Dissertation

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Regulation of taproot development and sucrose stabilization in sugar beet:
Influence of invertase inhibitors and occurrence of mitochondrial energy-dissipating proteins

Contents

1 Summary

Sugar beet (*Beta vulgaris*) is one of the few plants storing large amounts of sucrose. During the development of its large taproot, sucrose is accumulated inside the vacuoles, reaching concentrations of up to 20 % (w/w) in modern cultivars. In the course of this study, the expression of the major sucrolytic enzymes in sugar beet was determined. Invertase activity is negatively correlated with sucrose accumulation and during the major part of taproot development, only sucrose synthase isoforms and no acid invertases are expressed. After wounding, a cell wall and a vacuolar invertase are induced, of which the latter is of high importance for post-harvest sucrose losses. Efforts to silence this vacuolar invertase by overexpression of an invertase inhibitor from tobacco did not lead to a reduction of the wound-induced sucrose hydrolysis.

An endogenous invertase inhibitor of sugar beet, BvC/VIF1[1], was identified and functionally characterized. It is strongly expressed during taproot development and the recombinantly produced BvC/VIF1 protein has a strong affinity against acid invertases, especially against vacuolar invertases. In order elucidate the pyhsiological role of the inhibitor during taproot development, a transgenic approach to silence its expression was initiated. Furthermore, sugar beet plants overexpressing BvC/VIF1 have been generated and first tests with these plants have shown a significant reduction of vacuolar invertase activity in leaves.

With an antiserum raised against the BvC/VIF1 protein, two inhibitor proteins are detected in extracts from taproots, whereas in the cell wall of *Beta vulgaris* suspension culture cells only one protein is found. For all three proteins, partial peptide sequences were identified, which matched the known BvC/VIF1 sequence. In leaves of transgenic sugar beet plants overexpressing BvC/VIF1, but not in leaves of wildtype plants, two proteins of a similar size as observed in untransformed taproots were detected, suggesting that proteins of deviating size may arise from the translation of a single BvC/VIF1 gene.
The subcellular localization of the inhibitor proteins was studied using GFP fusions. The fusion proteins appeared either in the vacuole, indicating the presence of vacuolar sorting signals in the BvC/VIF1 sequence, or aggregated in vesicle-like structures. Furthermore, a proteolytic cleavage of the inhibitor-GFP fusion occurred, leading to the presence of free inhibitor protein.

In untransformed cells, the smaller of the two inhibitor proteins seems to be localized in the cell wall, as was deduced from differential extraction from taproots and suspension culture cells.
The interaction between invertases and inhibitors has been studied in detail. Therefore, a sugar beet invertase was produced recombinantly in bacteria and its enzymatic characteristics were studied. The K_M value of the invertase is in the low millimolar range and it shows

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[1]*Beta vulgaris* cell wall or vacuolar inhibitor of β-fructosidase

a maximal activity between pH 4 and 6. The interaction with the invertase inhibitor is also strongly pH dependent, showing maximum inhibition between pH 4 and 5. No inhibition and binding of the inhibitor was observed above pH 6. Since pH values in cellular compartments like the cell wall or the vacuole underly dynamic changes, this leaves open a potential regulatory mechanism of the invertase inactivation.

In a second project, first results about the presence and regulation of proteins regulating the efficiency of respiration have been obtained. Whereas two identified isoforms of uncoupling proteins are constitutively expressed in sugar beet, an induction of alternative oxidase proteins during wounding and storage was observed. Members of both gene families are potential target genes, which may influence sucrose losses during post-harvest storage. They serve as energy-dissipating systems and their activity can increase respiration rates and concomitantly sucrose breakdown. The obtained results provide first evidence, that at least the alternative oxidase shows a strong response to wounding and storage.

Zusammenfassung

Regulation der Saccharoseakkumulation und -stabilisierung in der Zuckerrübe: Einfluss von Invertaseinhibitoren und Auftreten von mitochondriellen, Energieüberschuss-abbauenden Proteinen

Die Zuckerrübe (*Beta vulgaris*) ist eine der wenigen Pflanzen, die große Mengen Saccharose speichert. Während der Entwicklung der Speicherwurzel akkumuliert Saccharose in den Vakuolen und kann in modernen Sorten einen Anteil von bis zu 20 % des Frischgewichtes erreichen. In der vorliegenden Arbeit wurde die Expression der wichtigsten Saccharose-spaltenden Enzyme bestimmt. Die Aktivität von Invertasen zeigt eine negative Korrelation zur Saccharoseanreicherung und während des Großteils der Speicherwurzelentwicklung werden lediglich Isoformen der Saccharose-Synthase und keine Invertasen exprimiert. Nach Verwundung werden eine Zellwand-lokalisierte und eine vakuolären Invertase induziert, wobei letztere für einen großen Teil der Saccharoseverluste nach der Ernte verantwortlich ist. Versuche die Aktivität dieser vakuolären Invertase durch Überexpression eines Invertaseinhibitors aus Tabak zu unterbinden erbrachten keine Verringerung der wundinduzierten Saccharosehydrolyse.

Ein endogener Invertaseinhibitor der Zuckerrübe, BvC/VIF1[2], wurde identifiziert und funktionell charakterisiert. Der Inhibitor zeigt eine starke Expression während der Speicherrübenentwicklung und das rekombinant hergestellte BvC/VIF1-Protein zeigt eine hohe Affinität zu sauren Invertasen, insbesondere zu vakuolären Invertasen. Um mehr über die physiologische Rolle des Inhibitors während der Entwicklung der Zuckerrübenspeicherwurzel herauszufinden, wurde Versuche zur Verringerung der Expression dieses Gens unternommen. Außerdem wurden Zuckerrübenpflanzen hergestellt, die BvC/VIF1 vermehrt exprimieren. Erste Untersuchungen an diesen Pflanzen zeigten, dass es hierdurch zu einer signifikanten Verringerung der vakuolären Invertaseaktivität in Blättern kommt.

Mittels eines gegen das BvC/VIF1-Protein erstelltem Antiserums wurden zwei Inhibitorproteine in Speicherwurzelextrakten nachgewiesen. Dahingegen trat in der Zellwand von *Beta vulgaris* Suspensionskulturzellen nur ein Protein auf. Von diesen drei Proteinen wurden partielle Peptidsequenzen ermittelt, die mit der bekannten BvC/VIF1-Sequenz übereinstimmten. In Blättern von transgenen, BvC/VIF1 überexprimierenden, Pflanzen wurden zwei Proteine beobachtet, die eine ähnliche Größe zeigen wie die in untransformierten Speicherwurzeln beobachteten und die in Blättern von Wildtyppflanzen nicht vorhanden sind. Dies deutet daraufhin, dass möglicherweise Proteine mit unterschiedlichen Größen durch die Translation eines einzelnen BvC/VIF1-Gens entstehen.

Die subzelluläre Lokalisierung der Inhibitorproteine wurde mittels GFP-Fusionen analysiert. Die Fusionsproteine wurden entweder in der Vakuole beobachtet, was auf das Vorhandensein von vakuolären Sortierungssignalen hindeutet, oder aggregierten in Vesikel-artigen

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[2]*Beta vulgaris* cell wall or vacuolar inhibitor of β-fructosidase

Strukturen. Weiterhin trat eine proteolytische Spaltung von Inhibitor-GFP Fusionen auf, die zum Nachweis von freiem Inhibitorprotein führte.

Mittels differentieller Extraktion aus untransformierten Speicherwurzeln und Suspensionskulturzellen konnte gezeigt werden, dass das kleinere der beiden Inhibitorproteine in der Zellwand lokalisiert zu sein scheint.

Um die Wechselwirkung zwischen Invertase und Inhibitor genauer zu untersuchen, wurde eine Zuckerrüben-Invertase rekombinant in Bakterien hergestellt und ihre enzymatischen Eigenschaften untersucht. Der K_M-Wert der Invertase liegt im unteren millimolaren Bereich und das Enzym zeigt eine maximale Aktivität zwischen pH 4 und 6. Die Wechselwirkung mit dem Invertaseinhibitor ist ebenfalls in hohem Maße pH-abhängig und zeigt ein Maximum zwischen pH 4 und 5. Oberhalb von pH 6 kommt es zu keiner Bindung des Inhibitors an die Invertase. Da die pH-Bedingungen von zellulären Kompartimenten wie der Zellwand oder der Vakuole dynamischen Schwankungen unterliegen, handelt es sich hierbei möglicherweise um einen Regulationsmechanismus der Invertaseinaktivierung.

In einem zweiten Projekt wurden erste Ergebnisse über das Vorhandensein und die Regulation von Proteinen, die die Effizienz der Atmung regulieren erzielt. Zwei Isoformen von sogenannten Uncoupling Proteinen werden konstitutiv in der Zuckerrübe exprimiert, während eine Induktion einer alternativen Oxidase nach Verwundung und Lagerung beobachtet wurde. Mitglieder beider Genfamilien sind mögliche Zielgene, die Saccharoseverluste während der Lagerung beeinflussen können. Sie dienen als Energie-Ableitungssysteme und ihre Aktivität kann die Respiration erhöhen, was einen vermehrten Saccharoseabbau zur Folge hat. Die ermittelten Ergebnisse weisen daraufhin, dass zumindest die alternative Oxidase eine starke Reaktion auf Verwundung und Lagerung zeigt.

2 Introduction

2.1 Sugar beet as a model system for sucrose storing plants

Sugar beet is besides sugarcane the only industrially exploited source of table sugar, the disaccharide sucrose. In most higher plants sucrose plays a central role as the transport form of assimilated carbon. Carbohydrates produced in photosynthetic active parts of the plant (source tissues) have to be transported to sink tissues, which require imported energy for consumption. In most plant species the imported sucrose is either metabolized or converted into storage compounds like starch, oil or storage proteins. Sugar beet plants however accumulate high levels of sucrose inside a large, vegetative storage root (taproot). At time of harvest, modern sugar beet cultivars reach sucrose contents of up to 20% of the total fresh weight. In order to accumulate and stabilize such a high concentration of the chemically and osmotically very active sucrose, sugar beet must have developed special mechanisms to fulfill this task.

In the first chapters, the role of sucrose and sucrose cleaving enzymes during plant development will be addressed. Later the current knowledge about these processes will be put into context with the only limited understanding on the process of sucrose accumulation and storage in sugar beet.

2.2 The role of sucrose during plant development

Most higher plants use sucrose as the transport form of carbohydrates from the organs of production (*sources*) to the energy consuming regions of the plant (*sinks*). During photosynthesis, the first stable form of fixed carbohydrates are triose phosphates produced in the Calvin cycle. In the chloroplasts the triose phosphates are either transiently stored as starch, or exported into the cytosol, where sucrose can be formed through the synthesis of sucrose-phosphate from UDP-glucose and fructose-6-phosphate by the enzyme sucrose-phosphate synthase (SPS, EC 2.4.1.14). The hydrolysis of sucrose-phosphate to sucrose by sucrose-phosphatase (SPP, EC 3.1.3.24) produces sucrose and drives the equilibrium of the previous reaction into the direction sucrose-phosphate production (Taiz & Zeiger, 2000).

2.2.1 Transport of sucrose involves phloem loading and unloading

The transport of photoassimilates from the place of production into sink tissues, where they are either rapidly consumed or actively stored, is managed via the phloem part of the plants conducting tissues. After sucrose is synthesized in leaf mesophyll cells, it diffuses symplastically towards the phloem (Williams et al., 2000).

Phloem loading

For the entrance of sucrose into the phloem, in many plant species an apoplastic step is required. The release of sucrose into the apoplast and the active uptake into the phloem helps to overcome the concentration difference of sucrose between the mesophyll cells (20-200 mM sucrose) and the sieve element-companion cell complex (300-1500 mM sucrose, Williams et al., 2000; Taiz & Zeiger, 2000). Sucrose released into the apoplast is not hydrolyzed into hexose before entering the phloem (Delrot, 1989). This assumption is supported by the severe distortions in carbon partitioning produced by the overexpression of yeast derived invertases in the apoplast of several plant species (e.g. Sonnewald et al., 1991; Heineke et al., 1992; Weber et al., 1998).

The process of phloem loading is an important regulatory step to determine the carbon partitioning between the source and sink tissues. Sugar beet is, due to the lack of symplastical connections between the mesophyll cells and the conducting complex, a typical example for apoplastic phloem loading. It was shown that this process is sensitive to anoxia and uncouplers, suggesting that sucrose is loaded into the phloem in an energy dependent process (Sovonick et al., 1974). For many plant species it has been shown, that sucrose is loaded into the phloem via an sucrose-H^+-symporter localized in the plasma membrane of phloem cells (Williams et al., 2000 and ref. therein). In sugar beet the activity of the sucrose symporter BvSUT1 is regulated by the availability of photoassimilates in the source tissue (Chiou & Bush, 1998). Externally fed sucrose leads to a decrease in transport activity of isolated membranes and a reduced expression of the sucrose symporter. The transporter is specifically localized in phloem companion cells and the loss of sucrose transport activity is accompanied by the loss of protein abundance as a result of decreased transcription (Vaughn et al., 2002). The response to sucrose accumulation is at least partially mediated by a protein kinase involved in down-regulating the transcription of the BvSUT1 gene. Conversely, the transcription can be induced via a protein phosphatase dependent mechanism (Ransom-Hodgkins et al., 2003). The authors propose, that this mechanism is a reaction of the plant to a decreased sink demand leading to sucrose accumulation in the vascular tissue. This accumulation is perceived by a yet unknown sucrose sensor in the phloem. Decreased phloem loading then leads to a feedback inhibition of photosynthesis in the leaf mesophyll cells. In contrary, a higher consumption of sucrose in sink tissues would lead to a induction of phloem loading and a higher efflux of photoassimilates from the leaves.

Inside the phloem, sucrose is transported via bulk flow. The transport is regulated according to the pressure flow hypothesis by Münch (1930) through the difference in solute concentration due to the loading into the sieve elements in source and the unloading in sink tissues.

Phloem unloading

Phloem unloading in sink tissues can either be symplastical or involve an apoplastic step. During symplastic unloading, sucrose leaves the phloem via plasmodesmatic connections with the surrounding cells. This has been shown for young leaves of some species like sugar beet (Schmalstig & Geiger, 1985, 1987) and tobacco (Taiz & Zeiger, 2000) and in the tips of primary roots of *A. thaliana* (Oparka et al., 1995). In order to sustain the concentration gradient between the phloem and the symplastically connected sink tissues, the imported sucrose is either metabolized or stored in the vacuole (Eschrich, 1989).

Alternatively, removal of sucrose from the phloem can involve an apoplastic step. During its transport through the phloem pathway, a constant concentration gradient between the apoplastic space surrounding the phloem and the content of the sieve tube is present and leakage of sucrose occurs frequently. In non-sink tissues the leaked sucrose is reloaded into the phloem via sucrose-H^+ symporters localized in the sieve element-companion cell complex and transport is maintained (Patrick, 1997 and ref. therein). In generative or vegetative storage tissues however, the sucrose released into the apoplastic space is drawn into surrounding parenchyma cells and not reloaded into the sieve element (Eschrich, 1989). The removal of sucrose into the parenchyma cells can either be realized by the direct uptake of sucrose via sucrose transporters or, seemingly more widely distributed, involve cleavage of sucrose in the apoplast. Cell wall bound invertases cleave sucrose and help to maintain a steep concentration gradient for sucrose between the phloem symplast and the surrounding apoplast. The resulting hexoses are then taken up by hexose-H^+ symporters specifically localized in sink cells (Williams et al., 2000). Inside the parenchyma cells the hexoses are either phosphorylated for further metabolism (storage as starch or sucrose or degradation) or taken up into the vacuole (Herbers & Sonnewald, 1998).

2.3 Sucrose hydrolyzing enzymes

In plants, sucrose cleavage is catalyzed by two known enzymes: sucrose synthase (SuSy) and invertase (Inv). The reactions catalyzed by the two enzymes are:

$$\text{sucrose} + \text{UDP} \xrightleftharpoons{\text{SuSy}} \text{UDPglucose} + \text{fructose}$$

$$\text{sucrose} + \text{H}_2\text{O} \xrightarrow{\text{Inv}} \text{glucose} + \text{fructose}$$
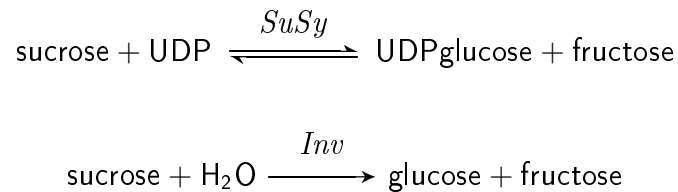
A major distinction between these two pathways is the amount of energy conservation: whereas during the SuSy reaction the most of energy of the bond between glucose and fructose is conserved by the formation of UDPglucose, invertase is a hydrolase and thus the energy is dissipated. Whereas hydrolysis by invertase is irreversible, the cleavage of sucrose is readily reversible (Geigenberger & Stitt, 1993). Additionally, SuSy generates only half as many hexoses as Inv (and no free glucose), which are involved in sugar sensing (see below and Koch, 2004).

2.4 Sucrose synthase

Although *in vitro* sucrose synthase (EC 2.4.1.13) is able to synthesize sucrose from UDPglucose and fructose (Nakai et al., 1997), *in vivo* it generally catalyzes the cleavage of sucrose. In general, SuSy is important for the entrance of the sucrose cleavage products into either anabolic reactions via intermediates from glycolysis or for biosynthetic reactions, especially the formation of cell wall polysaccharides (Koch, 2004).

During the synthesis of cellulose, SuSy, which is localized exclusively in the cytosol, is described to associate with the plasma membrane, the actin cytoskeleton (Doblin et al., 2002) and the cellulose synthase complex (Amor et al., 1995). The latter association supports the supply of UDPglucose for cellulose synthesis and at the same time the efficient recycling of the released UDP.

Apart from biosynthetic reactions, SuSy is important for anabolic processes (Sturm & Tang, 1999). UDPglucose is further metabolized inside the cytosol via UDP-glucose pyrophosphorylase (UGPase, EC 2.7.7.9):

$$\text{UDPglucose} + \text{PP}_i \xrightleftharpoons{\text{UGPase}} \text{glucose-6-P} + \text{UTP}$$
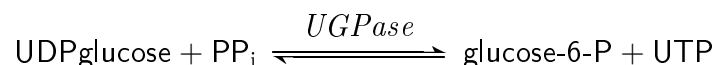
The formed UTP can be used for the phosphorylation of the previously released fructose and so both hexose-phosphates can enter glycolysis with the consumption of only one pyrophosphate (PP_i) molecule. The invertase pathway in contrast necessitates two ATP molecules for hexose phosphorylation. Since pyrophosphate is produced as a byproduct in many biosyn-

thetic reactions, the energy balance for the cell is even more favorable. SuSy activity has been described to be an important factor for sink strength, for example in tomato fruits (D'Aoust et al., 1999) and potato tubers (Zrenner et al., 1995). In carrot, inhibition of SuSy activity leads to a decrease in plant size, indicating a general role for plant growth (Sturm & Tang, 1999). The efficiency of the SuSy reaction especially comes to the fore during detrimental conditions for ATP synthesis like cold and oxygen deprivation, during which SuSy genes have been described to be induced (Zeng et al., 1999; Sturm & Tang, 1999; Hesse & Willmitzer, 1996). Inside bulky storage tissues like potato tubers oxygen depletion occurs (Geigenberger et al., 2000) and leads to the induction of SuSy genes (Bologa et al., 2003).

2.4.1 Regulation of SuSy activity

Sucrose synthase expression is regulated by various factors, of which sugar supply and oxygen deprivation have been studied extensively. Baud et al. (2004) comprehensively analyzed the six members of the SuSy gene family of *A. thaliana*. The expression of the individual members is partially redundant, but during stress treatments or developmental phases the isoforms show distinct responses. For maize, two SuSy isoforms have been described, which show contrary reactions to sugar supplies. Whereas one isoform is especially active during sugar depletion, the other isoform is induced during ample supply with carbohydrates (Koch, 1996). The presence of differentially regulated isoforms reinforces the need for co- and post-translational regulation of SuSy protein expression and activity. Indeed it has been shown, that SuSy is regulated cotranslationally, by changes in the subcellular localization and by regulation of protein stability through phosphorylation (Koch, 2004 and ref. therein).

2.5 Invertases

Plants possess several isoforms of invertase (EC 3.2.1.26). Usually they are classified according to their pH optimum into acid and neutral or alkaline invertases. A second level of classification comes from their subcellular localization, which greatly influences their importance during different developmental conditions. Neutral and alkaline invertases are localized in the cytosol of the cell and therefore are also referred as cytosolic invertases (CI). The acid invertases are further divided into cell wall (CWI or apoplastic invertase) and vacuolar invertases (VI). VI is also referred to as soluble invertase, since, in contrast to CWI, it does not bind to the insoluble cell wall material during extraction.

2.5.1 Cytosolic invertases

Invertase isoforms found in the cytosol of plant cells cleave sucrose at a pH optimum between 6.8 and 8.0 (Roitsch & Gonzalez, 2004). CIs are not glycosylated and are extremely unstable, which has hampered their detailed biochemical characterization (Sturm, 1999).

The available protein sequences of plant CIs are highly homologous to each other but not to acid invertases. Homologs have only been found in plants and photosynthetic bacteria leading to the speculation that they might have evolved from an ancestral prokaryotic gene after an endosymbiotic event (Vargas et al., 2003).

Due to the lack of biochemical and physiological data on CIs, no clear function during plant development has been assigned, but they are suspected to channel cytoplasmic sucrose into catabolic reactions (Sturm & Tang, 1999). Lee & Sturm (1996) showed, that neutral and alkaline invertases from *Daucus carota* are inhibited competitively by fructose (Ki=15 mM) and non-competitively by glucose (Ki=30 mM), whereas only alkaline invertase was inhibited by $CaCl_2$, $MgCl_2$ and $MnCl_2$. The feedback inhibition by their reaction product could pose and important regulation process, which adapts the cleavage of cytosolic sucrose to the consumption of glucose and fructose in cytoplasmic sucrose metabolism (Lee & Sturm, 1996).

2.6 Acid invertases

2.6.1 Structural features of vacuolar and cell wall invertases

Vacuolar and cell wall invertases share enzymatic and sequence similarities, like the acidic pH-optimum for sucrose cleavage and conserved sequence motifs. However, they possess distinct properties, which influence their role during plant development. The obvious dividing feature is the difference in the subcellular localization. Whereas the CWI probably reaches the cell wall via the secretory pathway with no additional sequence signal apart from the N-terminal signal peptide, VIs must contain an additional signal leading to a vacuolar localization. Unger et al. (1994) predicted a short C-terminal extension found only on vacuolar invertases to be a vacuolar sorting signal, but further experimental evidence was not provided for this. None of the described vacuolar invertases from *A. thaliana*, carrot or sugar beet contains a typical sequence motif of other protein localized in either protein storage vacuoles or lytic vacuoles (Matsuoka & Neuhaus, 1999; Vitale & Raikhel, 1999).

Acid invertases cleave sucrose from the fructose residue and thus are β-fructofuranosidases, which also cleave other oligosaccharides like raffinose and stachiose (Sturm, 1999). Indeed, recently it was shown by De Coninck et al. (2005), that at least two out of six genes annotated as cell wall invertases from *A. thaliana* do not cleave sucrose at all, but rather cleave fructans. Fructans are linear or branched polymers of fructose, which are synthesized from sucrose monomers (Van Laere & Van den Ende, 2002). They serve as storage polymers in about 15 % of all flowering plants, for example in grasses, onions and cereals (Vijn & Smeekens, 1999). Chicory (*Cichorium intybus* L.) is used commercially as a source of inulin-type fructans. Fructans are synthesized by sucrose:sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99) and fructan:fructan 1-fructosyltransferase (1-FFT, EC 2.4.1.100), which both seem to have evolved from vacuolar invertases (Van den Ende et al., 2002). The fructan degrading

fructan exohydrolases (FEH) however seem to be derived from cell wall invertases (Van den Ende et al., 2000) and have also been discovered in non-fructan storing plant species like sugar beet and *A. thaliana* (Van Den Ende et al., 2003; De Coninck et al., 2005). It was speculated, that in these plants they are either involved in the degradation of fructans from microbial pathogens or in the prevention of unwanted accumulation of endogenous fructans. All of the above enzymes belong to the GH32 family of glycoside hydrolases, which have been classified based on sequence homologies between those enzymes (Henrissat & Davies, 1997; Pons et al., 1998)[1]. In summary, the annotations in public sequence databases for acid invertases have to be taken with care, until their activity towards either sucrose or fructans has been shown.

