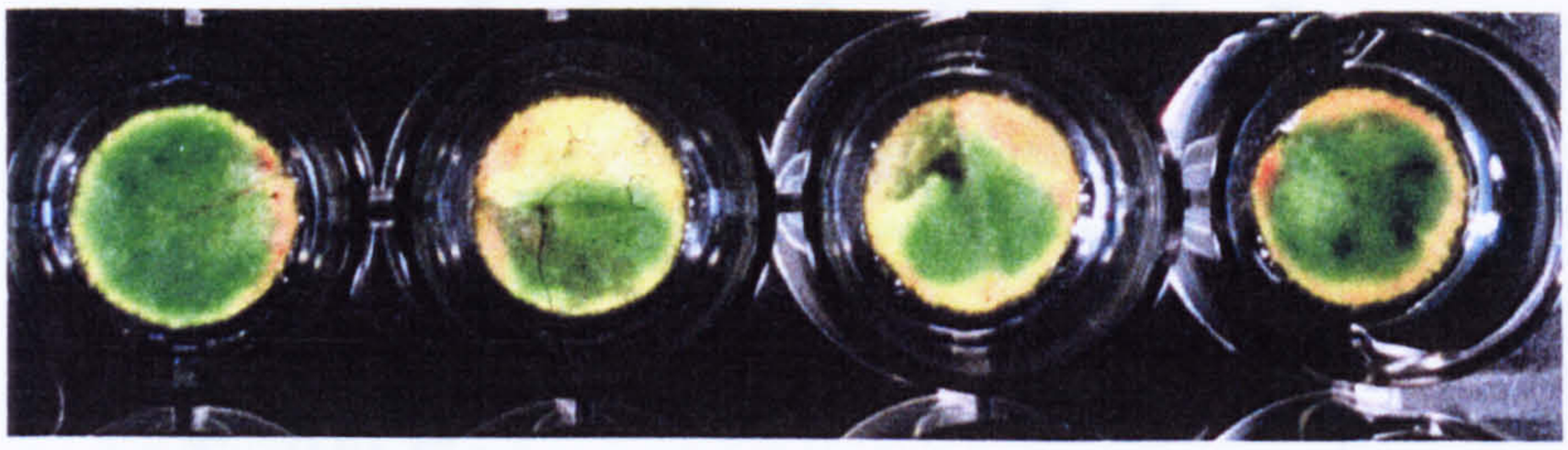
*The amount of chlorophyll in carbohydrate-fed leaf discs*

Figure 3.10a and b shows the amount of chlorophyll in leaf discs floated on 50 mM sucrose, glucose and in control leaf discs. The amount of chlorophyll in leaf discs floated on 50 mM glucose and sucrose for 7 d decreased over the time period compared to control leaf discs. The rate of decline was slower in leaf discs floated on 50 mM glucose than on leaf discs floated on 50 mM sucrose. The decline in the amount of chlorophyll in leaf discs floated on 50 mM sucrose was first measured 4 d after the

Control



50 mM sucrose



50 mM glucose

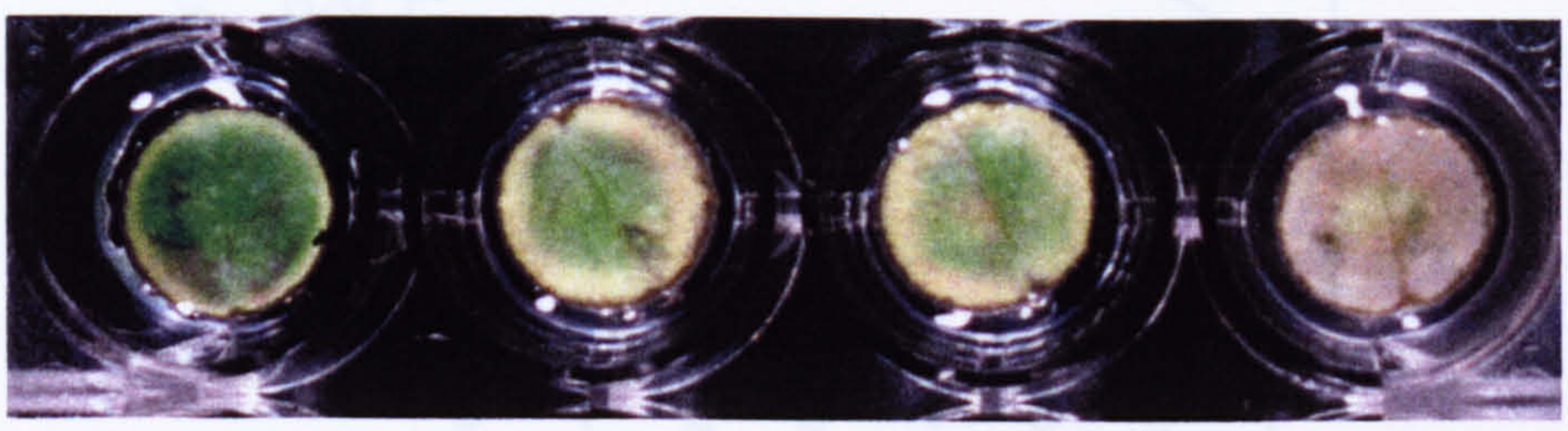


**Figure 3.9 Phenotype of *A. thaliana* leaf discs fed soluble carbohydrates for 7 days.** Leaf discs (0.95 $cm^2$) were floated on solutions of 50 mM sucrose, 50 mM glucose or distilled water and placed under continuous low light for 7 days.

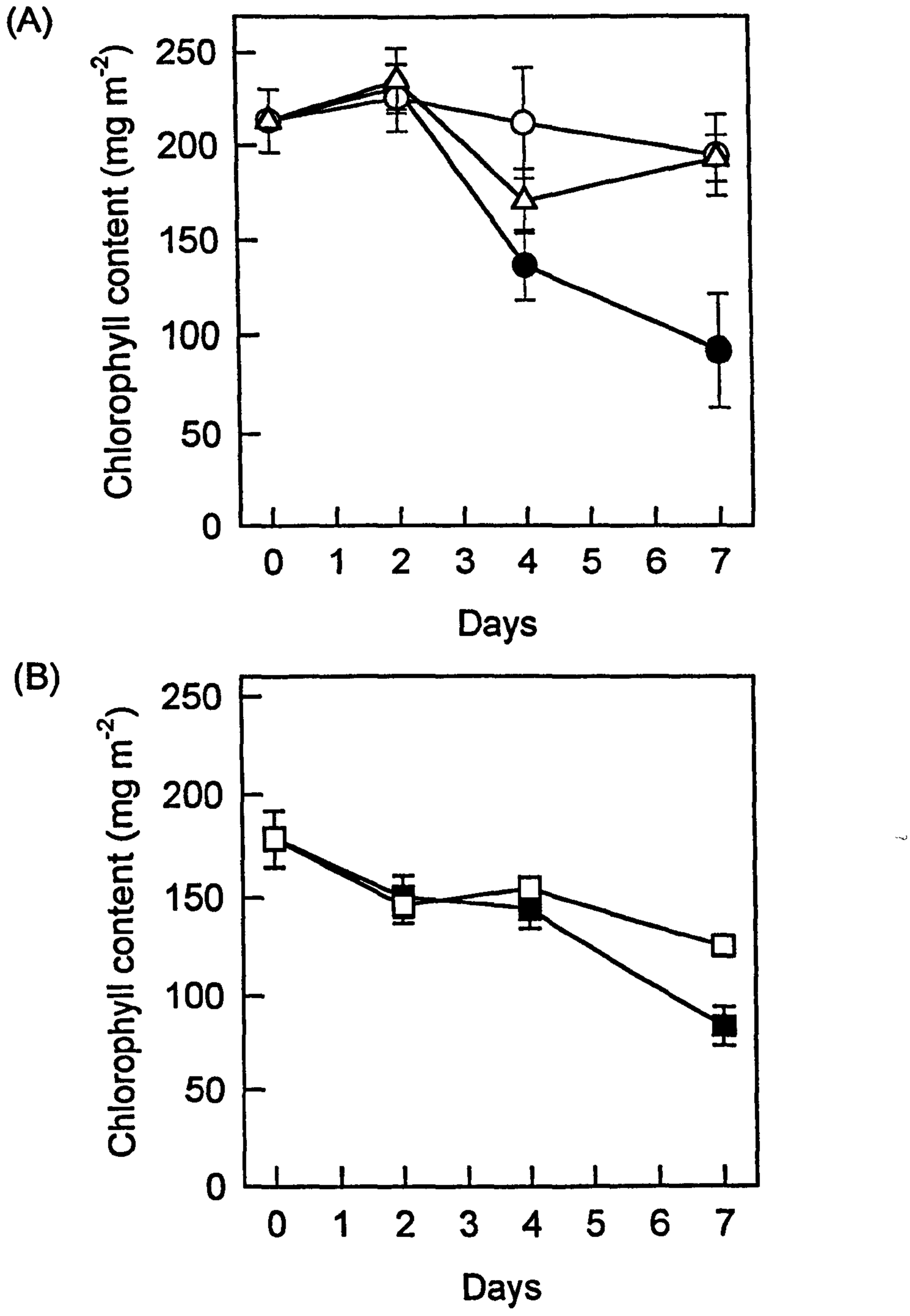



**Figure 3.10 Chlorophyll content of *A. thaliana* leaf discs fed soluble carbohydrate for 7 days.** leaf discs were fed either 50 mM sucrose (-●-), 50 mM sorbitol (-△-), or distilled water (-○-) (A); or 50 mM glucose (-■-) or distilled water (-□-) (B). Discs were taken for analysis of chlorophyll content after 0, 2, 4 and 7 d. The results are the mean ± S.E. of four replicate measurements.

beginning of the treatment and continued to decline to approximately half the initial amount by day 7 (Fig 3.10a). The amount of chlorophyll in leaf discs floated on 50 mM glucose declined gradually until 4 d after the beginning of the treatment. The rate of decline increased thereafter with the amount after 7 d half that at the beginning of the treatment (Fig 3.10b). There was a small decline in the amount of chlorophyll in control leaf discs floated on distilled water and 50 mM sorbitol during the treatment (Fig. 3.10 a and b).

***Soluble and storage carbohydrate in carbohydrate fed leaf discs***

Soluble carbohydrates accumulated in leaf discs fed 50 mM sucrose (Fig 3.11 a, b and c) and 50 mM glucose (Fig. 3.12 a, b and c) compared to control leaf discs floated on 50 mM sorbitol (osmotic control) or distilled water.

Sucrose-fed leaf discs accumulated glucose steadily throughout the treatment (Fig. 3.11a) while the amount of glucose in control leaf discs increased only slightly. By 7 d after the beginning of the treatment the amount of glucose in leaf discs fed 50 mM sucrose was approximately 17 mmol $m^{-2}$ or 24 times higher than in pre-treatment discs and 8 fold higher than leaf discs floated on distilled water for 7 d. Leaf discs fed 50 mM sucrose showed a similar pattern of fructose accumulation (Fig. 3.11b) although less than half the amount of fructose, compared with glucose, was measured at any time point. Leaf discs fed 50 mM sucrose also accumulated sucrose during the treatment (Fig. 3.11c) with the amount after 7 d 3 times higher than in pre-treatment discs. The maximum amount of sucrose in the sucrose-fed leaf discs was 1.8 mm $m^{-2}$, considerably lower than the amounts of glucose and fructose.

Similar patterns of soluble carbohydrate accumulation were measured in leaf discs fed 50 mM glucose (Fig. 3.12a, b and c). Leaf discs fed 50 mM glucose accumulated glucose until 4 d after the beginning of the treatment (Fig. 3.12a). However, by 7 d after the beginning of the treatment the amount of glucose in the leaf discs had dropped to control levels. The reason for this decline is unknown. In contrast leaf discs fed 50 mM glucose accumulated fructose throughout the treatment (Fig. 3.12b). The amount of glucose and fructose accumulating within the leaf discs fed 50 mM glucose was lower than in leaf discs fed 50 mM sucrose. The amount of sucrose in

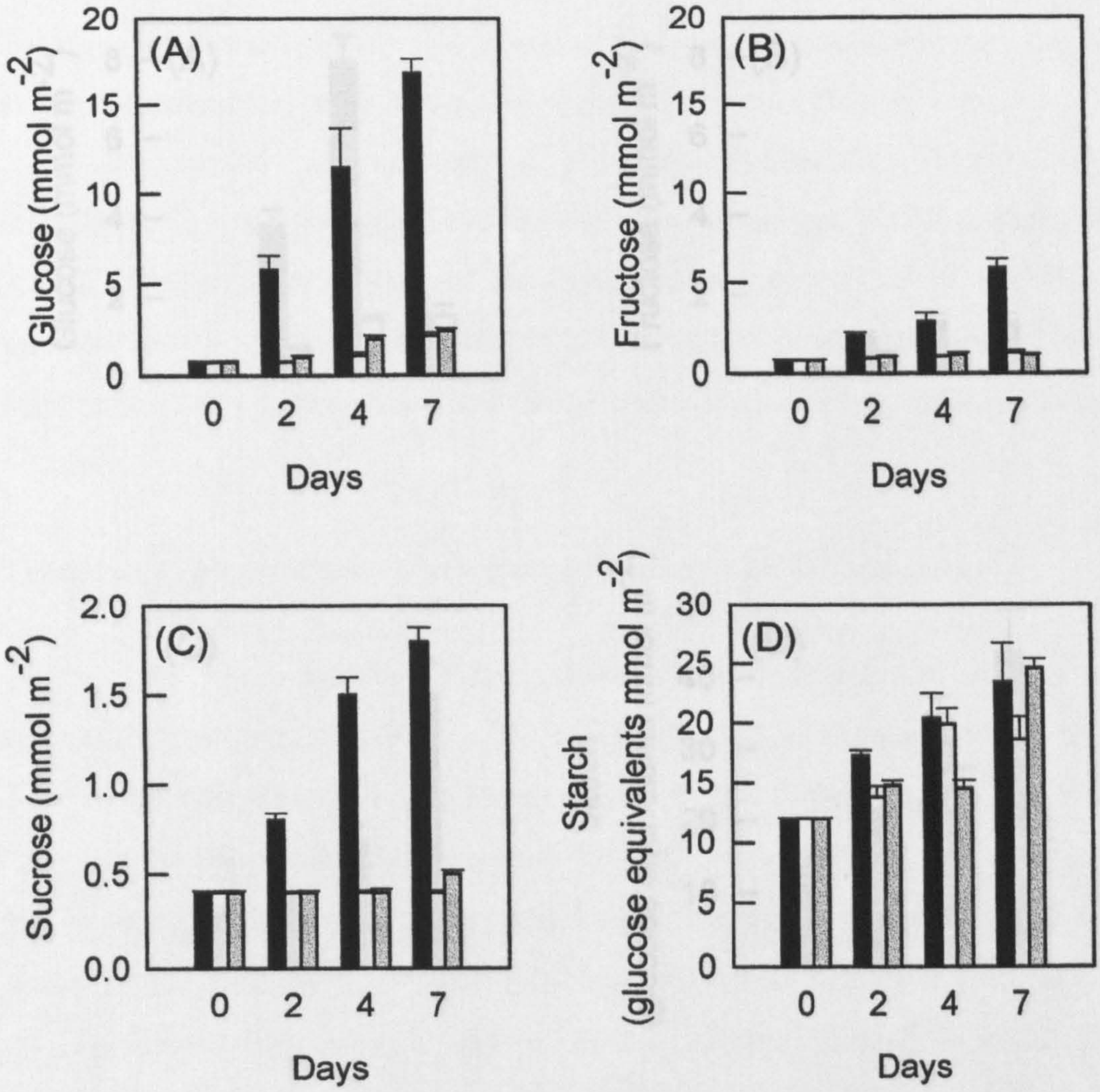



**Figure 3.11 Accumulation of soluble carbohydrates and starch in sucrose-fed *A. thaliana* leaf discs.** Glucose (A), fructose (B), sucrose (C) and starch (D) were measured in leaf discs floated on 50 mM sucrose (filled bars) and control leaf discs floated on distilled water (open bars) and 50 mM sorbitol (grey bars) 0, 2, 4 and 7 d after the beginning of the treatment. Results are the mean ± the standard error of four replicate samples.

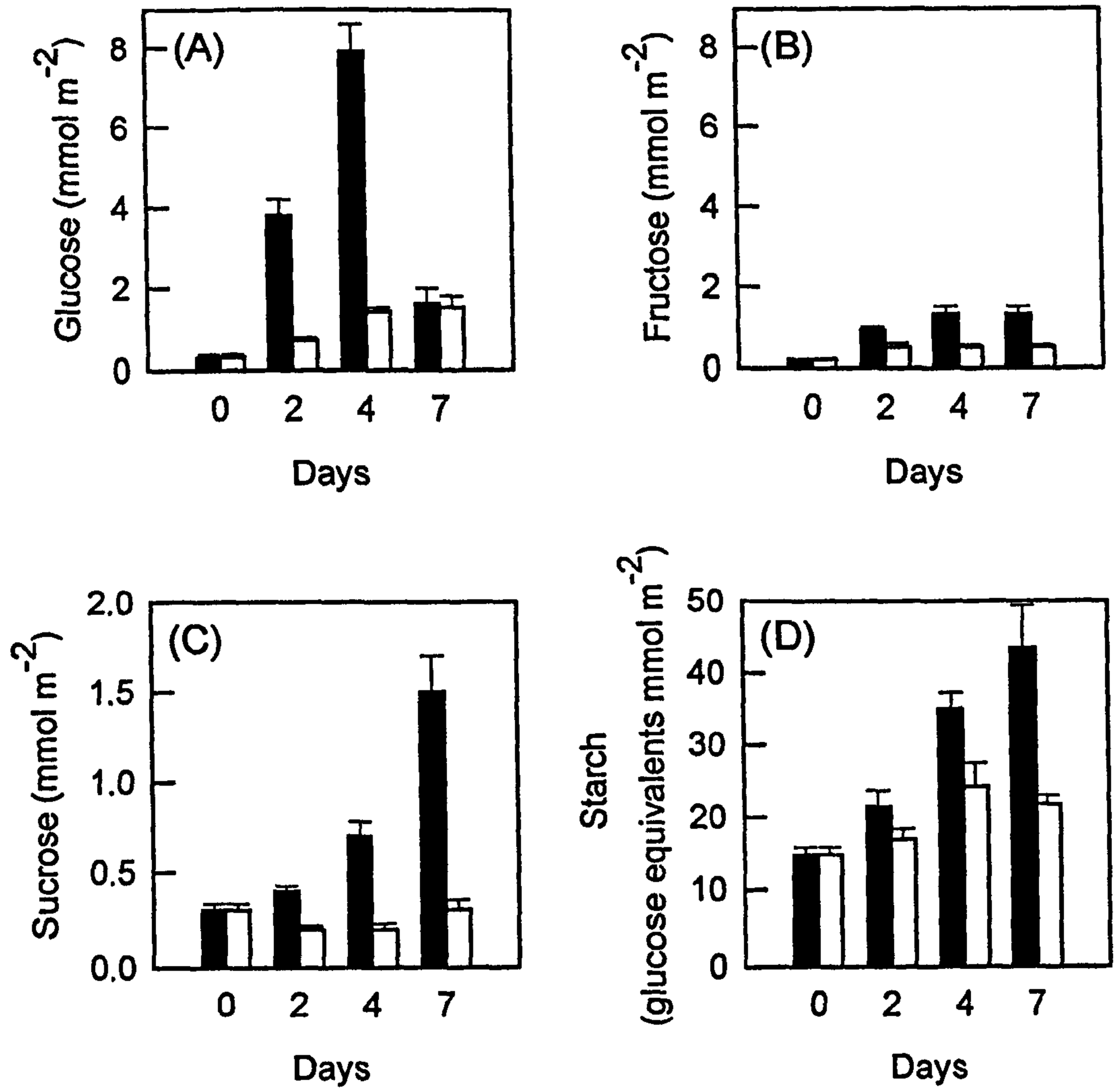



**Figure 3.12 Accumulation of soluble carbohydrates and starch in glucose-fed and control *A. thaliana* leaf discs.** Glucose (A), fructose (B), sucrose (C) and starch (D) were measured in leaf discs floated on 50 mM glucose (filled bars) and control leaf discs, floated on distilled water (open bars), 0, 2, 4 and 7 d after the beginning of the treatment. Results are the mean ± the standard error of four replicate measurements.

leaf discs fed 50 mM glucose also increased throughout the treatment with the amount of sucrose being comparable to that in sucrose-fed leaf discs (Fig. 3.12c).

Both control (water and sorbitol) and sucrose-fed leaf discs accumulated starch throughout the treatment with the amount of starch more than doubling after 7 d (Fig. 3.11d). Similarly, the amount of starch in control and glucose-fed leaf discs also increased during the treatment. However, the amount of starch in glucose-fed leaf discs was nearly double that in control leaf discs 7 d after the beginning of the treatment (Fig. 3.12d).

***Soluble and apoplastic invertase activity in carbohydrate fed leaf discs***

There was a transient but significant ($P < 0.05$) increase in soluble acid invertase activity in sucrose-fed leaf discs (Fig. 3.13a). This increase was first observed 2 d after the beginning of the treatment when activity was 66% higher than in control leaf discs but had declined by day 4 to 25% above control levels. Seven d after the beginning of the treatment soluble acid invertase activity in sucrose fed discs was similar to that in control leaf discs. Soluble acid invertase activity in leaf discs fed 50 mM sorbitol (osmotic control) and in control leaf discs floated on distilled water remained similar and stable throughout the treatment.

Soluble acid invertase activity increased transiently in glucose-fed leaf discs while activity remained constant in control leaf discs (Fig. 3.13b). The transient increase in activity in the glucose-fed leaf discs was first observed 2 d after the beginning of the treatment and was approximately 33% higher than that measured control leaf discs ($P < 0.05$). By 4 d after the beginning of the treatment soluble acid invertase activity in the glucose-fed leaf discs had declined and was similar to that in control leaf discs.

Apoplastic invertase activity increased 5 fold in both sucrose-fed and control leaf discs with maximum apoplastic invertase activity observed 4 d after the beginning of the treatment (Fig 3.13a). By 7 d after the beginning of the treatments the amount of apoplastic invertase activity had decreased slightly.

Apoplastic invertase activity in glucose-fed and control discs also increased during the treatments although this increase was not as marked as that observed in the sucrose feeding experiment (Figure 3.13b).