Both, CWI and VI, are characterized by their acidic pH optimum for the cleavage of sucrose, which is slightly more acidic (pH 3.5-5.0) for CWI than for VI (pH 5.0-5.5) (Roitsch & Gonzalez, 2004). Goetz & Roitsch (1999) showed that the different pH optimum of the two isoforms is determined by an amino acid exchange in the conserved WEC-P/V-D box, which is characteristic for all acid invertases. CWIs carry a proline residue at the fourth position where VIs have a valine residue (Roitsch & Gonzalez, 2004). By a proline to valine exchange in the extracellular invertase CIN1 from *Chenopodium rubrum*, the pH optimum of the heterologous expressed CIN1 was shifted from 3.75 to 4.4. Additionally, the cleavage rate of raffinose was lowered compared to the wildtype CIN1 protein (Goetz & Roitsch, 1999).

Another conserved sequence motif is the NDPNG motif close to the N-terminus of the mature polypeptide found in invertases from plant, bacteria and yeast. It forms an important part of the catalytic domain of acid invertases (Sturm, 1999).

CWI and VI are both synthesized as a prepropeptide, with a N-terminal signal peptide for the entry into the ER and a propeptide of over 100 amino acids. The propeptide is cleaved off in the mature form of the enzyme and is not conserved between invertases from different plant species (Sturm & Chrispeels, 1990; Unger et al., 1994). No clear function has been assigned to it yet. During their passage through the secretory pathway the acid invertases become glycosylated (Roitsch & Gonzalez, 2004) and it has been shown that this is important for protein stability (Pagny et al., 2003).

Recently the protein structure of a bacterial invertase and of a plant FEH has been solved (Alberto et al., 2004; Verhaest et al., 2005). Both proteins belong to the GH32 family (see above) and the overall three dimensional structure revealed to be very similar. The structure resembles an earlier described levansucrase (Meng & Fütterer, 2003) belonging to the related family GH68 of glycosyl hydrolases. It consist of a N-terminal five-bladed β-propeller, containing the active site, connected to a β-sandwich module. Three highly conserved amino acids, which are part of conserved motifs of the GH32 family, form a crucial part of the active site of the enzymes (NDPNG, FRDP, WECPD; identified amino acids in bold). The two β-sheets of the β-sandwich module are suspected to be involved in determining the substrate

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[1]`http://afmb.cnrs-mrs.fr/CAZY`

specificity of the hydrolases. In the invertase structure, the potential sucrose binding cleft is shielded in comparison to a more accessible situation in case of the fructan exohydrolase and this could influence the specificity of the enzymes (Verhaest et al., 2005).

2.6.2 Acid invertases are important for carbon partitioning and sugar composition during plant development

CWI activity is important for development of storage tissues

As noted before, cell wall invertases play a key role for apoplastic phloem unloading (see 2.2.1). This mechanism has been extensively studied during seed development. Since developing seeds are not symplastically connected to the maternal tissue, import of carbohydrates must involve an apoplastic step. It has been shown for the seeds of maize (Cheng et al., 1996), barley (Weschke et al., 2003), oilseed rape (Hill et al., 2003) and especially well for the seeds of *Vicia faba* (Weber et al., 1995; Wobus & Weber, 1999) that the first phases of seed development are characterized by a high hexose to sucrose ratio maintained by a high activity of acid invertases. Loss of CWI activity localized at the transfer layer between maternal and filial tissue in maize leads to a severe decrease in kernel size in the *miniature1* mutant (Cheng et al., 1996).

The released hexoses can be taken up by hexose transporters, which have been shown to be expressed in tissues in the vicinity of high CWI activity (Weber et al., 1997; Ehneß & Roitsch, 1997; Weschke et al., 2003). In carrot taproots, a vegetative storage tissue, antisense reduction of cell wall invertase activity leads to the abolishment of taproot formation (Tang et al., 1999). For tomato, Fridman & Zamir (2000) showed that sugar content of tomato fruits correlates with cell wall invertase activity. An amino acid change in the sequence of the CWI leads to a decreased K_M value and this is most likely responsible for the increased hexose content in these plants (Fridman et al., 2004).

Later phases of seed development, when cell division declines and storage products start to accumulate, are accompanied by a switch to a low hexose to sucrose ratio and a sharp decrease in invertase activity (Weber et al., 1995; Hill et al., 2003; Weschke et al., 2003). At least for fava bean seeds the parallel expression of sucrose transporters in the epidermal cells of the developing embryo imply, that sucrose is then taken up without prior cleavage in the apoplastic space (Weber et al., 1997). Inside the cells, sucrose is then metabolized via sucrose synthase and either polymerized into starch or converted into other storage compounds (Borisjuk et al., 2004 and ref. therein).

Isolated cotyledons of fava beans prolong their mitotic activity when incubated in hexose rich medium. In contrast to this, when bathed in high sucrose medium, they develop characteristics of cells already in the storage phase. This led to the postulation of the invertase control hypothesis of seed development (Weber & Wobus, 1997), emphasizing the role of the hexose to sucrose ratio that is to a substantial part regulated by the activity of cell wall

invertases.

Vacuolar invertases regulate the amount of sucrose stored in vacuoles

Vacuolar invertases (VI) regulate the ratio and levels of hexoses and sucrose, either stored temporarily or permanently, in vacuoles (Sturm & Tang, 1999). After cleavage by VI, the formed hexoses can be exported to the cytosol by hexose-H$^+$ symporters, where they are further metabolized. VIs are especially active in growing zones and expanding tissues like root tips, internodes and developing leaves. In these tissues they enhance cell enlargement via the increase in osmotic potential (Weschke et al., 2003) and probably also play a role in phloem unloading. In *A. thaliana* a quantitative trait locus (QTL) analysis showed, that length of roots and hypocotyls correlated with acid invertase activity and that a major locus for root length maps to one of two VI isoforms (at1g12240). A functional knock-out of this gene, leading to 50 % reduced VI activity, exhibited roots significantly reduced in length (Sergeeva et al., 2006)

A reduction of vacuolar invertase activity in transgenic carrot plants leads to a decrease in taproot size and to decreased levels of soluble sugars (Tang et al., 1999), indicating an important role for the expansion of the storage sink. In tomato fruits, the activity of vacuolar invertase determines whether sugars are stored as hexoses or as sucrose. Klann et al. (1996) showed, that by silencing VI transcription, hexose storing fruits are transformed into sucrose storing fruits without affecting the allocation of assimilates to the fruits.

2.6.3 Acid invertases are part of a complex regulatory system

During normal plant development, vacuolar and cell wall invertase activities are generally associated with developing tissues and a high mitotic activity. A strong down-regulation is observed, when tissues (e.g. seeds) develop into storage organs. Apart from this, acid invertases also show a strong induction in response to various stresses, which probably reflects a reaction of the plant to the need for changes in carbon partitioning (for review see Sturm, 1999; Roitsch et al., 2003).

Induction of CWI by mechanical or pathogen-induced wounding of leaves has been shown among others in carrot (Sturm & Chrispeels, 1990), pea (Zhang et al., 1996) and *Chenopodium rubrum* (Ehneß et al., 1997). The increase in apoplastic invertase activity probably reflects the energy demand of the cells affected by wounding, leading to the transformation into local sinks. By cleavage of extracellular sucrose the export from leaf tissues is interrupted and the carbon partitioning is changed so that the wounded tissue can respond to the stress with the induction of pathogenesis defence genes and synthesis of cell wall material for the closure of wounds. This is corroborated by the finding, that in *A. thaliana* sucrose and hexose transporters, which are normally restricted to sink tissues, are induced by wounding (Meyer et al., 2004; Truernit et al., 1996). However, the study by Quilliam et al. (2006) showed,

that the knock-out of the wound-induced CWI isoform AtcwINV1 does not hamper the creation of localized sink tissues in wounded *A. thaliana* leaves, indicating the presence of other mechanisms cooperating during this process.

Vacuolar invertases are regulated by abiotic stresses like drought, hypoxia and cold (Roitsch & Gonzalez, 2004). In maize, hypoxia leads to a rapid decrease in the expression and activity of two VI isoforms in roots (Zeng et al., 1999), whereas drought stress leads to the induction of a specific VI isoform (*Ivr2*) in leaves and root tips (Kim et al., 2000). The authors propose, that the induction of vacuolar sucrose cleavage leads to increased phloem unloading, osmotic adjustment and the generation of hexose signals that participate in a signal transduction cascade leading to increased resistance against drought stress. In contrast to this, drought stress during young ovary development leads to a down-regulation of acid invertases in maize kernels (Zinselmeier et al., 1995) and especially the down-regulation of *Ivr2* is correlated with seed abortion probably due to the reduction of symplastic phloem unloading in the affected areas (Andersen et al., 2002).

In stored potato tubers cold storage leads to increased starch degradation and resynthesis of sucrose, which is in turn cleaved by an induced VI (Sturm & Tang, 1999; Greiner et al., 1999). Furthermore, VI is also induced under wounding conditions (Rosenkranz et al., 2001; Roitsch & Gonzalez, 2004).

Acid invertases are also reported to be regulated by various phytohormones. The growth promoting hormones auxin, cytokinin and gibberellic acid, as well as abscisic acid, have been reported to promote invertase activity, whereas only the senescence promoting ethylene represses transcription of extracellular invertases (Roitsch et al., 2003 and ref. therein). Balibrea Lara et al. (2004) also showed, that senescence can be delayed in tobacco leaves by either application of cytokinins or the overexpression of a CWI under the control of a senescence induced promotor. The additional invertase activity seems to influence the carbon partitioning and thereby delays senescence effects.

Sucrolytic enzymes play a key role in sugar signaling

Apart from the metabolic and osmotic functions of the produced hexoses, they have also been described as a cellular signal molecule regulating the gene expression profile in a hormone-like fashion (Koch, 1996; Smeekens, 2000; Rolland et al., 2002). Most often the regulatory function of glucose has been addressed and the enzyme hexokinase and plasma-membrane localized transporter-like genes have been implicated as the initial hexose sensors (Jang et al., 1997; Rolland et al., 2002). Apart from hexose sensing, the regulatory function of sucrose has been shown (Chiou & Bush, 1998; Rook et al., 1998) and other products of sucrose metabolism have been proposed to be involved in signalling cascades (Rolland et al., 2002). The role of hexose and sucrose signals during the development of *Vicia faba* seeds (see 2.6.2) is another important system, where sugars have been proposed in regulating tissue development.

After the sugar signal is sensed, a signal cascade, which involves protein kinases and phosphatases, cytosolic Ca^{2+} concentration and other signal mediators is initiated (Smeekens, 2000) leading to transcriptional regulation of various genes.

Acid invertases themselves have been shown to be induced by hexoses (Ehneß et al., 1997; Tymowska-Lalanne & Kreis, 1998) and sucrose (Sinha et al., 2002; Tymowska-Lalanne & Kreis, 1998). Ehneß et al. (1997) showed, that induction of CWI by glucose is regulated through a different regulatory pathway than the induction of CWI and pathogenesis related proteins in reaction to pathogen attack or fungal elicitors. The observed upregulation of CWIs by hexoses might also be important for the amplification of other stimuli by a positive feed-forward mechanism through the reaction products (Roitsch & Gonzalez, 2004). In maize, a different reaction of two isoforms of vacuolar isoforms has been reported (Xu et al., 1996). Whereas *Ivr1* is repressed by sucrose, glucose and other sugars, *Ivr2* is upregulated by the same stimuli, indicating an isoform specific regulation. One vacuolar invertase isoform is generally active in times of high carbohydrate supplies, whereas a second isoform is active only during times when energy supply is limited. The same situation has been found for two isoforms of sucrose synthase ("feast and famine" enzymes, see Koch (1996)).

Mechanisms regulating the activity of acid invertases

As described above, the adaptation of carbon-partitioning by up or down-regulation of acid invertases is a key regulatory process during plant development. Most plant species contain several invertase isoforms. In the genome of *A. thaliana* six CWI and two VI isoforms have been annotated (Tymowska-Lalanne & Kreis, 1998; The Arabidopsis Genome Initiative, 2000), which differ remarkably in their expression pattern. Tymowska-Lalanne & Kreis (1998) and Sherson et al. (2003) analyzed the transcriptional regulation of some of the members of the invertase gene family and observed different spatial and temporal expression patterns for each member of the vacuolar and cell wall invertases, suggesting a complex regulatory system. It has to be noted however, that at least for two out of six members of the CWI gene family it has been shown that the gene product does not cleave sucrose, but fructans (see above and De Coninck et al., 2005).

Fast transcriptional up- and down-regulation of invertase isoforms has been described under various conditions and seems to be a general regulatory mechanism of these genes (Sturm & Chrispeels, 1990; Zhang et al., 1996; Ehneß et al., 1997; Zeng et al., 1999). For potato, a posttranscriptional regulation via exon-skipping of a CWI gene has been described (Bournay et al., 1996). The intron-exon gene structure of the invertase genes is conserved between monocots and dicots (Roitsch & Gonzalez, 2004) and all but one described invertase gene carry a small mini-exon of nine nucleotides, which encodes for the middle amino acids of the conserved NDPNG motif and represents one of the smallest exons described in plants (Simpson et al., 2000). In potato, aberrant splicing leading to the skipping of the mini-exon has been observed (Bournay et al., 1996), suggesting a possible regulatory mechanism.

However until now, no physiological relevance has been attributed to this process.

For *A. thaliana* a mechanism for the regulation of the targeting and the turnover of an VI protein has been proposed. Rojo et al. (2003) described the importance of the vacuolar processing enzyme VPEγ (at4g32940), a cystein protease, for the degradation of the vacuolar invertase AtFruct4 (at1g12240) during senescence. In null mutants of VPEγ, AtFruct4 protein accumulates during ageing of leaves and roots, whereas in WT plants the invertase protein is degraded. Even more intriguing, the authors showed that in young seedlings AtFruct4 colocalizes with VPEγ in so called precursor protease vesicles (PPV), a compartment originally described in seed storage tissues. Here PPVs contain precursor of cysteine proteases, which are activated during germination. Inside the PPV the VI is protected from degradation because VPEγ is autocatalytically activated only when delivered into the acidic lumen of the vacuole. Therefore, this mechanism seems to regulate the entry of the VI into the lumen of the vacuole and at the same time the subsequent turnover of the invertase (Koch, 2004).

2.7 Posttranslational regulation of invertases by proteinaceous inhibitors

An additional mechanism to efficiently silence invertase activity at certain stages of plant development is the posttranslational inactivation through invertase inhibitor proteins. The expression of specialized proteins, which specifically inhibit invertases, is probably based on the need to silence invertase activity efficiently and rapidly. Due to the high intrinsic stability of invertases as glycoproteins, compartimentalized into either the cell wall or the vacuole, transcriptional down-regulation and protein degradation alone seems not to be sufficient for this task. Although the presence of invertase inhibitors has been known for a long time and they have been described in various plant species, especially in storage organs of crop plants, their physiological significance is not yet fully understood. Albeit this, they have already been used successfully in biotechnological approaches to silence invertase activity in plants (Greiner et al., 1999; Balibrea Lara et al., 2004).

2.7.1 From the discovery of invertase inhibitors to the solution of their 3D structure

Presence in storage tissues

The first description of invertase inhibitors came from potato tubers, where a low molecular protein was described, that is bound to an endogenous invertase and lowers the activity of invertase preparations (Schwimmer et al., 1961; Pressey, 1966). Later the presence of similar proteins was also described in the storage tissues of sweet potato, red and sugar beet (Pressey,

1968) and in the endosperm of maize kernels (Jaynes & Nelson, 1971). Further studies were carried out on the invertase inhibitor from potato, which delivered biochemical data about the inhibitor and the mechanism of inhibition. The invertase inhibitors are non-glycosylated proteins, 15 - 23 kDa in size, showing an exceptional stability against treatment with heat and acidic conditions (Rausch & Greiner, 2004).

Cloning and analysis of NtCIF

The first sequence data of the inhibitor protein came form the purification and N-terminal sequencing of inhibitors in tomato (Pressey, 1994) and tobacco (Weil et al., 1994) and eventually the first invertase inhibitor was cloned from tobacco (Greiner et al., 1998). NtCIF (*Nicotiana tabacum* cell wall inhibitor of β-fructosidase) was purified from a cell-wall fraction of suspension culture cells in a stable complex with a CWI (Krausgrill et al., 1998). *In vitro* the NtCIF protein inhibits both, CWI, its probable native target enzyme, but also VI and inhibition also occurs with plant invertases from other plant species. However, for NtCIF and also for the inhibitor from maize, no inhibition of invertase from fungal sources is observed and thus a function in pathogen defence seems unlikely (Greiner, 1999; Pressey, 1967). The presence of divalent cations like Ca^{2+}, Mg^{2+} and Zn^{2+} interferes with the inhibition of NtCWI by NtCIF. A considerable pH dependency is observed: strongest inhibition occurs at pH 4.5, which is equivalent with the pH optimum of the CWI, while at pH 5.5 and 6.5 only a weak effect on CWI activity is observed (Weil et al., 1994). Since the apoplastic pH is reported to vary between pH 4.5 and 6.5 (Grignon & Sentenac, 1991), this could indicate an *in vivo* modulation of the inhibition by pH. The invertase inhibitors are sensitive to treatment with reducing agents (Pressey, 1967; Ovalle et al., 1995) due to the presence of two disulfide bridges stabilizing the structure of the molecules (see Greiner et al., 2000; Hothorn et al., 2004b and below).

Furthermore, NtCWI is protected from inhibition by presence of the substrate sucrose with half maximum protection at 1.2 mM sucrose (Weil et al., 1994), but this effect is not seen when NtCIF protein is incubated with VIs and CWIs and from other plant sources (Sander et al., 1996; Greiner, 1999), indicating that the availability of sucrose protection is an intrinsic quality of the respective invertase. Additionally, the complex formation between NtCIF and NtCWI is markedly slower than the binding of NtCIF to a VI preparation from tomato fruit (Sander et al., 1996). After the sequence of NtCIF was available, additional related genes were isolated and identified as invertase inhibitors. NtVIF, a second inhibitor isoform from tobacco was isolated and it was shown by heterologous overexpression in potato, that this inhibitor is localized inside the vacuole (see 2.7.3 and Greiner et al., 1999).

Invertase inhibitors form a gene family with pectin methylesterase inhibitors

With the onset of large-scale genome sequencing projects it became evident, that invertase inhibitor proteins are part of a larger gene family and are present in monocots and dicots.

In *A. thaliana* approximately 40 genes show a significant homology to invertase inhibitors (S. Greiner, personal communication) and two of these genes (AtC/VIF1 (at1g47960) and AtC/VIF2 (at5g64620)) have been functionally proven to inhibit invertases (Link et al., 2004). Recently the situation has been complicated by the observation that related proteins can also be ineffective against invertases and instead inhibit a family of cell-wall modifying enzymes called pectin methylesterases (PMEs). A PME inhibiting protein showing homologies to NtCIF was isolated from Kiwi fruit (Camardella et al., 2000) and two related genes (AtPMEI1 (at1g48020) and AtPMEI2 (at3g17220)) from *A. thaliana* were identified as inhibitors of pollen-expressed PMEs (Wolf et al., 2003; Raiola et al., 2004). All genes showing homologies to either PMEIs or invertase inhibitors have been integrated into a sequence family named PMEI-RPs (pectin methylesterase inhibitor-related proteins, Hothorn et al. (2004b)).

Molecular structure of NtCIF and AtPMEI

The general protein sequence homology of this gene family is not very high. Already the two identified invertase inhibitors from tobacco and *A. thaliana* show only 47% and 29% identical amino acids respectively and the general homology inside the PMEI-RP family is only between 20 and 35 %. However, all members possess 4 cysteine residues at conserved positions, which are important for the formation of two disulfide bridges. The principal similarity between the invertase inhibitors and PMEIs was shown by the crystallization and structure determination of the NtCIF (Hothorn et al., 2004a) and AtPMEI1 (Hothorn et al., 2004b) proteins. These studies showed, that the two proteins show, albeit the limited conservation of amino acids, a very similar overall fold, consisting of a four-helix bundle and an uncommon N-terminal extension. Each structure is stabilized by a disulfide bridge and it was shown, that the N-terminal extension of NtCIF is important for overall protein structure (Hothorn et al., 2004a). By comparing the structure of AtPMEI1 with NtCIF a different orientation of the N-terminal helical extension became obvious (see Fig. 2.1). In NtCIF this extension is directed towards the bundle core, whereas in AtPMEI1 it contacts a second inhibitor molecule leading to the formation of AtPMEI1-dimers (Hothorn et al., 2004b). By exchanging the N-terminal extension between the two inhibitors, the extension of AtPMEI1 was shown to be a key structure for PME inhibition, whereas for invertase inactivation the structure of the extension together with the bundle core is important. Although these two molecules have been studied in such detail, due to the low general sequence conservation inside the PMEI-RP family, a prediction of the target enzyme of a given PMEI-RP based on sequence data alone is not possible (Hothorn et al., 2004b). Thus, for every new PMEI-

RP sequence to be analyzed, the true function has to be proven by generating recombinant inhibitor protein and testing for inhibition activity on either invertases or PMEs. Until today, no PMEI-RP has been observed to be active against both potential target proteins (Rausch & Greiner, 2004).

Di Matteo et al. (2005) presented the structure of the complex between AcPMEI from kiwi (*Actinidia chinensis*) and a PME from tomato. Contradictory to the previously described results, the inhibitor contacts the target PME near the active site via the four-helix bundle and the N-terminal extension points away from the PME, making a critical role for the inhibition unlikely. Also no inhibitor dimers were observed. For the interaction of invertase inhibitors with invertases however, no structural model is available. As described in chapter 2.6.1 on page 10, 3D structures of invertases have been described, but no model of the complex between invertase and inhibitor is available yet and since PMEs and invertases show a completely different structure, no conclusions from the PME-PMEI complex described can be drawn.

Figure 2.1: Ribbon representation of a PMEI dimer (A) and NtCIF (B)
Both molecules feature a similar four-helix bundle core but the N-terminal extension in AtPMEI1 points outward and is in contact with a second molecule, whereas in NtCIF it shields a hydrophobic path in the bundle core. From Hothorn et al. (2004b).
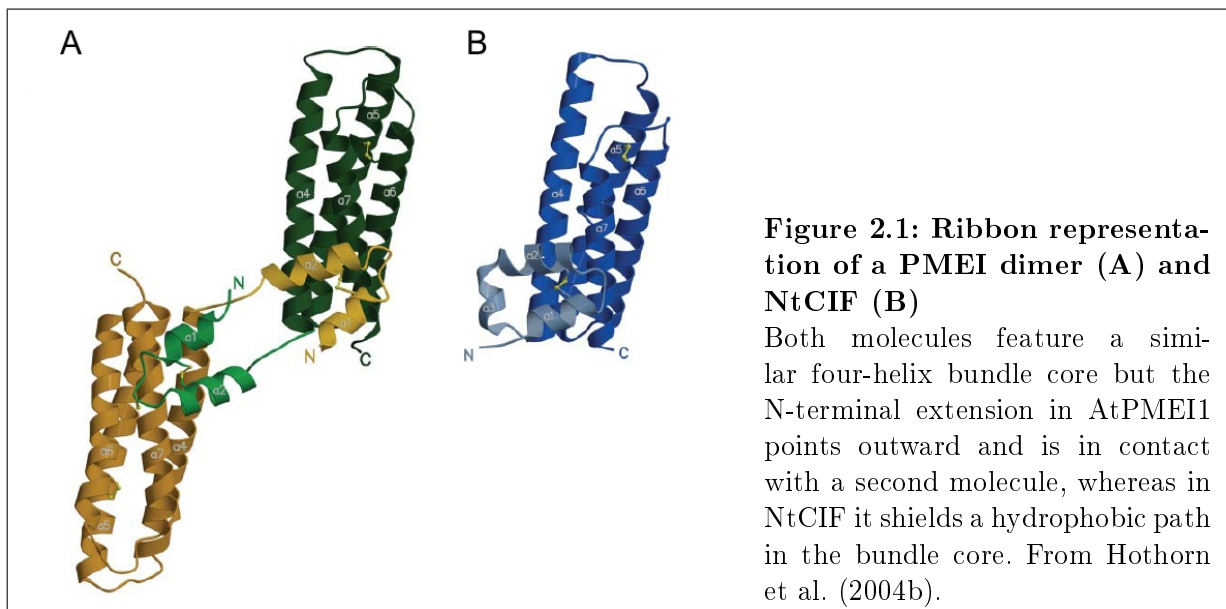
2.7.2 Expression and physiological functions of invertase inhibitors

Most of the early data on invertase inhibitors came from the purification from potato tubers. Here the inhibitor is found bound to a soluble invertase. The amount of inhibitor is variable between potato varieties and it can be induced by storage at elevated temperatures (Pressey, 1967). For tobacco, expression data are available for the expression of the cell wall inhibitor NtCIF. High amounts of NtCIF are found in suspension culture cells of tobacco, where it is expressed in parallel with CWI during most of the cultivation period, but due to the protective effect of sucrose on CWI inactivation, it probably only becomes active when sucrose in the medium is depleted (Krausgrill et al., 1998). Similarly, NtCIF-related proteins have been found in suspension culture cells of *Chenopodium rubrum* and *Daucus carota*,

both of which also show a high CWI expression (Greiner et al., 2000). In tobacco, NtCIF is most strongly expressed in floral tissues, but is also found in source and senescent leaves. In flowers, NtCIF is coexpressed with a CWI isoform during later stages of floral development (S. Bayer, personal communication). NtCIF shows an increased expression in response to ABA and PEG treatment, which simulate conditions of senescence and drought stress (Rausch & Greiner, 2004). This is in accordance with the data reported by Balibrea Lara et al. (2004) suggesting that CWI activity counteracts senescence processes.

In *A. thaliana*, the two described inhibitors AtC/VIF1 and AtC/VIF2 show a differential expression during plant development. AtC/VIF1, which seems to specifically inhibit VI *in vitro*, is expressed strongly in the vascular tissues of flowers, roots and senescent leaves. AtC/VIF2 however, shows a weaker still more broad expression in all analyzed tissues and the recombinant protein inhibits CWI and VI (Link et al., 2004).

In the early development of maize kernels, a cell wall localized invertase inhibitor (*ZmINV-INH1*) is expressed in the area surrounding the embryo, where it can interact with apoplastic invertases (Bate et al., 2004). Since the embryo at that phase develops much slower than the surrounding endosperm, the authors propose that the presence of *ZmINV-INH1* ensures that the embryo is exposed to lower hexose concentrations than the endosperm.

Several studies indicate the presence of invertase inhibitors in tissues, which simultaneously are characterized by the presence of invertases, but up to now no convincing physiological role of the inhibitors *in planta* could be provided. Although invertases and inhibitor proteins are frequently isolated as a complex from different plant tissues, it can never be completely ruled out, that this complexes arise post-extraction due to the disruption of spatial separations between the two proteins. Analysis of *A. thaliana* plants with reduced expression of AtC/VIF1 and 2 showed no obvious phenotypic difference in growth and only a minor increase in soluble invertase activity in the case of AtC/VIF1 (Link et al., 2004), most likely due to the presence of multiple inhibitor isoforms with redundant functions.

2.7.3 Biotechnological approaches using invertase inhibitors

Since acid invertases play a key role during the development of seeds and vegetative storage tissues, they are interesting targets for biotechnological approaches aiming to improve the performance of crop plants. During the early phases of development of such storage organs, invertases are a main factor for the establishment of sink strength and the maintenance of mitotic activity (Wobus & Weber, 1999; Koch, 2004). Therefore higher or longer persisting activities might enhance the import of photoassimilates or the proliferation of cells.

Whereas the overexpression of invertases on a whole plant level in either the cytosol, the apoplast or the vacuole led to adverse effects (von Schaewen et al., 1990; Sonnewald et al., 1991) a specific increase of apoplastic invertase activity during the development of potato tubers (Sonnewald et al., 1997) lead to an increase in tuber size, albeit a total reduction in tuber number. An increase in seed yield was observed when an apoplastic invertase was

overexpressed in meristematic tissues of *A. thaliana* (Heyer et al., 2004). As shown by the negative effect of invertase expression on whole plant level, the alteration of invertase activity via overexpression of heterologous genes might have negative effects and is, due to the high stability of the formed proteins, difficult to control. Another option to prolong invertase activity is the down-regulation of invertase-inhibitors described to be present in many storage tissues. An antisense approach to suppress NtCIF expression led to an increase in seed weight and oil content, concomitantly the number of seeds per flower and the seed size was not altered (Rausch, 2001). An extension of this approach onto seed crops like maize, soybean and rapeseed, which all are described to express invertase inhibitors during seed development, is a very promising target for the enhancement of these crops.

Contrary to the positive effect of invertase activity during development of storage tissues, the induction of invertases during certain stresses (see 2.6.3 on page 13) can be unfavorable for the accumulation of storage compounds. The cold-induction of vacuolar invertases in potato leads to the accumulation of reducing sugars, which deteriorate the quality of these potatoes at high processing temperatures during deep-frying. Via antisense repression of one soluble invertase isoform, Zrenner et al. (1996) could reduce the accumulation of hexoses during cold storage. However, the reduction of hexoses was only 34% compared to the wildtype in the lines with the strongest antisense effect. By the overexpression of NtVIF, the vacuolar invertase inhibitor from tobacco, Greiner et al. (1999) were able to reduce the hexose content of the tubers up to 75%. The stronger effect of the heterologous inhibitor might be due to its ability to inhibit more than one invertase isoform and thereby have an superior effect to the invertase antisense approach.

2.8 Sucrose accumulation and storage in the sugar beet taproot

2.8.1 The biology of sugar beet

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima* DÖLL) belongs to the family of Chenopodiaceae, which, apart from the *Beta vulgaris* varieties mangold (swiss chard), fodder and red beet, also contains spinach (*Spinacia oleraceae*) as another important crop species. It is assumed, that the cultivated sugar beet originates from the wild maritime beet (*Beta vulgaris* L. ssp. *maritima* (L.) TELL, Jung et al. (1993)). Sugar beet is a biennial plant. In the first year of development the sucrose storing taproot is developed. After overwintering in conditions without severe frost periods, a flowering stalk is developed from the taproot reaching heights of 1.2 m to 1.8 m, and seeds are produced (Canadian Food Inspection Agency, 2001).

2.8.2 Development of the sugar beet taproot

The sugar beet taproot is a vegetative storage organ formed by the main root and to a smaller extent of the hypocotyl, from which the leaf rosette is formed. The taproot shows an atypical growth with repeated concentric rings of cambium producing secondary phloem, xylem and parenchymous cells, leading to a potentially unlimited growth (Milford, 2006). Usually the root consists out of twelve to fifteen cambial rings and the six innermost account for around 75% of the mature storage organ (Schneider et al., 1999).

Sucrose accumulation inside the storage cells of the taproot

The mechanism of sucrose accumulation in sugar beet is not yet fully understood. The main controversial point is, whether sucrose incoming from the leaf rosettes is cleaved, prior to storage inside of the vacuoles. In sugarcane, which accumulates sucrose to similar concentrations inside the vacuoles of its internode cells, sucrose is cleaved by CWI and then resynthesized from the imported hexoses inside the cells. By this mechanism, the concentration gradient between the importing phloem and the surrounding apoplastic space can be maintained (Hatch et al., 1963; Glasziou & Gayler, 1972). Conversely, several studies in sugar beet show, that during the major period of taproot development imported sucrose is not cleaved prior to storage inside the vacuoles (Giaquinta, 1977, 1979; Wyse, 1979). The active process leading to sequestration of sucrose inside the vacuoles of the storage parenchyma is most likely carried out via an sucrose-H^+ antiporter localized in the tonoplast (Saftner et al., 1983; Briskin et al., 1985). Chiou & Bush (1996) described a tonoplast localized sugar transporter, that could be responsible for the described active uptake.

Activity of sucrose-hydrolyzing enzymes during taproot development

The activities of invertases and sucrose synthase during the development of the sugar beet taproot and its relation to the accumulation of sucrose have been addressed in several studies (Silvius & Snyder, 1979; Berghall et al., 1997; Klotz & Finger, 2002). In general, the course of activation of the different sucrolytic enzymes is in agreement with the model developed for the accumulation of storage products in seeds (see 2.6.2 on page 12). According to this model, VI and CWI activity are important for sink initiation and expansion and their activity is limited to the earlier phases of plant development. During initiation of storage compound accumulation, SuSy is the predominant source of sucrolytic activity (Koch, 2004), probably increasing the sink strength of the organ via a cycle of sucrose cleavage and resynthesis by SPS followed by transport into the vacuole (Hesse & Willmitzer, 1996). Both the studies of Berghall et al. (1997) and Klotz & Finger (2002) are in general accordance and show acid invertase activity only in the earlier phases of development, where they are accompanied by the highest relative growth rates. Silvius & Snyder (1979) showed, that acid invertase activity is localized differentially inside the taproot. High invertase activity is found at the

periphery of the taproot, where it influences the partitioning of photoassimilates between the taproot and the surrounding fibrous roots. As soon as the invertase activity drops, the cells start to accumulate sucrose and the amount of free hexoses is strongly reduced. During this time, SuSy is the main sucrose cleaving enzyme, with only a minor contribution of alkaline invertases. When the taproot reaches the mature phase, SuSy activity also decreases, probably reflecting the diminished energy demand of the plant (Klotz & Finger, 2002; Haagenson et al., 2006).

A distinctly different route for the sucrose accumulation in sugar beet is postulated in the study by Fieuws & Willenbrink (1990). The authors describe an preferential uptake of glucose via an H^+-glucose symport mechanism into isolated protoplasts from taproots and an additional uptake mechanism for fructose. Furthermore, considerable CWI and VI activity was found in the conducting and storage tissues, as well as SPS activity, which is required for the resynthesis of sucrose inside the cytosol. The proposed model emphasizes the apoplastic cleavage of sucrose and subsequent uptake of hexoses for the maintenance of a concentration gradient between the phloem and the surrounding parenchymous tissue resembling the mechanism described in sugar cane.

SuSy and invertase isoforms in *Beta vulgaris*

Despite the limited amount of sequence data available for sugar beet, sequences of several sugar beet isoforms of sucrose synthase and invertases are available in public databases. Two SuSy isoforms are described, designated as SBSS1 (sugar beet sucrose synthase 1, Genbank acc. X81974) and SBSS2 (AY457173). Hesse & Willmitzer (1996) described predominant expression of the SBSS1 mRNA in taproots. Cold and anaerobiosis induce its expression, whereas wounding of taproot slices leads to a repression. SBSS2 is also expressed mainly in root tissues and when both isoforms are compared, SBSS2 seems to be more strongly expressed in earlier phases of taproot development compared to SBSS1. The changes in transcript level are reflected on the protein level with significant delay, indicating posttranscriptional regulatory mechanisms (Haagenson et al., 2006).

Two CWI (BvCWI1=Bin35,AJ278531; BvCWI2=Bin46, X81797 and AJ277969) and two VI (BvVI1, AJ277457[2] and BvVI2=Bin44, X81796) isoforms have been described for sugar beet (Rosenkranz et al., 2001; Gonzalez et al., 2005). The cDNA denoted BvINV-V3 in Gonzalez et al. (2005) of a vacuolar isoform is highly homologous to the BvVI2 cDNA and therefore is probably an alternative allele of the same gene. Only few studies analyzed the isoform specific expression of these genes. In accordance with the observation of low invertase activities in the developing taproot, Gonzalez et al. (2005) found only weak expression of VI and CWI in the taproot tissue. For both VI isoforms they found a light dependent expression in leaf petioles, which was also reflected on the activity level and decreased with ageing of the leaves, indicating a possible role for the vacuolar invertases in assimilate partitioning.

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[2]falsely annotated as BvVI1 in the Genbank database

Invertase inhibitors in sugar beet

After first descriptions in other crop plants (see 2.7.1 on page 16), Pressey (1968) described the isolation of an invertase inhibitor protein with a molecular weight of 18.1 kDa from sugar beet. The inhibitor protein is active against soluble invertase preparations from potato, sweet potato and red beet. The inactivation of invertases is pH-dependent, showing a maximum inhibition at pH 4.5.

2.8.3 Postharvest sucrose losses during sugar beet storage

One major part of the BREATH-LESS GABI project (see 2.10) was aimed at the identification of processes influencing the amount of sucrose loss during storage of sugar beets before processing. In order to increase the profitability of sugar production, older sugar-producing factories are being closed and modern factories with a higher processing capability are preferentially developed. In order to amortize the investments in these new factories, a prolongation of campaign length is desirable. This implies the need for longer storage of sugar beets before processing. After harvest, beets are usually stored in covered piles and should be protected from severe frost, since subsequent thawing will lead to severe problems in processing due to rotting of the beets. During this period, the plants use up part of the stored sucrose, which leads to undesirable losses in yield. Earlier studies showed, that between different sugar beet breeding lines distinct differences exist in the amount of sucrose loss during that period and that genetic dispositions are attributable for this difference (Wyse et al., 1978). Most of these losses depend on the respiratory consumption of the stored sucrose (Vukov & Hangyal, 1985).

The loss due to respiration after harvest can be subdivided into two processes: reaction to mechanical wounding and basic respiration. The wounding of sugar beet during harvest is an inevitable side-effect of mechanical harvesting, mainly due to automatical removal of the leaves by decapitation. The major loss in the first days after harvest is due to the hydrolysis of sucrose and the subsequent wound reaction, which consumes energy and especially carbon skeletons for the wound closure (Campbell & Klotz, 2006). The second process is the predominant source of sucrose loss during prolonged storage, when the plant respires its sugar reserves to maintain vitality.

For both processes the mobilization of sucrose from its place of storage inside the vacuole is a key regulatory step. During most of the taproot development, sucrose synthase is the main source of sucrolytic activity. However, it is unclear whether SuSy is capable of accessing the sucrose pool inside the vacuole or if it only cleaves sucrose entering the cell from outside. In red beet, Etxeberria & Gonzalez (2003) described the possibility of ATP-stimulated efflux of sucrose across the tonoplast and the subsequent cleavage by a tonoplast associated form of SuSy, implying an invertase-independent efflux mechanism via a sucrose-transporter in the tonoplast. A further route of sucrose export is described by one of the authors proposing

the possibility of direct sucrose secretion via vesicles budding from the vacuole and fusing with the plasmalemma (Echeverria, 2000). Both mechanism have so far only been described for the mobilizing red beet hypocotyl parenchyma cells during sprouting and may be a very specific mechanism during this developmental process.

The induction of vacuolar invertases has an important function for the mobilization of sucrose from the vacuole, both during the wound reaction and also during basic respiration. Wyse (1974) and Berghall et al. (1997) both observed a decrease in SuSy activity and an increase of invertase activity during storage experiments, which was paralleled by an increase in reducing sugar content. During wounding of sugar beets, a remarkable increase in reducing sugars is observed. Rosenkranz et al. (2001) showed, that the induction of BvCWI1 precedes the induction of the BvVI1 isoform. The induction of BvVI1 however is accompanied by the largest generation of hexoses, identifying it as the major isoform contributing to sucrose losses after wounding. After cleavage, the generated hexoses are exported from the vacuole via hexose-carriers and can be metabolized inside the cytosol (Rausch, 1991). The large accumulation of hexoses after wounding indicates, that not all of the cleaved sucrose is later metabolized. Therefore down-regulation of this process should not be detrimental to the performance of the plant, making BvVI1 an appealing target for biotechnological manipulation.

2.9 Proteins regulating the efficiency of respiration

The respiration rate of non-photosynthesizing tissues like stored sugar beet taproots might not only be controlled by the re-mobilization of sucrose and its subsequent catabolism via glycolysis and the citric acid cycle, but can also be influenced by the efficiency of the reactions in the respiratory chain. Plants possess two type of proteins, alternative oxidases (AOX) and uncoupling proteins (UCP), which influence the coupling ratio of mitochondria. Both enzymes dissipate the energy of the electron transport chain without the formation of a proton motif force, thereby uncoupling the action of the electron transport chain from the ATP-synthase activity, leading to a decrease in ATP synthesis (Vercesi et al., 2006). Although both, AOX and UCP, have the same principal function, namely preventing an over-reduction of the mitochondrial respiratory chain and minimizing formation of reactive oxygen species (ROS), they are unrelated proteins with entirely different mechanisms.

UCPs catalyze the non-energetic transfer of protons

UCPs are integral membrane proteins of about 30 - 33 kDa residing in the inner mitochondrial membrane. They are also referred as PUMP (plant uncoupling mitochondrial proteins) and catalyze a free fatty acid activated transfer of protons from the intermembrane space back into the mitochondrial matrix, thereby decreasing the generated protonmotive force (PMF) without ATP synthesis. The activation by fatty acid is due to the importance of these

for the UCP mechanism for which two models have been proposed (Vercesi et al., 2006). In one model, UCPs catalyze the transfer of anionic fatty acids from the mitochondrial matrix through the membrane and their spontaneous back-transfer after protonation in the intermembrane space. Alternatively, the fatty acids could serve as a prosthetic groups of the UCP without being transported through the membrane.

Originally UCPs were characterized in brown adipose tissue of newborn and hibernating mammals, where they are involved in non-shivering thermogenesis. In plants they were first discovered in potato mitochondria (Vercesi et al., 1995) and *A. thaliana* (Maia et al., 1998). With the finding of UCP homologs in various other animal and fungal species, it became evident that UCPs are widely distributed in eukaryotes (Borecky et al., 2006; Vercesi et al., 2006 and ref. therein).

The AOX protein is a non-protonmotive terminal oxidase

The alternative oxidase (AOX) is an additional terminal oxidase, which catalyzes the reduction of oxygen to water without the concomitant proton transfer observed during the action of the cytochrome c oxidase. The electrons are taken from the ubiquinone pool of the electron transfer chain. In contrast to cytochrome c oxidase, AOX activity is not inhibited by cyanide. Initially, the AOX protein was purified and characterized in the thermogenic Arum species *Sauromatum guttatum* (Elthon & McIntosh, 1986) and *Arum maculatum* (Bonner et al., 1986). Later AOX homologs were found in all higher plants, fungi, non-fermentative yeasts and trypanosomes (Moore et al., 2002), indicating further functions apart from thermogenesis. The AOX protein is not membrane spanning and occurs as a dimer, which can either be non-covalently linked (reduced) or covalently linked via a disulfide bridge (oxidized form). The oxidized form is less active than the reduced form, posing a potential regulatory mechanism (Vanlerberghe & McIntosh, 1997). The existence of the inactive form of the AOX protein is however questioned and might only arise during extraction (Millenaar & Lambers, 2002).

The activity of the AOX protein is stimulated by α-keto acids like pyruvate and the reduction state of the ubiquinone pool, probably reflecting its role as an overflow mechanism during conditions of limited activity of the cytochrome pathway to allow continuous activity of the TCA cycle and to reduce the production of reactive oxygen species (Moore et al., 2002). The AOX protein is induced upon various stresses, e.g. cyanide-addition, chilling, wounding and pathogen attack and is often expressed in a tissue specific manner, with varying responses of the members of the AOX multigene family (Saisho et al., 1997; McCabe et al., 1998).

AOX and UCP have a similar physiological function but are not redundant

Both, AOX and UCP, serve as energy dissipating systems in plants, that are able to uncouple the reduction of oxygen from oxidative phosphorylation and lead to a decrease in ATP synthesis. Although under certain conditions, like cold storage of potato tubers (Calegario et al., 2003), both activities are induced, the differential expression patterns of the individual members of the respective gene families points out, that the activities are not merely redundant, but have different physiological functions (Vercesi et al., 2006). Expression analysis of all putative *A. thaliana* AOX and PUMP genes during chilling revealed, that each isoform shows a specific regulation, often in opposition to other members with the same presumed function (Borecky et al., 2006), indicating the involvement of multiple regulatory mechanisms. Differences in the regulation of individual isoforms show, that the physiological functions of the individual genes might well be variable *in planta*. Both enzymes are implicated in the protection against oxidative stress, but they are stimulated by different factors (**UCP**: high PMF, ROS; **AOX**: high O_2 concentration, excess of reduced ubiquinone). Furthermore both are able to allow continuous action of the TCA cycle during conditions, when either ADP supply or ATP consumption are limited, which can occur during conditions of high biosynthetic demand for the carbon skeletons produced in the TCA cycle concomitantly to reduced energy consumption.

Due to the energy wasting effect of both pathways, fine-control of their action can influence the rate of breakdown of storage products during post-harvest storage of crops and variations in the expression and activity of both genes might influence the amount of economic losses during inevitable storage periods.

2.10 Research objective

This thesis was prepared as part of the joint project *"BREATH-LESS GABI: Molecular physiology of storage organs: integrating genetics and genomics to reduce sucrose respiration losses during storage of sugar beet"* between the KWS SAAT AG (Einbeck), Südzucker AG (Mannheim) and the University of Heidelberg. The main aim of this project was the evaluation of sucrose losses during post-harvest storage of sugar beet and the characterization of underlying genetic mechanisms. In this thesis, the biotechnological part of the project was carried out and fundamental research about the regulation of sucrolytic enzymes during taproot development was undertaken.

Regulation of sugar beet respiration during wounding and storage
For the analysis of sugar beet lines differing in post-harvest sucrose losses, possible target genes regulating the efficiency of respiration should be identified. Focus was directed on uncoupling proteins and alternative oxidase isoforms. The presence of those genes was investigated and then their expression during taproot development was analyzed, emphasizing

possible regulations during wounding and storage of taproots.

Biotechnological approaches

In order to decrease sucrose losses due to the induction of vacuolar invertases after wounding, transgenic sugar beet plants were generated, which constitutively express a vacuolar invertase inhibitor from tobacco. These sugar beet plants were analyzed based on the following criteria:

- Which effects does the heterologously expressed inhibitor have on the wound-induced invertase?

- Does the inhibitor lead to a decrease in sucrose loss during wounding?

- Is the overall performance of the plants altered?

Mechanisms for sucrose stabilization in sugar beet

Previous studies indicated the presence of invertase inhibitors in sugar beet taproots. At the beginning of this thesis a sequence with homologies to invertase inhibitors from other plants was available (BvC/VIF1). In order to investigate, if BvC/VIF1 is involved in regulation of invertase activity during taproot development, the following questions were addressed:

- Does the BvC/VIF1 sequence code for an invertase inhibitor?

- Where and when during plant development is the inhibitor expressed?

- Where in the cell is the inhibitor localized?

- Which are the target proteins for the inhibitor and how is the interaction regulated?

- Is the inhibitor a potential target gene for biotechnological approaches?

Furthermore, by producing recombinant invertase protein, the interaction between invertases and their inhibitory proteins should be studied in detail.

3 Results

3.1 The life cycle of *Beta vulgaris* shows a distinct regulation of sucrolytic enzymes

3.1.1 Expression of invertase isoforms

During the development of the sugar beet taproot high amounts of sucrose are accumulated and stored in the vacuole. Since sucrose is cleaved by acid invertases, localized either in the cell wall or inside the vacuole, in an irreversible reaction, these enzymes have to be tightly controlled during plant development. Figure 3.2 shows the expression analysis of three acid invertase isoforms from sugar beet. Whereas the cell wall invertase BvCWI1 is only present in six week old roots and after wounding, BvVI1 is strongly expressed also in floral and leaf tissues. The second vacuolar invertase isoform BvVI2 is only detected in floral tissues. For a second CWI isoform (BvCWI2) no transcript was detected in the analyzed developmental stages. From week six to eight after germination, depending on the growth conditions, the storage phase of the taproot begins (Berghall et al., 1997; Klotz & Finger, 2002) and no acid invertases can be detected in the main part of the taproot tissue. Only in the cortex of the taproot, which is exposed to the soil, a weak expression of BvVI1 can be detected (see Fig. 3.1 for sample sources of taproot cross-section).

3.1.2 Expression of sucrose synthase isoforms

Figure 3.3 shows the expression of the two sucrose synthase isoforms SBSS1 and SBSS2 described for sugar beet (Hesse & Willmitzer, 1996; Haagenson et al., 2006). The sucrose

Figure 3.1: Cross-section of *Beta vulgaris* taproot indicating the position of samples taken
The picture shows a cross-section of a typical taproot used for sampling. Clearly visible are the concentric rings of vascular tissues (phloem and xylem). Indicated are the positions where the samples for the Northern blots in the following figures are taken from.

Figure 3.2: Transcript analysis of the cell wall invertase BvCWI1 and the vacuolar invertases BvVI1 and BvVI2.
In each lane, approximately 15 µg total RNA was loaded and after blotting hybridized with biotinylated probes against the indicated genes. The taproot cross-section and the wounding stages came from 28 week old, harvested taproots. Above each film panel, the EtBr stained gel bands of the 28S rRNA are shown as a loading control.

synthase genes show a complementary expression pattern to the acid invertases in figure 3.2. Both isoforms are expressed in the later phases of the taproot development and can be detected across the complete cross-section of the mature taproot. SBSS2 seems to have a much higher expression level than SBSS1, since the exposure time of the northern blot film was significantly shorter (2 min in contrast to 3 d exposure for SBSS2 and SBSS1 respectively). After wounding, SBSS1 seems to be further down regulated, whereas SBSS2 is slightly induced.

3.1.3 Induction of acid invertases after wounding leads to major sucrose losses

As described in Rosenkranz et al. (2001), wounding of taproot tissues leads to an induction of acid invertases. This leads to a major breakdown of sucrose and to the accumulation of hexoses, especially glucose (Figure 3.4.B). Although the increase of cell wall invertase activity precedes the increase in vacuolar invertase activity, major sucrose losses appear only after two to three days, when the soluble invertase activity exceeds cell wall bound activity. This is also reflected in the protein levels detected by western blot analysis (Figure 3.4.C), where the amount of vacuolar invertase protein has its maximum at three and five days after wounding. Only at these time-points the typical fragments of the vacuolar invertase

Figure 3.3: Northern blot of two sucrose synthase isoforms from sugar beet (SBSS1 and SBSS2).
In each lane, approximately 15 µg total RNA was loaded and after blotting hybridized with a biotinylated probe. The taproot cross-section and the wounding stages came from 28 week old, harvested taproots. Above each film panel, the EtBr stained gel bands of the 28S rRNA are shown as a loading control. The exposure time of the film was 3 days for SBSS1 and 2 minutes for SBSS2.

at 30 and 45 kDa can be detected. On transcript level, the isoforms BvVI1 and BvCWI1 are already detected 1 d after wounding (Fig. 3.2). Earlier results however showed, that BvCWI1 transcript is already present 10 h after wounding, whereas BvVI1 is only detected beginning from 1 d after wounding (Rosenkranz et al., 2001). Taken together, these data emphasize the importance of the VI for sucrose degradation, which is further supported by the colocalization of the invertase with most of the stored sucrose in the vacuole.