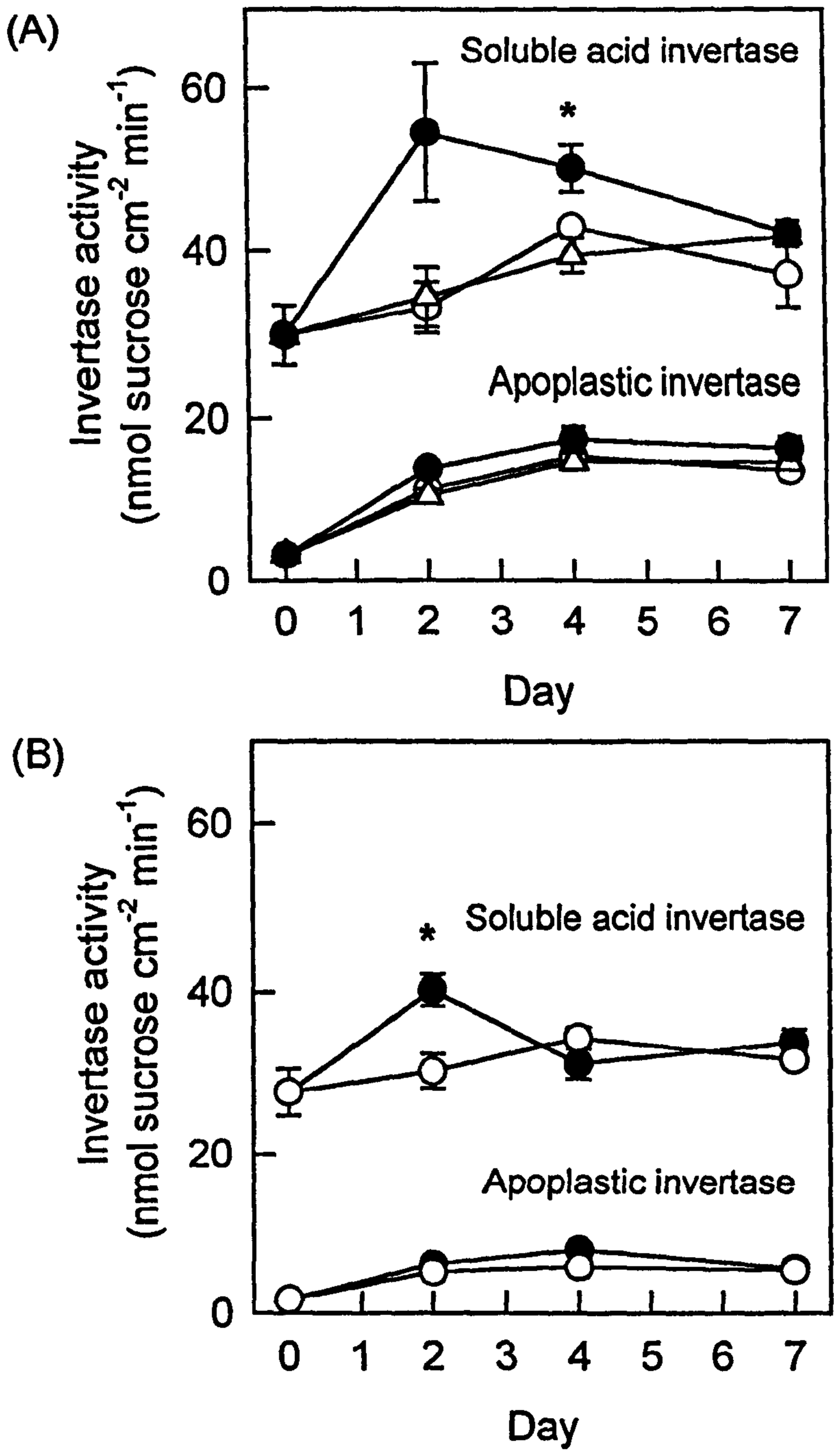



**Figure 3.13 Soluble acid and apoplastic invertase activity in sucrose and glucose-fed leaf discs.** Leaf discs from *A. thaliana* plants were floated on 50 mM sucrose or distilled water or sorbitol (A) or 50 mM glucose or distilled water (B) and sampled for measurements of soluble and apoplastic invertase activity. Samples were taken from sucrose and glucose-fed leaf discs (-●-)and from control leaf discs floated on 50 mM sorbitol (-△-) or distilled water (-O-) 0, 2, 4 and 7 d after the beginning of the treatments. The results are the mean ± the standard error of four replicate measurements. * indicate that data are significantly different ($P<0.05$) from comparable control treatments.

*Soluble invertase isoforms in carbohydrate fed leaf discs*

The soluble invertase isoform pattern of glucose-fed leaf discs did not alter during the time course of the experiment and was similar to that observed in control leaf discs (Fig 3.14). Four major and one minor isoform were observed, with isoelectric points of 4.65, 4.70, 4.75, 4.85 and 4.90. The soluble invertase isoform pattern of sucrose-fed leaf discs was similar to that observed for glucose-fed discs (data not shown).

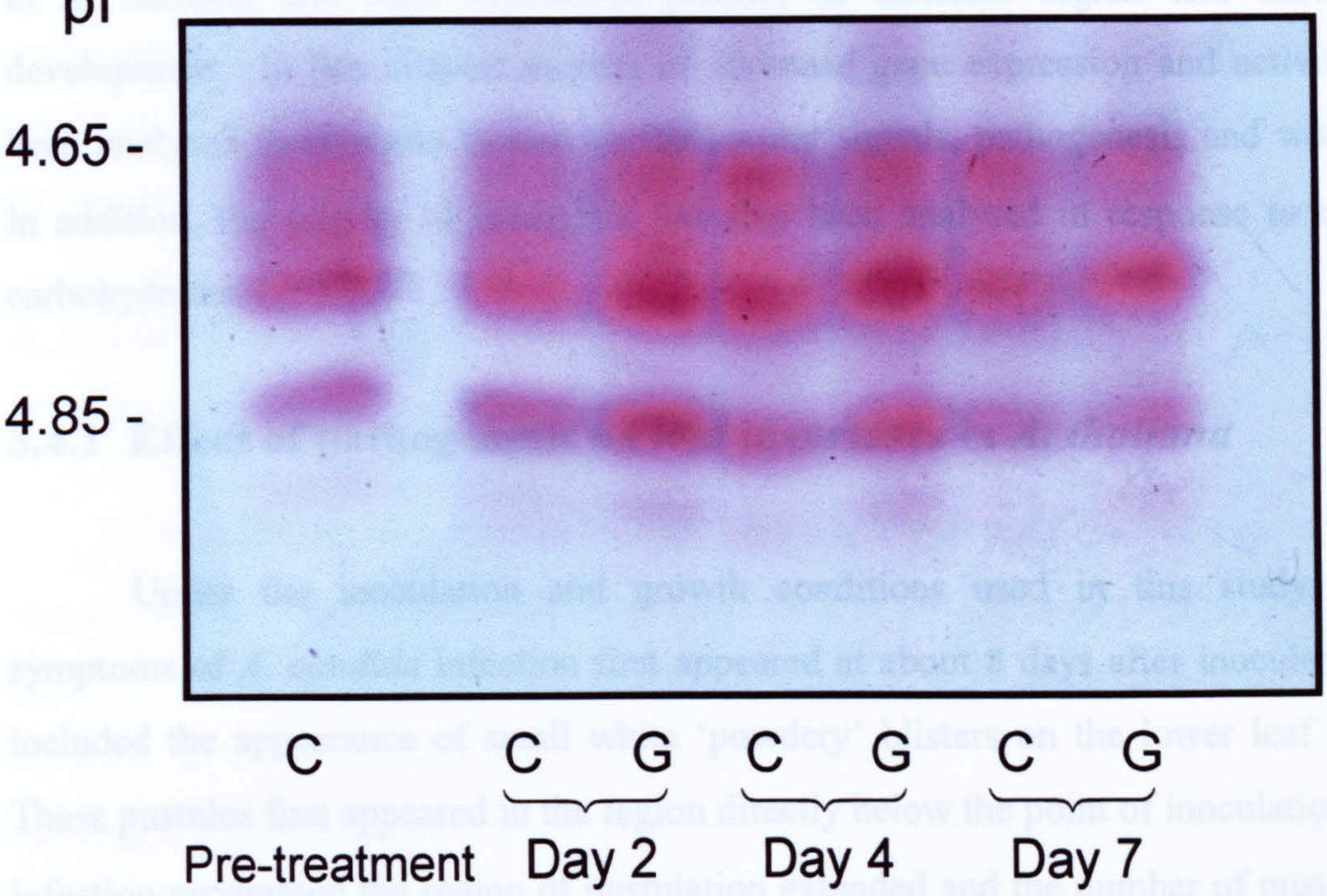



**Figure 3.14 Soluble invertase isoforms in glucose-fed and control *A. thaliana* leaf discs.** Soluble invertase isoforms were separated by isoelectric focussing over a pH range of 4.0 - 6.5. Invertase activity was visualised by staining reducing sugars with 2,3,5 tetrazolium chloride. Samples were prepared from leaf discs fed 50 mM glucose (G) or from control leaf discs floated on distilled water (C). Samples were prepared from leaf discs 0, 2, 4 and 7 days after the beginning of the treatment. Samples were loaded on an equal activity basis.

## 3.4 DISCUSSION

Chapter 2 contained a detailed characterisation of five different invertase genes in *A. thaliana* and their expression profiles in different organs and during leaf development. In this chapter aspects of invertase gene expression and activity have been analysed in response to two environmental signals, pathogenesis and wounding. In addition, the activity of invertases has also been analysed in response to elevated carbohydrates.

### 3.4.1 Effect of pathogenesis on leaf invertases in *A. thaliana*

Under the inoculation and growth conditions used in this study, visible symptoms of *A. candida* infection first appeared at about 8 days after inoculation and included the appearance of small white 'powdery' blisters on the lower leaf surface. These pustules first appeared in the region directly below the point of inoculation but as infection progressed the region of pustulation extended and the number of pustules per unit area increased. The degree of pustulation is an indication of the host/pathogen compatibility, and the interaction between *A. candida* and the *A. thaliana* ecotype OY0 is fully compatible. Other ecotypes of *A. thaliana* show a range of interactions from fully resistant to compatible (Holub *et al.*, 1995).

About 11 d after inoculation yellow-green patches could be seen on the upper leaf surface. This region of discoloration represented a loss of chlorophyll from the leaf, a phenomenon that has previously been reported for this particular host/pathogen interaction (Dang *et al.*, 1992; Tang *et al.*, 1996). The loss of chlorophyll from leaves has also been observed following infection by a range of biotrophic fungi for example, in wheat leaves infected with the powdery mildew fungus *Erysiphe graminis* (Wright *et al.*, 1995) and bluebell leaves infected with the rust fungus *Uromyces muscari* (Scholes and Farrar, 1985).

During the latter stages of infection the visible symptoms of the disease also included the appearance of purple regions around the pustules. This coloration was probably due to the production of anothocyanins by the host, a common sign of stress in *A. thaliana* plants.

Chou (1997) studying the same host/pathogen interaction and using the same inoculation procedure examined the spread of the fungal mycelium in regions A and B of inoculated leaves as infection progressed. This author decolourised infected leaves in a solution of 80% (v/v) ethanol and then stained the mycelium using a solution of lactophenol/cotton blue. This work revealed that region B of the infected leaves remained free of fungal mycelium for the duration of the experiment and that symptom development was synonymous with the spread of the mycelium in region A.

***A. candida infection results in the induction of the host ATβFRUCT 1 apoplastic invertase gene***

The infection of *A. thaliana* leaves with the fungal biotroph *A. candida* resulted in a stimulation of cell-wall associated invertase activity. This increase in activity was restricted to region A of infected leaves indicating that the induction of activity only occurred in close proximity to the fungal mycelium. The induction of cell-wall associated invertase activity was first observed 6-8 d after inoculation and rose steeply thereafter such that by 13 d after inoculation activity was approximately 10-11 fold higher than in regions C and D of control leaves and region B of infected leaves ($P < 0.05$). These data confirm work by Tang *et al.* (1996) who also observed an increase in cell-wall associated invertase activity in the same host-pathogen system and Chou (1997) who localised this increase in activity to region A of infected leaves.

Despite general agreement that the interaction of *A. candida* and *A. thaliana* results in the induction of cell-wall associated invertase activity it is still a matter of debate as to the origin of this increase, host or fungal. Tang *et al.* (1996) suggested that the increase in activity was of host origin as there was no change in the apoplastic invertase isoform pattern during infection. However, this thesis provides the first definitive evidence that most, if not all, of the increase in cell-wall associated invertase activity was of host origin. This evidence was gained by analysing the expression of the host apoplastic invertase gene ATβFRUCT 1 during infection. This work revealed that there was a massive induction of ATβFRUCT 1 gene expression mirroring the observed increase in cell-wall associated invertase activity. As expected, there was no stimulation of ATβFRUCT 1 gene expression in region B of the infected leaves.

The induction of host apoplastic invertase activity and/or gene expression

during pathogenesis has been observed by a number of authors studying a range of host-pathogen systems. For example, Sturm and Chrispeels (1990) examining carrot tap roots infected with the bacterial pathogen *Erwinia carotovora* observed a rapid induction in host apoplastic invertase gene expression within 2 h of inoculation. In this interaction these authors speculated that the increase in apoplastic invertase activity may help to provide the hexoses needed to mount an effective defence response.

Benhamou *et al.* (1991) used an antibody raised against deglycosylated apoplastic invertase from carrot to study, by immuno-gold labelling, the distribution of apoplastic invertase in tomato roots infected with *Fusarium oxysporum*. These authors found that in plants of a susceptible cultivar apoplastic invertase accumulated in the cell walls of colonised cells within 72 h of inoculation. However, when plants from a resistant cultivar were examined in the same manner apoplastic invertase accumulated more rapidly and in cells not directly colonised by fungal hyphae. This differential response led the authors to speculate that in resistant plants the successful detection of the invading pathogen led to the stimulation of a defence response. Again, increased apoplastic invertase activity may have helped to provide the hexoses needed to mount this response.

One interesting point to come from the study of Benhamou *et al.* (1991) is that in resistant versus susceptible cultivars the rate and extent of apoplastic invertase accumulation was very different and that the increase in apoplastic invertase in the susceptible cultivar was only induced in cells directly surrounded or invaded by fungal hyphae. I hypothesise that a similar induction is occurring in *A. thaliana* leaves infected with *A. candida* as the timing and the magnitude of the increase in ATβFRUCT 1 gene expression (and cell-wall associated invertase activity) in region A showed a striking correlation with the amount and location of fungal mycelium within the host leaf. Tang *et al.* (1996) used measurements of the fungal carbohydrate trehalose as a biochemical marker indicative of the amount of fungal mycelium in the leaf (Whipps *et al.*, 1992). These measurements indicated that there was a dramatic increase in the biomass of the pathogen from 8 d after inoculation. It therefore appears that induction of ATβFRUCT 1 gene expression closely correlated with the growth of the fungus within the leaf. Whether direct contact between *A. candida* hyphae and host cells proceeds induction of ATβFRUCT 1 gene expression is unknown. This could

form an interesting area of future work. Equally interesting are the potential signals stimulating ATβFRUCT 1 gene expression and whether these differ between compatible and resistant interactions.

***Potential signals inducing the stimulation of ATβFRUCT 1 gene expression***

Work presented in this chapter demonstrated that the induction of ATβFRUCT 1 gene expression was localised and only occurred in regions of the leaf directly colonised by the fungal mycelium. This induction may have been initiated in two ways. (i) The direct release of a substance from the fungal hyphae resulting in a stimulation of ATβFRUCT 1 gene expression. (ii) Host recognition of a fungal 'signature' indicating the presence of a pathogen. There is a subtle distinction between these two types of signal. The first may represent a possible nutritional strategy of the pathogen i.e. increased apoplastic invertase activity leading to an accumulation of carbohydrate within the leaf. The second may represent a possible defence strategy of the host i.e. increased apoplastic invertase activity for mounting an energetically costly defence response.

If ATβFRUCT 1 gene expression is directly stimulated by a compound produced by *A. candida* then one potential signal is the phyto-hormone cytokinin (Fig. 3.15①). This hormone has been shown to be produced by a number of fungi (Pegg, 1981). Ehness and Roitsch (1997) showed that the application of cytokinin to suspension cultured cells of *Chenopodium rubrum* resulted in the direct stimulation of CIN 1 apoplastic invertase gene expression. We have already seen that CIN 1 and ATβFRUCT 1 show a high degree of regulatory similarity.

One potential fungal 'signature' ('elicitor' on Fig 3.15) that could initiate the stimulation of ATβFRUCT 1 gene expression in *A. thaliana* are cell wall fragments such as oligogalacturonides, chitosans and pectic substances (Fig. 3.15②). The application of these substances to a number of species has been demonstrated to stimulate the production of a range of wound induced and pathogenesis related proteins including invertase (Walker-Simmons *et al.*, 1984; Ehness *et al.*, 1997). Ehness *et al.* (1997) demonstrated that the application of chitosans to suspension cultured cells of *Chenopodium rubrum* resulted in the stimulation of CIN 1 apoplastic invertase gene

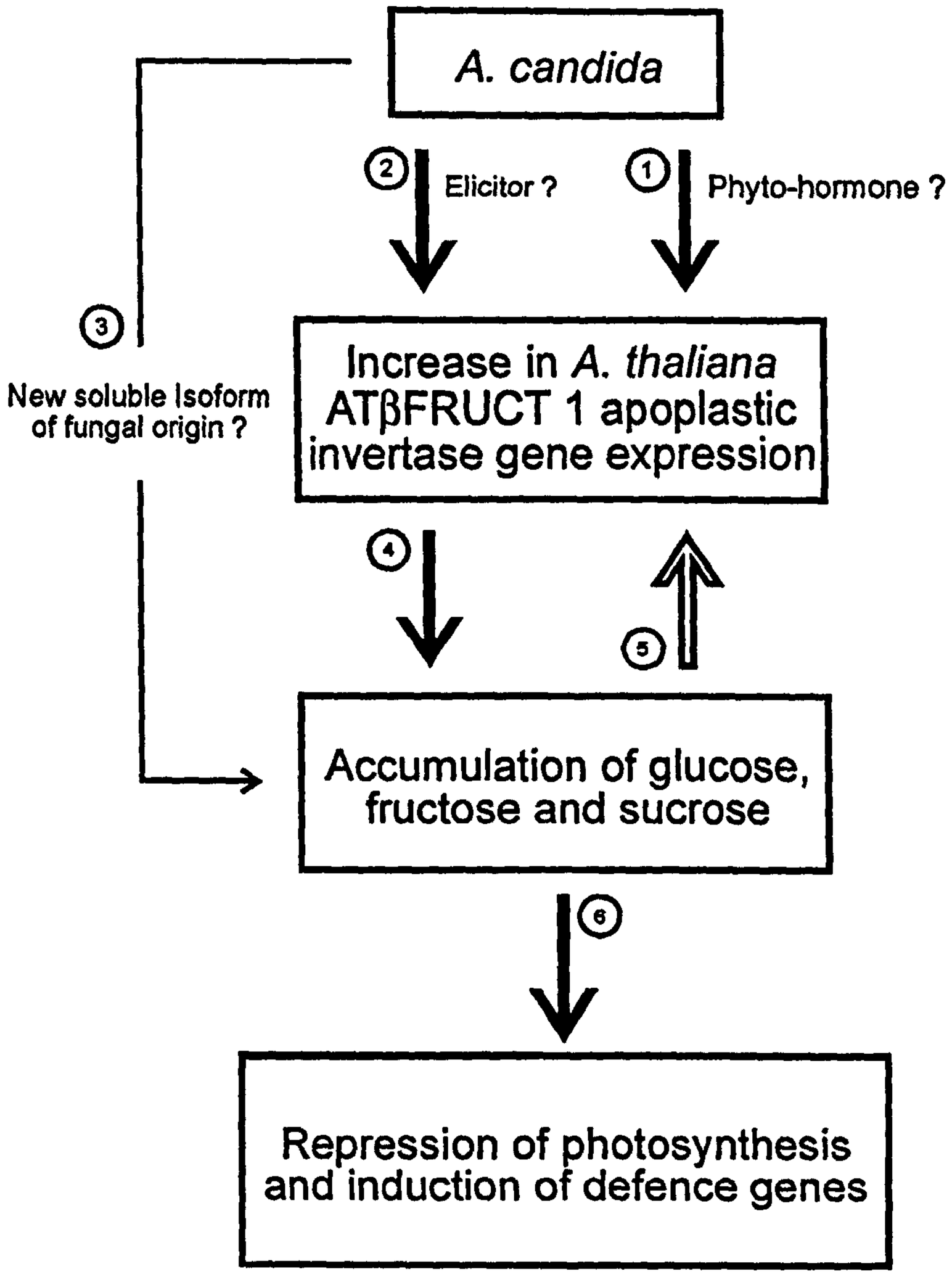



**Figure 3.15 Potential signals regulating ATβFRUCT 1 gene expression during infection of *A. thaliana* leaves by *A. candida*.** The induction of ATβFRUCT 1 gene expression during infection may occur in several ways, for example, the production of an elicitor molecule such as host or fungal cell wall fragments ① and/or the release of a phytohormone from the fungal hyphae ②. The appearance of a new soluble invertase isoform during infection, possibly of fungal origin, also contributes to the total invertase activity of the infected leaf ③. Increases in acid invertase activity during infection are thought to lead to the accumulation of soluble carbohydrates within the leaf ④. This in turn may further induce ATβFRUCT 1 gene expression via a feed-forward mechanism ⑤. The accumulation of soluble carbohydrate within the leaf may also effect the expression of a range of other genes ⑥.

expression. In another study the application of pectic substances (PGA) to suspension cultured cells of tomato resulted in the stimulation of the Lin6 apoplastic invertase gene. As the infection of *A. thaliana* by *A. candida* includes hyphal penetration of host cell walls it is possible that this could result in the release of cell wall fragments leading to a stimulation of ATβFRUCT 1 gene expression. This is consistent with nature of the induction of ATβFRUCT 1 gene expression in infected leaves which appears to increase in direct proportion to the amount of mycelium (and hence the number of cells penetrated) present within the leaf. In addition, Baydoun and Fry (1985) have shown that cell wall fragments are non-mobile within plant tissue which is consistent with the localised nature of ATβFRUCT 1 induction observed.