Figure 3.4: Wound induced CWI and VI activity leads to sucrose degradation in taproots

A: Invertase activity of the soluble and the combined salt-eluted and cell wall pellet fractions, measured at pH 4.6. Error bars indicate the standard deviation of triplicates. **B:** Sucrose hydrolysis and accumulation of glucose and fructose after wounding. Soluble sugars were extracted in 80% ethanol and measured using a coupled enzymatic assay. Error bars indicate the standard deviation of triplicates. **C:** Vacuolar and cell wall invertases are induced in taproot tissues by wounding. Soluble (VI) and cell wall bound (CWI) protein extracts from taproot tissues were detected with the antiserum raised against BvVI1 and NtCWI. Soluble protein extracts were acetone precipitated and 20 μg protein was loaded. For CWI the cell wall pellet was boiled in SDS sample buffer and the equivalent of 40 mg fresh weight was loaded.

3.2 Occurrence of proteins regulating respiration efficiency

This thesis was prepared as part of the so-called "BREATH-LESS GABI" project, a joint project between the industrial partners Südzucker AG (Mannheim), KWS AG (Einbeck) and the university of Heidelberg. One aim of this project was to identify sugar beet breeding lines differing in their post-harvest sucrose loss. The post-harvest respiration of these breeding lines was analyzed by Südzucker and KWS with the aim to identify high and low respiring lines. After the identification of lines with significant differences, those lines can be further characterized with respect to differences in the expression or activity of potential target genes influencing the respiration rate of the stored plants. Apart from sucrose-hydrolyzing enzymes, which are important for the provision of carbon sources, the role of proteins influencing the efficiency of the respiratory chain in mitochondria was investigated.

After harvest, the taproots first show a strong increase in respiration due to the wound reaction in bruised parts of the taproot. After this initial increase, the respiration rate declines again to a relatively stable level (K. Harms, Südzucker, personal communication). In order to test the influence of the wounding process, the oxygen consumption of taproot slices was measured at different time-points (Fig. 3.5). All samples show a strong increase after wounding with the maximum activity 24 hours after wounding. Later the oxygen consumption decreases and stabilizes at three to five days after wounding.
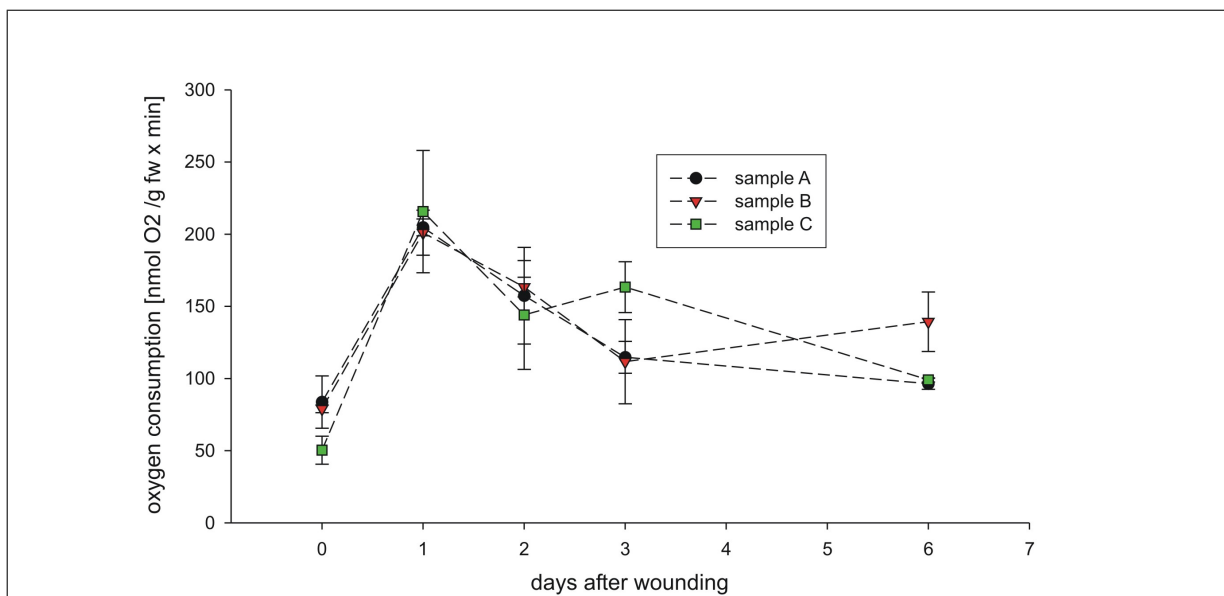
Figure 3.5: Oxygen consumption of three taproot samples at different time points after wounding.
Oxygen consumption was measured electrochemically using Clark-type electrodes. Each value is the mean of three repetitive measurements, error bars indicate the standard deviation.

3.2.1 Expression of uncoupling proteins and alternative oxidases in sugar beet

In order to investigate the presence of proteins regulating the respiration efficiency in sugar beet, cDNAs for two putative uncoupling proteins (BvUCP1 and BvUCP2) and an isoform of alternative oxidase (BvAOX1) were identified in an EST database. The matching cDNA-clones were sequenced and analyzed. For the UCP isoforms, both cDNA clones contained a putative full-length open reading frame, whereas the AOX isoform was incomplete. Therefore the missing cDNA ends were determined by 5' and 3' RACE using a cDNA prepared from unwounded taproots. The sequence data for the genes can be found in the appendix (see page B).

Northern blot experiments were carried out with various tissues from *Beta vulgaris*. The expression analysis for BvUCP2 is shown in figure 3.6. BvUCP2 shows an evenly expression in all tissues tested, wounding does not seem to alter the expression of the uncoupling protein. For BvUCP1 only very weak signals, which also were present in all tested tissues, were detected in the Northern blot (data not shown).

With the probe against the alternative oxidase isoform BvAOX1 no signals were detected in the Northern blot. In order to test whether the protein is present in *Beta vulgaris* mitochondria, Western blots were carried out using an monoclonal antiserum raised against the alternative oxidase of *Sauromatum guttatum* (Elthon et al., 1989). Due to the high conservation of AOX proteins between different plant species, this antiserum has been used successfully in a variety of species (McDonald et al., 2002). Mitochondria from leaf, wounded and unwounded taproot were isolated and used for Western blotting. Figure 3.7 shows, that in all tested tissues at least one band approximately 30 kDa in size appears. At one and three days after wounding a distinct second band is detectable, probably representing a second AOX isoform, which is upregulated by wounding. At six days after wounding, this second isoform is not detected anymore. In the last lane a mitochondrial extract from a taproot, which had been stored for more than a year in a cold room at 4°C is loaded. This sample shows a very strong signal, that is also composed of more than one protein band. In most of the lanes a second signal appears at approximately 65 kDa, most likely due to the presence of covalently linked AOX protein dimers. It is well known that AOX activity is regulated by dimerization via an intermolecular disulfide bridge (Vanlerberghe & McIntosh, 1997). This disulfide bridge is usually broken under the reducing conditions used for the SDS-gels, however here it might have remained partially intact by incomplete reduction of the sample. In further western blotting experiments the intensity of the dimer band increases strongly, when the protein samples are loaded without a reducing agent onto SDS-gels (data not shown).

Figure 3.6: Transcript analysis of the mitochondrial uncoupling protein BvUCP2
In each lane, approximately 15 μg total RNA was loaded and after blotting hybridized with a biotinylated probe. Above the film panel, the EtBr stained gel bands of the 28S rRNA are shown as a loading control.

Figure 3.7: Detection of AOX isoforms in different tissues of *Beta vulgaris*
Mitochondria were purified and in each lane 20 μg mitochondrial protein was loaded. AOX proteins were detected with the monoclonal antiserum against the *Sauromatum guttatum* AOX protein.

3.3 Overexpression of the tobacco vacuolar invertase inhibitor NtVIF in sugar beet

In order to suppress sucrose losses caused by the induction of vacuolar invertases after wounding, transgenic sugar beets were generated overexpressing the vacuolar invertase inhibitor NtVIF from tobacco. Previously it was shown in potato, that ectopic overexpression of this inhibitor protein drastically reduced the production of hexoses during cold storage (Greiner et al., 1999).

Transgenic sugar beet plants were generated in cooperation with KWS. For transformation, the same binary vector construct as in Greiner et al. (1999) was used, featuring the tobacco NtVIF cDNA expressed under the control of a CaMV 35S promoter, which should lead to strong expression in all vegetative tissues. In addition, the vector confers kanamycin resistance to the transformed plants.

3.3.1 In adventitious roots, no clear effect of the NtVIF transgene is observed

After the petiol transformation using agrobacteria, plantlets were regenerated in sterile culture. At this stage, individuals can be propagated leading to several clonal plants of each line. After the plantlets are transferred to soil, they develop a large adventitious root, which accumulates comparable amounts of sucrose as seed-grown taproots. They also posses the typical concentric arrangement of conducting tissues found in sugar beet taproots.

Initially 19 individual transgenic lines were produced by KWS. Thirteen lines belonged to the genotype 8T0015 and six lines were transformed in genotype 6B2840. After a first round of analyses, three lines from genotype 8T0015 were chosen for further characterization. These lines (designated 2-72, 4-39 and 4-74) showed a strong expression of the transgene and a reduction in vacuolar invertases activity after wounding. These results however were, due to the lack of more plant material, based on only three individual plants per line. The number of T-DNA insertions in the individual lines was determined via Southern blotting using a NtVIF specific probe. Line 2-72 carried three, line 4-39 one and line 4-74 two DNA-insertions (data not shown). None of the six lines from genotype 6B2840 showed any observable effect of the transgene compared to the wildtype of the same genotype and these lines were omitted from further analyses.

Figure 3.8 shows the expression of the NtVIF transcript in different individuals of the three chosen lines. The RNA was isolated from adventitious root tissue, which had been wounded for three days. It is noteworthy, that although the used CaMV 35S promoter should not respond to environmental stresses like wounding, a clear induction of the NtVIF transcript was observed when unwounded and wounded root materials were compared (Figure 3.9).

Figure 3.10.A and B show the weight of the adventitious roots at harvest and the activity of
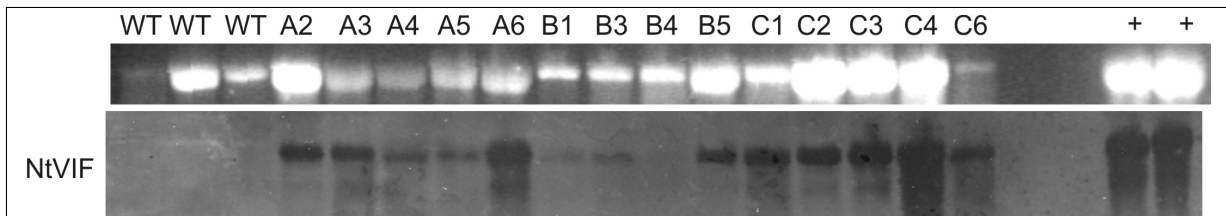
Figure 3.8: Detection of NtVIF transcript in wounded adventitious roots of primary transformants
In each lane, approximately 15 µg total RNA from wounded taproot tissue was loaded and hybridized with a biotinylated probe against NtVIF. Above the film panel, the EtBr stained bands of the 28S rRNA are shown as a loading control. RNAs from three wildtype (WT) are loaded together with RNAs of four to five individuals from each line (A (2-72), B (4-39) and C (4-74)). + indicates earlier tested overexpressing plants.
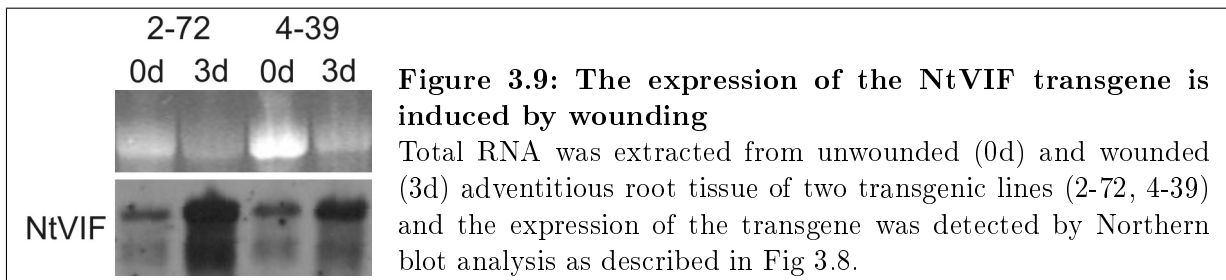
Figure 3.9: The expression of the NtVIF transgene is induced by wounding
Total RNA was extracted from unwounded (0d) and wounded (3d) adventitious root tissue of two transgenic lines (2-72, 4-39) and the expression of the transgene was detected by Northern blot analysis as described in Fig 3.8.

the vacuolar invertase three days after wounding. In Figure 3.10.C the sucrose concentration before and after wounding in the same experiment are shown. None of the three lines showed a significant decrease neither in invertase activity, nor in the loss of sucrose after wounding. Line A and C show a small decrease in invertase activity and sucrose loss, but due to the high variation between individual plants no significant effect is present.

Figure 3.10: Weight of adventitious roots at harvest, activity of vacuolar invertase after wounding, sucrose concentrations and calculated sucrose losses in adventitious root tissues A: Weight of roots at harvest. **B:** Soluble invertase activity of transformants and wildtype three days after wounding. Invertase activity was measured after acetone precipitation of the soluble fraction. **C:** Sucrose concentration before (0d) and after (3d) wounding of wildtype and transformants were determined enzymatically after ethanolic extraction. The diamond symbols and the given values display the relative loss of sucrose three days after wounding.
2-72, 4-39, 4-74: transgenic lines; WT: wildtype Each value is the mean of ten plants (2-72, 4-39, WT) or five plants (4-74), error bars indicate the standard deviation.

3.3.2 Seed grown NtVIF overexpressing sugar beets show no reduction of wound induced VI activity

Since the previous results were only based on plants generated from *in vitro* culture, seeds of the three lines were produced and new experiments were carried out with seed grown taproots. These taproots were bigger and accumulated more sucrose compared to the previously analyzed adventitious roots (see Figure 3.11). The presence of the transgene was tested via Northern blot analysis and plants without expression of the transgene were omitted from further measurements. Again all three transgenic lines showed a smaller root size and no general reduction in wound induced invertase activity. Furthermore, the sucrose loss in the transgenic lines was higher than in the control line (data not shown).

Figure 3.11: Weight of seed grown taproots at harvest and activity of vacuolar invertase after wounding
A: Weight of taproots at harvest.
B: Soluble invertase activity of transformants (2-72, 4-39, 4-74) and wildtype (WT) three days after wounding. Invertase activity was measured after acetone precipitation of the soluble fraction of the wounded taproot tissues.
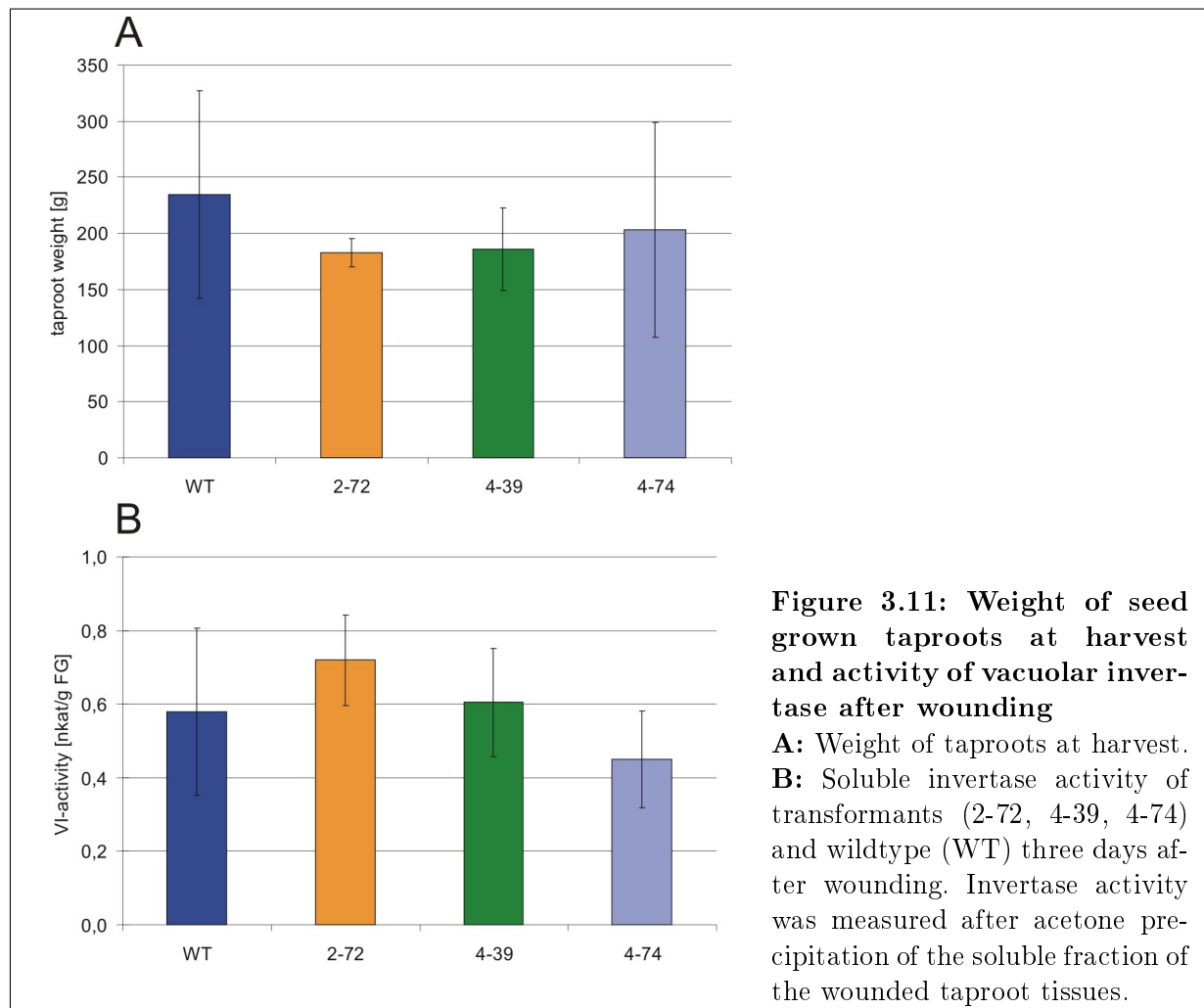
3.4 Recombinant overexpression and characterization of a sugar beet vacuolar invertase

Since the characterization and crystallization of the invertase inhibitor NtCIF from tobacco (Greiner et al., 1998; Hothorn et al., 2004b), it was possible to produce further proteins of the PMEI-RP protein family recombinantly in bacteria and to provide proof of their function as either invertase or PME inhibitors (Wolf et al., 2003; Link et al., 2004). One major obstacle for the more intense characterization of the interaction between the inhibitors of the PMEI-RP protein family and their target proteins was the lack of pure preparations of target proteins. Most of the functional characterization of the inhibitors was carried out with more or less crude plant extracts containing either PME or invertase activity. These extracts however can contain several isoforms of the target protein and often include endogenous inhibitor proteins bound to part of the tested target enzymes, thereby hampering the interpretation of the observed activity data. Only recently the heterologous production of invertases (Huang et al., 2003; De Coninck et al., 2005) and PMEs (Peng et al., 2005) was described in the eucaryotic expression host *Pichia pastoris*.

In order to produce pure invertase protein, several expression systems and invertase isoforms were tested until the expression of the *Beta vulgaris* vacuolar isoform BvVI1 in *E. coli* was successful. BvVI1 is the major vacuolar invertase isoform in *Beta vulgaris*, it is expressed in leaf tissue and young roots and its induction after wounding of taproots is responsible for a major part of the sucrose losses after harvest (see 3.1.1 and 3.1.3).

3.4.1 Overexpression and purification of the vacuolar invertase BvVI1 in *E. coli*

First experiments with the overexpression of the tobacco cell wall invertase NtCWI and BvVI1 in the methylotrophic yeast *Pichia pastoris* using the vector pPICZαA (Invitrogen) did not succeed in production of active invertase protein.

A new expression strategy for these proteins was initiated using the pETG-vector series[1] (EMBL, Heidelberg) in *E. coli*. These vectors are based on the pET vector system (Novagen), that uses the strong viral T_7 promoter in combination with the *E. coli* lac-operator (Dubendorff & Studier, 1991). Expression can be induced by the addition of IPTG or other galactosides. The pETG vectors contain attR recombination recognition sites, so that they can be used with Gateway-Technology (Invitrogen, Hartley et al., 2000) and are constructed to allow the expression of the gene of interest in frame with a variety of N-terminal fusion tags. It has been shown for several proteins, that the addition of these tags can promote solubility and proper folding of foreign proteins in *E. coli*. The used vectors and the results obtained with the tobacco and the sugar beet invertase are summarized in table 3.1. Whereas

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[1]http://www.embl.de/ExternalInfo/protein_unit/draft_frames/index.html

Vector	N-terminal Fusion tag	NtCWI expression	BvVI1 expression
pETG-10A	6xHis	-	+
pETG-20A	6xHis-TrxA	-	+
pETG-30A	6xHis-GST	-	+
pETG-50A	6xHis-DsbA	-	+
pETG-52A	6xHis-llDsbA	-	+
pETG-60A	6xHis-NusA	-	+

Table 3.1: Overview of the used expression vectors and the obtained results with the NtCWI and BvVI1 gene (6xHis: hexa-histidine tag; TrxA: ThioredoxinA-tag; GST: Glutathione-S-transferase-tag; DsbA: DsbA-tag; llDsbA: leaderless DsbA-tag, protein is not secreted into the periplasmic space; NusA: N utilization substance A-tag). No expression was observed with NtCWI, whereas all tested constructs with BvVI1 lead to overerexpressed protein.

BvVI1 was expressed in all vectors used, no overexpressed NtCWI protein was detected. In order to increase the amount of soluble protein, the expression was carried out after induction with 0.2 mM IPTG at 18 °C overnight. Also the addition of 2.5 % glucose to the growth medium led to higher yields of soluble protein, whereas addition of sucrose had no significant effect.

All constructs contain a 6xHis-tag allowing purification via immobilized metal affinity chromatography (IMAC) using Nickel resins. Since a recognition site for the TEV protease was incorporated into the constructs, all fusion tags can be removed after purification by treatment with recombinant TEV-protease (also containing a 6xHis-tag) and a subsequent second IMAC step. In this step, the fusion tag and the TEV-protease are removed (see scheme in Fig. 3.12.A). The recombinant invertase protein is now free of any additional tags and is collected in the flow-through of the second IMAC column. Figure 3.12.B gives a typical purification of the BvVI1 protein expressed in the pETG-30A vector leading to a GST-BvVI1 fusion protein. The pETG-30 vector was used, because it gave the highest amount of soluble fusion protein compared to the other pETG-vectors (not shown). After purification of the fusion protein, the GST-Tag is cleaved off together with the 6xHis-Tag using TEV-protease.

Figure 3.12: Overexpression and purification of BvVI1
A: Schematic diagram showing the functional domains of the expressed fusion proteins: His-Tag (6xHis), GST-tag (GST), recombination sites (attR1, attR2), TEV-cleavage site and the BvVI1-ORF (without signal peptide).
B: Typical purification of BvVI1-protein from bacteria with subsequent cleavage of the fusion protein by TEV protease. On the first column, the GST-BvVI1 fusion protein is purified from the bacterial extract and eluted (E1-E4, M: protein standard, T: total protein sol: soluble protein). After dialysis, the protein containing fractions (- TEV) are treated with TEV protease (+ TEV) and then passed over a second Nickel-column. The cleaved BvVI protein is retained in FT-2. After further washing (W I to W IV), the TEV-protease and the GST-tag (not visible) is eluted (E TEV).

3.4.2 Characterization of the recombinant BvVI1 protein

After purification, the BvVI1 protein was usually dialyzed into an acidic buffer for activity measurements. Invertase activity was determined in an enzymatical-optical test according to Weil & Rausch (1990), in which the released glucose is converted in a reaction coupled to the reduction of $NADP^+$. All reactions were carried out at 37℃. The activity of the recombinant protein showed a linear dependency on the incubation time and the amount of protein used (Figure 3.13). The enzyme showed a broad pH optimum with the maximum activity at pH 5.1 (Figure 3.14.A). In order to determine the K_M value of the enzyme, the reaction was carried out at different substrate concentrations (Figure 3.14.B). The recombinant invertase protein shows a typical Michaelis-Menten kinetic for the substrate sucrose. To determine the K_M value, the substrate concentration and the reaction velocity were plotted reciprocally in a Lineweaver-Burk diagram (Lineweaver & Burke, 1934). Linear regression analysis yielded a K_M value for sucrose of 3.7 mM. For comparison, the data were also plotted according to Hanes and Woolf (Hanes, 1932) and calculated via best fit curve to the Michaelis-Menten equation. With these methods a K_M value of 4.3 mM and 4.0 mM sucrose respectively was determined. The V_{max} value was calculated as 0.55 nkat per µg enzyme.

Figure 3.13: Time (A) and dose (B) dependency of the recombinant BvVI1 protein
In A, 1 pmol of BvVI1 protein were incubated in buffer (50 mM sodium acetate, 300 mM NaCl, pH 5) containing 33 mM sucrose and the reaction was stopped at the indicated time-points. In B, increasing amounts of BvVI1 were incubated in the same buffer and the reaction was stopped after 60 minutes.

Figure 3.14: pH dependency (A) and K_M determination (B) for recombinant BvVI1 protein
A: Relative BvVI1 activity at different pH. The activity at pH 5.1 was set as 100% B: K_M determination: the plot shows the reaction velocity (v_0) plotted against the used sucrose concentration [S]. The small diagram in the inset shows a Lineweaver-Burk plot of the obtained data.

3.5 BvC/VIF1 is a potent inhibitor of CWI and VI

The presence of proteinaceous inhibitors for invertases in the sugar beet taproot has been described already several decades ago (see 2.8.2). However, no sequence data of the gene coding for this inhibitor were available previously. By a BLAST homology search using the invertase inhibitor NtCIF as a template sequence, a cDNA clone with homologies to PMEI-RPs was found in a sugar beet EST library. The gene was named BvC/VIF1 for *Beta vulgaris* cell wall or vacuolar inhibitor of β-fructosidase, reflecting its homology to other invertase inhibitors. Since the PMEI-RPs characterized previously either code for inhibitors of PME or invertase, a functional characterization of the recombinant protein was carried out first.

3.5.1 Features of the BvC/VIF1 cDNA

An alignment of the predicted BvC/VIF1 protein and other functionally characterized inhibitors of PMEs and invertases is given in figure 3.15. Noticeable is the conservation of four cysteine residues, which have been shown to be important for the overall structure of the inhibitor proteins by stabilizing the structure of the N-terminal hairpin (by linking helices 1 and 2) and the bundle core (connection between helix 5 and 6) via two disulfide bridges (see Hothorn et al. (2004b) and figure 2.1 on page 19).

3.5.2 The BvC/VIF1 cDNA encodes an invertase inhibitor protein

In order to confirm, that the obtained BvC/VIF1 cDNA sequence indeed codes for an invertase inhibitor, the protein was overexpressed in a bacterial system. The cDNA without the predicted signal peptide was cloned into the vector pQE30 (Qiagen) and transformed into *E. coli*. The expression of the target gene in the pQE30-vector can be induced by the addition of IPTG and the protein is fused to a N-terminal 6xHis-tag. The *E. coli* expression host Rosetta-gami (Novagen) was used, which contains an oxidizing cytosol promoting the formation of disulfide bridges in order to facilitate the formation of correct folded inhibitor proteins. In order to increase the amount of soluble protein, the expression was carried out at 18 °C instead of 37 °C in TB-medium with the addition of one percent glucose to reduce leaky expression of the promoter in the uninduced state.

Figure 3.16.A shows a typical purification of the BvC/VIF1-protein. The elution fractions containing most of the BvC/VIF1 protein were combined and dialyzed into an acidic buffer. To test whether the purified protein contained disulfide bridges, it was analyzed by SDS-PAGE in the presence and absence of reducing agents (Fig. 3.16.B). The protein in the reduced form runs shortly above the 20 kDa marker band, whereas the corresponding unreduced protein band runs clearly below this band, indicating a more compact structure due to the presence of disulfide bridges. The unreduced sample contains an additional band at about 35 kDa, which is not present when the same sample is loaded in reduced state. This sec-
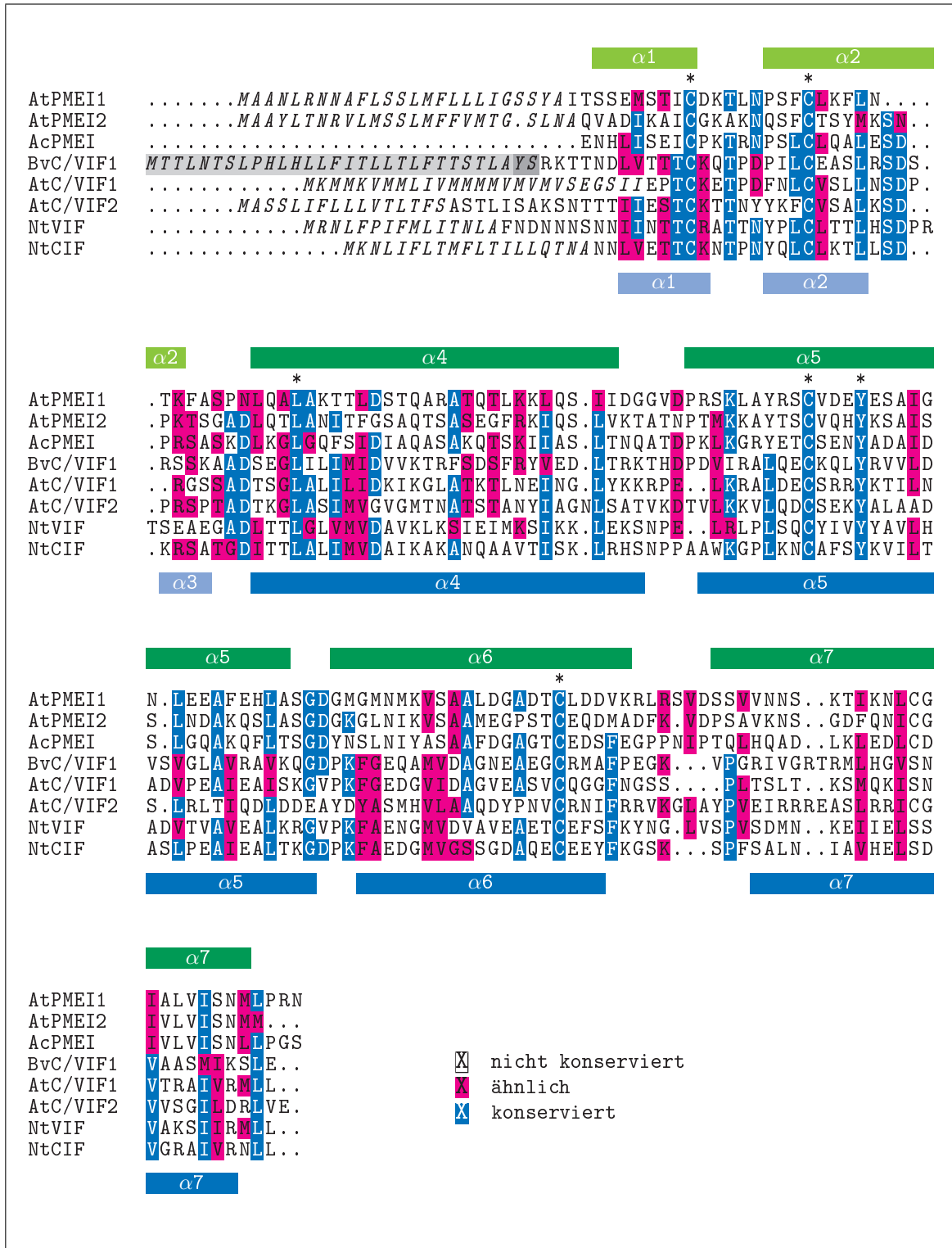
Figure 3.15: Alignment of PME and invertase inhibitor proteins
Alignment was carried with ClustalW (Thompson et al., 1994). Predicted signal peptides are shown in italics, for BvC/VIF1 the signal peptide is predicted to include either amino acids 1 to 28 or 1 to 30 (shaded in grey). Indicated are the α-helical parts of AtPMEI1 (first row) and NtCIF (last row) numbered according to Hothorn et al. (2004a,b). Residues conserved between all proteins are marked with an asterisk.

ond band probably consists of BvC/VIF1 dimers, which are covalently linked via a disulfide bridge. Further indications for this come from the results of size exclusion chromatography experiments (see 3.7.2).
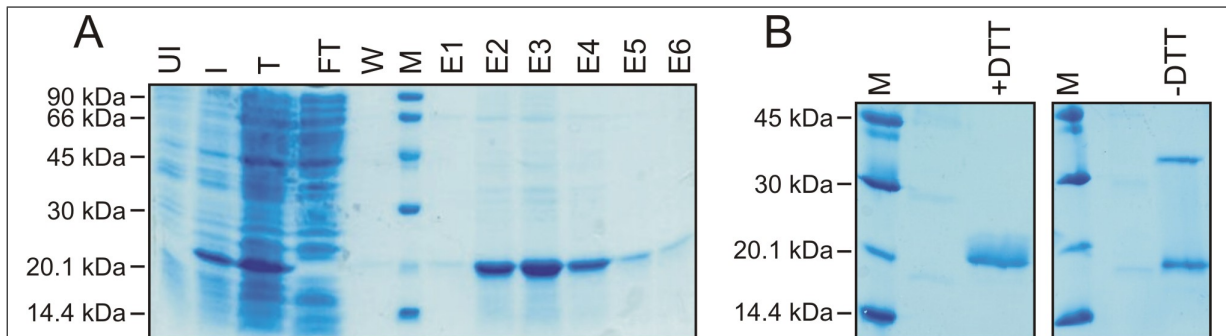
Figure 3.16: Overexpression of BvC/VIF1 in *E. coli* and purification via Nickel-affinity chromatography (A), and analysis of the reduction state of the recombinant protein (B).
A: The BvC/VIF1 protein (without the signal peptide) was overexpressed with a 6xHis-tag in the *E. coli* strain Rosetta-gami (Novagen) and purified using a Ni-NTA matrix (Qiagen). UI and I show the undinduced and induced bacterial samples, T the total protein after extraction. After centrifuging the soluble protein was bound to the Nickel matrix. FT shows the unbound bacterial proteins. After washing (W) the proteins were eluted in different fractions (E1-E6) with a buffer containing 250 mM imidazole (M, protein standard).
B: Recombinant BvC/VIF1 protein is present as monomer and dimer. Purified BvC/VIF1 protein was loaded onto a SDS-gel under reducing conditions (+DTT) and non reducing conditions (-DTT). Without a reducing agent, part of the recombinant protein runs at a molecular size of approximately 35 kDa, indicating the presence of dimers, which are linked via disulfide bridges. Note that the monomer fraction runs at a lower molecular weight than the reduced protein, indicating the presence of intramolecular disulfide bridges (M, protein standard).

Figure 3.17 shows a comparison of the inhibitory capacity of the recombinant BvC/VIF1 protein in comparison with the two described tobacco inhibitors NtCIF and NtVIF (Rausch & Greiner, 2004). Preparations of vacuolar and cell wall invertases from tobacco and sugar beet leaves, two recombinant vacuolar invertases (see section 3.4) and vacuolar invertase from wounded taproots were used as target proteins. The invertase inhibitor from sugar beet and NtCIF show a comparable inhibition of all vacuolar invertases and also a comparable inhibition of the CWI preparations. Increasing amounts of the respective inhibitor proteins led to a progressive reduction of invertase activity. However, both proteins do not inhibit the crude CWI preparations completely.

When comparing preparations of BvC/VIF1 and NtVIF, the vacuolar inhibitor from tobacco, only a weak inhibition of VI extracted from wounded *Beta vulgaris* taproots is observed with NtVIF, whereas BvC/VIF1 is able to inhibit the same preparation completely (Fig. 3.17.E,F). The NtVIF protein is however able to inhibit the invertase from tobacco leaves.
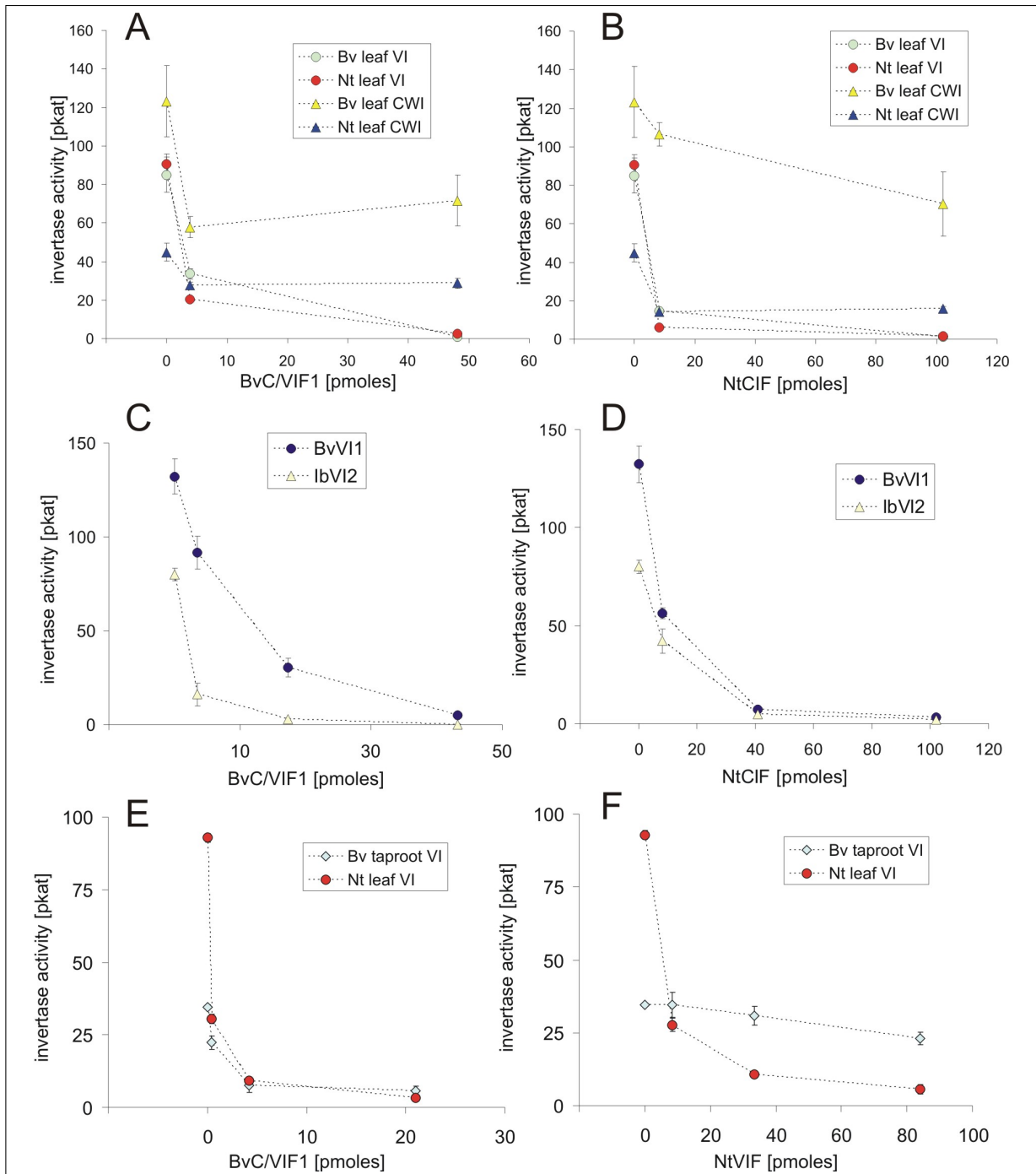
Figure 3.17: Inhibition of plant extracted and recombinant invertases by BvC/VIF1 and the cell wall and vacuolar invertase inhibitor from tobacco (NtCIF and NtVIF).
Extracts containing mainly vacuolar invertases from sugar beet leaves (A,B), wounded sugar beet taproots (E,F) or tobacco leaves (A,B,E,F) and the recombinant invertase IbVI2 and BvVI1 (C,D) were treated with different amounts of recombinant inhibitor proteins. BvC/VIF1 and NtCIF were purified in soluble form from the bacteria, whereas NtVIF was renatured from inclusion bodies. BvC/VIF1 and NtCIF completely inhibit all vacuolar invertase preparations tested. In contrast, NtVIF is only able to inhibit the VI from tobacco leaves, whereas the VI from wounded taproots is only weakly inhibited.

3.5.3 Genomic organization and expression analysis of the BvC/VIF1 gene

The presence of isoforms with a high degree of sequence homology to the BvC/VIF1 sequence was tested via Southern blotting. *Beta vulgaris* genomic DNA was isolated and digested with restriction enzymes and hybridized with a probe against the coding region of the BvC/VIF1 cDNA (Fig. 3.18). No other closely related isoform is detected, since only one band appears after EcoRI digestion, whereas two bands appear with the restriction enzymes BamHI and HindIII, each recognizing one cleavage site inside the sequence fragment detected by the BvC/VIF1 probe. Due to the low sequence conservation of invertase inhibitors in other plant species, the presence of additional inhibitor isoforms not detected under the washing conditions used, can however not be ruled out completely.

Figure 3.18: Southern blot analysis of BvC/VIF1-sequences in the sugar beet genome
In each lane 10 µg genomic DNA, which was digested with the indicated restriction enzymes, was loaded and hybridized with a 500 bp probe against the coding region of the BvC/VIF1 cDNA. Note that either BamHI and HindIII cut inside the region, which was amplified for the generation of the probe.
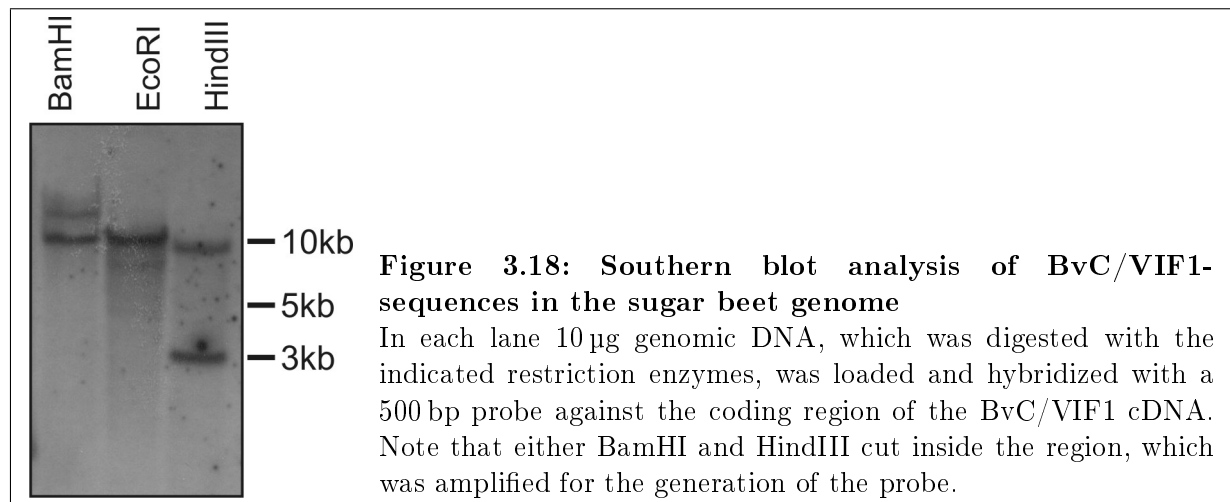
To gather more information about the importance of the BvC/VIF1 gene, a transcription profile was created by Northern blotting (see Fig. 3.19). The results indicate a strong expression of the gene in *Beta vulgaris* suspension culture and in taproot tissues. A weaker expression was detected in floral tissues. No BvC/VIF1 expression was found in leaves. In the developing taproot, BvC/VIF1 only shows a weak expression in six weeks old roots. In older taproot stages the expression increases, reaching its maximum at the end of the vegetation period, where a strong signal is present throughout the whole taproot cross-section. During formation of the taproot BvC/VIF1 shows an inverse regulation of expression compared to the acid invertases (Fig. 3.2 on page 30).

Surprisingly, during wounding of the taproot the expression of the inhibitor stays at a high level and even shows a slight increase during the procedure. During this physiological state the inhibitor and its putative target proteins are expressed in parallel.

Figure 3.19: Northern blot of the BvC/VIF1 gene.
In each lane, approximately 15 µg total RNA was loaded and after blotting hybridized with a
biotinylated probe. The taproot cross-section and the wounding stages came from 28 week old,
harvested taproots. Above the film panel, the EtBr stained bands of the 28S rRNA are shown
as a loading control.
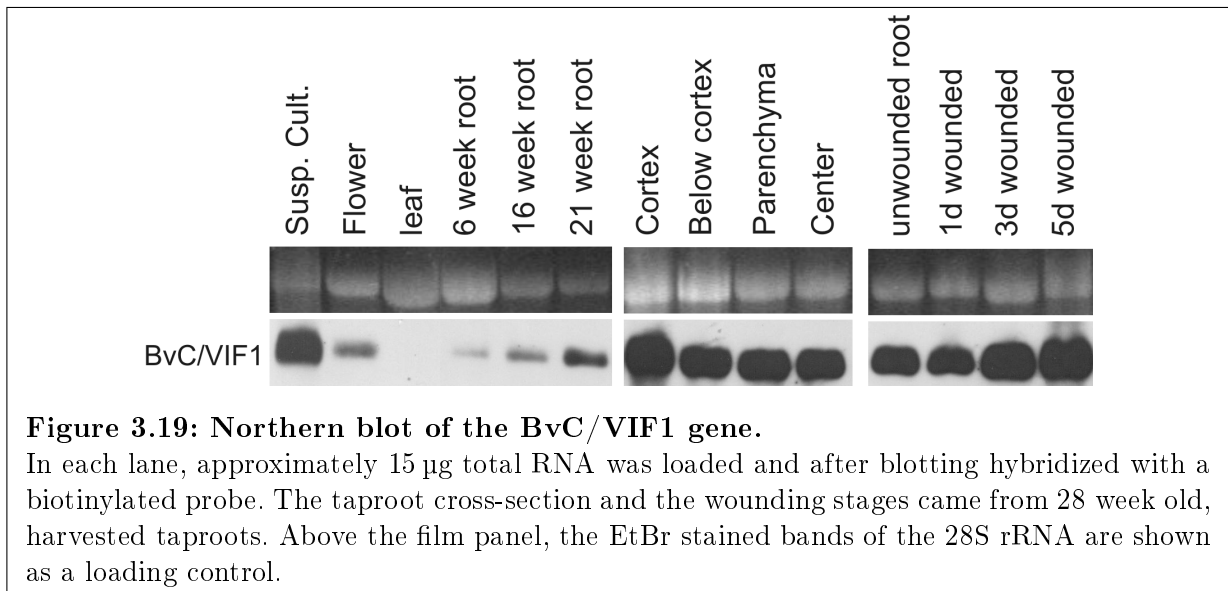
3.5.4 Subcellular localization of BvC/VIF1:GFP reportergene fusions

Invertase inhibitors have been described to be localized either in the apoplast or in the vac-
uole of plant cells (Rausch & Greiner, 2004). The subcellular localization of the inhibitor
proteins defines their target enzymes and since cell wall and vacuolar invertase have different
functions during plant development, the localization of the BvC/VIF1 protein has an im-
portant implication for its role during taproot development. Using the predicted BvC/VIF1
protein sequence as an input, bioinformatic tools for the prediction of transit peptides recog-
nize a N-terminal transit peptide for the co-translational entrance into the secretory pathway.
However, two possible cleavage sites are predicted by the individual programs. The program
PSort (Nakai & Horton, 1999 settings: plant sequence)[2] identifies the most likely cleavage
site after amino acid number 30 of the BvC/VIF1 sequence (YS-RK, see Fig. 3.15), whereas
SignalP3.0 (Bendtsen et al., 2004)[3] predicts the cleavage site to be after amino acid 28 (LA-
YS, see Fig. 3.20 for the graphical output of the prediction). The cleavage site after amino
acid 30 is however also recognized at a lower score.

In contrast to the well characterized structure of signal peptides for the co-translational
entrance into the ER, knowledge about vacuolar sorting signals in plants is limited. Although
several possible motifs have been identified, often, a prediction whether a protein is sorted
into the vacuole or is secreted into the apoplastic space can not be made based on sequence
data alone (Matsuoka & Neuhaus, 1999).

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[2]http://psort.nibb.ac.jp/
[3]http://www.cbs.dtu.dk/services/SignalP-3.0

Figure 3.20: Graphical output of the signal peptide prediction for the BvC/VIF1 protein sequence using SignalP3.0 (Bendtsen et al., 2004).
The program predicts the presence of signal peptidase I cleavage sites. In addition, for each amino acid it calculates the probability to be part of either the N-terminal, the central hydrophobic or the C-terminal region of a signal peptide.