Roitsch *et al.* (1995) showed that the application of glucose to suspension cultured cells of *Chenopodium rubrum* resulted in a stimulation of CIN 1 apoplastic invertase gene expression. During the latter stages of *A. candida* infection of *A. thaliana* leaves there were measurable increases in the amount of hexoses within the leaf (Fig. 3.15④). This has the potential to further stimulate apoplastic invertase gene expression via a feed-forward induction loop (Fig. 3.15⑤). However, as ATβFRUCT 1 gene expression was not induced in region B of the infected leaves, which also showed accumulation of hexoses, this feed-forward mechanism may not be important.

***How does A. candida alter the soluble invertase activity and isoform pattern of its host?***

In *A. thaliana* leaves infected with *A. candida* soluble acid invertase activity was higher in region A than in region B 13 d after inoculation, possibly indicating a stimulation of activity by the pathogen. However, unusually soluble acid invertase activity also differed between regions C and D of control leaves thus masking any potential pathogen effects. The reason for the difference in activity between regions C and D is unknown as soluble acid invertase activity does not normally vary significantly across the surface of a mature, healthy *A. thaliana* leaf. However, previous studies using the same host-pathogen system have reported an increase in soluble acid invertase activity in infected leaves compared to control leaves. Tang *et al.* (1996) saw a 1.5 fold stimulation of activity in whole infected leaves 14 d after inoculation. Chou (1997) localised this increase in activity to region A of infected leaves revealing that the

increase only occurred in regions of the leaf in close proximity to the fungal mycelium.

Increases in acid invertase activity have been reported to occur in a large number of biotrophic fungal infections but due to the extraction procedure used many of these studies do not differentiate between increases in cell-wall associated invertase activity and soluble acid invertase activity. However, increases in soluble acid invertase activity have been reported in range of host-pathogen systems. For example, in maize leaves infected with corn smut (*Ustilago maydis*) soluble acid invertase activity increased during infection and was 21 fold higher than in control leaves 10 d after inoculation (Billett *et al.*, 1977). Increases in soluble acid invertase activity have also been seen in flax with the rust *Melampsora lini* (Clancy and Coffey, 1980), wheat infected with *Puccinia graminis* (Heisteruber *et al.*, 1994) and barely infected with *Puccinia hordei* (Tetlow and Farrar, 1992).

Tang *et al.* (1996) showed that the increase in soluble acid invertase activity in *A. thaliana* leaves infected with *A. candida* was accompanied by changes in the soluble invertase isoform pattern. Isoelectric focussing and subsequent activity staining revealed that in healthy leaves there were at least four major soluble invertase isoforms with pIs of 4.65, 4.70, 4.75 and 4.85. During infection the activity of the four major isoforms decreased while a new isoform with a pI of 5.1 appeared. Further investigation of this phenomenon by Chou (1997) revealed that the appearance of this novel isoform was restricted to region A. Data presented in this thesis confirms the work by Tang *et al.* (1996) and Chou (1997). Isoelectric focussing of soluble invertase extracts from control and infected leaves 13 d after inoculation again revealed the presence of a new isoform. The timing of the appearance of the new isoform and the apparent loss of activity of the other four isoforms may explain why the amount of soluble invertase activity in infected leaves tends to vary from experiment to experiment. This variability could be due to subtle changes in the activity of the individual isoforms during pathogenesis. The appearance of the new isoform in region A of the infected leaf suggests that its appearance is linked to the close proximity of the fungal mycelium. This raises the inevitable question as to the origin of this new isoform, host or fungal?

If the new isoform is of host origin then it has either resulted either from the post-translational modification of an existing soluble invertase isoform or it represents

the expression of a hitherto unidentified host invertase gene. However, as the decrease in the host soluble invertase isoforms during pathogenesis coincides with the appearance of the new isoform in regions of the leaf invaded by the fungal mycelium a simple hypothesis is that the new isoform is of fungal origin.

If the new isoform is of fungal origin then its role in the metabolism of the infected leaf would critically depend upon its sub-cellular location. If located within the fungal hyphae then its ability to alter the carbohydrate metabolism of the host would be limited as it would not be able to hydrolyse sucrose in the extra-haustorial matrix prior to uptake. However, if it was bound either to the hyphal membrane or excreted it would further contribute to the invertase activity already present within the host.

The controversy surrounding the origin of increases in soluble invertase activity during pathogenesis, regardless of the presence of a new isoform *per se*, is reflected in other host-pathogen interactions. Billet *et al.* (1977) suggested that in maize leaves infected with corn smut the *Ustilago maydis* the increase in soluble invertase activity was of host origin. These authors based this conclusion on a comparison of the electrophoretic properties of soluble invertases extracted from diseased and healthy leaves with an invertase extracted from the haploid spores of the biotroph. In contrast Callow *et al.* (1980) investigating the same host-pathogen interaction suggested that the increase in activity was of fungal origin as haploid spores of the fungus contained an invertase identical to that isolated during pathogenesis. In oat leaves infected with crown rust *Puccinia coronata* there were increases in both apoplastic and soluble invertase activity during infection (Greenland and Lewis, 1983). Most of the invertase from diseased leaves appeared as a new peak when separated by gel filtration. This new peak had a higher Km for sucrose and was more sensitive to the inhibitor, aniline, than the others. Again it is not clear whether the origin of this new activity was host or fungal but the striking differences in its reaction kinetics suggest that it originated from the pathogen. Ruffner *et al.* (1992) were able to separate two distinct invertase activities in *Botrytis cinera* infected grape berries on the basis of their substantially different chromatographic behaviour. When these activities were compared to those isolated from axenically grown cultures of *B. cinera* it was estimated that in infected grape berries (one week after inoculation) approximately one third of the soluble invertase activity was of fungal origin.

***How may increases in invertase activity affect carbohydrates in the infected leaves?***

Hexoses accumulated in regions A and B of *A. thaliana* leaves infected with *A. candida*. This accumulation was first observed 8 d after inoculation and rose steeply thereafter such that by 13 d after inoculation the amount of hexose in each region was more than double that in equivalent control regions. Infected leaves also accumulated sucrose, although this was primarily restricted to region A.

The accumulation of glucose, fructose and sucrose has also been reported in a number of other foliar biotrophic pathogen infections (for review see Whipps and Lewis, 1981). In barley leaves infected with the powdery mildew *Erysiphe graminis* there was a massive increase in the amount of glucose and fructose during infection and a tripling in the amount of sucrose by 8 d after inoculation (Scholes *et al.*, 1994). Hexoses and sucrose also accumulated in wheat and pea leaves infected with powdery mildew *Erysiphe graminis* (Wright *et al.*, 1995; Hall *et al.*, 1992), vine leaves infected with *Unicula necator* (Brem *et al.*, 1986), *Senecio squalidus* infected with *Albugo tragopoginis* (Long and Cooke, 1974; Long *et al.*, 1975), *A. thaliana* infected with *A. candida* (Tang *et al.*, 1996; Chou, 1997).

As previously discussed, cell-wall associated invertase activity in region A of *A. thaliana* leaves inoculated with *A. candida* increased as infection progressed. We have already seen that this increase was almost totally attributable to the stimulation of the host ATβFRUCT 1 apoplastic invertase gene. Interestingly, the rate of increase in cell-wall associated invertase activity closely mirrored the accumulation of hexoses in the infected leaves. This observation suggests that the accumulation of glucose and fructose in region A may be partly due to the stimulation of invertase activity. A correlation between increasing invertase activity and the accumulation of hexoses was also observed in the other host-pathogen systems listed above.

In a further line of evidence linking increases in invertase activity to the accumulation of hexoses Brem *et al.* (1986) infected vine leaves with two biotrophs *Unicula necator* (powdery mildew) and *Plasmopara viticola* (downy mildew). Infection by *U. necator* resulted in a stimulation of acid invertase activity and a concomitant accumulation of hexoses while infection by *P. viticola* did not result in a stimulation of invertase activity and the amount of hexoses in the leaf actually declined.

Previously, Chou (1997) observed that *A. thaliana* leaves inoculated with *A. candida* only accumulated hexoses and sucrose in region A of the infected leaf; the region where both the mycelium and increases in invertase activity were also located. This present study revealed there was an accumulation of hexoses in both region A and B of infected leaves; this is the first time that this has been observed in this host-pathogen association. The reason for this more widespread accumulation of hexoses is unknown although it could be due to the relative strength of the fungal sink. Variations in fungal sink strength could have been caused by a number of reasons. For example, a high density of spore inoculum could have led to a more rapid and dense growth of fungal mycelium. Alternatively, differences in the growth conditions and/or health of experimental plants could also have affected the growth of *A. candida.*

It is perhaps surprising that sucrose accumulated in region A of *A. thaliana* leaves infected with *A candida* during infection given that there was a stimulation of invertase activity in this region and the appearance of a new soluble invertase isoform of unknown cellular location. Similar accumulations of sucrose in *A. thaliana* infected with *A. candida* were also observed by Tang *et al.* (1996) and Chou (1997). The accumulation of sucrose during other biotrophic infections is variable but has been reported in *Senecio squalidus* infected with *Albugo tragopogonis* (Long and Cooke, 1974) and in powdery mildew infections of barely, wheat, vine and pea (Scholes *et al.*, 1994; Wright *et al.*, 1995; Brem *et al.*, 1986 and Hall *et al.*, 1992). The impact of invertase activity on the accumulation of sucrose may depend upon a number of factors including the rate of sucrose synthesis and degradation, import, export and fungal sequestration. It must also be remembered that sucrose may be located in different sub-cellular compartments or cell types. Interestingly however transgenic plants over-expressing a yeast-derived invertase in the apoplast (Schaewen *et al.*, 1990), cytosol (Sonnewald *et al.*, 1991) and vacuole (Heinke *et al.*, 1994) all accumulated sucrose in the leaves.

### 3.4.2 The effect of leaf wounding on invertases in *A. thaliana*

Mature leaves of six week old *A. thaliana* plants were wounded in a consistent and reproducible manner, as described in the Materials and Methods, and sampled after 8, 24, 48 and 72 hours. Samples were analysed for apoplastic and soluble acid

invertase activity and ATβFRUCT 1 gene expression and compared with samples from the leaves of non-wounded plants.

Wounding resulted in a significant increase in apoplastic invertase activity in the affected region which was mirrored by an increase in ATβFRUCT 1 gene expression. Soluble acid invertase activity was unaffected by wounding.

***The ATβFRUCT 1 apoplastic invertase gene is wound inducible***

To date the effect of wounding on invertase gene expression or activity has only been examined in a few species, these include *Beta vulgaris* (Bacon *et al.*, 1965; Leigh *et al.*, 1979), sweet potato (Matsushita and Uritani, 1974), carrot (Sturm and Chrispeels, 1990), pea (Zhang *et al.*, 1996), tomato (Godt and Roitsch, 1997) and *Chenopodium rubrum* (Ehness *et al.*, 1997).

In *A. thaliana*, Tymowska-Lalanne *et al.* (1996) analysed a 0.4 kb fragment of the 5‘ region of the ATβFRUCT 1 apoplastic invertase gene, located immediately upstream of the transcription start site and found a putative wound-responsive element. This wound responsive element (TTGTGGAAACAAC) was located between –202 and –190 and was similar to that previously identified in the 5’ flanking region of the *wun 1* gene in potato (Siebertz *et al.*, 1989) and the carrot cell wall invertase gene (Ramloch-Lorenz *et al.*, 1993). We have shown, for the first time, that the ATβFRUCT 1 apoplastic invertase gene is indeed regulated by a wounding stimuli and have examined its expression over a 72 h period.

ATβFRUCT 1 gene expression in wounded leaves was analysed using semi-quantitative RT-PCR. This analysis revealed that expression was first elevated 24 h after wounding and continued to be elevated above that observed in non-wounded leaves until 72 h (the last time point examined). Unfortunately, due to the failure of the internal standard to amplify correctly, exact quantification of gene expression was not possible.

When wound-induced changes in apoplastic gene expression in *A. thaliana* are compared with those observed in other species it is apparent that they all exhibit slightly different induction kinetics. For example, in wounded pea leaves an increase in apoplastic invertase gene expression was first measured after a lag of 3 h and was

maximally induced by 12 h, thereafter expression declined over the next 36 h (Zhang *et al.*, 1996). In wounded *Chenopodium rubrum* leaves expression of the CIN 1 apoplastic invertase gene was maximally induced by 3 h and remained at that level until 47 h, the final time point measured (Ehness *et al.*, 1997). In wounded carrot tap root the expression of an apoplastic invertase gene was first detected after a lag of 2 h and was maximally induced by 12 h, thereafter expression declined over the next 60 h (Sturm and Chrispeels, 1990). In wounded tomato leaves the Lin6 apoplastic invertase gene was first induced after 24 h, was maximally induced after 33 h and declined to pre-wounding levels by 48 h (Godt and Roitsch).

Apoplastic invertase activity in the wounded *A. thaliana* leaves began to increase after a lag of about 8 h and was maximally induced after 24 h. Activity at this point was approximately 1.5 fold higher than that measured in control leaves and remained at about the same level until 72 h, the last time point measured. There was a clear correlation between the first measurable increase in apoplastic invertase activity and the induction of ATβFRUCT 1 gene expression, with both occurring at about the same time.

The reason for the difference in induction kinetics is not known but could be due to species variations. Alternatively, Zhang *et al.* (1996) suggested that the difference in induction kinetics could be due to a combination of factors including the intensity of the wound and the organ affected. These authors analysed the induction of the PcI-1 apoplastic invertase gene in both attached and detached stems, roots and leaves of pea. Stems and leaves showed similar induction kinetics, although expression was four to five times higher in the leaves than stems, the authors attributed this difference to the severity of the wound. In wounded roots expression was maximally induced by 3 h but had declined 50% by 6 h, a very different response to that seen in the aerial parts of the plant. Sturm and Chrispeels (1990) observed that the induction of apoplastic invertase gene expression in carrot tap root infected with the bacterial pathogen *Erwinia carotovora* was considerably faster and more transient than that observed following wounding. These authors suggested that different *trans*-acting factors could be induced by different environmental stimuli with different induction kinetics and that each of these factors could be binding to an independent *cis*-regulatory element of the gene.

Soluble invertase activity did not increase in *A. thaliana* leaves in response to wounding. This is in contrast with other species studied to date, for example red beet tubers and carrot tap roots (Matsushita and Uritani, 1974; Sturm and Chrispeels, 1990), where soluble invertase activity was induced concomitant with increases in apoplastic invertase activity. In tomato leaves induction of the TIV1 vacuolar invertase gene was observed as soon as 1 h after wounding but declined concomitant with the induction of the Lin6 apoplastic invertase gene 23 h after wounding. Haouazine-Takvorian *et al.* (1997) examined the 5' non-coding region upstream of the translation start site of both the ATβFRUCT 3 and ATβFRUCT 4 soluble invertase genes in *A. thaliana* and located putative auxin, abscisic acid and ethylene responsive elements. While only detailed promoter analysis can confirm if these elements have a regulatory function it is perhaps surprising that soluble invertase activity did not increase in response to wounding as abscisic acid has been shown to induce invertase activity in pea (Zhang *et al.*, 1996) and is thought to play a role in the wound induced signal transduction pathway (Hildmann *et al.*, 1992; Pena-Cortes *et al.*, 1995).

***Why does apoplastic invertase activity increase in wounded tissue?***

Plants are frequently subjected to the stress associated with mechanical injury. This injury is either inflicted by abiotic forces such as the wind or by biotic forces such as herbivory by grazing animals or insect pests. In response to mechanical injury or herbivory plants mount a complex defence response involving the induction of many genes and the *de novo* synthesis of a range of proteins (Hildmann *et al.*, 1992). The induction of widespread protein synthesis leads to such defence responses as the deposition of lignin, callose and the production of phytoalexins and lytic enzymes (Collinge and Slusarenko, 1987). One of the responses of plants to wounding is an increase in invertase gene expression and activity (Matsushita and Uritani, 1974; Sturm and Chrispeels, 1990; Zhang *et al.*, 1996; ).

In stress situations such as wounding cells have a high demand for hexoses to fulfil the energy and carbon requirements needed to mount an adequate defence response (Herbers *et al.*, 1996). Indeed it has been shown that the respiratory activity of wounded sweet potato doubles within 20 hours and is marked by an increase in the

number of mitochondria within affected cells (Asahi *et al.*, 1966; Greksak *et al.*, 1972). An increase in invertase activity may play an important role in the supply of hexoses to these cells. Indeed Matsushita and Uritani (1974) reported that the wound induced increase in invertase activity in sweet potato was accompanied by a decline in sucrose and an increase in the hexose content of the tissue.

How does a wound induced increase in invertase activity supply damaged tissue with hexoses? It has been demonstrated by *in situ* hybridisation that wounding results in a substantial increase in apoplastic invertase mRNA within the phloem of wounded pea leaves and stems (Zhang *et al.* (1996) with the majority of the mRNA in the leaves being found in the mid-veins, particularly toward the tips. These authors suggest that the increase in invertase activity in this region may greatly inhibit sucrose export from wounded leaves. Recently, Truernit *et al.* (1996) examined the expression of a monosaccharide $H^+$ symporter, STP4, in *A. thaliana*. These authors found that the expression of this gene was associated with the sink regions of plant suggesting a role in the supply of hexoses to developing or metabolically active tissue. When plants were wounded or subjected to pathogen attack STP4 gene expression in the affected regions was up-regulated within 24 h. STP4 promoter-GUS fusion experiments in *A. thaliana* revealed that wounding resulted in a marked stimulation in GUS activity in cells adjacent to the damaged region. These two pieces of evidence suggest that wounding results in a co-ordinated response in which sucrose is retained in the damaged region by hydrolysis and the resulting hexoses are actively absorbed by symporters thus supplying cells with the requirements needed for the enhanced respiratory activity measured during stress.

***Potential wounding signals resulting in the stimulation of ATβFRUCT 1 gene expression***

Wounding results in the stimulation of a raft of defence related genes. These genes are either systemically induced such as proteinase inhibitors (Green and Ryan, 1972) or they are induced in a localised manner such as ATβFRUCT 1 in *A. thaliana* and the PcI-1 apoplastic invertase gene in pea (Zhang *et al.*, 1996).

The systemic induction of certain wound-induced genes led to the search for a

'wound-hormone'. One approach to identifying this 'hormone' was to prepare extracts from wounded leaves and then to feed this extract, via the petiole, to detached tomato leaves. This assay revealed that the active component of the wounded leaf juices were pectic polysaccharides (Ryan, 1974). However, work by Baydoun and Fry (1985) revealed that these substances were not the wound messenger *per se* as they were generally non-mobile in leaf tissue. However, as the wound induction of ATβFRUCT 1 is not systemic it is possible that these substances could potentially form the first step in the signal transduction pathway leading to its accumulation.

An alternative theory was proposed by Pena-Cortes *et al.* (1995) who suggested that mechanical injury may generate changes in plasma membrane potential that propagate throughout the plant leading to the induction of wound induced genes. The highly mobile polypeptide systemin has also been implicated in initiating the wound response (Pearce, 1991; Farmer and Ryan, 1992).

Whatever the initial signal/s leading to the stimulation of wound induced gene expression it is clear that the hormones abscisic acid (ABA) and jasmonic acid (JA) play a pivotal role in the signal transduction pathway (Pena-Cortes *et al.*, 1989, 1992, 1993; Farmer and Ryan, 1990). Both of these hormones have been shown to initiate transcription of certain wound induced genes and recently Zhang *et al.* (1996) found that applications of these hormones induced apoplastic invertase gene expression in pea. However, if ABA and JA do form part of the signal transduction pathway leading to increases in ATβFRUCT 1 gene expression it is not clear why the induction is not systemic as was seen for another wound inducible gene, proteinase inhibitor II gene in potato (Hildmann *et al.*, 1992; Pena-Cortes *et al.*, 1995).

Recent work has shown that two interconnecting 'wounding' signal transduction pathways may operate in plants and that these might better be termed the 'insect damage' and 'mechanical injury' pathways. Korth and Dixon (1997) showed that an elicitor in insect regurgitant elicited induction of certain defence related genes faster than mechanical wounding and that the application of regurgitant to wounded leaves caused transcript accumulation profiles to shift paralleling those in insect damaged tissue. Whether insect damage or mechanical injury have a differential effect on ATβFRUCT 1 gene expression could form an area of future work.

### 3.4.3 The effect of sugar feeding on invertases in *A. thaliana*

Changes in the amount of sucrose (substrate) or glucose and fructose (products) within leaf tissue have the potential to regulate soluble and apoplastic invertase activity either at the level of gene expression (Xu *et al.*, 1996; Ehness and Roitsch, 1997) or via competitive inhibition of the mature protein (Sampietro *et al.*, 1980; Lopez *et al.*, 1988; Isla *et al.*, 1991; Burch *et al.*, 1992).

In order to examine the potential for substrate or product effects on invertase activity in *A. thaliana*, leaf discs were floated on solutions of 50 mM sucrose or 50 mM glucose. Control leaf discs were placed on solutions of distilled water or 50 mM sorbitol (osmotic control). The leaf discs were placed under continuous low light in order to prevent the accumulation of internally generated carbohydrates within the leaf discs.

***Changes in the phenotype of sugar fed leaf discs***

Elevated hexoses are thought to repress photosynthetic gene expression via a sugar sensing mechanism (Jang and Sheen, 1994) and one of the genes repressed by elevated hexose is chlorophyll a/b binding protein (Krapp *et al.*, 1993). In confirmation of this *A. thaliana* leaf discs fed 50 mM glucose or 50 mM sucrose showed a measurable loss of chlorophyll from 4 d after the beginning of the treatment indicating that hexoses were accumulating within the tissue. The rate of chlorophyll loss accelerated as the treatment continued and became visible 5 d after the beginning of the treatment with chlorosis visible around the edge of the discs. This zone of chlorosis extended toward the centre of the discs as the treatment continued.

Chlorophyll was not lost from control discs placed on distilled water or 50 mM sorbitol which remained green for the duration of the treatment. This indicated that under the low light conditions provided hexoses were not accumulating.