Targeting of BvC/VIF1:GFP fusions to the vacuole

To elucidate the subcellular localization of the BvC/VIF1-protein, two constructs were generated for the expression of the BvC/VIF1-protein as an N-terminal fusion with the green fluorescent protein (GFP) under the control of the CaMV 35S promoter. One construct was generated in the vector pK7FWG2 (Karimi et al., 2002) using Gateway technology, the second in the vector pFF19-GFP (Wachter et al., 2005). pK7FWG2 is a binary vector, which can be used for plant transformation using agrobacteria. The second construct was generated, because the pFF19-vector is due to its smaller size better suited for usage in particle bombardments. Both constructs were used to transiently transform onion epidermal cells. Onion epidermis is a favorable system, since the epidermal cells are easily removed from the leaf and consist of large, non-colored cells, which can be analyzed by light microscopy. As a control, the cells were always cotransformed with a second construct leading to the localization of a red fluorescent protein (RFP) in either the cytosol or in plastids.

Figure 3.21 shows two transformed onion epidermal cells, in which the BvC/VIF1:GFP protein is localized inside the large central vacuole. The cell in Fig. 3.21.C to E has been partially plasmolyzed using 1M mannitol. The plasma membrane is partially detached from the cell wall and the central vacuole has separated into two smaller ones. The RFP control construct in B and E labels the plastids. These are restricted to the cytoplasmic layer surrounding the vacuole. Figure 3.22 shows again an epidermal cell analyzed using confocal laser scanning microscopy (CLSM), now transformed with the pFF19-BvC/VIF1:GFP construct. In the analyzed optical plane the clear differentiation between the cytosol labeled with a

RFP construct and the GFP labeled central vacuole can be observed.

It has to be noted however, that not all transformed cells showed a clear vacuolar local-
ization of the GFP-fluorescence, often only the ER of the cells was labeled with GFP (data
not shown).

In tobacco leaves, overexpressed BvC/VIF1:GFP fusion proteins are cleaved proteolytically

A different result was obtained, when the BvC/VIF1:GFP fusion construct was used to
transiently transform tobacco leaves by infiltration with agrobacteria (Fig. 3.23). Here the
GFP-fluorescence was localized in vesicular structures at the borders of the cells and no
vacuolar fluorescence was observed.

When differentially extracted protein samples from the infiltrated leaves were analyzed
with the BvC/VIF-antiserum (see ch. 3.5.5), two specific signals were observed. In the soluble
and the salt-eluted fraction, a protein was detected, which showed a similar size to the
BvC/VIF1 signals observed in taproots (e.g. Fig. 3.25). When the remaining cell wall material
was boiled in SDS-containing buffer, a signal of approximately 45 kDa was detected. Since
this is the size expected for the BvC/VIF1:GFP fusion protein, this protein is probably the
inhibitor-GFP fusion protein. This was proven by performing a Western blot with a GFP
antiserum with the same samples. Here only the putative fusion protein was detected, but
not the inhibitor alone. This indicates a proteolytic processing event, which separates the
GFP-tag from the inhibitor found in soluble extracts. When the invertase activity of the
soluble and the cell wall bound fraction was analyzed in these samples, a clear reduction of
the soluble invertase activity was observed, whereas the cell wall bound activity showed no
clear alteration compared to uninfiltrated plants.
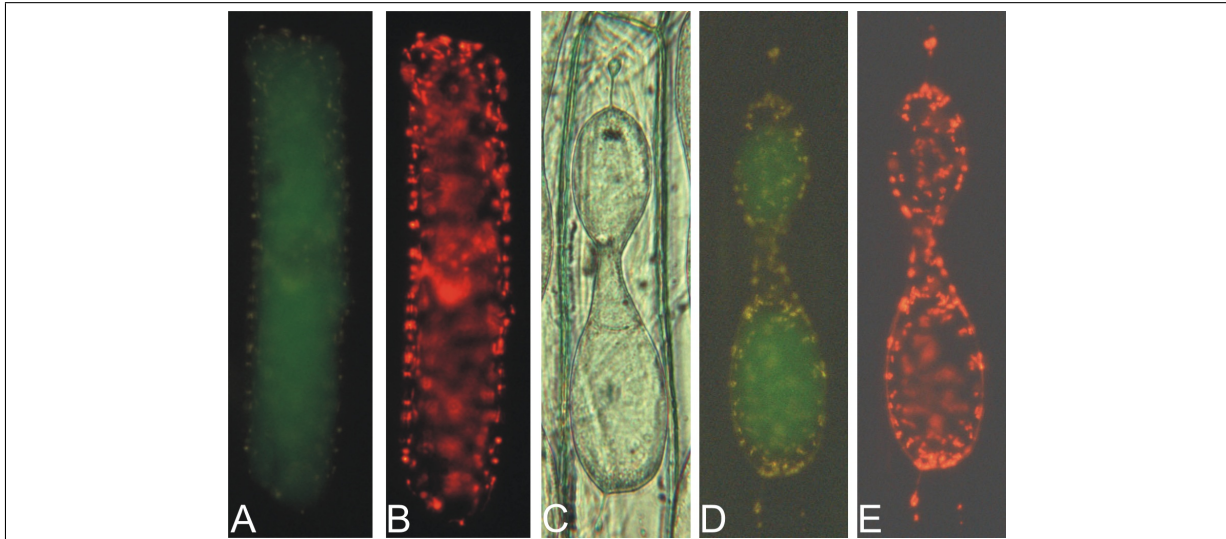
Figure 3.21: *In vivo* localization of the pK7FWG2-BvC/VIF1-encoded BvC/VIF1:GFP fusion protein in onion epidermal cells after transient transformation

Onion epidermal cells were transiently transformed by particle bombardment with the pK7FWG2-BvC/VIF1 construct and the pFF19-AtGSH1-TP:RFP (Wachter et al., 2005), which is exclusively localized in plastids, and analyzed by fluorescence microscopy. A and D show the GFP fluorescence of the BvC/VIF1:GFP construct in the central vacuole, whereas the RFP-labeled plastids are restricted to the small cytosolic layer at the boundary of the cell (B and E). The epidermal cell in C-E was partially plasmolyzed by the treatment with 1 M mannitol, which led to the constriction of a second vacuole. C shows the cell from D and E in transmitted light.
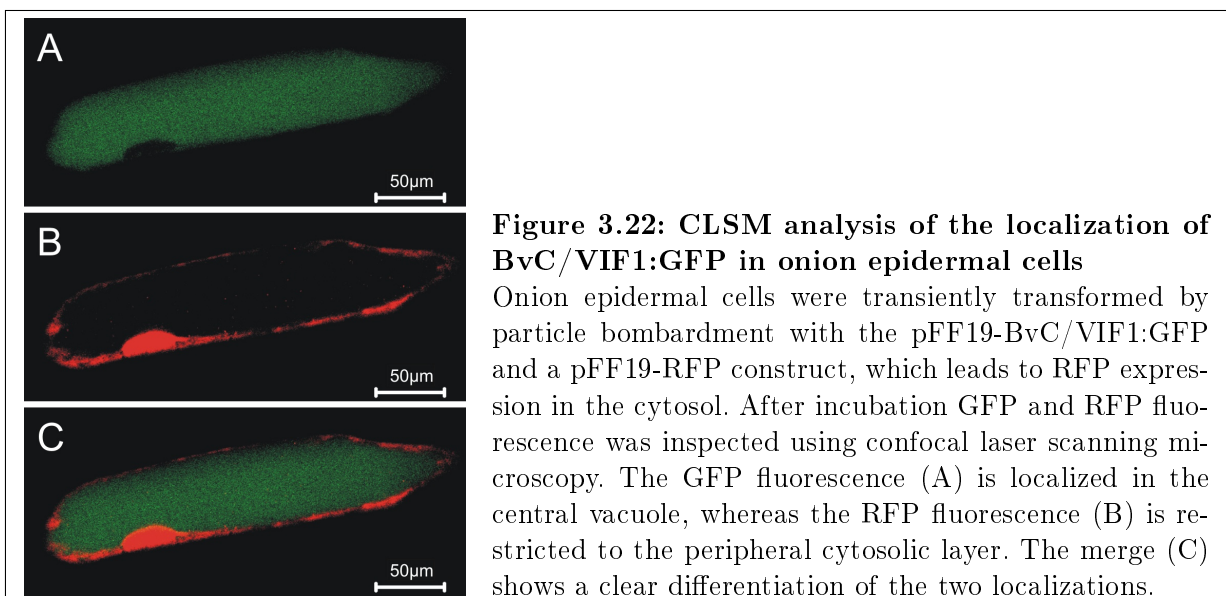
Figure 3.22: CLSM analysis of the localization of BvC/VIF1:GFP in onion epidermal cells

Onion epidermal cells were transiently transformed by particle bombardment with the pFF19-BvC/VIF1:GFP and a pFF19-RFP construct, which leads to RFP expression in the cytosol. After incubation GFP and RFP fluorescence was inspected using confocal laser scanning microscopy. The GFP fluorescence (A) is localized in the central vacuole, whereas the RFP fluorescence (B) is restricted to the peripheral cytosolic layer. The merge (C) shows a clear differentiation of the two localizations.

Figure 3.23: Detection of cleaved BvC/VIF1 and BvC/VIF-GFP in transiently transformed tobacco leaves
Tobacco leaves were infiltrated with agrobacteria containing the pK7FWG2-BvC/VIF1 construct and analyzed 48 h after infiltration. **A-F**: Confocal images of infiltrated leaves **A,D**: transmitted light; **B,E**: GFP fluorescence; **C,F**: overlay; **G**: soluble and cell wall bound invertase activity of uninfiltrated (WT) leaves and leaves transformed with the BvC/VIF1:GFP construct; **H**: Western Blot of soluble (sol.), salt-eluted (cw salt) and residual proteins extracted by boiling the cell wall remnants in SDS sample buffer (cw boiled). The samples were analyzed with the BvC/VIF and a GFP-antiserum. The BvC/VIF1:GFP fusion protein detected with both antisera is marked with an asterisk, the cleaved BvC/VIF1 protein by an arrow.

3.5.5 Production and affinity purification of the BvC/VIF antiserum

In order to examine the expression of BvC/VIF1 on protein level, an antiserum was raised against the recombinant inhibitor protein. A characterization of the different bleedings obtained from the immunization procedure can be found in the appendix, chapter 8.2.

In order to reduce the presence of unspecific bands, the generated antiserum had to be affinity purified against recombinant BvC/VIF1 protein (Figure 3.24). The protein was covalently coupled to a sepharose matrix and used to bind specific antibodies from the immune serum. After washing, the antibodies were eluted by a change to acidic pH. As shown in figure 3.24, it was possible to remove two strong unspecific bands (present only in flow-through) and to increase the strength of the putative inhibitor signal. Another putative unspecific band at 45 kDa is still present in the affinity purified serum.

Figure 3.24: Affinity purification of the BvC/VIF antiserum against recombinant BvC/VIF1 protein.
Different amounts of recombinant BvC/VIF1 protein (0,1=0.1 μg, 0,02=0.02 μg) were loaded together with a leaf extract (**l** - 5 mg f.w. equivalent), an extract from Beta suspension culture cells (**cc** - 12.5 mg f.w. equivalent) and a wounded taproot extract (**tr_w** - 12.5 mg f.w. equivalent)and detected with the unpurified final bleeding, the affinity purified serum and the flow-through of the affinity purification (FT). A closed arrow indicates the size of the correct band of the recombinant protein. The dashed arrow marks the corresponding BvC/VIF bands from taproot and cell culture tissues. The strength of the immune reaction could be enhanced by the affinity-purification and concomitantly two major unspecific signals could be removed and appear only in the flow-through of the affinity column.

3.5.6 Detection of two inhibitor proteins in taproot tissues

Figure 3.25.A shows the detection of the putative inhibitor signals in a cross-section of a sugar beet taproot and in a salt-eluted cell wall fraction of the *Beta vulgaris* suspension culture. Since the used gel showed a higher size resolution than the gels depicted before (Fig. 3.24) the previously observed putative inhibitor signal in the extracts from taproot tissues was separated into two bands of a size between 17 and 19 kDa. In the bark of the taproot, the upper signal seems to be more pronounced, whereas in a salt-eluted fraction from *Beta*

vulgaris suspension culture, which is strongly enriched in cell wall localized proteins, only the lower signal is present.

After a sequential extraction of the soluble and the cell wall proteins from taproot tissue (Figure 3.25.B) a differential localization of the two proteins became evident. In the soluble fraction, both proteins are detected, whereas in the fraction containing the cell wall bound proteins only the lower signal is present. These data indicate the presence of at least two structurally related inhibitor proteins in the sugar beet taproot, of which the lower one seems to be at least partially localized in the cell wall.

Figure 3.25: Detection of multiple BvC/VIF proteins in cross-sections of taproot tissues.
A: Soluble extracts from a cross-section (**0-4, bark**) of a taproot and the non thickened part of the same taproot. **cc** - cell wall is a salt-eluted cell wall extract from *Beta vulgaris* suspension culture cells (25 mg f.w. each). In the soluble extracts from the taproot, two bands of a size of around 16 and 18 kDa are detected. In the cell culture cell wall fraction, only the lower band is present. **B:** soluble fraction (25 mg f.w.) and cell wall fraction (50 mg f.w., extracted with SDS-sample buffer after removal of soluble proteins) of unwounded taproot interior. Only the lower band is present in the cell wall fraction.

3.5.7 The BvC/VIF proteins form stable complexes with acid invertases

In mature taproots strong inhibitor signals are detected (Fig. 3.25), but no acid invertase expression is detected (Fig. 3.2). However, during wounding the inhibitor proteins and their potential target invertases are coexpressed. If this expression occurs in the same cell types, at least part of these proteins should be present as invertase-inhibitor complexes. To examine the presence of these complexes, extracts from wounded taproots were separated via a size exclusion chromatography (SEC) and the obtained fractions were then analyzed for the presence of invertases and their inhibitor proteins in the same fractions. In comparison, the same was done for extracts from unwounded taproots, where no invertase expression is detectable.

As shown in figure 3.26.A, in unwounded taproot extracts the inhibitors elute from the column at elution volumes of 92 to 100 ml. From runs with marker proteins on the same
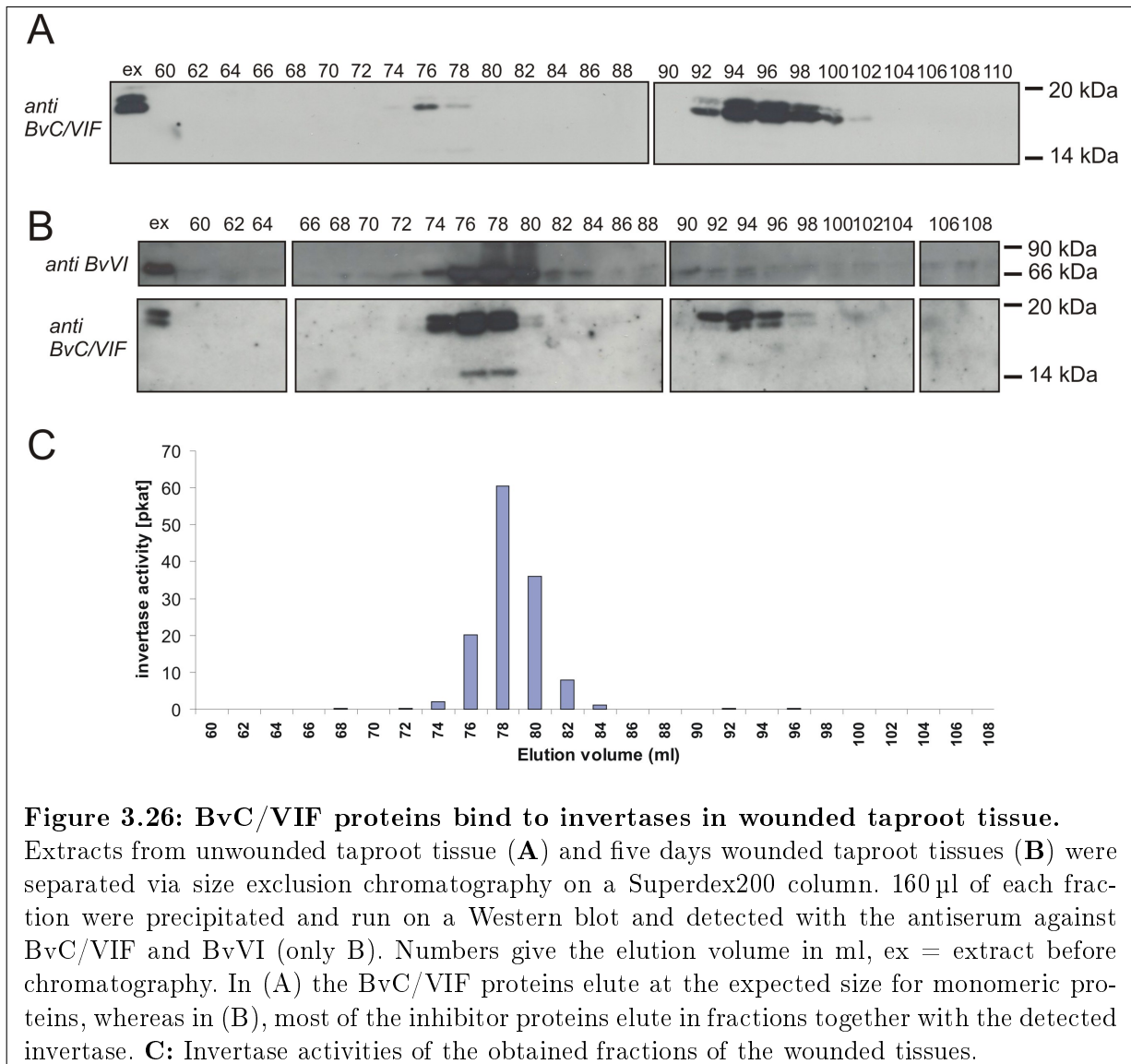
Figure 3.26: BvC/VIF proteins bind to invertases in wounded taproot tissue.
Extracts from unwounded taproot tissue (**A**) and five days wounded taproot tissues (**B**) were separated via size exclusion chromatography on a Superdex200 column. 160 μl of each fraction were precipitated and run on a Western blot and detected with the antiserum against BvC/VIF and BvVI (only B). Numbers give the elution volume in ml, ex = extract before chromatography. In (A) the BvC/VIF proteins elute at the expected size for monomeric proteins, whereas in (B), most of the inhibitor proteins elute in fractions together with the detected invertase. **C:** Invertase activities of the obtained fractions of the wounded tissues.

column, this elution volumes correspond for globular proteins around 16 kDa in size. A weak inhibitor signal is also present around an elution volume of 72 ml.

In Fig. 3.26.B the same experiment is carried out with an extract from taproots five days after wounding. Here the inhibitor mainly elutes between 74 and 78ml, which is in range of the observed size of the smallest peak of the complexes formed by recombinant BvC/VIF1 and BvVI1 protein (see Figure 3.30 on page 65). The Western blot developed with the antiserum against the vacuolar invertase and the activity assay in 3.26.C show, that the vacuolar invertase mainly elutes at an elution volume between 74 and 82ml. The highest invertase activity is observed at 78ml, indicating, that either the invertase is present in surplus to the inhibitor, or that the inhibitor does not completely inhibit the invertase activity.

3.6 Purification of BvC/VIF proteins from *Beta vulgaris* taproot tissue and suspension culture

The antiserum raised against the recombinant BvC/VIF1 protein detects two putative inhibitor proteins present in sugar beet taproot tissues (see Fig. 3.25). In order to proof the nature of these bands, the corresponding proteins were purified from *Beta vulgaris* tissues. The purified cross-reacting proteins were then partially sequenced via mass spectroscopy (carried out at the ZMBH, Heidelberg) to test, whether the obtained protein sequences fit to the BvC/VIF1 cDNA or belong to a further inhibitor isoform.

3.6.1 In *Beta* suspension culture cells, the BvC/VIF protein is present as a complex with a cell wall invertase

In the *Beta vulgaris* suspension culture only the lower of the two observed BvC/VIF bands is observed in Western blots (see Figure 3.25.A, page 56). This protein is localized in the cell wall, since it can be removed from the cells by addition of $0.5\,M$ sodium chloride, which elutes ionically bound proteins from the cell wall, leaving the cell membrane intact. A similar approach was originally used to purify and identify the NtCIF protein from a tobacco cell culture (Weil et al., 1994).

Figure 3.27 shows, that in the *Beta vulgaris* suspension culture the observed inhibitor forms a complex with a cell wall invertase. During a lectin chromatography with a concanavalin A (ConA) matrix, the invertase inhibitor, which is itself not glycosylated, is found both in the lectin unbound fraction (cc ConA-), and also in the lectin bound fraction (cc ConA+). The appearance of the inhibitor in this fraction is most likely due to the binding of a part of the present inhibitor proteins to the cell wall invertase, which is strongly enriched in the ConA+ fraction. This finding is further supported by the observation, that during size exclusion chromatography the observed inhibitor signal, like in the described experiment with wounded taproot tissue (Figure 3.26), is partially present in the same elution volumes like the cell wall invertase. Only part of the inhibitor proteins present are not bound to an invertase and elute later at elution volumes corresponding to its monomeric size (data not shown).

This hypothesis was later proofed in a diploma thesis (Claussen, 2005), in which the BvC/VIF protein could be purified and partially sequenced. The protein was purified via a sequential lectin and size exclusion chromatography of a cell wall enriched protein sample from the *Beta vulgaris* suspension culture cells. The resulting peptide fragments are included in table 3.2 on page 60. Out of four different peptide sequences generated, two fit perfectly to the predicted protein sequence of the BvC/VIF1-cDNA.

Figure 3.27: In *B.v.* suspension culture cells, BvC/VIF forms a complex with a cell wall invertase
Soluble and salt eluted samples from taproots (tr) and cell culture (cc) were detected with the antiserum against BvC/VIF and against cell wall invertase (anti NtCWI, cc samples only). In the soluble fraction from taproots (**tr sol**) both BvC/VIF bands are present, whereas in the cell wall sample (**tr cw**) only the lower band appears. In salt eluted fractions from suspension culture cells (**cc cw**) only the smaller BvC/VIF band appears. After lectin chromatography, this signal appears in the unbound fraction (**cc ConA-**) and in the lectin bound fractions (**cc ConA+**), where it elutes together with the cell wall invertase. (M, protein standard)

3.6.2 Purification of both BvC/VIF proteins via affinity chromatography with recombinant BvVI1 protein

The identification of peptide fragments from the cell wall of the suspension culture cells matching the BvC/VIF1 protein sequence indicate a cell wall localization of the inhibitor protein. However, these data contradicted the partial vacuolar localization of the BvC/VIF1-GFP fusion protein in chapter 3.5.4. In samples from unwounded taproots the lower of the two proteins detected is also at least partially bound to the cell wall (Fig. 3.25.B) and corresponds in size to the protein isolated from the suspension culture. This led to the assumption that this immuno signal corresponds to the BvC/VIF1 protein localized in the cell wall of suspension culture cells. The second, approximately 1 to 1.5 kDa larger signal is only found in soluble extracts and could either be a closely related second inhibitor isoform with a different localization, or a different processing product of BvC/VIF1. In order to get more insight into the nature of these signals, both proteins were purified from taproot tissues and again partially sequenced.

First, the strongest expression of both inhibitor proteins is observed in unwounded taproot tissue. Since in this tissue, no or very little invertase is present, the purification strategy used before for the inhibitor from cell culture was not feasible. Therefore a new strategy was developed using the recombinantly expressed BvVI1 protein (see ch. 3.4) as an affinity partner for the purification of inhibitor proteins from crude taproot extracts (Figure 3.28).

First, BvVI1 protein was covalently coupled to CNBr-sepharose, since this coupling is stable over the used pH-range. Second, the taproot extract (prepared at pH 5, sugars present were removed by ammonium sulfate precipitation) was passed over the column at a very

low flow rate, allowing the inhibitor proteins to bind to the invertase. Then the column was washed with buffer at pH 5 followed by a change to pH 8.5. In preliminary experiments and by size exclusion chromatography (Fig. 3.31) it had been shown, that this pH change into the basic range leads to the loss of binding between the invertase and the inhibitor.

The purification was monitored by Western blotting of the different fractions with the BvC/VIF antiserum (Fig. 3.28.A). Most of the inhibitor proteins present in the initial extract (ex) did not bind to the invertase column and are detected in the flow-through (FT). However, a strong signal for both inhibitor proteins appeared in the elution fractions (E). The elution fraction was further precipitated and loaded onto a preparative SDS gel and stained with colloidal coomassie (Fig. 3.28.B). In comparison with a Western blot carried out with the same sample, two bands (marked with 1 and 2) could be identified corresponding in size to the observed immuno signals. These two bands were used for peptide sequencing via nano-ESI-qTOF, which lead to the identification of peptide fragments specific corresponding to the BvC/VIF1 sequence (Fig. 3.28.C). These peptides were sequenced from both protein bands and their position in the BvC/VIF1 sequence is limited by trypsin cleavage sites. Further peptide sequences were only identified for the gel sample surrounding the upper band 1, but these showed no significant homology to any known sequence (Table 3.2).