***Carbohydrate accumulation in sugar-fed leaf discs***

Glucose, fructose and sucrose accumulated in the sugar-fed leaf discs during the

treatment. Sucrose-fed leaf discs accumulated glucose to a greater extent than fructose. The reason for this is unknown but was probably due to the differential utilisation of the hexoses within the tissue. Data also revealed that the majority of the soluble sugar accumulating within the sucrose-fed discs were hexoses not sucrose. This was probably the result of either sucrose hydrolysis in the apoplast, prior to uptake, or the immediate hydrolysis of the disaccharide once within the cells. This hydrolytic step may account for the higher soluble sugar accumulations within the leaf discs floated on 50 mM sucrose than those floated on 50 mM glucose; the hydrolysis of one sucrose molecule results in two molecules of hexose within the tissue. Soluble carbohydrates did not accumulate in the control leaf discs.

Starch accumulation was observed in sucrose and glucose-fed leaf discs. The accumulation of large quantities of soluble carbohydrate within the cytosol of the leaf discs probably favoured the formation of starch within the chloroplasts. Starch accumulation was also observed in leaf discs floated on 50 mM sorbitol and distilled water, particularly the controls for the sucrose feeding experiment. Although the light conditions were adjusted to prevent the accumulation of soluble carbohydrate within control leaf discs the prevention of export and a low level of photosynthesis may have combined to favour the production of starch. Increases in soluble carbohydrates have been shown to induce the expression of the gene encoding ADP-glucose pyrophosphorylase, a key enzyme in starch synthesis (Koch, 1996).

*Changes in invertase activity in sugar fed leaf discs*

There was a transient but significant stimulation of soluble acid invertase activity in sucrose and glucose-fed leaf discs. This increase in activity was first apparent 2 d after the beginning of the treatments but had declined to control levels in the glucose-fed leaf discs by day 4 of the treatment. The stimulation of soluble acid invertase activity in sucrose-fed leaf discs was greater than that seen in glucose-fed discs but had declined to control levels by day 7 of the treatment.

These data show that soluble acid invertase activity was stimulated by an accumulation of soluble carbohydrate within the leaf discs. Theoretically this could have occurred in two ways. (i) Activation or repression of invertase gene expression.

(ii) Alterations in the activity of the mature protein.

Sugar-induced changes in invertase activity have been reported in a number of other species. Kaufman *et al.* (1973) observed that soluble acid and apoplastic invertase activities in *Avena* stems were affected by the exogenous supply of sucrose and hexoses. Supplying *Avena* stems with pulses of sucrose or glucose and or fructose resulted in a fluctuation of invertase activity during the following 70 hour period. The time scale of these changes suggests an alteration in the *de novo* synthesis of invertase protein. There is also evidence that in a number of species soluble acid invertase activity is reduced directly by end product inhibition. For example, a soluble acid invertase from potato leaves and tubers has been shown to be inhibited by fructose in a competitive manner and is thought to modulate activity by interaction with two reaction sites. The same enzyme is also repressed by glucose in a non-competitive manner (Isla *et al.*, 1991; Burch *et al.*, 1992). A similar regulatory mechanism was found for a soluble invertase purified from *Carica papaya* fruits (Lopez *et al.*, 1988).

More recent work has examined the affect of sucrose and glucose on invertase activity and gene expression in a number of species (Xu *et al.*, 1996; Ehness *et al.*, 1997). This work has shown that invertase genes can be modulated by the intercellular carbohydrate concentration. In maize, two soluble invertase genes, *Ivr I* and *Ivr 2* were found to be differentially modulated by glucose. *Ivr1* was found to be repressed by increasing amounts of glucose and up-regulated by carbohydrate depletion while *Ivr 2* was found to be up-regulated by increasing carbohydrate supply (Xu *et al.*, 1996). These authors suggest that the differential regulation of the two soluble invertase genes in maize provides a way for controlling subtle changes in the supply and demand of carbohydrate within the leaf and that this provides a way for balancing the essential needs of metabolism for hexoses under all physiological conditions.

The reasons for the stimulation of soluble acid invertase activity in sucrose and glucose-fed *A. thaliana* leaf discs cannot be discerned until there is a more detailed examination of the changes in gene expression in response to carbohydrate supply. This work would need to examine both ATβFRUCT 3 and ATβFRUCT 4 gene expression and may find that they a differentially modulated by hexoses as in maize.

It is interesting that there was no change in the soluble invertase isoform pattern in sucrose or glucose-fed leaf discs. At least four major isoforms could be detected

with pIs of 4.65, 4.70, 4.75 and 4.85. This is in agreement with the work of Tang *et al.* (1996). It appears as though the increase in soluble acid invertase activity during the initial stages of sugar feeding did not coincide with the appearance of any new isoforms and suggests that the increase in activity resulted from existing isoforms.

It is also interesting that sugar feeding did not induce the appearance of the new isoform seen during infection of *A. thaliana* leaves with *A. candida* (pI 5.1). As previously discussed, infection by this pathogen resulted in an accumulation of carbohydrate within the infected leaf. If the new soluble invertase isoform is of host origin then it is not induced by increases in the concentration of leaf carbohydrate.

Apoplastic invertase activity increased in the leaf discs from all treatments, sugar-fed and control discs. This increase in activity was more marked in the sucrose feeding experiment (sucrose-fed and control) than in the glucose feeding experiment. It appears as though apoplastic invertase activity increased as a response to wounding. In *Chenopodium rubrum* an apoplastic invertase gene, CIN 1, was found to be up-regulated by glucose while the soluble invertase genes were unaffected (Ehness and Roitsch, 1997). While in contrast Sturm and Chrispeels (1990) found that expression of an apoplastic invertase gene in carrot suspension cultures was not regulated by glucose, fructose or sucrose. Due to the wound response seen in the *A. thaliana* discs from all the treatments the effect of sugar feeding on apoplastic invertase could not be discerned.

### 3.4.4 Conclusion and future work

Work presented in this chapter shows that the ATβFRUCT 1 apoplastic invertase gene is wound and pathogen responsive. This adds to the work in the previous chapter which showed that this gene was also expressed in a wide range of sink tissues. Together these two chapters indicate the importance of apoplastic invertase activity for the supply of hexoses to tissues under a spectrum of developmental and environmental conditions.

Specifically this chapter has demonstrated that:

- Increases in cell-wall associated invertase activity during infection of *A. thaliana*

leaves with *A. candida* resulted from increases in ATβFRUCT 1 gene expression and are thus of host origin.

- Induction of ATβFRUCT 1 gene expression during *A. candida* infection are localised to the region of the leaf invaded by the fungal mycelium. This has implications for the potential signals stimulating this increase.

- Induction of ATβFRUCT 1 gene expression and increases in apoplastic invertase activity correlate closely with the accumulation of hexoses also observed during infection.

- ATβFRUCT 1 gene expression is induced by wounding.

- The induction kinetics of ATβFRUCT 1 gene expression in response to wounding differ from those elicited by pathogenesis.

- Semi-quantitative RT-PCR is a versatile tool for the measurement of low abundance mRNAs.

- Glucose and sucrose feeding of *A. thaliana* leaf discs resulted in a transient stimulation of soluble acid invertase activity. This indicated that internal as well as environmental stimuli influence invertase activity in *A. thaliana*.

For future work I would want to examine the effect of wounding and pathogenesis on transgenic *A. thaliana* plants lacking apoplastic invertase activity. This would help to elucidate the role of this enzyme under different environmental conditions. This would also help to clarify if other sucrolytic enzymes were able to restore the function of the missing activity.

I would like to localise wound and pathogen induced increases in ATβFRUCT 1 gene expression to particular cell and tissue types. I would achieve this by an *in situ* approach and would hope to gain valuable insight into the relationship between the localisation and role of this enzyme.

Finally, I would like to examine the sugar feeding system in more detail with a characterisation of soluble invertase gene expression. I hypothesise that the two soluble acid invertase genes in *A. thaliana* are differentially regulated by hexoses as was observed in maize (Xu *et al.*, 1996).

In the next chapter the role of soluble acid invertases in the regulation of carbohydrate metabolism will be explored in more detail. To achieve this transgenic tomato plants with reduced soluble acid invertase activity will be examined. Particular emphasis will be placed on the way reduced soluble acid invertase activity affects the metabolism of mature leaves.

# Chapter 4

# The impact of reduced vacuolar invertase activity on the carbohydrate metabolism of tomato (*Lycopersicon esculentum*)

## 4.1 INTRODUCTION

In the previous two chapters I have examined the regulation of five invertase genes in *A. thaliana* in response to developmental and certain environmental stimuli. This work has revealed some interesting findings. Firstly, that each of the five invertase genes were differentially regulated in response to developmental stimuli. Some genes appeared to be regulated in an organ specific manner (ATβFRUCT 2 and 3) while some were regulated in a developmental manner (ATβFRUCT 1 and 4). Secondly, that one particular apoplastic invertase gene , ATβFRUCT 1, was wound and pathogen responsive. Expression of this gene was elevated in response to both of these stimuli although the timing of these increases differed.

Work presented in this thesis shows that high levels of invertase gene expression and activity in *A. thaliana* are generally associated with sink tissues or tissues in which sink metabolism has been induced in order to mount a defence response. A survey of the literature also reveals that more is known about the role of invertases in sink metabolism. For example, in fava bean, apoplastic invertase in the seed coat is thought to result in a high concentration of hexoses which are taken up by the developing embryo (Weber *et al.*, 1995, 1996). In the roots of certain plant species apoplastic invertase activity is thought to facilitate phloem unloading by maintaining a steep sucrose gradient between source and sink regions of the plant (Eschrich, 1980, Morris and Arthur, 1985). In other species vacuolar invertase activity has been implicated in the maintenance of sink strength (Sturm *et al.*, 1995). Recently, vacuolar invertase activity has been reduced in the fruit of tomato using antisense technology in order to examine the role of vacuolar invertase activity in determining the sugar composition of tomato fruit (Ohyama *et al.*, 1995). Red ripening tomatoes accumulate hexoses as a result of hydrolysis of sucrose by invertase. In transgenic tomato fruit where acid invertase activity was low sucrose content was markedly elevated when compared to wild-type fruit, clearly showing that the high activity of acid invertase in ripening fruit prevents the accumulation of sucrose. An antisense approach has also been used to reduce acid invertase activity in potato tubers (Zrenner *et al.*, 1996). In these plants vacuolar invertase appears to control the hexose:sucrose ratio in cold-stored tubers.

Whilst there is a clear correlation between acid invertase activity and sugar metabolism in many sink tissues, their role in source tissues such as leaves is less clear. This was highlighted in chapter two when invertase gene expression and activity were examined in developing *A. thaliana* leaves. Here I found that expression of both the ATβFRUCT 4 (vacuolar) and ATβFRUCT 1 (apoplastic) invertase genes was highest in the youngest leaf class examined (L1) but expression of both genes declined as the leaves matured. However, interestingly, corresponding measurements of invertase activity showed that considerable apoplastic and vacuolar invertase activity remained even in the oldest leaf class examined. The role of this remaining activity is confusing and even surprising given that leaves are the primary source tissue and that hydrolysis of sucrose here could influence export from the leaf.

The presence of vacuolar invertase activity in the mature leaves of *A. thaliana* plants is not unique but it appears to be species dependent. Huber (1989) reported activities ranging from 5 μmol $(g\ FW)^{-1}\ h^{-1}$ in broad bean to 168 μmol $(g\ FW)^{-1}\ h^{-1}$ in soybean, and demonstrated that in many species the extractable invertase activity was negatively correlated with sucrose content. Thus vacuolar invertase may play a role in (i) in determining the extent to which sucrose or starch accumulates during photosynthesis – it has long been known that different species store different amounts of sucrose and starch – and (ii) in regulating the amount of sucrose available for export. Huber (1989) and Huber and Goldschmidt (1992) postulated a futile cycle of sucrose synthesis and degradation in plants with high soluble invertase activity. They proposed that sucrose which moves into the vacuole is rapidly hydrolysed to hexoses which, because they do not usually accumulate to high levels in plants, move back into the cytoplasm for phosphorylation and subsequent metabolism. Such a cycle also has implications for the long-term down-regulation of photosynthetic metabolism since phosphorylation of hexoses by hexokinase is thought to be involved in the repression of photosynthetic gene expression (Sheen, 1990; Jang and Sheen, 1994, Jang 1997). Hexoses accumulated to high levels in leaves of tobacco plants which over-expressed yeast invertase in their vacuoles. Phenotypically, these plants were stunted, leaves exhibited lower rates of photosynthesis than wild-type plants (Sonnewald *et al.*, 1991).

Over-expression of chimearic yeast invertase genes in the apoplast of tobacco and *A. thaliana* (von Schaewen *et al.*, 1990), tomato (Dickinson *et al.*, 1991) and potato

(Heinke *et al.*, 1992) and in the apoplast, vacuole and cytoplasm of tobacco plants (von Schaewen *et al.*, 1990, Sonnewald *et al.*, 1991) has yielded much information on the role of the different cellular compartments in sucrose metabolism.

In order to further examine the role of vacuolar invertase activity in mature leaves transgenic tomato plants with reduced vacuolar invertase activity will be examined. These plants were prepared by Zeneca at Jealott's hill (Zeneca Plant Science, Jealott's Hill Research Station, Bracknell, Berkshire) and contain a co-suppression construct which, it is hoped, will reduce activity in successfully transformed plants. Here I report the invertase activity, growth, photosynthesis and carbohydrate content of these plants.

## 4.2. MATERIALS AND METHODS

### 4.2.1. Construction of the gene for co-suppression of soluble acid invertase and plant transformation

The preparation of the co-suppression construct and the production of transgenic tomato plants were carried out by Zeneca Plant Science (Robin Wilde, Zeneca Plant Science, Jealott's Hill Research Station, Bracknell, Berks., UK). Standard procedures were used for recombinant DNA work as described in sections 2.2.7 to 2.2.20 and Sambrook *et al.* (1989). Constructs were prepared in *E. coli* (DH5α). A 2.2 Kb fragment containing the entire soluble acid invertase cDNA (InvLe31, see Table 1.1) (Elliot *et al.*, 1993) of *Lycopersicon esculentum* cv. UC82B was excised from pTOM3-LI with the restriction enzymes XhoI and NotI and blunt-ended. The fragment was ligated into PstI-digested and blunt-ended pCaMCCN which contained the Cauliflower Mosaic Virus 35S promoter and nopaline synthase terminator (Pharmacia, LKB Biotechnology, Piscataway, NJ, USA). The resulting plasmid was digested with PstI and religated to remove a 181-bp internal invertase fragment which rendered the plasmid incapable of expressing a functional invertase protein. A 3.0-Kb fragment containing the expression cassette was released by digestion with BglII and partial digestion with XbaI. The fragment was then ligated into BamHI-XbaI-digested pBIN19 (Bevan, 1984) producing the co-suppression construct pWC1 (Fig. 4.1). pWC1 was transferred from *E. coli* into *Agrobacterium tumefaciens* strain LBA4044 by triparental mating (Bevan, 1984). A commercial processing variety of tomato (*Lycopersicon esculentum* L.) was then transformed by *Agrobacterium*-mediated gene transfer (Bird *et al.*, 1988).

### 4.2.2. Selection of lines for further analysis and growth of plants

The initial transformants ($T_0$) were allowed to set seed. The fruit of 50 independent lines ($T_1$) derived from these plants were screened by Zeneca Plant Science for elevated sucrose content using a linked enzyme assay as described in section 2.2.2.

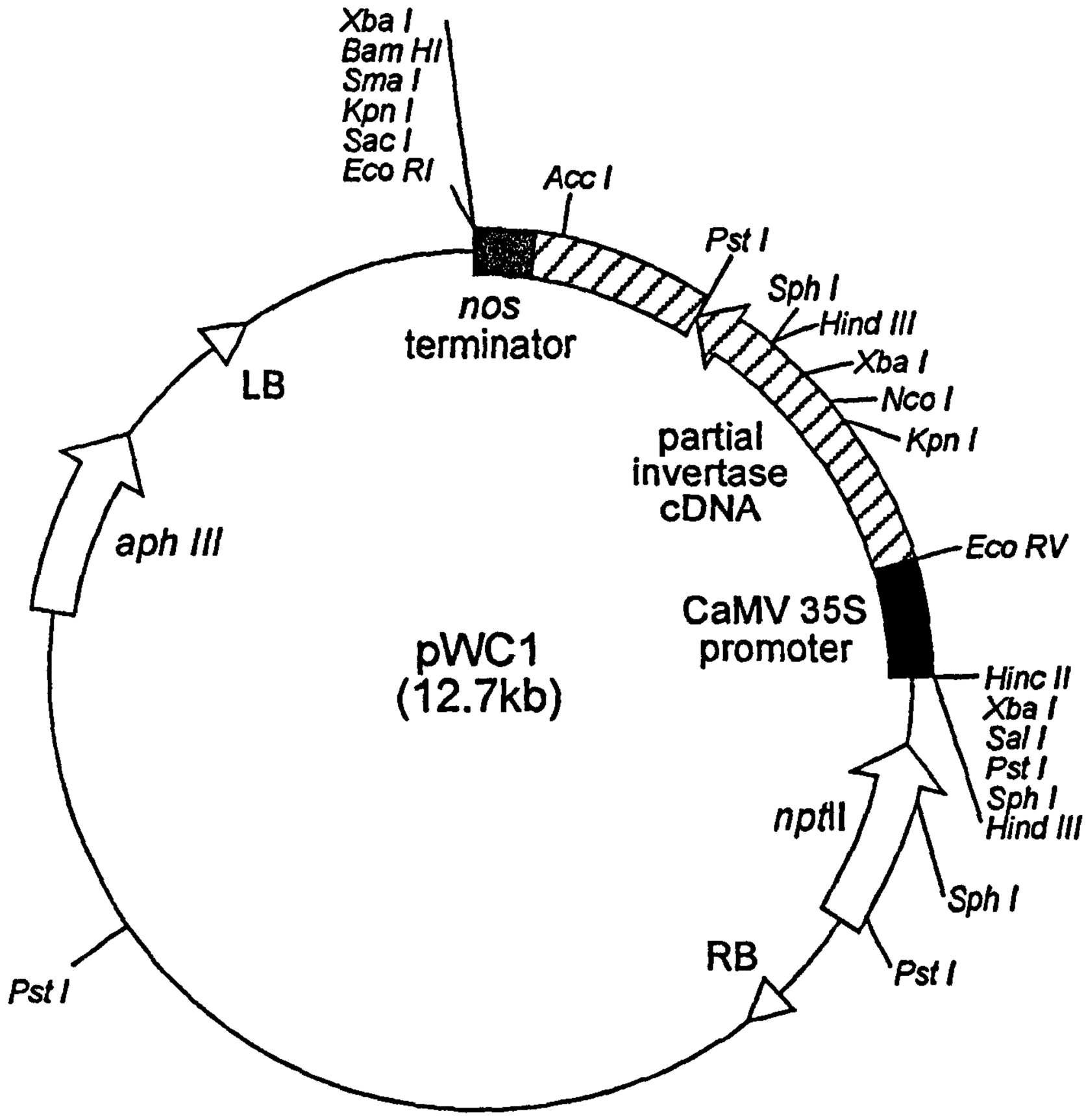



**Figure 4.1 Construction of chimearic gene used for co-suppression of vacuolar invertase activity.** Arrows indicate open reading frames. *LB* and *RB* are the left and right hand borders of the T-DNA. 181 bp of the vacuolar invertase cDNA has been excised from the PstI site. *CaMV 35S* is the cauliflower Mosaic Virus 35S promoter; *nos* is the nopaline synthase gene terminator. *nptII* is a neomycin phosphotransferase II gene conferring kanamycin resistance on the transgenic plants. The vector is pBIN19.

Sucrose content as a percentage of total sugar varied from 0% (equivalent to the wild type) to 80%. On the basis of elevated fruit sucrose content two lines from the $T_2$ generation, designated INV-A and INV-B, were selected for further study at Sheffield.

*Growth of plants*

Tomato seeds were germinated in darkness on moist filter paper for 7 d and then transplanted to 22 cm diameter plastic pots containing 2 L of peat-based compost (M3; Fisons, Loughborough, Leicestershire, UK) and 1.5 g of nutrient pellets (Osmocote, Zeneca, Bracknell, UK). The seedlings were placed in a transgenic greenhouse and covered with transparent plastic pots for 3 d to allow establishment. The plants were grown for seven weeks (July-August, 1995) after which measurements were made. All measurements were made on the uppermost fully-expanded leaves.

## 4.2.3 Measurement of invertase activity, carbohydrates, hexose phosphates, photosynthetic pigments and photosynthesis

*Measurement of invertase activity*

Soluble and apoplastic invertase activity was extracted as described in Ohyama *et al.* (1995). A 7.4 $cm^2$ sample of leaf material was homogenised in 1 ml of ice-cold extraction buffer (50 mM Hepes-KOH, pH 8.3, 2 mM EDTA, 2 mM EGTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 6 mM DTT) and centrifuged at 28 000 *g* for 15 min at 4 °C. The supernatant containing the soluble invertase was decanted into a fresh eppendorf tube and placed on ice temporarily until use. The insoluble pellet was washed four times in 1 ml of extraction buffer and then incubated, with shaking, for 4 h in ice-cold extraction buffer containing 1 M NaCl. Following centrifugation at 28 000 *g* for 15 min at 4 °C the supernatant contained apoplastic invertase activity . Invertase activity was extracted from 0.4 g of root tissue in the same manner.

Samples were measured for soluble and apoplastic invertase activity as described in section 2.2.4, with the following alterations. Aliquots of the samples were incubated for 30 min at 37 °C in 100 mM sucrose and either 200 mM Na-acetate buffer,

pH 4.5 (for apoplastic and soluble invertase) or 200 mM Na-phosphate, pH 7.5 (for alkaline invertase).

For samples with very low invertase activity, 500 µl of soluble extract was desalted by applying to a Sephadex G-25 column (NAP-5, Pharmacia Biotech, St Albans, Herts, UK) equilibrated in extraction buffer. The sample was then concentrated to a volume of 250 µl using a centrifugal microconcentrator (Centricon-30, Amicon, Stonehouse, Gloucestershire, UK). A 100 µl aliquot of this concentrated extract was incubated with 400 µl of 100 mM sucrose, 200 mM Na-acetate buffer (pH 4.5) at 37 °C. Every 15 min, 100 µl aliquots were withdrawn and boiled to stop the reaction. Glucose and fructose were measured using an enzyme-linked assay as described in section 2.2.2.

***Measurement of soluble carbohydrates and starch***

Leaf discs (0.95 $cm^2$) were taken before dawn and late in the afternoon, and immediately frozen in liquid nitrogen. Soluble carbohydrates and starch were extracted and assayed as described in section 2.2.2 and section 2.2.3.

***Measurement of hexose phosphates***

Leaf discs (0.95 $cm^2$) were taken late in the afternoon and frozen immediately in liquid nitrogen. The samples were transferred to a mortar containing liquid nitrogen and a pellet of 0.8 ml 1 M $HClO_4$. These were ground to a fine powder and allowed to thaw. Once thawed, the sample was transferred to an eppendorf tube and the mortar washed with 200 µl of 0.1 M $HClO_4$. The pooled samples were centrifuged at 2500 *g* for 3 min and the supernatant decanted into a fresh eppendorf tube, this was neutralised to pH 7.0 using 5 M $K_2CO_3$. A 100 µl aliquot of a suspension of activated charcoal (100 mg $ml^{-1}$) was added to the neutralised supernatant (to remove pigments) which was then centrifuged at 2500 *g* for 3 min. Hexose phosphates were measured in aliquots of the supernatant using an enzyme linked assay (Scholes *et al.*, 1994).

### *Measurement of photosynthesis and photosynthetic pigments*

Assimilation of $CO_2$ and transpiration were measured using an infra-red gas analyser (IRGA) (Model LCA4 with PLC4 leaf chamber, Analytical Development Company, Hoddesdon, Kent, UK). Actinic light was provided by 150 W tungsten halogen lamp (Schott KL1500T). All measurements were made at room temperature (23 °C) and atmospheric $CO_2$ concentration. The uppermost, fully-expanded leaf was placed in the IRGA chamber and maintained in darkness for 10 min to measure the rate of respiration. The leaf was then illuminated at an irradiance of 180 μmol $m^{-2}$ $s^{-1}$ until photosynthetic induction was complete (approximately 20 min). The steady state rate of photosynthesis was then recorded and the irradiance increased to 1000 μmol $m^{-2}$ $s^{-1}$. The new rate of photosynthesis was then recorded after 10 min.

Chlorophyll and carotenoids were extracted and assayed as described in section 3.2.3. Total chlorophyll, the chlorophyll *a*: chlorophyll *b* ratio and total carotenoid content were calculated from the equations of Lichtenthaler and Wellburn (1983).

### *Statistical analysis*

Where samples exhibited equal variance, one-way Analysis of Variance (ANOVA) was performed using Minitab 9.2 (Minitab Inc, State College, USA). Where variances were unequal, two-way Student's *t*-tests (assuming unequal variance) was performed using Microsoft Excel 5.0 (Microsoft, Corporation, Redmond, Wash., USA).

## 4.3 RESULTS

### 4.3.1 Invertase activity in the leaves of co-suppressed plants

Two lines of transgenic tomato plants (designated INV-A and INV-B) contained the co-suppression construct pWC1 (Fig. 4.1). Work at Zeneca showed that the genome of line INV-A plants contained the co-suppression construct located at a single locus and that they were homozygous for this event. Line INV-B plants also contained the expression cassette at a single locus but were heterozygous for this event; progeny from this line would therefore segregate with respect to pWC1.

Transgenic and wild type (WT) tomato plants were grown in a greenhouse for seven weeks. Leaf discs were taken from the uppermost fully-expanded leaves and assayed for both soluble acid (vacuolar) and apoplastic invertase activity. There was no significant difference in the activity of WT and INV-A plants whereas INV-B plants exhibited either reduced or no detectable leaf vacuolar invertase activity (Table 4.1). A list of the plants assayed and the amount of vacuolar and apoplastic invertase activity contained in the leaf is shown in Fig. 4.2. There was considerable variation in the invertase activity of WT and INV-A plants. This variation did not arise from experimental error or differences in sampling as when the plants were re-assayed, activities did not differ by more than 5%. Two plants from line INV-B exhibited intermediate levels of leaf vacuolar invertase activity. These plants may have arisen from the segregation of the pWC1 co-suppression cassette and are designated INV-B*.

Figure 4.2 shows that six plants from line INV-B exhibited no detectable leaf vacuolar invertase activity. Originally invertase extracts from all the plants were assayed for the generation of hexoses using DNSA reagent (see materials and methods section 2.2.4). As this method is relatively insensitive at detecting low amounts of reducing sugar, invertase extracts from line INV-B plants were re-assayed using the more sensitive enzyme-linked assay (see materials and methods section 2.2.2). Even after extended incubation of INV-B invertase extracts in the presence of sucrose, no reducing sugars could be detected thus verifying the absence of leaf vacuolar invertase activity in these plants. The detection limit using the enzyme-linked assay was 0.25 nmol suc $cm^{-2}$ $min^{-1}$ (approximately 1% of WT).

**Table 4.1. Leaf vacuolar and apoplastic invertase activities.** Activities were extracted from different lines of tomato plants (mean ± SE). n.d., not detectable.

| Plant line | *n* | Leaf invertase activity (nmol suc $cm^{-2}$ $min^{-1}$) | |
|---|---|---|---|
| | | Vacuolar | Apoplastic |
| WT | 5 | 26.46 ± 2.10 | 2.21 ± 0.26 |
| INV-A | 5 | 29.83 ± 3.51 | 2.00 ± 0.10 |
| INV-B* | 2 | 17.96 ± 0.34 | 1.99 ± 0.99 |
| INV-B | 6 | n.d. | 1.69 ± 0.08 |

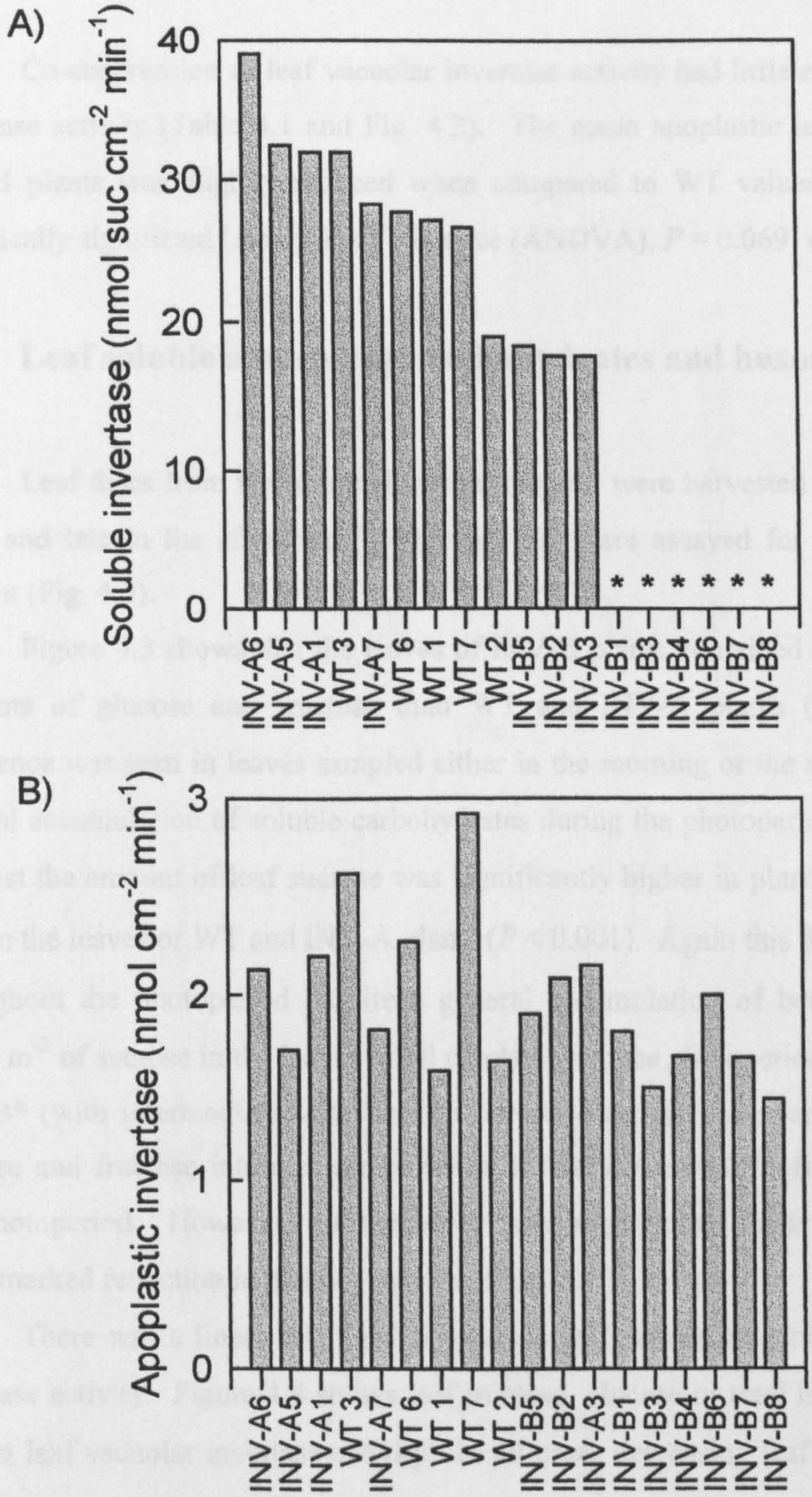



**Figure 4.2 Soluble acid and apoplastic invertase activities in the leaves of wild type and transgenic tomato plants.** Measurements of soluble acid (A) and apoplastic (B) invertase activity were taken from the first fully-expanded leaves of wild type (WT), INV-A and INV-B tomato plants.

Co-suppression of leaf vacuolar invertase activity had little effect on apoplastic invertase activity (Table 4.1 and Fig. 4.2). The mean apoplastic invertase activity of INV-B plants was slightly reduced when compared to WT values but this was not statistically significant [Analysis of Variance (ANOVA), $P = 0.069$] (Table 4.1).

### 4.3.2 Leaf soluble and storage carbohydrates and hexose phosphates

Leaf discs from the plants shown in Fig. 4.2 were harvested before dawn (5:30 a.m.) and late in the afternoon (5:00 p.m.) and were assayed for their carbohydrate content (Fig. 4.3).

Figure 4.3 shows that the leaves of INV-B plants contained significantly lower amounts of glucose and fructose than WT and INV-A plants ($P < 0.05$). This difference was seen in leaves sampled either in the morning or the evening despite the general accumulation of soluble carbohydrates during the photoperiod in all plants. In contrast the amount of leaf sucrose was significantly higher in plants from line INV-B than in the leaves of WT and INV-A plants ($P < 0.001$). Again this difference remained throughout the photoperiod despite a general accumulation of between 0.6 and 1.1 mmol $m^{-2}$ of sucrose in the leaves of all plants during the photoperiod. Plants from line INV-B* (with intermediate leaf vacuolar invertase activity) showed accumulations of glucose and fructose intermediate between WT/INV-A and INV-B plants throughout the photoperiod. However, the leaves of these segregating plants exhibited a much more marked reduction in glucose than fructose.

There was a linear relationship between leaf hexose content and leaf vacuolar invertase activity. Figure 4.4 shows leaf fructose, glucose or total leaf hexoses plotted against leaf vacuolar invertase activity. In all cases decreasing leaf vacuolar invertase activity correlated with a decline in leaf hexoses both in pre-dawn or end of photoperiod samples. Leaves which contained no detectable vacuolar invertase activity had a total hexose content which was less than 10% of that of leaves with high invertase activity. Leaves with intermediate invertase activity had an intermediate hexose content.

The relationship between leaf vacuolar invertase activity and the sucrose content of leaves was not linear. Plants with no detectable leaf vacuolar invertase activity

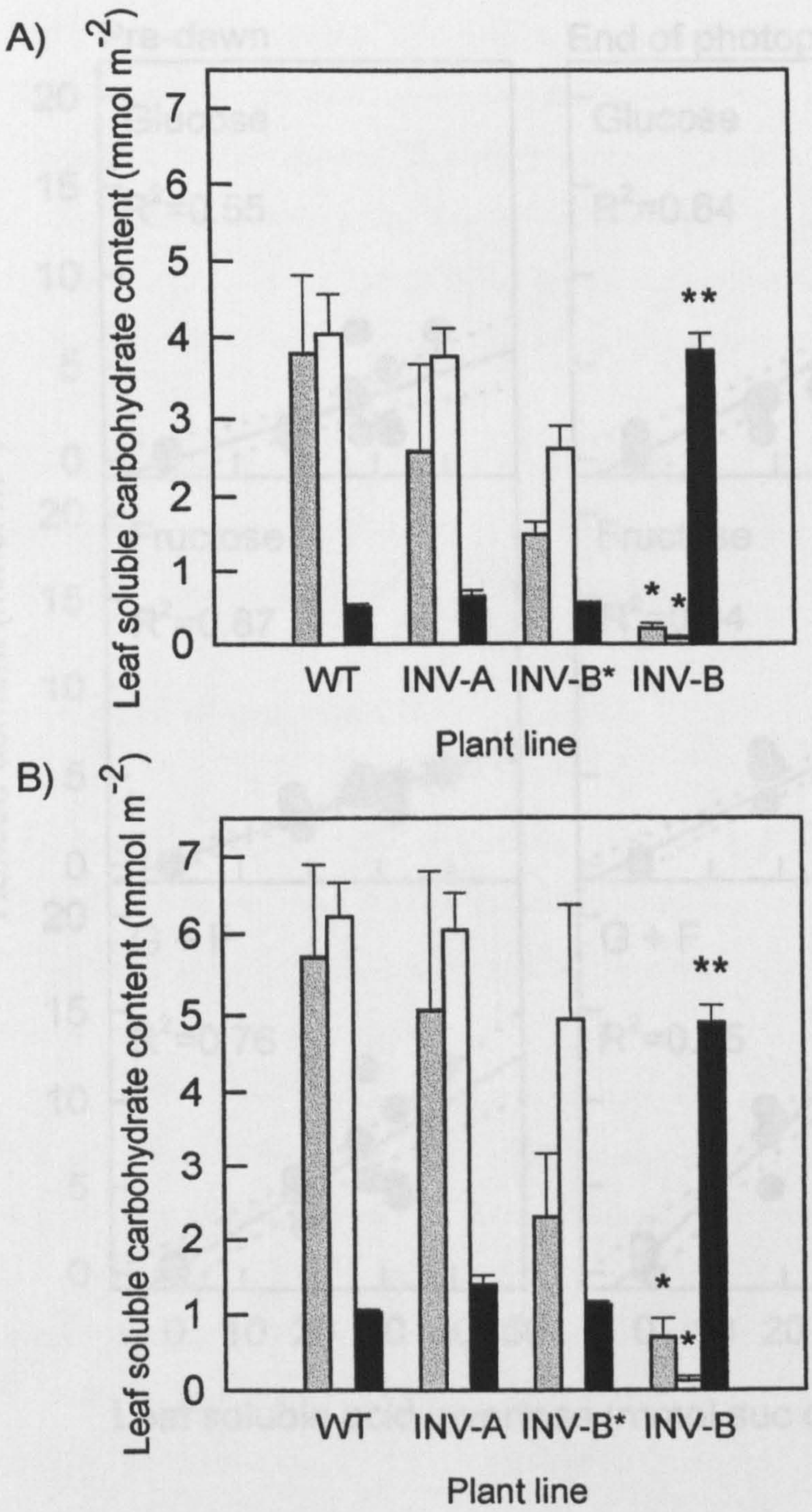



**Figure 4.3 Carbohydrate content of leaves of different lines of tomato plants.** Samples were harvested pre-dawn at 5:30 a.m. (A) and late afternoon at 5:00 p.m. (B). Leaf discs from the first-fully expanded leaves were harvested and measured for glucose (shaded bar), fructose (unfilled bar) and sucrose (filled bar). Measurements are the mean ± SE. Numbers of plants sampled are shown in Table 4.1. Columns marked * and ** are significantly different from the comparable carbohydrate in WT and INV-A plants, $P < 0.05$ and 0.001 respectively.

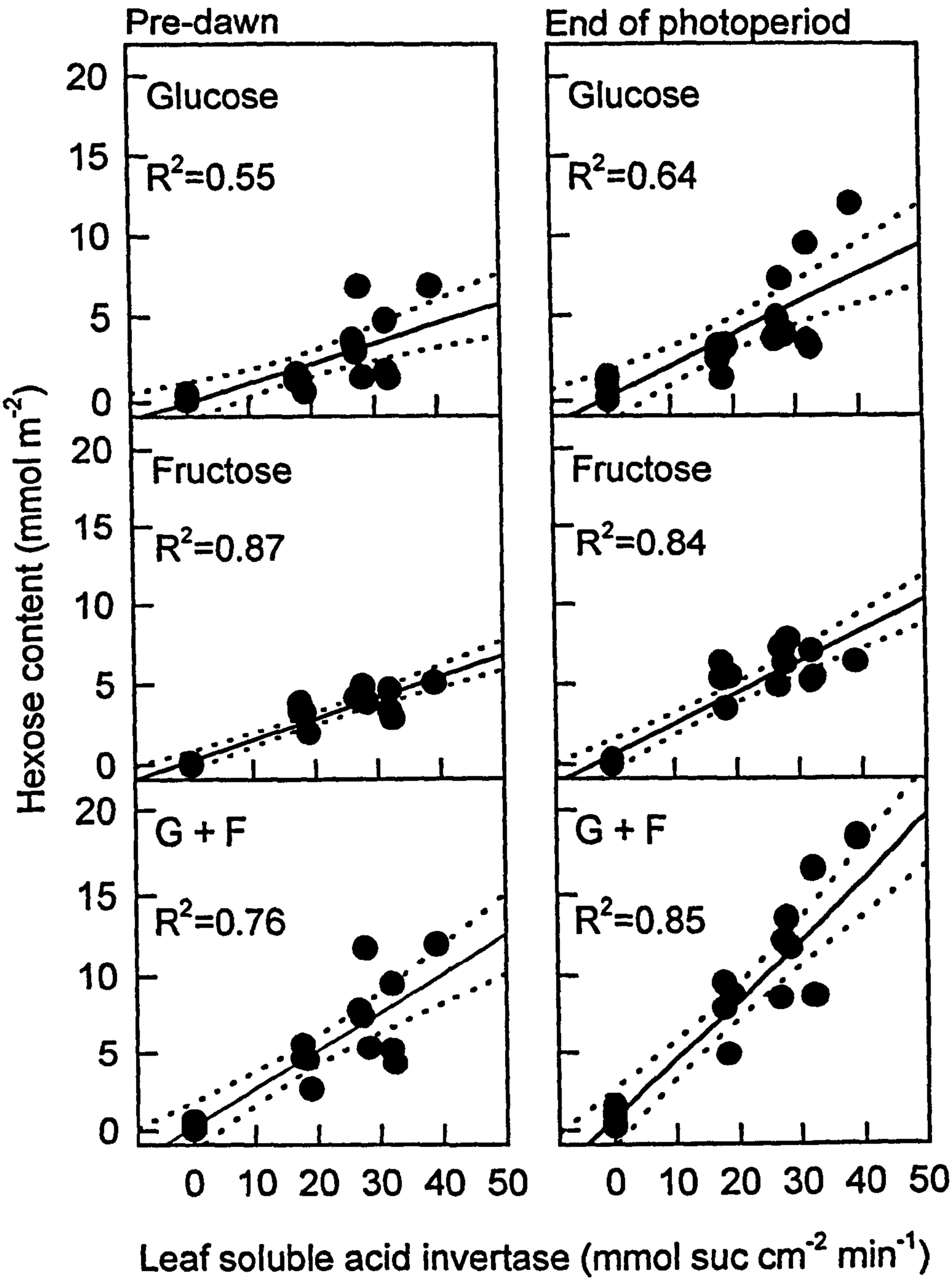



**Figure 4.4 Correlation between leaf soluble acid invertase activity and leaf hexoses.** The leaf glucose, fructose and glucose + fructose (G + F) content of leaf discs from the first full-expanded leaves of wild type, INV-A, INV-B* and INV-B tomato plants were sampled pre-dawn (5:30 a.m.) and late afternoon (5:00 p.m.) and correlated with the soluble acid invertase activity from the same plant. A linear regression (solid line) with 95% confidence intervals (dotted line) is shown.

contained higher amounts of sucrose compared to those with higher invertase activity. However, above a certain threshold increasing invertase activity was not associated with a further decline in leaf sucrose. This relationship was maintained throughout the photoperiod despite the accumulation of sucrose in the leaves of all plants (Fig. 4.5).

Starch accumulated in the leaves of all plants throughout the photoperiod but there was no significant difference in the amount of starch between any of the lines (Fig. 4.6).

Leaves were harvested late in the afternoon for measurement of hexose-phosphate content. There was no significant difference in the glucose-6-phosphate or fructose-6-phosphate content of any of the lines (Table 4.2).

### 4.3.3 Growth and photosynthesis

Co-suppression of leaf vacuolar invertase activity had no effect on shoot growth. Plant height and leaf area and the dry weight of leaves and stems did not alter significantly between any of the lines of plants nor was there an effect on the fresh weight of leaves, stems or flowers (Table 4.3).

Co-suppression of leaf vacuolar invertase activity did not appear to effect the photosynthetic apparatus of any of the lines. The amount of total leaf chlorophyll, the chlorophyll a : b ratio and the amount of leaf carotenoids did not differ significantly between any of the lines (Table 4.4). In addition to the lack of effects on photosynthetic pigments measurements of $CO_2$ assimilation and transpiration rates did not differ significantly between any of the lines either at 180 μmol $m^{-2}$ $s^{-1}$ or under saturating light (1000 μmol $m^{-2}$ $s^{-1}$). In addition, the rate of respiration (measured in darkness) did not differ between lines (Table 4.4).

### 4.3.4 Invertase activities in the roots

Invertase activities were also measured in the roots of plants from each line. In addition to the vacuolar and apoplastic invertase activity found in the leaves , roots also contain a soluble invertase located in the cytoplasm, with a neutral pH optimum. Leaf extracts contain relatively little invertase activity when measured at pH 7.5, generally

**Figure 4.5 Correlation between leaf soluble acid invertase activity and leaf sucrose.** The leaf sucrose content of leaf discs from the first full-expanded leaves of wild type, INV-A, INV-B* and INV-B tomato plants were sampled pre-dawn (5:30 a.m.) and late afternoon (5:00 p.m.) and correlated with the soluble acid invertase activity from the same plant.