Source	Obtained fragments	Match with BvC/VIF1
Suspension culture cells (cell wall fraction)	`[KQ]TPDPXXCEASXR`	x
	`YVEDXTR`	x
	`[KQ]XVG[FMox]ESVNXK`	-
	`YVXVSD[FMox]ESESCK`	-
	`YVXVSD....`	-
	`YVXSD[FMox]E...`	-
Taproot Band 1	`...PDPXXCEASXR`	x
	`YVEDXTR`	x
	`.........SVGHNK`	-
	`...DSAAVNSVGHNK`	-
	`VVXDVWGXGXR`	-
Taproot Band 2	`...PDPXXCEASXR`	x
	`YVEDXTR`	x

Table 3.2: Sequences of the obtained peptide fragments from the purified proteins
Amino acids in square brackets can not be differentiated by mass spectroscopy (carried out by Dr. Th. Ruppert, ZMBH, Heidelberg). (Mox= oxidized methionine; X: L or I; . : unidentified amino acid)

Figure 3.28: Purification of BvC/VIF proteins from sugar beet taproot tissue.
A: Western blot monitoring the purification of the BvC/VIF proteins via an invertase affinity column. An extract from sugar beet taproot (ex) was passed over a column onto which recombinant BvVI1 protein was covalently coupled. Although most of the inhibitor proteins did not bind to the column (FT, flow-through), after washing (WI, WII) BvC/VIF proteins could be eluted by a pH change to 8,5 (E). **B:** Elution fraction E on a colloidal coomassie stained gel. The two protein bands partially sequenced by nano-ESI-QTOF are marked with arrows. (M, protein standard) **C:** BvC/VIF1 sequence showing the identified peptides from both protein bands in bold. The predicted signal peptide is in italics (see also Tab. 3.2).

3.7 Characterization of the interaction between the recombinant invertase BvVI1 and the inhibitor BvC/VIF1

In the course of this thesis it was possible to produce highly active preparations of a recombinant invertase (BvVI1, see 3.4) and an invertase inhibitor from the same plant species (BvC/VIF1, see 3.5.2) for the first time. This opened the possibility for more detailed studies of the mechanism underlying the posttranslational silencing of invertases by proteinaceous inhibitors.

3.7.1 Inhibition of BvVI1 by BvC/VIF1 is strongly pH dependent

Earlier studies (Weil et al., 1994) observed a strong pH-dependency during the inhibition of invertases through inhibitor proteins. To test, whether this effect can be reproduced with the pure protein preparations, the inhibition of the recombinant invertase was measured at different pH values using increasing amounts of the recombinant inhibitor protein.

Figure 3.29: pH dependent inhibition of BvVI1 by BvC/VIF1
At each measured pH, the activity of the invertase was measured without and with increasing amounts of recombinant BvC/VIF1 protein. Before addition of sucrose, the invertase and inhibitor were incubated for 30 minutes to allow the complex to form. Each value is a mean of three measurements, error bars indicate the standard deviation.
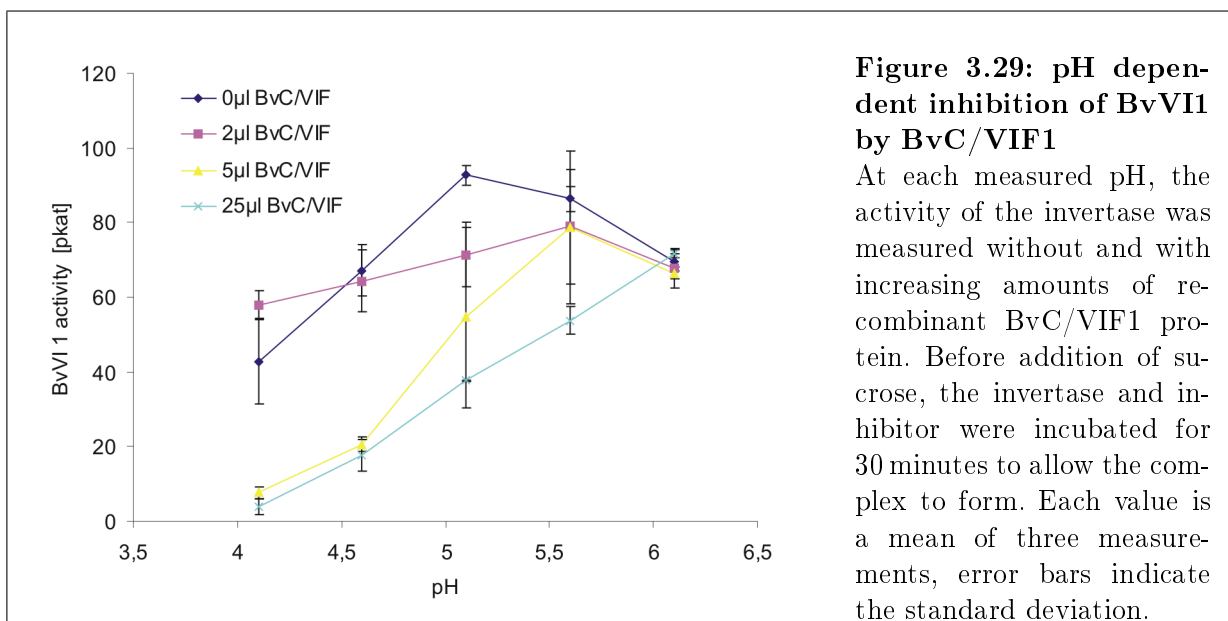
As shown in figure 3.29, at pH 5.6 the invertase is still highly active, but the effect of the inhibitor is markedly decreased. At pH 6.1 no inhibitory effect on the invertase is observed, although the invertase without inhibitor possesses still 75 % of the maximal activity observed at pH 5.1. The strongly reduced inhibition at elevated pH values might be physiological relevant during *in vivo* changes of the apoplastic and vacuolar pH. An increase in the pH might lead to reduced inactivation of the invertase albeit the presence of inhibitor proteins. At pH 4.1 and 4.6 near complete inhibition of the invertase is observed.

3.7.2 Complex formation between recombinant BvVI1 and BvC/VIF1

As previously described (Fig. 3.17.C on page 48), BvC/VIF1 inhibits recombinant BvVI1 protein. In order to study this interaction more closely, size exclusion chromatography experiments were carried out using a Superdex200-column (Pharmacia). The recombinant invertase and its inhibitor were run separately and in combination on the column and the elution of the proteins was monitored by UV-absorption. The recombinant proteins differ widely in there content of UV-absorbing aromatic acids, therefore the high UV-absorbtion of the BvVI1 protein had to be decreased mathematically to fit the absorption of both proteins into one diagram. Figures 3.30 and 3.31 show the UV-absorption traces of several runs under different conditions. After purification, the recombinant inhibitor protein is partially present in a dimeric state (see Fig. 3.16.B on page 47), most likely due to the formation of a intermolecular disulfide bridge. This is corroborated by the elution profile of the inhibitor passed individually over the column. In figure 3.30.A two peaks, corresponding in size to proteins of 17 and 36 kDa respectively, can be observed (labeled 1 and 2).

In contrast to this, the BvVI1 protein alone runs as a single peak (3) under all tested conditions, indicating the presence of only monomeric proteins. The peak is situated at an elution volume of 82.5 ml. In comparison with the elution volumes of the standard proteins used for column calibration, this indicates a size of approximately 42 kDa (Tab. 3.3), whereas the calculated molecular mass of the recombinant protein is 60.5 kDa. This elution behavior indicates a very compact fold of the protein, leading to a retarded elution volume.

When both proteins are combined in acidic buffer, the elution profile is altered dramatically. A two-fold excess of BvC/VIF1 molecules was added to BvVI1 on a molar basis[4]. The peaks observed with the individual proteins disappear and three new peaks at earlier elution volumes (4,5,6) appear, probably due to the formation of higher molecular weight complexes between the invertase and the inhibitor. This profile is not affected by the presence of sucrose during the column run (Fig. 3.30.B).

At pH 7.5 conditions no complex formation is observed (Fig. 3.31), emphasizing the strong pH dependency of inhibition observed earlier (see Fig. 3.29 on page 62).

The elution volumes of the peaks 4, 5 and 6 indicate the formation of complexes with different composition. Table 3.3 gives an overview of the theoretical molecular weights for the observed elution peaks, calculated from a standard curve based on the elution of standard proteins. The peak with the highest elution volume (4) corresponds in size to approximately 65 kDa (calculated from the running behavior of the marker proteins), indicating a one to one complex of monomeric inhibitor and the invertase. In contrast to this, the first peak (6) elutes at the same volume as the 150 kDa marker protein. This leads to the interpretation, that this complex must consist of two invertase proteins linked by a dimerized inhibitor. The

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[4]1 µg BvC/VIF1 = 52.3 pmoles; 1 µg BvVI1 = 16.5 pmoles

minor middle peak (5) might be an intermediate between these two states.

This interpretation is strengthened by the detection of the monomeric inhibitor protein on SDS gels (Figure 3.32) only in the fraction corresponding to elution volume of peak 4. The dimeric inhibitor, which can be visualized under non-reducing conditions is present preferentially in earlier fractions. The invertase protein is present in all fractions, indicating that the observed peaks indeed are formed by complexes between the two proteins.

peak number	Ve [ml]	MW calculated [kDa]	Sample
1	92.3	19.7	BvC/VIF1
2	83.0	40.2	BvC/VIF1
3	82.3	42.4	BvVI1
4	77.2	62.6	BvVI1 + BvC/VIF1
5	73.5	83.2	BvVI1 + BvC/VIF1
6	67.8	128.7	BvVI1 + BvC/VIF1

Table 3.3: Elution volumes and molecular weights (MW) calculated for the peaks labeled in Fig. 3.30 and 3.31
MW was calculated based on a standard curve prepared with the elution volumes of the standard proteins on the same SEC-column.

Invertase activity of fractions obtained after SEC

Invertase activity of all protein containing fractions from the SEC run at pH 5 containing BvVI1 and BvC/VIF1 was measured. The profile of the invertase activity followed closely the profile of the protein content of the fractions, indicating that not all the invertase present in the respective fraction is inactivated by the inhibitor (see Fig. 3.33). The total activity of all fractions was approximately one third of the activity of the same amount of invertase when incubated without inhibitor. The sample loaded on the column showed only a very low invertase activity, indicating that at this point nearly all invertase was inactivated.

Figure 3.30: Recombinant BvVI1 and BvC/VIF1 proteins form stable complexes, which can be separated by size exclusion chromatography

The recombinant proteins were passed separately and combined over a Superdex200 column and protein content was detected photometrically at 280 nm. The elution volumes and molecular masses of the used standard proteins are also given (A, β-Amylase; B, Alcohol Dehydrogenase; C, BSA; D, Carbonic Anhydrase; E, Cytochrome C) For the enumeration of the peaks please refer to the text.

A: SEC without sucrose present (running buffer: 50 mM sodium acetate, 300 mM NaCl pH 5).
B: SEC with the addition of 30mM sucrose to the buffer from A.

Figure 3.31: At neutral pH, no complex between BvVI1 and BvC/VIF1 is observed. The experiment was carried out as in Fig. 3.30 in a neutral running buffer (50 mM sodium phosphate, 300 mM NaCl pH 7.5) Here no complex formation of BvVI1 and BvC/VIF1 can be observed. Only a small new peak appears in the combined run (Ve=43 ml), which elutes in the void volume (V_0) of the column.

Figure 3.32: Detection of BvVI1 and BvC/VIF1 proteins in the elution fractions of combined SEC run

The elution volumes of the major peaks of the SEC run shown in 3.30.A were separated on a SDS-gel either in reduced (+DTT) or non-reduced form (-DTT). The BvVI1 protein (arrowhead) runs similarly under both conditions, whereas the invertase inhibitor is in the non-reduced stated either present as a dimer (dotted arrow) or as an monomeric protein (closed arrow).(M, protein standard; S, sample; elution volumes in ml)

Figure 3.33: Protein content and invertase activity profile of the SEC run shown in Fig. 3.30.A containing 410 µg BvVI and 250 µg BvC/VIF1
After the SEC run, invertase activity of each protein containing fraction was determined.

3.8 Characterization of sugar beet plants with increased or down-regulated BvC/VIF1 expression

The initially generated transgenic sugar beet plants, which overexpressed the vacuolar invertase inhibitor NtVIF from tobacco, did not lead to a significant decrease in the activity of vacuolar invertases after wounding (see 3.3).

After the discovery and characterization of the endogenous sugar beet inhibitor BvC/VIF1, it became evident, that this inhibitor had, compared to NtVIF, a much stronger activity towards vacuolar invertases from wounded sugar beet taproots (see Fig. 3.17.E and F on page 48).

In order to pursue the aim of decreasing the postharvest sucrose losses due to hydrolysis by acid invertases, a new biotechnological approach using altered expression of the endogenous inhibitor BvC/VIF1 was initiated. This approach should also deliver valuable insights concerning the still not comprehensively understood subcellular localization of the BvC/VIF1 protein *in planta* and its role during taproot development. The following constructs were generated and used for the *Agrobacterium* mediated transformation of *Beta vulgaris*:

- **p70S-BvC/VIF1:** in this construct, the complete coding region with 29 bp of the 5' UTR of the BvC/VIF1-cDNA is expressed under the control of the constitutive CaMV 35S promoter. To increase expression, the sequence of the promoter has been duplicated leading to a so called double 35S (p70S) promoter.

- **p2-1-48-BvC/VIF1:** here the BvC/VIF1-cDNA is expressed under the control of the primarily taproot specific 2-1-48 promoter, isolated and characterized by the work group of Prof. Hehl (Braunschweig, Hehl et al. (2002); Oltmanns et al. (2006)). The 2-1-48 promoter (Genbank accession: AX449164) belongs to the primarily taproot specific *Mll* gene, coding for a homologue of the major latex-like protein from *Mesembryanthemum crystallinum* (Kloos et al., 2002). This additional overexpression approach is based on the assumption, that a decrease of acid invertase activity in all plant tissues could lead to restrictions of the overall performance of the plants.

- **p70S-RNAi-BvC/VIF1:** in order to down-regulate the expression of the invertase inhibitor in the taproot, this construct was generated to knock-down the native BvC/VIF1 expression through a RNAi approach. A removal of BvC/VIF1 expression in the taproot could increase the understanding of the physiological role of invertase inhibitors. The construct consists of approximately 260 bp of the BvC/VIF1 coding region first in antisense and then in sense direction, linked by intron 2 of the *A. thaliana* gene AtAAP6 (at5g49630), and is expressed under the control of the constitutive doubled CaMV 35S promoter

All constructs are based on the vector p70S, which was provided by Dr. D. Stahl (Plant GmbH, Einbeck). The transformations and regeneration of positive plantlets were again

carried out by KWS. During the preparation of this thesis, only *in vitro* regenerated leaf material of the transgenic plants was available for characterization. Of the first construct, 20 individual lines were generated, whereas for the taproot specific and the RNAi construct only 11 and 4 lines respectively have been generated so far.

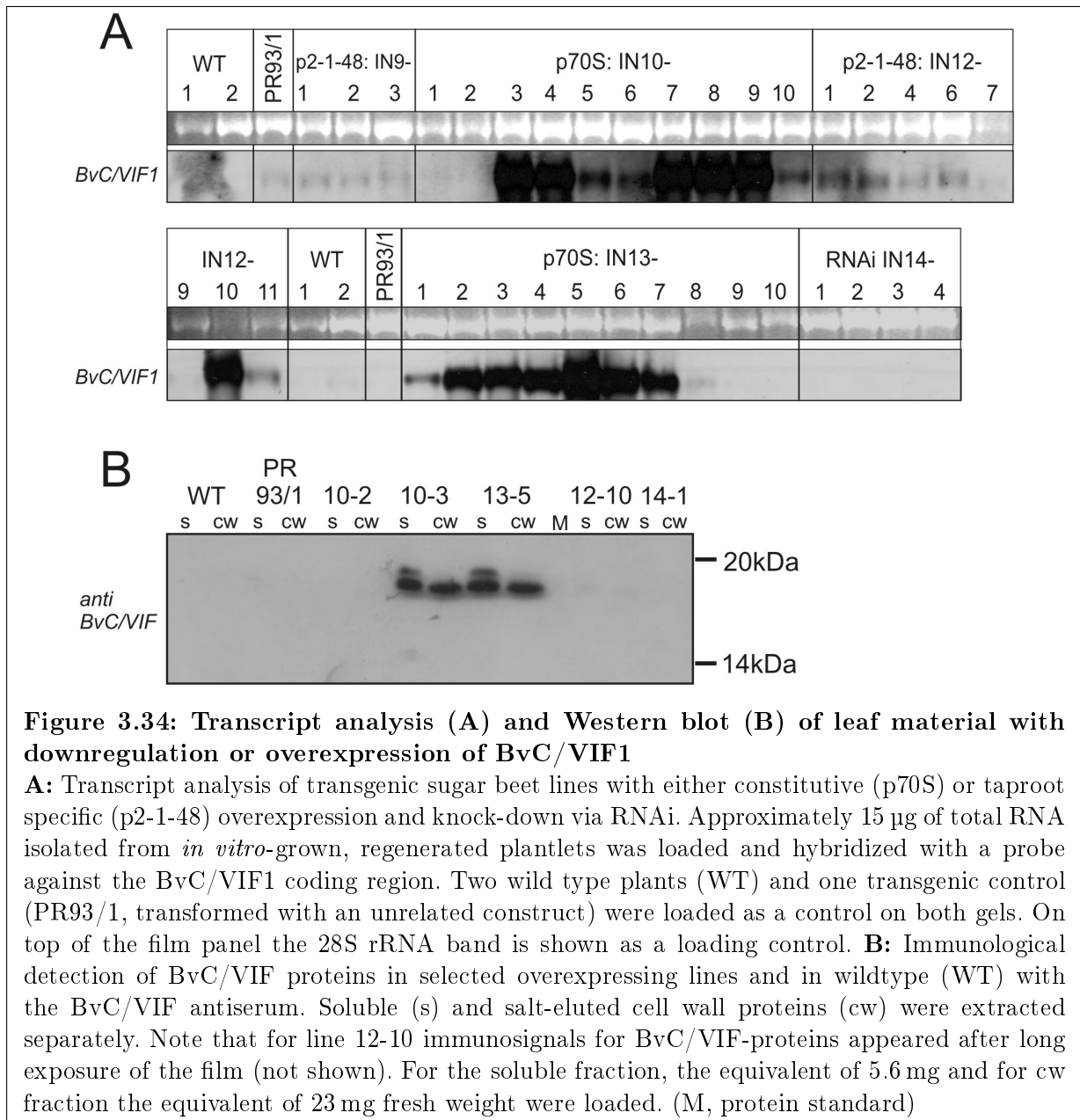### Detection of two protein in leaves of sugar beet plants overexpressing BvC/VIF1

For a first identification of transgenic plants with an altered BvC/VIF1 expression, Northern and Western blots were carried out using a probe against the BvC/VIF1 cDNA or the BvC/VIF antiserum, respectively. Figure 3.34.A shows, that in most of the p70S-BvC/VIF1 lines a strong signal is observed in Northern blots, indicating the presence of high amounts of BvC/VIF1 transcripts. As the wildtype plants (WT) and a transgenic line transformed with a control construct (PR93/1) show only a very weak expression, it can be concluded, that the p70S-BvC/VIF1 construct leads to a strong overexpression in the analyzed tissue. The different lines however show a variable level of overexpression, including lines like e.g. IN10-1 and IN13-8 with no clear overexpression.

The strong overexpression in most of the lines was further supported by the detection of strong signals in Western blots of the same tissues. As shown in Figure 3.34.B, the BvC/VIF antiserum produces strong signals with the overexpressing plants, but not with the wildtype. Surprisingly, the soluble protein fraction shows two strong immuno signals corresponding in size to the observed signals in the untransformed taproots (see for instance Fig.3.25 and ch. 3.6.2). This observation strongly supports the idea, that both signals arise from one gene.

For the two other transformed constructs, which should either lead to a taproot specific expression via the 2-1-48 promoter, or to a reduction of BvC/VIF1 expression via RNAi, the analysis is hampered by the restriction on leaf material. Since the wildtype leaves show hardly any detectable BvC/VIF1 expression in leaves, the RNAi effect can only be observed when transformed root material is available, which shows a strong endogenous expression of the inhibitor. For the p2-1-48-BvC/VIF1 constructs, the Northern blots in figure 3.34.A shows some lines, IN12-1, 2 and 10, with a significantly increased BvC/VIF1 expression compared to the wildtype controls. Since the aim of this construct is to specifically overexpress the inhibitor in taproots, the selection of promising lines should also be based on the analysis of taproot material, by which lines with a strong expression in taproots and no expression in leaves can be identified.

Vacuolar invertase activity is reduced in transgenic leaves

Figure 3.35 shows the invertase activity of the soluble and the cell wall bound fractions of the leaf material from selected lines of the BvC/VIF1 transformants together with the obtained signals for the BvC/VIF1 transcript in Northern blots. The activity of the highest WT sample was arbitrarily set to 100 %. Transgenic lines with a strong expression show a

Figure 3.34: Transcript analysis (A) and Western blot (B) of leaf material with downregulation or overexpression of BvC/VIF1
A: Transcript analysis of transgenic sugar beet lines with either constitutive (p70S) or taproot specific (p2-1-48) overexpression and knock-down via RNAi. Approximately 15 µg of total RNA isolated from *in vitro*-grown, regenerated plantlets was loaded and hybridized with a probe against the BvC/VIF1 coding region. Two wild type plants (WT) and one transgenic control (PR93/1, transformed with an unrelated construct) were loaded as a control on both gels. On top of the film panel the 28S rRNA band is shown as a loading control. **B:** Immunological detection of BvC/VIF proteins in selected overexpressing lines and in wildtype (WT) with the BvC/VIF antiserum. Soluble (s) and salt-eluted cell wall proteins (cw) were extracted separately. Note that for line 12-10 immunosignals for BvC/VIF-proteins appeared after long exposure of the film (not shown). For the soluble fraction, the equivalent of 5.6 mg and for cw fraction the equivalent of 23 mg fresh weight were loaded. (M, protein standard)

reduced invertase activity in the soluble fraction. The activity of the cell wall fraction seems to be unaltered.
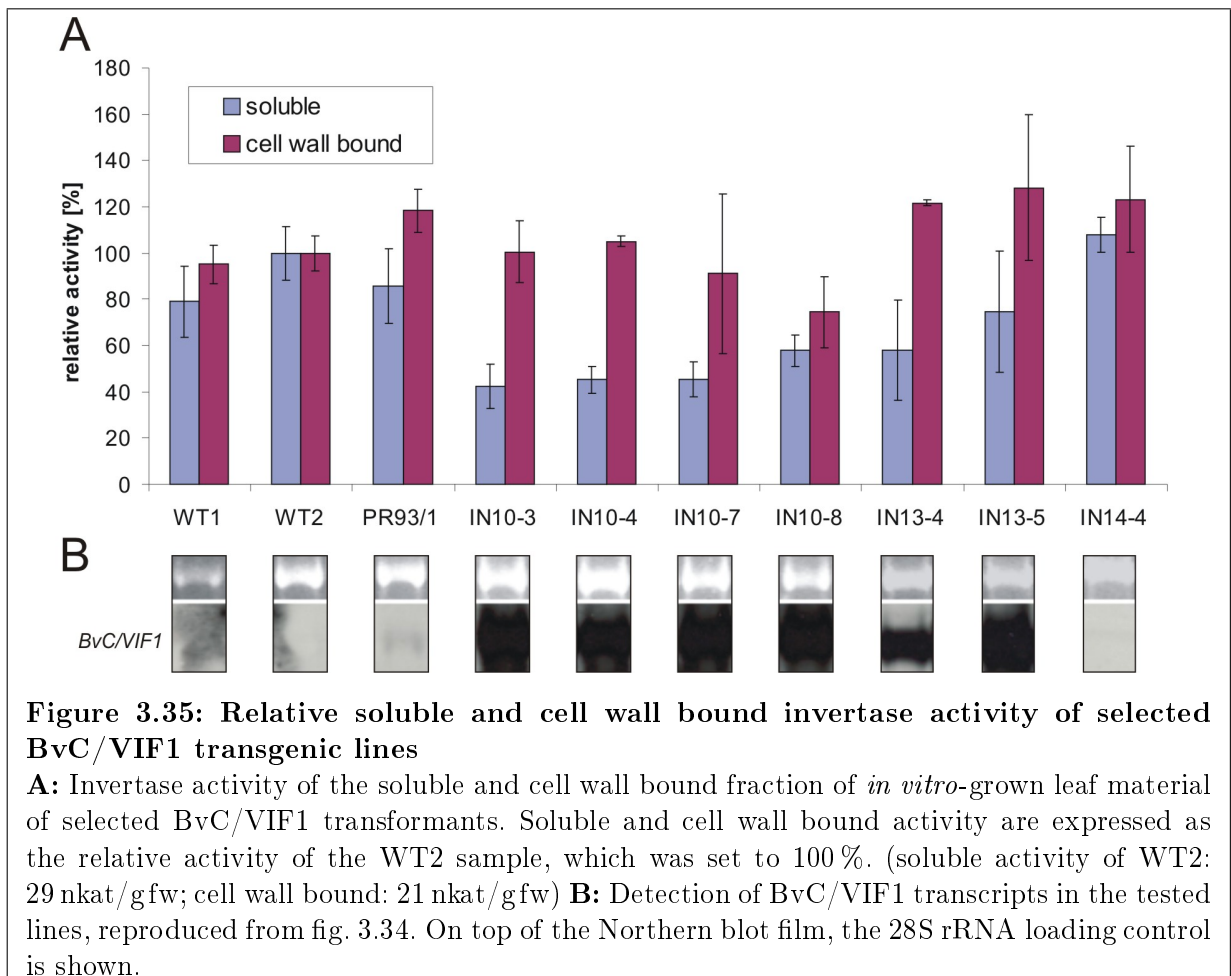
Figure 3.35: Relative soluble and cell wall bound invertase activity of selected BvC/VIF1 transgenic lines
A: Invertase activity of the soluble and cell wall bound fraction of *in vitro*-grown leaf material of selected BvC/VIF1 transformants. Soluble and cell wall bound activity are expressed as the relative activity of the WT2 sample, which was set to 100 %. (soluble activity of WT2: 29 nkat/gfw; cell wall bound: 21 nkat/gfw) **B:** Detection of BvC/VIF1 transcripts in the tested lines, reproduced from fig. 3.34. On top of the Northern blot film, the 28S rRNA loading control is shown.