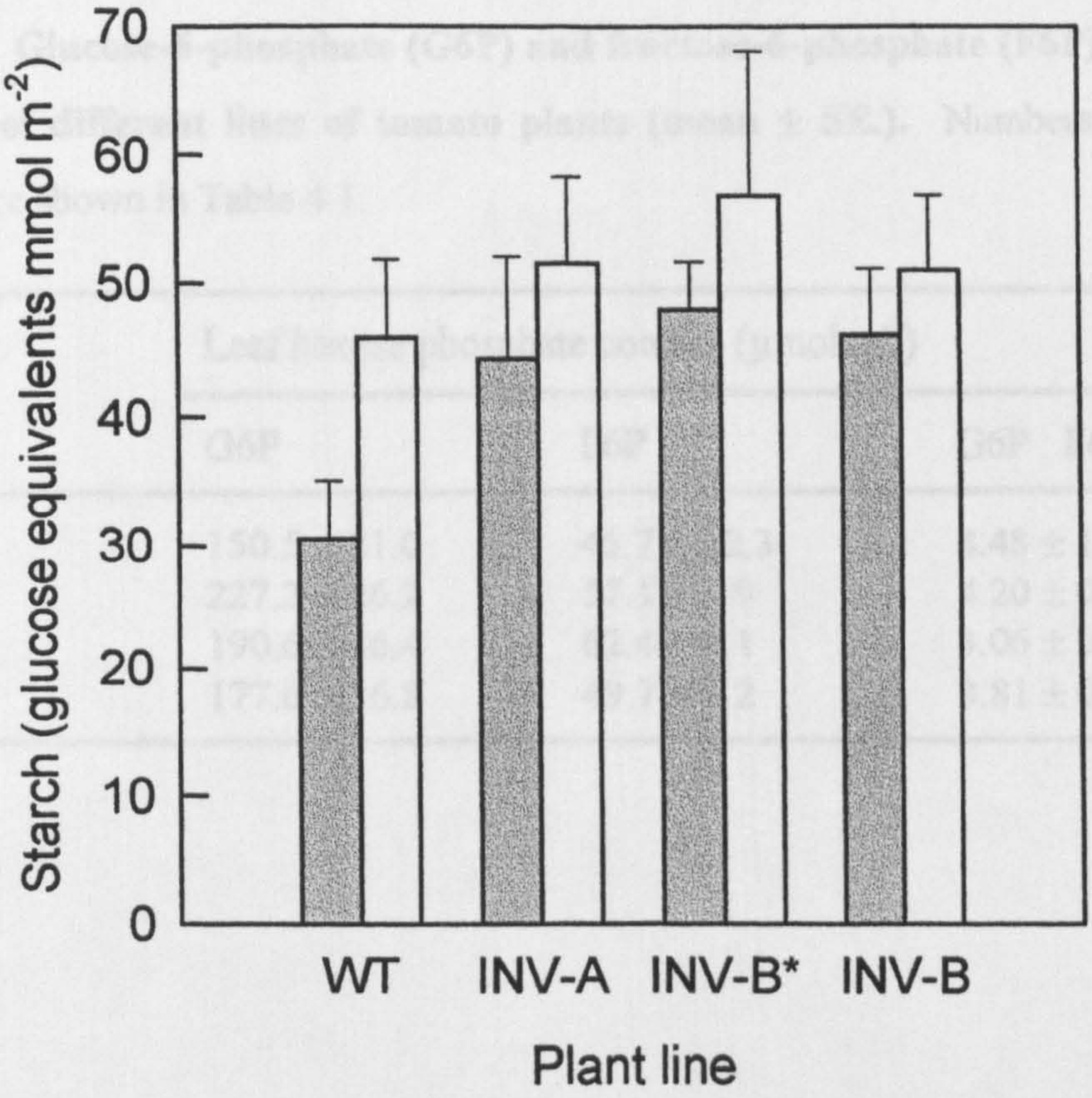



**Figure 4.6 Starch content of leaves of different lines of tomato plants.** Samples were harvested pre-dawn, 5:30 a.m. (shaded bar) and late in the afternoon, 5:00 p.m. (unshaded bar). Measurements are the mean ± SE. Numbers of plants sampled are shown in Table 4.1.

**Table 4.2 Glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) contents of leaves of different lines of tomato plants (mean ± SE.).** Numbers of plants sampled are shown in Table 4.1.

| Plant line | Leaf hexose phosphate content (μmol $m^{-2}$) | | |
|---|---|---|---|
| | G6P | F6P | G6P : F6P |
| WT | 150.5 ± 21.0 | 45.7 ± 12.3 | 4.48 ± 1.57 |
| INV-A | 227.2 ± 26.2 | 57.1 ± 5.9 | 4.20 ± 0.70 |
| INV-B* | 190.6 ± 16.4 | 62.4 ± 0.1 | 3.06 ± 0.27 |
| INV-B | 177.6 ± 16.8 | 49.7 ± 5.2 | 3.81 ± 0.65 |

**Table 4.3 Measurements of shoot growth, dry weight (DW) and fresh weight (FW) from lines of tomato plants with different leaf vacuolar invertase activities.** Results of are the means ± SE. Numbers of plants sampled are shown in Table 4.1.

| Plant line | Plant height (cm) | Leaf area (cm) | Leaf DW (g) | Stem DW (g) | Leaf +stem DW (g) | Leaf FW (g) | Stem FW (g) | Flower FW (g) | Leaf + stem + flower FW (g) |
|---|---|---|---|---|---|---|---|---|---|
| WT | 63.8 ± 1.6 | 1741 ± 83 | 5.3 ± 0.4 | 5.7 ± 0.4 | 11.0 ± 0.9 | 43.2 ± 2.5 | 62.9 ± 3.4 | 3.4 ± 1.1 | 35.7 ± 7.0 |
| INV-A | 62.8 ± 1.4 | 1535 ± 203 | 5.4 ± 1.5 | 6.7 ± 1.8 | 12.2 ± 3.3 | 40.5 ± 8.2 | 64.6 ± 13.6 | 13.6 ± 1.2 | 35.4 ± 8.5 |
| INV-B* | 66.0 ± 2.0 | 757 ± 92 | 3.1 ± 0.0 | 4.1 ± 0.2 | 7.2 ± 0.2 | 26.2 ± 0.4 | 42.5 ± 0.5 | 0.5 ± 1.1 | 23.2 ± 7.6 |
| INV-B | 62.8 ± 0.7 | 1298 ± 237 | 4.7 ± 0.7 | 6.1 ± 0.8 | 10.8 ± 1.5 | 36.6 ± 5.1 | 57.2 ± 6.7 | 6.7 ± 1.8 | 31.9 ± 6.1 |

**Table 4.4 Measurement of photosynthetic pigments, respiratory and photosynthetic rates and transpiration rates in the leaves of tomato plants with different leaf vacuolar invertase activities.** The rates of $CO_2$ assimilation were measured in the dark (L=0)(respiratory rate), under low light (L=180 μmol $m^{-2}$ $s^{-1}$) and under saturating irradiance (L=1000 μmol $m^{-2}$ $s^{-1}$). The transpiration rate was measured under the same light conditions. Results are the means ± SE. Numbers of plants sampled are shown in Table 4.1.

| Plant Line | Chlorophyll (mg $m^{-2}$) | Chlorophyll a/b | Carotenoids (mg $m^{-2}$) | Assimilation rate (L=0 μmol $m^{-2}$ $s^{-1}$) | Assimilation rate (L=180 μmol $m^{-2}$ $s^{-1}$) | Assimilation rate (L=1000 μmol $m^{-2}$ $s^{-1}$) | Transpiration rate (L=0 mmol $m^{-2}$ $s^{-1}$) | Transpiration rate (L=180 mmol $m^{-2}$ $s^{-1}$) | Transpiration rate (L=1000 mmol $m^{-2}$ $s^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| WT | 276 ± 21 | 3.10 ± 0.07 | 61 ± 1 | -1.9 ± 0.3 | 7.4 ± 0.5 | 11.9 ± 0.4 | 2.9 ± 0.2 | 2.4 ± 0.2 | 3.0 ± 0.2 |
| INV-A | 260 ± 20 | 3.05 ± 0.10 | 58 ± 2 | -2.2 ± 0.1 | 6.0 ± 0.5 | 12.6 ± 0.4 | 2.8 ± 0.2 | 1.6 ± 0.3 | 3.2 ± 0.2 |
| INV-B* | 262 ± 10 | 3.32 ± 0.20 | 55 ± 3 | -1.9 ± 0.1 | 7.2 ± 0.1 | 10.3 ± 1.6 | 2.7 ± 0.5 | 2.3 ± 0.2 | 2.5 ± 0.7 |
| INV-B | 281 ± 14 | 3.02 ± 0.06 | 59 ± 1 | -1.8 ± 0.2 | 7.3 ± 0.2 | 13.0 ± 0.5 | 2.5 ± 0.4 | 2.4 ± 0.1 | 3.1 ± 0.2 |

less than 10% the activity measured at pH 4.5. In contrast, the cytoplasmic invertase has a pH optimum close to neutral and exhibits little activity at acidic pH (Chen and Black, 1992). Therefore, assays conducted at pH 7.5 will largely reflect the activity of the cytoplasmic enzyme.

Surprisingly, there was little difference in root vacuolar invertase activity between plants with or without detectable leaf vacuolar invertase activities. Analysis of the different lines (Fig. 4.7 and Table 4.5) revealed that although there was a reduction in the mean root vacuolar invertase activity in plants from line INV-B this was not statistically significant (one-way ANOVA, $P = 0.236$). As in the leaves the mean root apoplastic invertase activity was slightly reduced in plants from line INV-B but again this was not statistically significant (one-way ANOVA, $P = 0.058$). Roots also contained significant soluble invertase activity when extracts were assayed at pH 7.5. The majority of this probably represents the activity of the cytoplasmic enzyme. There was no significant differences in alkaline invertase activity between any of the groups of plants (Table 4.5).

### 4.3.5 Soluble and storage carbohydrates in the roots

There was a slight reduction in the amount of hexoses in the roots of plants from line INV-B compared to WT and INV-A plants (Table 4.6). However, these differences were not significant. The amount of sucrose in the roots of plants from line INV-B was slightly elevated compared to WT and INV-A plants (Table 4.6). Again, these differences were not significant. There was no difference in the amount of root starch between the different lines of plants (Table 4.6).

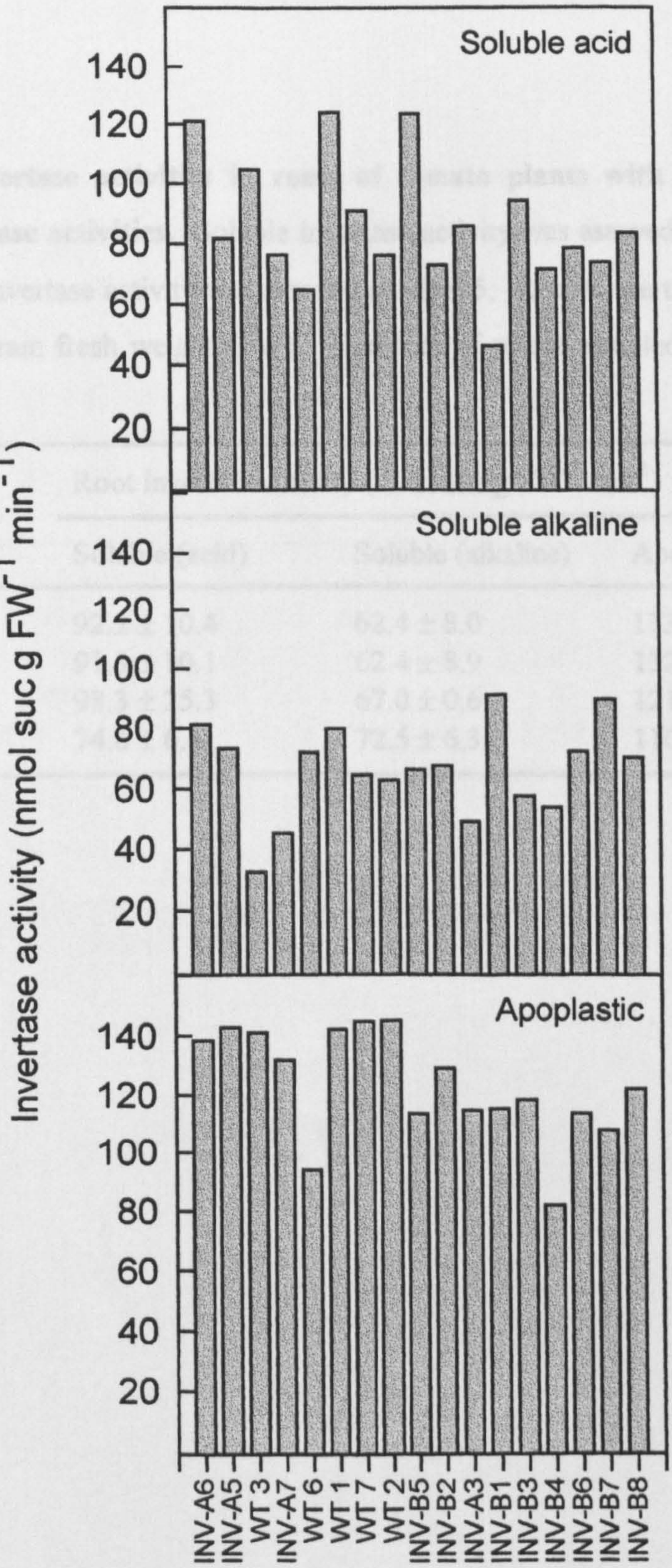



**Figure 4.7 Soluble and apoplastic invertase activities in the roots of wild type and transgenic tomatoes.** Measurements of soluble acid, soluble alkaline and apoplastic invertase activity were taken from the roots of wild type (WT), INV-A, INV-B* and INV-B tomato plants.

**Table 4.5 Invertase activities in roots of tomato plants with different leaf vacuolar invertase activities.** Soluble invertase activity was assayed at pH 4.5 and 7.5, apoplastic invertase activity was assayed at pH 4.5. Results are the means ± SE expressed per gram fresh weight (FW). Numbers of plants sampled are shown in Table 4.1.

| Plant line | Root invertase activity (nmol suc g $FW^{-1}$ $min^{-1}$) | | |
|---|---|---|---|
| | Soluble (acid) | Soluble (alkaline) | Apoplastic |
| WT | 92.2 ± 10.4 | 62.4 ± 8.0 | 133.5 ± 9.9 |
| INV-A | 91.3 ± 10.1 | 62.4 ± 8.9 | 132.2 ± 6.1 |
| INV-B* | 98.3 ± 25.3 | 67.0 ± 0.6 | 121.6 ± 7.8 |
| INV-B | 74.6 ± 6.6 | 72.5 ± 6.3 | 110.1 ± 5.9 |

**Table 4.6 Carbohydrate contents of roots from lines of tomato plants with different leaf vacuolar invertase activities.** Results are the means ± SE expressed per gram fresh weight (FW). Numbers of plants sampled are shown in Table 4.1.

| Plant line | Root carbohydrate content (μmol g $FW^{-1}$) | | | |
|---|---|---|---|---|
| | Glucose | Fructose | Sucrose | Starch (glucose equivalents) |
| WT | 7.84 ± 0.70 | 15.39 ± 1.92 | 12.89 ± 1.21 | 13.95 ± 0.72 |
| INV-A | 9.56 ± 1.21 | 19.00 ± 1.89 | 14.85 ± 1.84 | 14.83 ± 0.96 |
| INV-B* | 6.24 ± 1.15 | 15.11 ± 1.29 | 10.81 ± 2.09 | 14.86 ± 0.78 |
| INV-B | 4.94 ± 1.17 | 13.54 ± 1.89 | 17.98 ± 1.33 | 14.88 ± 0.61 |

## 4.4 DISCUSSION

*Impact of co-suppression construct on invertase activity in tomato*

An invertase co-suppression construct was introduced into a domestic tomato (*L. esculentum*) variety in order to reduce vacuolar invertase activity in the resulting transformants. At present the mechanism of co-suppression is not fully understood although the use of aberrant sense transgenes to silence the expression of homologous endogenous genes is a well documented phenomenon (for review see Meyer, 1995). In most cases where a chimaeric transgene has been introduced only some of the resulting plants exhibit gene silencing. This suggests that in addition to the transgene itself, additional factors are required. The importance of these additional factors was demonstrated when the co-suppression of nitrate reductase gene expression was transmitted from silenced tobacco stocks to non-silenced scions expressing the corresponding transgene (Palauqui *et al.*, 1997).

It has been proposed that the induction of co-suppression only occurs when the amount of a particular mRNA reaches a certain 'threshold' level. This may occur when the 'normal' export of aberrant transgene mRNA from the nucleus is blocked due to structural abnormalities (Metzlaff *et al.*, 1997). Once this 'biochemical switch' has been activated it may trigger the specific degradation of all homologous mRNA in the cytoplasm (Elmayan and Vaucheret, 1996). This theory is consistent with the fact that transgenes expressed under the control of a 35s promoter with a double enhancer are more likely to be silenced than transgenes under the control of weaker promoters (Palauqui and Vaucheret, 1995). However, instances have been recorded where gene silencing has occurred in plants harbouring promoterless transgenes (van Blockland *et al.*, 1994).

The mechanism for mRNA degradation is not full understood although it has been proposed that transgene mRNA pairs with endogene mRNA and that this induces endonucleolytic cleavage at specific sites. These cleaved products can then either be exported from the nucleus or act as substrates for further cleavage reactions (Metzlaff *et al.*, 1997).

The introduction of the co-suppression construct into tomato successfully

reduced vacuolar invertase activity below the level of detection in the leaves of some plants derived from line INV-B. However, of the eight plants examined from this line two plants still exhibited substantial activity (INV-B*). This was probably due to a segregation effect as the transformants were heterozygous for the co-suppression construct. Leaf vacuolar invertase activity of INV-A and WT plants showed a great deal of variation. The reason for this variation between plants is unclear, but it was reproducible; within any one plant invertase activity did not vary significantly between young fully-expanded leaves. As apoplastic invertase activity was not significantly altered by the presence of the co-suppression construct any changes in carbohydrate metabolism resulted from the changes in vacuolar invertase activity alone.

Two recent studies have used an antisense approach to reduce invertase activity in tomato. In the first study by Ohyama *et al.* (1995), fruit vacuolar invertase activity in some lines was reduced to below the level of detection (0.05% of WT). In these lines the amount of sucrose in red-ripe fruit was markedly elevated while the amount of hexoses was reduced compared to control fruit. The introduction of the antisense vacuolar invertase gene also reduced apoplastic invertase activity in the fruit to 1% of wild type levels.

In the leaves, the impact of the antisense construct was less apparent than in the fruit. Here, vacuolar invertase activity was reduced to about 20% of wild type. The reduction of apoplastic invertase activity in the leaves was also less marked, typically 40-80% of wild type. However, despite less marked reductions in acid invertase activity in the leaves the amount of sucrose was still elevated above that observed in control leaves.

The second study expanded upon that just described by examining the secondary generation of transformants throughout fruit development (Klann *et al.*, 1996). These authors found that the final size of fruit from transgenic tomato plants with reduced vacuolar invertase activity and elevated sucrose were 30% smaller than wild type fruit.

The different patterns of invertase activity between the plants containing the invertase co-suppression construct and the antisense construct may be due to a number of factors, including the developmental stage of the plants when sampled, and the invertase DNA sequence used in the binary vector. A comparison of the tomato

vacuolar invertase gene sequence with four apoplastic invertase clones recently isolated from tomato show that all five sequences share 75% to 79% homology (Godt and Roitsch, 1997). In the antisense approach of Ohyama *et al.* (1995) the entire vacuolar gene was introduced in the antisense orientation whereas the co-suppression construct used in this study had a 181 bp region deleted from a region known to share homology with other apoplastic invertase genes. This may explain why the co-suppression construct had little effect on apoplastic invertase gene expression.

To date, only one vacuolar invertase gene (TIV1) has been isolated from tomato (Elliot *et al.*, 1993). It is therefore possible that vacuolar invertase activity throughout the plant is derived from the expression of a single gene. If this is the case then it is perhaps surprising that there was little reduction in vacuolar invertase activity in the roots of co-suppressed plants, particularly those which contained no detectable vacuolar invertase activity in the leaves. A similar loss of co-suppression was also observed by Metzlaff *et al.* (1997) who examined transgenic petunia plants containing chalcone synthase co-suppression constructs. Chalcone synthase is a key enzyme in the anthocyanin biosynthesis pathway which, if blocked, leads to the loss of anthocyanin pigment in the petals and white flowers are produced instead of the normal purple. These authors found that the flowers of some co-suppressed lines contained distinct sectors of white and purple pigmentation, indicating variable co-suppression efficiency. Jorgensen *et al.* (1995) showed that this loss of co-suppression might be the result of epigenetic changes occurring in the floral meristem, a region where resetting of nuclear organisation and transgene or endogene chromatin occurs preferentially. As roots also arise from an independent meristem this may explain the lack of vacuolar invertase co-suppression observed in the roots of the tomato plants used in this study.

Alternatively, it is possible that the lack of co-suppression of vacuolar invertase activity in the roots may have been due to the expression of a second, as yet unidentified, vacuolar invertase gene.

***The impact of reduced vacuolar invertase activity on leaf carbohydrate metabolism***

Previous work has suggested the possibility of 'futile' cycling of sucrose in some species. For example, Geigenberger and Stitt (1991) using $^{14}C$ pulse-chase

experiments found that in *Ricinus communis* seedlings there was a cycle of sucrose synthesis and degradation in the cotyledons. By removing the hypocotyl and thus blocking sucrose export these authors found that the unidirectional rate of sucrose synthesis in the cotyledons decreased while that of degradation increased. These changes were also accompanied by an increase in starch in the cotyledons. Interestingly, the switch from sucrose export to starch storage was accompanied by only a small increase in the sucrose concentration of the cotyledon. This led the authors to speculate that energy dependent futile cycling of sucrose allows for sudden and sensitive regulation of sucrose metabolism in response to environmental, physiological and developmental change. Futile cycling of sucrose has also been observed in *Chenopodium rubrum* (Dancer *et al.*, 1990) and sugar cane cell suspension cultures (Wendler *et al.*, 1991) and in banana fruit (Hubbard *et al.*, 1990).

Huber (1989) speculated that sucrose may also be cycled in the mature leaves of some species and that this cycling was linked to the activity of vacuolar invertase. This author examined ten species that differed in their ability to accumulate leaf sucrose during the photoperiod and found that it was critically dependent on the amount of leaf vacuolar invertase activity present within the leaf. In species with low invertase activity (e.g. broad bean, spinach and pea) sucrose accumulated in the vacuole. In contrast, in species with high vacuolar invertase activity (e.g. tomato and tobacco) sucrose did not accumulate. When this relationship was plotted there was a linear relationship between leaf vacuolar invertase activity and leaf sucrose. In leaves containing vacuolar invertase activity sucrose enters the vacuole where it is rapidly hydrolysed to glucose and fructose which then moves into the cytoplasm for re-phosphorylation by hexokinase (see Fig. 4.8). Heinke *et al.* (1994) have shown that in plants with high vacuolar invertase activity the majority of the sucrose is present within the cytoplasm.

By examining transgenic plants of a single species with widely varying invertase activities it has been possible to gain evidence to support the proposal of futile cycling of sucrose in mature leaves. In the leaves of tomato plants derived from line INV-B with no detectable vacuolar invertase activity sucrose accumulates to much higher levels than in WT plants, and hexoses are barely detectable. In these plants it is likely that sucrose enters the vacuole where it accumulates because it cannot be hydrolysed. Measurement of the sucrose content of these leaves in the morning and late in the

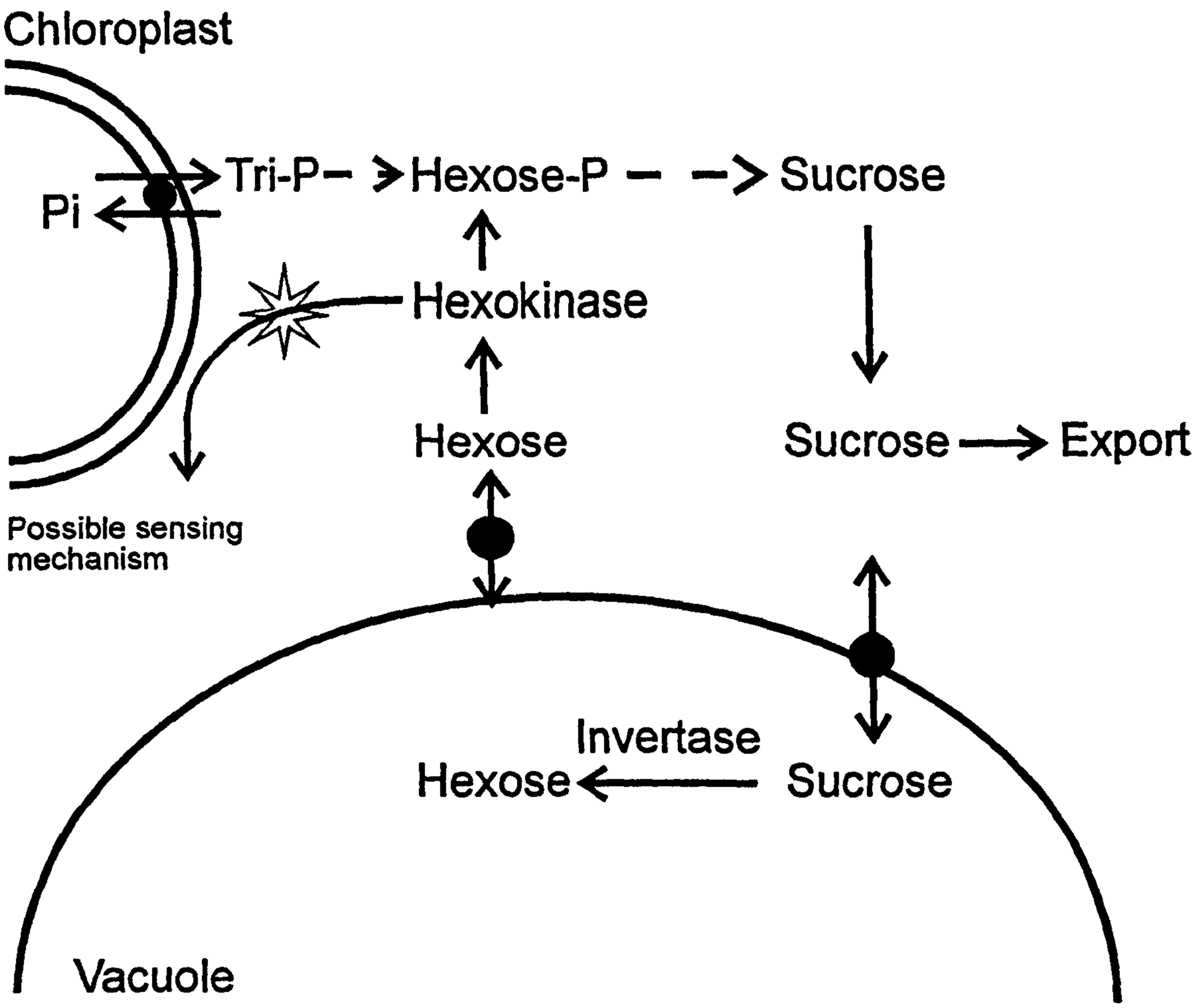



**Figure 4.8. Diagrammatic representation of leaf vacuolar sucrose cycling and possible involvement of a hexokinase sensing mechanism.** Sucrose entering the vacuole is hydrolysed by invertase. The resulting hexoses move into the cytoplasm were they are re-phosphorylated to hexose-phosphate (hexose-P) and metabolised. This phosphorylation step, catalysed by hexokinase, may lead to a signal transduction cascade resulting in altered patterns of gene expression. A reduction in sucrose export may lead to an increase sucrose cycling through the vacuole. *Tri-P* (triose-phosphate); *Pi* (inorganic phosphate).

afternoon did show that there was a small increase in the sucrose of leaves during the day. This may reflect a very small amount of residual invertase activity but, more likely, reflects changes in cytoplasmic sucrose content. The magnitude of accumulation of sucrose during the day was similar in both INV-B plants and plants showing high vacuolar invertase activity. Few species show any diurnal regulation of invertase activity and light has been shown not to affect invertases in tomato fruit or leaves (Guan and James, 1991, Kingston-Smith *et al.*, 1998).

A threshold relationship between invertase activity and sucrose content was observed by Goldschmidt and Huber (1992) in a comparative study of 12 different species with naturally varying invertase activity. Species such as spinach with low vacuolar invertase activity accumulated sucrose in the leaves whereas those with high activity such as tomato did not. Sunflower represented a species whose leaf vacuolar invertase activity was on the edge of the threshold. Under normal conditions sucrose did not accumulate in the leaves of this species. However, upon girdling, which led to a very small decrease in invertase activity, large amounts of sucrose accumulated in the leaves. Girdling did not affect the leaf sucrose content of species with higher invertase activity. In this present study such a threshold effect has been observed in a single species. Only the complete removal of vacuolar invertase activity led to an accumulation of sucrose.

As shown in Fig. 4.4 there was a linear relationship between leaf vacuolar invertase activity and hexose content. Thses data imply that invertase activity in the vacuole was not in excess. As the concentration of vacuolar invertase increased so did the rate of sucrose hydrolysis. This is in agreement with Huber (1989) who suggests that there is just sufficient invertase activity to hydrolyse sucrose entering the vacuole.

Interestingly, the linearity of this relationship was maintained in both morning and evening samples (Fig. 4.4) even though the amount of hexoses differed. This possibly indicates that the rate of import of sucrose into the vacuole is also important in determining the rate of sucrose hydrolysis. At present little is known about sugar transporters in the tonoplast membrane or the factors that may regulate their activity. Recently, it has been shown that in many species there are two tonoplast-associated primary $H^+$ pumps, an $H^+$-transporting ATPase and a $H^+$-transporting inorganic pyrophosphatase (Sze *et al.*, 1992; Rea and Poole, 1993). In theory these pumps could

be used to drive the active uptake of sucrose into the vacuolar compartment; the active uptake of sucrose into the vacuoles of red beet has already been demonstrated (Getz, 1991). However, Milner *et al.* (1995) demonstrated that in tomato fruit sucrose, glucose and fructose were likely to be transported into the vacuole via facilitated diffusion as was the case in mesophyll cells of barley (Kaiser and Heber, 1984).

Glucose and fructose produced as a result of futile cycling of sucrose through the vacuole may be expected to produce an increase in the amount of hexose phosphates relative to plants with no vacuolar invertase activity. However, as there was no difference in the hexose-phosphate content of the different lines, the majority of the hexose phosphates present were most probably derived directly from photosynthetic sucrose synthesis. This is consistent with the fact that the plants used for this study were grown under high irradiance and were photosynthesising and growing rapidly. It would be expected that sucrose cycling through the vacuole becomes increasingly important as sucrose export is reduced. Foyer (1987) proposed that sucrose hydrolysis by invertase in either the apoplast or vacuole may result in sucrose cycling under conditions of reduced sink capacity as was subsequently demonstrated by Geigenberger and Stitt (1991). The increase in hexose resulting from this sucrose cycling may play an important role in the regulation of photosynthetic gene expression.

It has already been demonstrated that hexoses play an important role in the regulation of photosynthesis. In one study the addition of glucose to a maize protoplast transient expression assay resulted in a decrease in the expression of seven photosynthetic genes (Sheen, 1990). In another approach, plants over expressing a yeast derived invertase in the apoplast or vacuole resulted in the accumulation of soluble carbohydrate within the leaves and the down-regulation of photosynthesis (Schaewen *et al.*, 1990; Dickinson *et al.*, 1991; Sonnewald *et al.*, 1991).

Recently, it has been shown that it is not the build-up of hexoses *per se* that critically affect photosynthetic gene expression rather it is the flux of hexoses through the enzyme hexokinase (Jang and Sheen, 1994; Smeekens and Rook, 1997). This enzyme has been proposed as a hexose sensor that elicits a signal transduction chain ending in the repression of photosynthetic gene expression. Transgenic *A. thaliana* plants expressing antisense or sense hexokinase genes display sugar responses consistent with hexokinase being the hexose sensor. Plants containing the antisense

construct were generally hypersensitive to sugar whereas plants over-expressing hexokinase were generally sugar hypersensitive (Jang *et al.*, 1997).

Under conditions of reduced export where high fluxes of sucrose are cycling through the vacuole the generation of hexoses may elicit the down-regulation of photosynthetic gene expression via the hexokinase sensor (Fig. 4.8). This would provide a sensitive mechanism for the adjustment of photosynthetic capacity under different growth conditions. Goldschmidt and Huber (1992) reduced export in twelve species that exhibited a range of leaf vacuolar invertase activities by hot wax girdling. These authors found that the only link between reduced export and the down regulation of photosynthesis was plants with high levels of vacuolar invertase activity and low leaf sucrose. This work provides powerful evidence for the role of sucrose cycling under certain conditions.

The reduction in vacuolar invertase activity in tomato had no effect on the rate of photosynthesis or on shoot growth. Given the alterations in carbohydrate metabolism in these leaves this is not surprising.

***Impact of the co-suppression construct on root carbohydrate metabolism***

The invertase construct had much less of an effect on invertase activity in the roots; potential reasons for this are discussed above. There was a small reduction in root soluble acid and apoplastic invertase activity between WT and INV-B plants but this was not statistically significant. Roots from plants derived from line INV-B did exhibit some reduction in hexoses and a slight elevation in sucrose but again this was not statistically significant. The impact of the co-suppression construct on carbohydrate content of roots was similar to that of leaves but effects were much less marked.

### 4.4.1 Future work

The reduction in leaf vacuolar invertase activity in plants derived from line INV-B provides a powerful tool to examine the role of futile cycling of sucrose through the vacuole under different physiological and environmental conditions. It has been proposed here that in plants with no leaf vacuolar invertase activity there is little futile

cycling of sucrose and no accumulation of hexoses. Under normal conditions this is not important as the majority of the sucrose is exported from the leaves to sink regions of the plant. However, under conditions of reduced sucrose export, futile cycling of sucrose may become relatively more important.

In order to examine the effect of reduced export, plants would be girdled or leaf discs floated on different solutions of soluble carbohydrate. Under these conditions I hypothesise that: (i) In plants containing leaf vacuolar invertase activity the accumulation of carbohydrate resulting from blocked export would lead to cycling of sucrose through the vacuole and the initiation of a hexokinase mediated signal transduction pathway leading to the down-regulation of certain photosynthetic genes. (ii) In plants with no detectable vacuolar invertase activity sucrose would accumulate due to the of low rate of sucrose hydrolysis. As the production of hexoses under these conditions would be low, and hence little hexokinase activity, there may be a delay in the repression of photosynthetic gene expression compared to plants with high leaf vacuolar invertase activity. (iii) Hexose feeding of leaf discs taken from plants with no detectable leaf vacuolar invertase activity would by-pass the need for vacuolar sucrose hydrolysis and lead to the expected repression of photosynthetic gene expression.

# Chapter 5

# General Discussion

## 5.1 GENERAL DISCUSSION

Previous work has revealed the presence of an invertase gene family in the cruciferous plant *A. thaliana*. In total five invertase genes have been identified in this species. Two of these genes, ATβFRUCT 1 and 2, encode apoplastic invertases (Schwebel-Dugué *et al.*, 1994; Mercier and Gogarten, 1995) while a further two, ATβFRUCT 3 and ATβFRUCT 4, encode soluble acid invertases with a probable vacuolar localisation (Haouazine-Takvorian *et al.*, 1997). A fifth sequence, ATβFRUCT 5, with unusual characteristics, was located using the Expressed Sequence Tag database and shows close sequence homology to apoplastic invertases while predicting a protein with an acidic pI, more usual for vacuolar invertases. The large number of *A. thaliana* invertase genes identified so far and the availability of appropriate gene probes made this species an ideal candidate for the exploration of the role and regulation of plant invertases.

Invertase gene families have been identified in a wide range of species including the dicotyledonous species carrot (Sturm and Chrispeels, 1990; Ramloch-Lorenz *et al.*, 1993; Unger *et al.*, 1994; Lorenz *et al.*, 1995) and tomato (Klann *et al.*, 1992; Elliot *et al.*, 1993; Godt and Roitsch, 1997) and monocotyledonous species such as maize (Xu *et al.*, 1995; Koch *et al.*, 1995; Shanker *et al.*, 1995; Cheng *et al.*, 1996). Exactly why evolutionary pressure should have favoured the possession of a multigene family can only be speculated upon but it probably arose due to the sessile nature of plants and the need to control vital aspects of carbohydrate metabolism under different environmental and development conditions. This view is certainly supported by promoter analysis of *A. thaliana* invertase genes which show a range of different regulatory sequences (Tymowska-Lalanne *et al.*, 1996; Haouazine-Takvorian *et al.*, 1997).

The first remit of this thesis was to study the regulation of invertase gene expression in *A. thaliana* in response to developmental and certain environmental signals. The developmental aspect of this study broadened those done previously by Tymowska-Lalanne *et al.* (1996) and Haouazine-Takvorian *et al.* (1997). Work presented in this thesis has shown that each of the compartment specific invertases has a unique role in the regulation of carbohydrate metabolism during development and under different environmental stimuli. The analysis of all five genes in *A. thaliana*

allowed these roles to be examined at greater depth and showed that each gene was regulated in a highly co-ordinated manner.

Perhaps the most interesting invertase gene examined in *A. thaliana* was the apoplastic invertase gene ATβFRUCT 1. This gene was analysed using the highly sensitive technique RT-PCR. Inclusion of an internal standard utilising the same primer binding sites as the ATβFRUCT 1 primers enabled semi-quantitative data to be obtained; this is the first report in which the expression of an invertase gene has been analysed in this manner.

ATβFRUCT 1 was expressed in all the tissues examined but showed a strong element of developmental regulation. Expression of this gene appeared to be linked to the tissues status as either a source or sink for carbon compounds. This relationship was clearly demonstrated in the leaves in which expression was high in young sink leaves but declined in older source leaves. Expression was particularly high in seedling roots, a strong sink for carbon compounds.

It has been suggested that apoplastic invertase activity in sink tissues functions by mediating the transfer of sucrose from the phloem by a hydrolytic step. The hydrolysis of sucrose by apoplastic invertase and the rapid uptake of the resulting hexoses by membrane bound transporters may help to create a strong source to sink sucrose gradient thus supplying the metabolically active or storage tissue with carbohydrates (Eschrich, 1980; Truernit *et al.*, 1997). The role of apoplastic invertase in the supply of carbohydrate to sink tissues has been positively identified in maize kernels deficient in apoplastic invertase activity (Miller and Chourey, 1992; Cheng *et al.*, 1996). Data presented here showed that there was a broad correlation between ATβFRUCT 1 gene expression and measurements of apoplastic invertase activity in sink tissues. However, in the leaves it was found that considerable activity remained long after ATβFRUCT 1 gene expression had declined. The role of this remaining activity is not known and may critically depend upon its tissue localisation. Studies of pea leaves by *in situ* hybridisation showed that wound induced increases in apoplastic invertase gene expression were associated with the vasculature (Zhang *et al.*, 1996). If this is also the location of apoplastic invertase activity in a healthy unwounded leaf than it is possible that activity in this region could play a role in scavenging for sucrose that leaks from the mesophyll cells or sieve elements during phloem loading of sucrose.

The proposed role of ATβFRUCT 1 in the supply of hexoses to tissues was further strengthened by examination of its expression in response to the environmental signals of pathogenesis and wounding. The infection of *A. thaliana* with the fungal biotrophic pathogen *A. candida* resulted in a massive localised stimulation of apoplastic invertase activity during the course of infection. Analysis revealed that this change in activity was closely co-ordinated with a stimulation in ATβFRUCT 1 gene expression. Speculation as to the origin of increases in apoplastic invertase activity during pathogenesis have been a matter of some debate. Data presented in chapter 3 showed that the majority of the stimulation of activity could be traced to the induction of host apoplastic invertase activity. The wounding of *A. thaliana* leaves also resulted in a localised stimulation of apoplastic invertase activity. Again, this increase was due to the stimulation of ATβFRUCT 1 gene expression.

It has been suggested that the induction of apoplastic invertase in response to wounding and pathogenesis may result in the formation of a localised sink for carbohydrate. This induction could help to supply tissues with the hexoses needed to mount an effective defence response (Sturm and Chrispeels, 1990; Ehness *et al.*, 1997; Zhang *et al.*, 1996). However, the situation during pathogenesis is likely to be more complicated. In an incompatible interaction the rapid induction of invertase activity may supply the hexoses required to mount an effective defence response (Benhamou *et al.*, 1991) while in a compatible interaction invertase activity is induced during rapid fungal growth through the tissue and may be involved in supplying hexoses required to sustain such growth.

Work presented in this thesis has characterised the expression profile of ATβFRUCT 1 in response to a number of different stimuli. These responses are strikingly similar to those of the LIN6 apoplastic invertase gene in tomato. A comparison of these responses can be seen in Tables 5.1, 5.2 and 5.3. Both genes are active in sink tissues and both genes increase in response to wounding and pathogenesis signals. This suggests that an apoplastic invertase gene regulated by an array of diverse stimuli may be present in many plant species.

Future work could aim to produce transgenic *A. thaliana* plants with reduced apoplastic invertase activity. This would help to clarify the role of this enzyme in processes such as phloem unloading and hexose supply. It could be expected that the

**Table 5.1 Expression of invertase genes in thale cress (*A. thaliana*) and tomato (*L. esculentum*) in different vegetative organs during development.** The expression of each gene is designated as either very high, high, medium, low, very low, N/D (not detectable) or – (not examined). These designations can be used to compare the expression of each individual gene in different organs but cannot be used to compare the expression of different genes within an organ. Data for tomato is taken from Godt and Roitsch (1997).

| Plant species | Invertase gene | Gene expression in different tissues | | | | |
|---|---|---|---|---|---|---|
| | | Seedling roots | Mature roots | Seedling cotyledons | Sink leaves | Source leaves |
| *A. thaliana* | **Apoplastic** | | | | | |
| | ATβFRUCT 1 | Very high | High | Medium | Low | Very low |
| | ATβFRUCT 2 | N/D | N/D | N/D | N/D | N/D |
| | **Vacuolar** | | | | | |
| | ATβFRUCT 3 | High | Medium | Low | N/D | N/D |
| | ATβFRUCT 4 | High | Very low | High | Low | Very low |
| | ATβFRUCT 5 | N/D | N/D | N/D | N/D | N/D |
| Tomato | **Apoplastic** | | | | | |
| | LIN5 | N/D | N/D | - | - | N/D |
| | LIN6 | High | N/D | - | - | N/D |
| | LIN7 | N/D | N/D | - | - | N/D |
| | LIN8 | N/D | N/D | - | - | N/D |
| | **Vacuolar** | | | | | |
| | TIV1 | Low | Low | - | - | Low |

**Table 5.2 Expression of invertase genes in thale cress (*A. thaliana*) and tomato (*L. esculentum*) in different floral organs during development.** The expression of each gene is designated as either high, medium, low, N/D (not detectable) or – (not examined). These designations can be used to compare the expression of each individual gene between different organs but cannot be used to compare the expression of different genes within an organ. Data for tomato is taken from Godt and Roitsch (1997). [a] data obtained not quantitative. [b] Haouazine-Takvorian (1997) reported expression in this tissue in contrast to results presented in this thesis. [c] Data obtained from Haouazine-Takvorian (1997).

| Plant species | Invertase gene | Gene expression in different tissues | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total inflorescence | Stems | Small flower buds | Large flower buds | Whole flowers | Gynoecia | Stamens | Petals | Immature fruit | Ripe fruit |
| *A. thaliana* | **Apoplastic** | | | | | | | | | | |
| | ATβFRUCT 1 | Low | Low [c] | - | - | - | - | - | - | - | - |
| | ATβFRUCT 2 | Expressed [a] | | - | - | - | - | - | - | - | - |
| | **Vacuolar** | | | | | | | | | | |
| | ATβFRUCT 3 | N/D [b] | Medium [c] | - | - | Medium [c] | - | - | - | - | - |
| | ATβFRUCT 4 | High | High [c] | - | - | High [c] | - | - | - | - | - |
| | ATβFRUCT 5 | N/D | - | - | - | - | - | - | - | - | - |
| Tomato | **Apoplastic** | | | | | | | | | | |
| | LIN5 | - | - | Low | Low | Low | High | Low | N/D | Low | Low |
| | LIN6 | - | - | High | Low | Low | - | - | - | N/D | N/D |
| | LIN7 | - | - | N/D | Medium | High | Low | High | N/D | N/D | N/D |
| | LIN8 | - | - | N/D | N/D | N/D | - | - | - | N/D | N/D |
| | **Vacuolar** | | | | | | | | | | |
| | TIV1 | - | - | Low | Low | Low | Low | Medium | High | N/D | High |

**Table 5.3 Expression of invertase genes in thale cress (*A. thaliana*) and tomato (*L. esculentum*) in response to soluble carbohydrates, *A. candida* infection, the pathogen elicitor polygalacturonic acid (PGA), wounding, cytokinin and gibberellic acid.** Tomato data was obtained from Godt and Roitsch (1997). [a] Vacuolar activity increased but gene expression not examined. [b] Vacuolar invertase activity increased in some reports (Tang *et al.*, 1996) but not conclusive in this thesis, gene expression not examined. [c] Vacuolar invertase activity unchanged but gene expression not examined. – Effect of condition on gene expression unknown. N/D Expression not detected.

| Plant species | Invertase gene | Carbohydrates | | Pathogenesis | | Wounding | | Plant growth regulators | |
|---|---|---|---|---|---|---|---|---|---|
| | | Glucose | Sucrose | PGA | *A. candida* | Induction (h) | Maximum expression (h) | Cytokinin | Gibberellic acid |
| *A. thaliana* | **Apoplastic** | | | | | | | | |
| | ATβFRUCT 1 | - | - | - | Massive induction over 13 d | 8 h? | 24 h? | - | - |
| | ATβFRUCT 2 | - | - | - | - | - | - | - | - |
| | **Vacuolar** | | | | | | | | |
| | ATβFRUCT 3 | a | a | | b | c | c | - | - |
| | ATβFRUCT 4 | a | a | | b | c | c | - | - |
| | ATβFRUCT 5 | a | a | | b | c | c | - | - |
| Tomato | **Apoplastic** | | | | | | | | |
| | LIN5 | N/D | - | N/D | - | N/D | N/D | N/D | N/D |
| | LIN6 | Stimulated | - | Stimulated | - | 24 h | 33 h | Stimulated | No effect |
| | LIN7 | N/D | - | N/D | - | N/D | N/D | N/D | N/D |
| | LIN8 | N/D | - | N/D | - | N/D | N/D | N/D | N/D |
| | **Vacuolar** | | - | | | | | | |
| | TIV1 | Repressed | - | Stimulated | - | 1 h | 3 - 24 h | N/D | No effect |

complete removal of apoplastic invertase activity in the roots would have a devastating effect on plant growth. However, surprisingly, Duke *et al.* (1991) found that a lack of invertase activity in the roots of inbred maize (Oh 43) had no effect on growth. It is possible that the function of invertase in these plants was restored by the activity of another enzyme with a similar physiological role. If transgenic *A. thaliana* with reduced apoplastic invertase activity were produced then it would be interesting to examine which, if any, enzymes were restoring the function of apoplastic invertase activity in sink tissues. Perhaps more interesting would be to examine the impact of reduced apoplastic invertase activity in mature leaves as at present its role in these tissues is unknown.

The expression of a second apoplastic invertase gene, ATβFRUCT 2, was examined in different organs of *A. thaliana* using non-quantitative RT-PCR. This gene showed a highly organ specific pattern of expression; being found only in the inflorescence. This is the first report in which the expression of this gene has been positively identified Although the exact tissue location of this expression was not determined it is likely to have been in the floral organs themselves. Apoplastic invertase genes in other species have also been shown to have highly organ specific patterns of expression. Table 5.2 shows that in tomato two apoplastic invertase genes, LIN5 and LIN7, show highly organ specific expression patterns. The expression of LIN5 was found mainly in the gynoecia while that of LIN7 was found mainly in the stamens. These two genes are probably analogous in role to ATβFRUCT 2 and supply specific floral organs with hexoses required for growth.

The vacuolar invertase genes ATβFRUCT 3 and 4 were differentially regulated during development. ATβFRUCT 3 showed a highly organ specific pattern of expression being expressed only in seedling roots and to a lesser extent seedling cotyledons. Previous studies (Haouazine-Takvorian *et al.*, 1997) showed that this gene may also be expressed in the leaves but at a very low level. In contrast expression of ATβFRUCT 4 showed a developmental pattern of expression being expressed in all the tissues examined. The expression of this gene appeared to be closely linked to the status of the tissue as a sink for carbohydrates. This was clearly demonstrated in the leaves where expression declined as they matured and became a source of carbon compounds. Expression of this gene was also high in seedling roots, cotyledons and

inflorescence all classic sinks for carbon compounds. The expression pattern of the TIV1 vacuolar invertase gene in tomato shows certain similarities to ATβFRUCT 3 and 4. This gene is also expressed in roots, source leaves and developing floral organs (Table 5.1 and 5.2). Recently, Sturm *et al.* (1995) suggested that vacuolar invertase activity may help to maintain a steep source to sink sucrose concentration gradient by the hydrolysis of incoming sucrose. Other possible roles for vacuolar invertase include the regulation of hexose content in developing fruit (Yelle *et al.*, 1991; Klann *et al.*, 1993).

Recently Xu *et al.* (1995) showed that in maize two vacuolar invertase genes *Ivr1* and *Ivr2* were differentially regulated by glucose. Increased supplies of glucose repressed expression of *Ivr1* while inducing *Ivr2* while under conditions of starvation the *Ivr1* gene continued to be expressed while *Ivr2* was repressed. These authors suggest that this differential regulation allows the supply of hexoses to be tightly controlled under a range of developmental and environmental conditions. Evidence in support of this came from Geigenberger and Stitt (1991) who showed that blocked export led to increased hydrolysis sucrose by vacuolar invertase in *Ricinus communis* cotyledons. Whether or not ATβFRUCT 3 and 4 are differentially regulated by hexoses is not clear although sucrose and glucose feeding of *A. thaliana* leaf discs resulted in a transient increase in vacuolar invertase activity. This increase may have been the net result of increased expression of one gene versus the repression of the other. Future work would attempt to examine this more closely by examining the expression of ATβFRUCT 3 and 4 in response to hexoses and sucrose.

Leaf vacuolar invertase activity in *A. thaliana* showed little change in response to the environmental stimuli of pathogenesis and wounding; it was therefore decided not to examine the expression of the corresponding vacuolar invertase genes (ATβFRUCT 3 and 4) under these conditions. The minimal impact of these stimuli on vacuolar invertase activity in *A. thaliana* is in contrast to that often observed in other species under similar conditions. For example, maize leaves infected with corn smut (*Ustilago maydis*) showed an increase in soluble acid invertase activity during infection (Billet *et al.*, 1977; Callow *et al.*, 1980) as did barley leaves infected with brown rust (*Puccinia graminis*) (Tetlow and Farrar, 1992) and oat infected with crown rust (*Puccinia coronata*) (Heisteruber *et al.*, 1994). In suspension cultured cells of tomato

the expression of the TIV1 vacuolar invertase gene was massively induced by the application of the fungal elicitor PGA (Table 5.3). To date, almost all examinations of wounding report increased vacuolar invertase activity or gene expression in the affected region (Matsushita and Uritani, 1974; Sturm and Chrispeels, 1990). For example, in tomato (Table 5.3) the TIV1 vacuolar invertase gene was rapidly induced in response to wounding (Godt and Roitsch, 1997).

Measurements of invertase activity in *A. thaliana* showed that considerable activity remained in mature leaves long after expression of ATβFRUCT 4 ceased. High levels of vacuolar invertase activity have also been observed in the mature leaves of a number of other species including tobacco, cotton and cucumber (Huber, 1989). The role of vacuolar invertase in these species is not fully understood but it appears that there is a strong correlation between activity and the amount of sucrose found within the leaf. Species with high leaf vacuolar invertase activity contain low amounts of sucrose. In order to investigate the role of this enzyme in mature leaves further transgenic plants with reduced leaf vacuolar invertase activity were examined.

In transgenic tomato plants with no leaf vacuolar invertase activity there were no measurable accumulations of hexoses and increased accumulation of sucrose. This strongly suggests that vacuolar invertase activity is not only important in the generation of leaf hexoses but that they are produced as sucrose cycles through the vacuole. The cycling of sucrose through the vacuole may have important metabolic consequences under conditions of reduced export. Under these conditions sucrose accumulating within the leaf may be hydrolysed in the vacuole by invertase and move into the cytoplasm whereupon they are immediately phosphorylated by hexokinase. The increased flux of hexoses through this phosphorylation step may lead to the repression of photosynthetic gene expression via the hexokinase sensing mechanism proposed by Jang and Sheen (1994) and Jang *et al.* (1997). Evidence in favour of this hypothesis was presented by Goldschmidt and Huber (1992) who found that blocking the export of sucrose from the leaves by girdling led to the long term reduction of photosynthetic capacity ($A_{max}$) in those plant species which retained vacuolar invertase activity in the mature leaves. Future work would attempt to establish the role of vacuolar invertase in sucrose cycling and photosynthetic gene expression by examining transgenic and wild type tomato plants girdled to block export.

### 5.1.1 Environmental stimuli and the transduction pathways that influence invertase gene expression

This thesis has shown that the environmental stimuli wounding and pathogenesis led to the localised induction of ATβFRUCT 1 apoplastic invertase gene expression, this response is similar to that reported in a number of other species, as previously discussed.

Wounding and pathogenesis are amongst the most severe forms of environmental stress to which plants may be subjected. For this reason plants have evolved a range of extremely complex defence mechanisms designed to repair wounded tissue and to protect against infection. In addition to the induction of defence related proteins such as ammonia lyase, chalcone synthase and phytoalexins, stress stimuli also induce the induction of sink metabolism of which a key enzyme is apoplastic invertase. It is commonly believed that the induction of apoplastic invertase activity leads to an enhanced flow of hexoses to cells mounting an energetically costly defence response.

The signal transduction pathway leading to the induction of defence related genes is extremely complex and despite extensive research is still poorly understood. Some of the proposed steps can be seen in Fig. 5.1. It must be noted that the exact sequence of events is not necessarily known.

Plants respond differentially to wounding and pathogenesis. Wound induced signals are thought to be transmitted through a jasmonic acid (JA) mediated pathway (Fig 5.1a-e). In contrast, in plants undergoing a defence response the signal transduction pathway is thought to involve salicylic acid (SA) (Fig. 5.1f-i) (Sano *et al.*, 1996). Evidence suggests that SA and JA interact with one another in such a way that signals are transmitted down the appropriate transduction pathway (Seo *et al.*, 1997).

The initial steps in the transduction of wounding stimuli probably involve the release of pectic substances from the cell walls of damaged tissues (Fig. 5.1a). The effect of these substances has been shown to elicit the classic wounding response when fed artificial to healthy leaves (Ryan, 1974). It has been speculated that physical damage may also result in the propagation of an electrical signal throughout the affected plant (Wildon *et al.*, 1992). How these electrical signals arise and whether they are elicited by pectic substances is not fully understood but the stimulation of

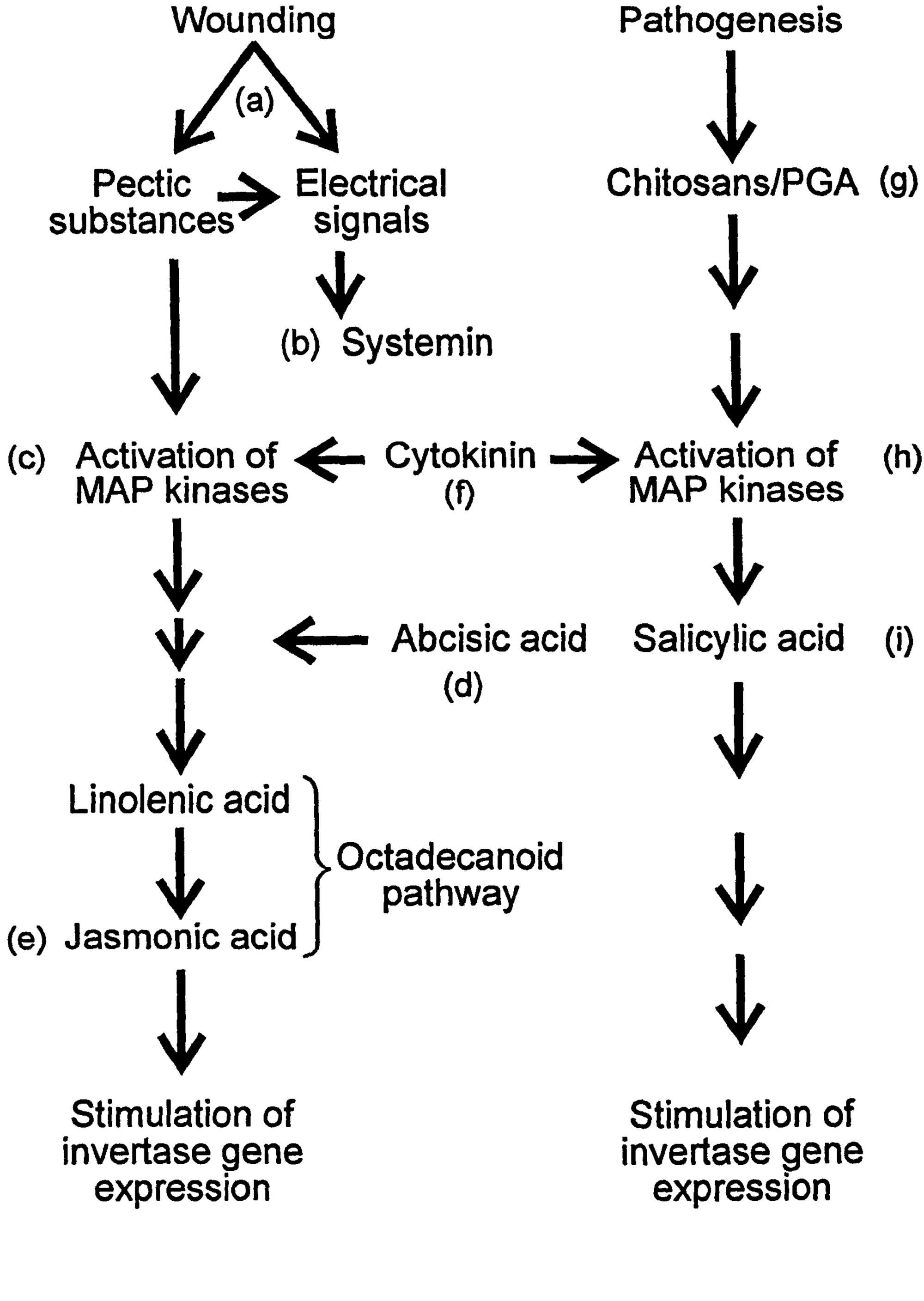



**Figure 5.1 Potential signal transduction pathways elicited by wounding and pathogenesis that may lead to the induction of invertase gene expression.**

tomato and potato leaves with a small electrical current resulted in the induction of a systemic wound response (Wildon *et al.*, 1992; Herde *et al.*, 1996).

During a classic wound response the transcription of two classes of enzymes are induced. The first class is induced systemically while the second class is only induced in the locality of the wound itself. An example of a systemically induced enzyme is the protease inhibitor II gene (Pin2) of tomato and potato (Hildmann *et al.*, 1992; Peña-Cortés *et al.*, 1996). Invertase genes, however, have been shown in to be induced in a highly localised manner in a range of species including pea (Zhang *et al.*, 1996), and in this thesis *A. thaliana*. At present it is not understood how these two transcriptional classes of enzyme are differentially induced by wounding. Recently it has been shown that wounding stimulates the release of a small highly mobile 18 amino acid polypeptide called systemin (Pearce *et al.*, 1991). It is possible that this hormone activates cell receptors that transduce the wounding signal to systemically activated genes (Fig. 5.1b) while locally induced genes such as invertase are stimulated directly by the release of pectic substances as has been shown for other genes (Bergey *et al.*, 1996). Pectic substances are good candidates for this role as they have previously been shown to be immobile in plant tissue (Baydoun and Fry, 1985).

In animals and yeast stress signals have been shown to be mediated by mitogen-activated protein (MAP) kinase cascades (Hirt, 1997). Recently, analogous protein kinases have been cloned from different plants (Jonak *et al.*, 1994) including nine from *A. thaliana* (Mizoguchi *et al.*, 1996; Shinozaki *et al.*, 1996) and several authors have demonstrated the importance of protein phosphorylation in the activation of certain plant defence responses (Dietrich *et al.*, 1990; Felix *et al.*, 1991). Ehness *et al.* (1997) showed that wounding and pathogenesis stimuli resulted in the stimulation of the CIN1 apoplastic invertase gene in *Chenopodium rubrum* autotrophic cell suspension cultures. These authors found that this stimulation was accompanied by the activation of 44 and 46 KD protein kinases (Fig. 5.1c). The addition of the protein kinase inhibitor staurosporine to the cell suspension culture blocked the wound and pathogen stimulated induction of the CIN1 gene and demonstrated for the first time that MAP-kinases play a vital part in the induction of plant invertase genes in response to stress.

The plant hormones abscisic acid (ABA) and jasmonic acid (JA) are thought to play a key role in the induction of wound responsive genes in a range of plants

including the much studied Pin2 gene in tomato and potato (Hildmann *et al.*, 1992; Peña-Cortés *et al.*, 1996; Titarenko *et al.*, 1997) (Fig 5.1d, e). Apoplastic invertase gene expression has also been shown to be stimulated by the application of ABA and JA to the leaves of pea plants (Zhang *et al.*, 1996). Plants deficient in ABA do not transduce wounding signals. The application of JA to these plants results in the stimulation of wound responsive genes indicating that ABA acts before JA in the signal transduction pathway (Herde *et al.*, 1996). Exactly how JA accumulation results in changes in gene expression is unclear. This hormone may interact directly with nuclear binding factors or may transduce the signal to another set of down-stream components. Recently, Birkenmeir and Ryan (1998) argued against the direct role of ABA in the transduction of the wounding signal suggesting instead that this hormone is involved in the maintenance of healthy plant physiology, which itself facilitates a normal wound response.

Evidence suggest that the signal transduction pathway elicited by pathogenesis is different from that elicited upon wounding (Hare *et al.*, 1997; Zhang and Klessig, 1997). It is thought that the initial signals of this pathway (Fig. 5.1g) are mediated by chitosans and/or polygalacturonic acid (PGA) released from the fungal cell wall or plant cell wall, respectively (Chappell and Hahlbrock, 1984; Ehness *et al.*, 1997). These signals have been shown to stimulate a MAP-kinase cascade in a similar manner to the wounding stimuli although it is thought that different MAP-kinases are involved (Zhang and Klessig, 1997) (Fig 5.1h). It has been shown that cytokinin can act as a molecular switch that can direct stress stimuli from the wounding pathway to the pathogenesis pathway (Figure 5.1f) indicating that the two pathways are closely co-ordinated (Sano *et al.*, 1996; Hare *et al.*, 1997). Transgenic tobacco plants expressing a gene for the small GTP binding protein responded abnormally to wounding by producing proteins more commonly associated with pathogenesis than wounding. Analysis revealed that these plants contained six fold greater concentrations of cytokinin than wild type plants and that the cytokinin antagonist 2-chloro-4-cyclohexylamino-6-ethylamino-s-triazine erased these affects.

Although very little is known about the signal transduction pathway elicited by pathogenesis it is known that salicylic acid is an important mediator of this defence response (Ryals *et al.*, 1994; Sano *et al.*, 1996; Durner *et al.*, 1997; Zhang and Klessig,

1997)(Fig 5.1i). However, evidence presented in this thesis suggests that the stimulation of ATβFRUCT 1 gene expression upon infection of *A. thaliana* by *A. candida* did not result from the elicitation of a classic defence response. Not only was the interaction of *A. thaliana* and *A. candida* a compatible one, and therefore not likely to elicit a defence signal transduction pathway, but the increase in apoplastic invertase activity in region A observed during infection showed similar characteristics to other compatible host-pathogen interactions. For example, in resistant tomato plants infected with the necrotrophic pathogen *Fusarium oxysporum* there was a massive and widespread accumulation of apoplastic invertase in the infected roots. However, when susceptible tomato plants were infected with the same fungus apoplastic invertase accumulated gradually and only in cells in direct contact with fungal hyphae. This second response was similar to that observed in *A. thaliana* infected with *A. candida* where increases in apoplastic invertase activity were initially slow but increased in proportion with the growth of the fungal mycelium and where the response was localised observed only in regions of the leaf invaded by the fungal hyphae. The stimulation of this gene could either have resulted from the production of a phytohormone by the pathogen or could conceivably have resulted from the release of cell wall fragments as the pathogen penetrated the host apoplast prior to haustorial formation.

### 5.1.2 Future work

Work presented in this thesis has provided a great deal of information about the regulation and role of plant invertases. However, this work has also resulted in the generation of a number of new questions. In the previous chapters I have discussed future work as these questions have arisen. In this section I would like to pose a few questions that particularly interest me and briefly discuss how they could be answered.

- What is the role of apoplastic invertase activity in mature leaves?

**Approach:** To generate transgenic *A. thaliana* plants with reduced leaf apoplastic invertase activity and examine the impact of these changes on the carbohydrate

metabolism, photosynthesis and growth of the resulting transformants. In chapter 4 I successfully used this same approach to examine the role of leaf vacuolar invertase activity. Having carried out a thorough characterisation of the physiology of these plants I would use them to answer some further questions. For example, how does reduced leaf apoplastic invertase activity influence the growth and infection of *A. candida*? Under normal physiological conditions I have demonstrated that infection of *A. thaliana* leaves with *A. candida* results in the induction of host apoplastic invertase activity. If this response did not occur in transgenic plants then how might it affect fungal growth?

- What is the tissue localisation of apoplastic and vacuolar invertase?

**Approach:** One of the least investigated aspects of invertases in plants is their tissue localisation and yet, as I have investigated these enzymes during the course of this thesis, I have realised that this is very important in order to understand their role in metabolism. To date information on the localisation of the different invertases is patchy and often not reliable due to the techniques used. The approach I would take is to create a series of probes suitable for *in situ* hybridisation. Using this approach I would aim to localise expression of each of the invertase genes in a range of organs including the leaves, roots and floral organs.

- What is the signal transduction pathway leading to increase in ATβFRUCT 1 gene expression during wounding and pathogenesis?

**Approach:** I would examine the impact of wounding and *A. candida* infection on ATβFRUCT 1 gene expression in a range of mutant *A. thaliana* plants. These plants would included ABA deficient and ethylene insensitive mutants.

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