4 Discussion

4.1 Post-harvest sucrose losses in sugar beet are multi-causal

Sugar beet is one of the youngest crop plants grown on large scale in modern agriculture. Breeding of fodder beet for sugar content began only at the end of the 18th century by Franz Karl Achard and since then, the sugar content has been increased from originally 5 to 6 % sucrose (w/w) to over 20 % in modern sugar beet varieties (Pennington & Baker, 1995). While breeding has put much focus on the increase of sugar content in individual plants, little attention has been drawn on sucrose losses after harvest. This is in part due to difficulties in assessing this trait as part of a breeding program.

Several factors can influence the amount of sucrose lost during storage, i.e. the initial amount of sucrose present, harvest techniques as well as the conditions and temperature of storage (Burba, 1976). Also, earlier studies indicated significant differences in post-harvest sugar losses between different sugar beet varieties (Wyse et al., 1978; Burba, 1976; Akeson & Widner, 1981).

Physiologically, the loss of sucrose can be regulated at multiple levels. Initially sucrose, the main source of carbon and energy in a decapitated taproot after harvest, has to be remobilized from its place of storage inside the vacuole and cleaved. After this, the resulting hexose sugars can be used in various processes of the cell's metabolism, but further regulatory steps are likely to be involved.

The development of sugar beet lines with decreased post-harvest sucrose losses is a promising new aim of plant breeding. Therefore crosses with the most efficient post-harvest metabolism have to be identified. Ideally, this breeding lines should only consume the minimal amount of stored sucrose necessary to maintain the integrity of the taproot during storage periods. The identification and characterization of key genes for the consumption or stabilization of sucrose, which can either be used as marker genes in breeding or as targets for biotechnological manipulations, is of high importance for this task.

The focus of this thesis was drawn on the regulation of sucrose-hydrolyzing enzymes, which are important for the cleavage of stored sucrose. Additionally, genes influencing the efficiency of mitochondrial respiration have been identified and their expression in sugar beet has been addressed.

4.1.1 Acid invertase and sucrose synthase show an opposite regulation during development of sugar beet taproots

In relation to the expression of sucrolytic enzymes, the development of the sugar beet taproot can be divided into three phases.

The first phase of development is characterized by a high expression of vacuolar and cell wall invertases. The near complete cleavage of sucrose incoming from the shoot leads to the establishment of the root sink. In other developing plant tissues it has been shown, that VI and CWI are important for the expansion of new tissues and that the high concentration of hexoses generated serves as a metabolic signal, leading to high mitotic activity (reviewed in Koch, 2004).

In the second phase, starting around eight weeks after germination, the taproot develops and sucrose accumulation begins (Berghall et al., 1997; Klotz & Finger, 2002). This is accompanied by a drop in detectable invertase activity and no acid invertase expression can be detected in the major part of the root (see Fig. 3.2). During this phase, two sucrose synthase enzymes are expressed, which is also reflected in increasing SuSy activity during taproot development (Klotz & Finger, 2002). A similar course of invertase and SuSy activity has been observed during seed development in several plant species (Weber & Wobus, 1997; Hill et al., 2003) and seems to be a general model during the development of plant storage tissues (Koch, 2004).

Two sucrose synthase isoforms are described for sugar beet (Hesse & Willmitzer, 1996; Haagenson et al., 2006). Expression studies using isoform specific 3' UTR probes show a stronger expression for SBSS2 than for SBSS1 in taproots (see Fig. 3.3). SBSS2 is already present in six weeks old roots, whereas SBSS1 is detected only in older roots, in accordance with other data showing a stronger SBSS2 expression in young roots (Haagenson et al., 2006). Further SuSy isoforms are likely to exist in sugar beet, since e.g. for *A. thaliana* six (Baud et al., 2004) and for pea and maize, at least 3 SuSy isoforms with a different regulation are known (Barratt et al., 2001; Carlson et al., 2002). The expression of both SBSS-genes in the taproot points towards an important role for the efficient accumulation of sucrose during the major part of taproot development. The precise role of sucrose synthase in taproots is however still unclear. On the one hand, it could be important for the maintenance of sink strength by cleaving incoming sucrose before storage in the vacuole, as has been observed for potato (Zrenner et al., 1995) and tomato (Wang et al., 1993). On the other hand, its activity might only be important for the basic metabolism of the cell, whereas sucrose accumulation is mainly dependent on the efficient sequestration of sucrose in the vacuoles without the need for prior cleavage.

One reason for the switch from an invertase based metabolic pathway to the more energy conserving sucrose synthase pathway might be the hypoxic conditions developing in the bulky storage tissues. Oxygen concentrations sharply decline in the interior of sugar beet taproots,

with only 3.4% O_2 in a depth of $2.5\,cm$ (Schirmer, 2004). Incubation of potato tubers in sub-ambient oxygen concentrations leads to a repression of invertase and an induction of SuSy genes. Furthermore, ectopic overexpression of invertases in the potato tuber leads to decreased oxygen concentration and reduced starch formation in the tubers (Bologa et al., 2003), emphasizing the role of SuSy activity for tuber development.

The third phase of taproot development is initiated by the harvest of the plants. Inevitably the plants are wounded. During the storage period preceding the processing in sugar factories, sucrose is constantly lost. A close study of the expression of acid invertase isoforms (Fig. 3.2) and the resulting soluble and cell wall bound enzyme activities during wounding (Fig. 3.4) shows, that a cell wall (BvCWI1) and a vacuolar invertase (BvVI1) are induced. The expression of two known SuSy isoforms (SBSS1 and SBSS2) however is not influenced by the treatment. The induction of cell wall bound activity is already prominent at one day after wounding, whereas the BvVI1 protein is only detected strongly three days after wounding. Simultaneously a strong accumulation of hexoses is observed, emphasizing the role of BvVI1 as a key enzyme for sucrose mobilization (Rosenkranz et al., 2001). The characterization of the recombinant BvVI1 protein showed (see 4.2.2), that the determined K_M value of the enzyme (around $4\,mM$ sucrose) is well below the sucrose concentration in a mature taproot of up to $600\,mM$ sucrose. It is active over a broad pH range between pH 4 and 6, indicating that the enzyme's activity will not be limited by the availability of substrate or the vacuolar pH.

4.1.2 Induction of alternative oxidases during wounding and storage of taproots

Wounding and storage increase the respiration of sugar beet taproot material. A prominent rise in O_2 consumption of taproot disks is seen already one day after wounding (Fig. 3.5). During the course of the wound reaction, the respiration rate declines again, albeit to an increased level compared to the unwounded material. The same behavior has been observed during the storage trials carried out on larger scale by Südzucker and others (K. Harms, personal communication and Campbell & Klotz (2006)).

Respiration in mitochondria can be influenced by the activities of alternative oxidase (AOX) and uncoupling proteins (UCP), which influence the efficiency of the electron transport chain. For both, sequences of high homology to isoforms in other plants have been found in sugar beet EST collections. Expression of both gene families was detected during taproot development.

BvUCP2 transcripts are present in all tissues analyzed (see Fig. 3.6), but showed no significant change during wounding. The second isoform, BvUCP1, showed a similar ubiquitous, but much lower expression. Both genes seem to be part of a general defence mechanism against over-reduction of the electron transport chain and reactive oxygen species that is ac-

tive during all plant stages. It is however most likely, that the activity of the UCPs is strongly regulated, for example by the presence of reactive oxygen species as has been observed in other plants (Considine et al., 2003). In addition, for *Arabidopsis thaliana* differential expression of the five members of the AtPUMP gene family has been observed (Vercesi et al., 2006), leaving open the possibility of further *Beta vulgaris* isoforms, which are induced during stress responses.

An induction of alternative oxidase activity during ageing and wounding has been reported in various species, e.g. in potato tubers (Hiser & McIntosh, 1990) and red beet (Potter et al., 2000). The analysis of AOX protein expression in mitochondria from wounded sugar beet slices showed a differential induction of at least two AOX-isoforms (Fig. 3.7). In the unwounded taproot, only a single band is detected with the AOX antiserum, whereas 1 d and 3 d after wounding, a second, slightly larger protein is detected. In source leaves, a protein of the same size is present, whereas in sink leaves only the smaller one is observed. The induction of the second AOX protein shows, that *Beta vulgaris* possesses multiple AOX genes with distinct regulation patterns. Induction of the second isoform after wounding most likely leads to an increased capacity of the alternative respiration pathway. An assignment of the observed protein bands to the corresponding genes is so far not possible, since up to now only one AOX gene (BvAOX1) has been identified on a sequence basis. In Northern blots, the BvAOX1 gene could not be detected, indicating a relatively low gene expression at the mRNA level. However, expression in taproots was proven by reverse transcription PCR (RT-PCR).

4.2 BvC/VIF1: a potential key factor for the regulation of invertase activity in sugar beet

The presence of proteins with invertase inhibiting activity in *Beta vulgaris* was already published in 1968. Pressey (1968) describe the isolation of a protein, 18.1 kDa in size, from fresh sugar beet taproots by acid treatment, sequential precipitation and size exclusion chromatography. The protein is active against potato leaf invertase and inhibition is most pronounced between pH 4 and 5. Through the characterization of the recombinantly expressed BvC/VIF1 protein, expression analysis and studies of the complex formation between BvC/VIF1 and plant invertases, increasing evidence accumulated during the preparation of this thesis, that BvC/VIF1 is the gene corresponding to the earlier described invertase inhibitor.

The BvC/VIF1 sequence was identified from a sugar beet EST library due to its homology to described invertase inhibitors from tobacco (Greiner et al., 1998) and *A. thaliana* (Link et al., 2004). Compared to other members of the respective gene family, the predicted BvC/VIF1 protein sequence shows in a protein alignment (Fig. 3.15) typical features of the PMEI-RP gene family, containing inhibitors of pectin methylesterase and invertase. It

possesses four cysteine residues at conserved positions, which have been shown to be important for the structural integrity of the inhibitor proteins (Hothorn et al., 2004a). The protein without the predicted signal peptide carries an basic isoelectric point (pI = 8.4) and a molecular weight of approximately 17 kDa.

An assignment, whether BvC/VIF1 codes for an invertase or PME inhibitor, was however not possible based on sequence data alone, due to the general low sequence conservation of the protein family (Hothorn et al., 2004b). To elucidate the *in vivo* target enzyme of the inhibitor, the protein was recombinantly produced and tested against invertase preparations.

4.2.1 Recombinant BvC/VIF1 protein shows a broad specificity for plant invertases

The BvC/VIF1 protein was expressed in bacteria without the predicted signal peptide and could be purified in soluble form. It is active against various plant invertases and shares a comparable inhibition capacity with NtCIF (Fig. 3.17), the cell wall localized invertase inhibitor from tobacco (Greiner et al., 1998). Vacuolar invertases, either extracted from leaves of sugar beet and tobacco or from wounded *Beta vulgaris* taproots are inhibited completely by increasing amounts of the inhibitor protein.

Whereas all vacuolar invertase preparation tested were fully inhibited, the protein showed divergent activity against different CWI isoforms. While CWI preparations from leaves are only inhibited to around 50 % of their basic activity, a CWI-preparation extracted from sugar beet suspension culture cells and purified by lectin chromatography was inhibited completely by the protein (data not shown). This indicated, that BvC/VIF1 shows a different specificity on CWI preparations, probably depending on the presence of different CWI isoforms. This is also shown by the effect, that already low amounts of BvC/VIF1, as well as NtCIF, inhibited the leaf CWI preparation by 50 %. Higher amounts of inhibitor led to no further reduction of the activity, indicating the presence of multiple invertase isoforms deviating in their ability to be inhibited by the used inhibitor proteins (Fig. 3.17.A and B).

4.2.2 Production of recombinant sugar beet invertase and characterization of its interaction with BvC/VIF1

For a closer characterization of the interaction between invertases and their inhibitors, the activity of the BvC/VIF1 protein was tested against recombinant plant invertases. In contrast to assays using invertase preparations extracted from plants, the usage of recombinant invertases possesses several advantages. Crude plant extracts often contain a mixture of different invertase isoforms and other contaminating proteins. Especially the presence of endogenous inhibitor proteins, which remain bound to part of the prepared enzymes, hampers a detailed analysis of the interaction with added recombinant inhibitor protein. During the preparation

of this thesis, publications appeared describing the expression of vacuolar invertases from sweet potato (Huang et al., 2003; Wang et al., 2005) and cell wall invertases from *A. thaliana* (De Coninck et al., 2005) in the yeast *Pichia pastoris*. In order to obtain recombinant invertases from sugar beet and tobacco, the *Pichia pastoris* expression system was tested for the production of recombinant NtCWI and BvVI1 proteins. Although multiple constructs deviating in the length of the expressed protein were tested, no expression of active invertase protein was achieved.

Recombinant BvVI1 protein shows the typical activity of plant invertases

After the unsuccessful expression in yeast, an alternative invertase expression strategy in *E. coli* was used. This led to the production of active BvVI1 protein. After testing of several tags to increase solubility of the expressed protein in the bacteria, best expression yields were obtained by expressing the protein in fusion with a N-terminal GST-tag, which could be removed after purification of the fusion protein. Addition of glucose to the medium enhanced the yield of the recombinant enzyme, possibly by a stabilizing effect on the invertase. A promoting effect of the substrate sucrose itself was not noticed (data not shown). The production of BvVI1 in *E. coli* is at present the only published expression of a plant invertase in bacteria and will prove helpful for further analysis of the posttranslational regulation of these important enzymes of plant primary metabolism.

BvVI1 is the major vacuolar invertase isoform in the life cycle of sugar beet, having a high expression in leaves, young roots and in wounded taproots (Fig. 3.2 and Rosenkranz et al., 2001). Regulation of its activity is therefore of high interest for the understanding of sucrose accumulation and re-mobilization in sugar beet. The recombinant BvVI1 protein, albeit lacking glycosylation due to the procaryotic expression hosts, shows a K_M value and pH optimum (Fig. 3.14) comparable to other vacuolar invertase preparations (Masuda et al., 1988; Wang et al., 2005), which usually lies in the range between 1 and 30 mM for sucrose (BRENDA database[1], Schomburg et al., 2002). The enzyme shows a maximal activity between pH 4.5 and 6, above pH 6.5 the activity sharply declines. The observed pH optimum is in accordance with values described for other plant VIs, which usually have a higher pH optimum than CWIs (Roitsch & Gonzalez, 2004).

The BvVI1 protein is inhibited by recombinant invertase inhibitors

When incubated together with increasing amounts of BvC/VIF1 and NtCIF, recombinant BvVI1 is fully inhibited. A similar behavior was observed, when either of the two inhibitors was incubated with the IbVI2 protein, a VI isoform from sweet potato (Wang et al., 2005) heterologously expressed in *Pichia pastoris* (Fig. 3.17.C and D). Since IbVI2 is produced in glycosylated form in the eucaryotic expression system and is inhibited in a similar manner

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[1]`http://www.brenda.uni-koeln.de/`

as BvVI1, an influence of the post-translational glycosylation on the interaction between invertase and inhibitor seems unlikely. Also an increased instability of the recombinant BvVI1 protein due to the lack of glycosylation was not observed, since the recombinant protein showed an linear time dependency of its activity for at least 2 h at 37 °C (Fig. 3.13) and the protein kept its activity during storage for several days at 4 °C.

Inhibition of BvVI1 is strongly pH dependent

A strong pH-dependency was observed for the inhibition of BvVI1 by BvC/VIF1. When incubated together with BvVI1 at different pH values between 4.1 and 6.1, a strong inhibition was only observed below pH 5.1 (Fig. 3.29). Above these pH values, the VI is still active, but no inhibition occurs, opening the possibility of a regulation of inhibition through changes in vacuolar or apoplastic pH. A similar behavior was reported for the inactivation of a tobacco CWI by NtCIF (Weil et al., 1994), with the distinction that the tested CWI showed without inhibitor a stronger intrinsic decrease of activity due to elevated pH, and for the invertase inhibitor isolated from sugar beets (Pressey, 1968). At neutral pH, no complex formation between the recombinant proteins occurred, as it was shown by size exclusion chromatography (Fig. 3.31). Hothorn & Scheffzek (2006) determined the crystal structure of the tobacco inhibitor NtCIF at different pH values. The protein showed no major rearrangements at different pH values and it is proposed, that the pH-dependency of the inhibition is most likely caused by changes in the surface charge of both interacting proteins.

The exact pH values and induced changes in the subcellular compartments of plants are not clearly defined. Felle (2001) reviewed reports about measured values and changes in the cell wall's pH. In roots, the apoplastic pH usually lies between 4.5 and 6 and is maintained by active regulation through the plant (Taylor et al., 1996; Felle, 1998). Plant vacuoles in general are also assumed to be acidic (Marty, 1999), but existence of acidic and non-acidic vacuoles together in one cell or in different cell types have been described (Di Sansebastiano et al., 1998). Also the vacuolar proton pool is involved in regulations of cytosolic pH and increases of the vacuolar pH due to acidification of the cytosol have been reported (Roos et al., 1998). The rapid loss of efficient inhibition at increased pH values indicates a further potential regulatory mechanism of the inactivation of plant invertases through transient changes of the ambient pH. Co-expression of invertase and inhibitor together in a given compartment alone might therefore be not sufficient for inactivation of the invertase, but it is also dependent on the prevailing pH. When changes in the metabolic state of the cell are reflected via pH changes of the vacuole or the apoplast (e.g. by affecting the activity of proton pumps in the apoplast or tonoplast), the cleavage of sucrose and the release of hexoses from the vacuole (or the uptake from the apoplast), could be fine-tuned to the cellular demand.
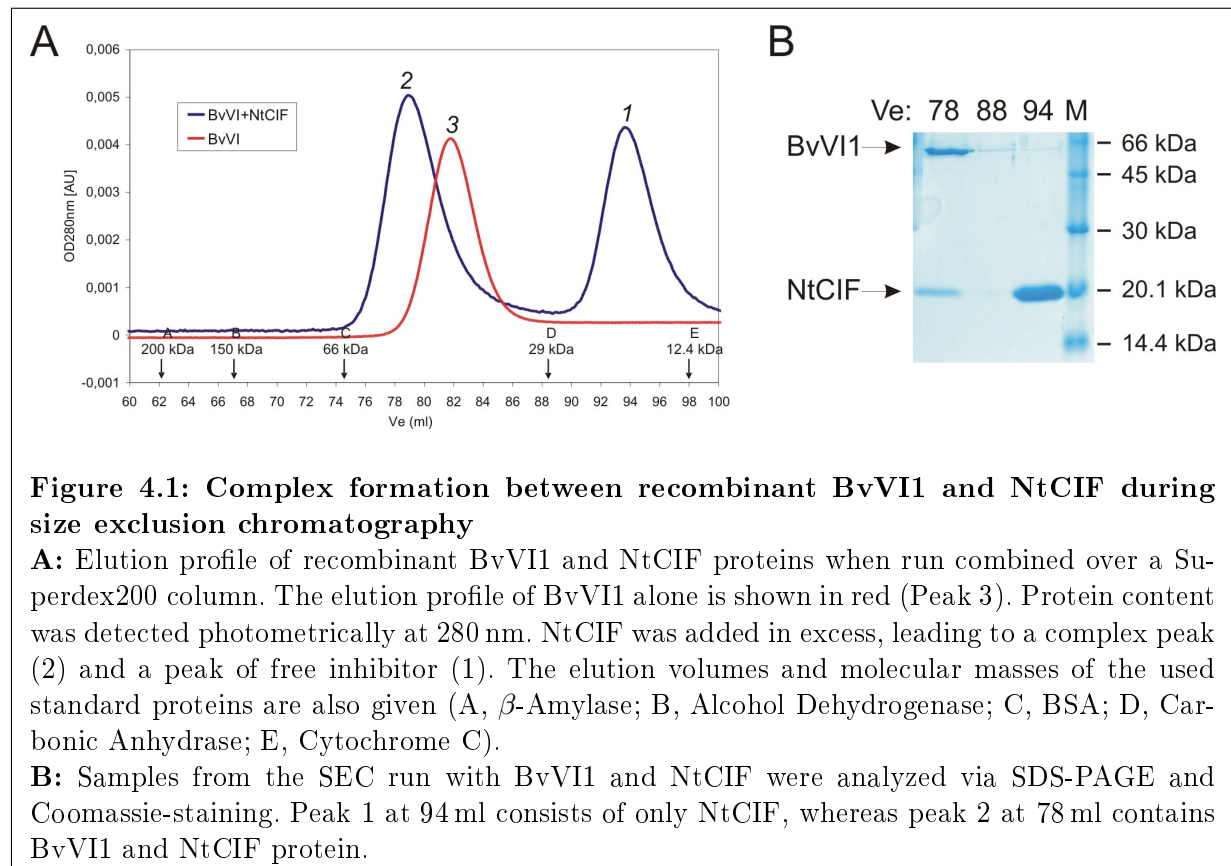
BvVI1 and BvC/VIF1 form tight complexes

Invertase inhibitors are known to form tight 1:1 complexes with their target proteins (Krausgrill et al., 1998). The properties of the BvVI1-BvC/VIF1 complex were further analyzed by size exclusion chromatography. The BvC/VIF1 protein alone shows two elution peaks, corresponding to the presence of monomeric and dimeric forms of the protein (Fig. 3.30, peak 1 and 2). The inhibitor dimer purified from *E. coli* is covalently connected via a disulfide bridge, which can be opened by addition of reducing agents (Fig. 3.16.B). *In planta*, invertase inhibitors have only been observed as monomers and also the elution volumes of the BvC/VIF-proteins from unwounded taproot tissues (Fig. 3.26.A) corresponds to the elution volume of the monomeric recombinant inhibitor proteins. Therefore the presence of dimeric inhibitor protein after overexpression in bacteria is most likely an artefact of the heterologous expression host.

The recombinant invertase BvVI1 alone showed only a single peak at an elution volume corresponding to a molecular weight of only 42 kDa (Table 3.3 on p. 64), as compared to the elution of standard proteins on the same column. The molecular weight of the recombinant BvVI1 protein is however 60 kDa and this molecular weight was also observed during SDS-PAGE of the purified protein. The protracted elution from the SEC-column indicates a very compact fold of the recombinant protein. A similar behavior was observed during the purification of the invertase from *Thermotoga maritima* and the authors proposed a nonspecific interaction of the protein with the Sephadex column to be the cause for the protracted elution (Alberto et al., 2004). The *T. maritima* invertase is, like the BvVI1 protein, present as a monomer in solution, in contrast to yeast invertases that form dimer and even larger oligomers (Kern et al., 1992).

When the inhibitor is combined with the BvVI1 protein in acidic pH, the proteins elute together in a broad peak with three maxima consisting of complexes with different composition. Either due to the relatively small size differences between the different complexes, or to a dynamic equilibrium of the complexes, a clear separation of the individual species is not possible. By analyzing the corresponding fractions via SDS-PAGE in reduced and non-reduced state (Fig. 3.32) it became obvious, that monomeric inhibitor is only present in the peak at 77 ml elution volume. The calculated molecular weight for a complex of this elution volume is approximately 63 kDa (see Table 3.3) corresponding to the sum of the molecular weights for the peaks observed for the monomeric inhibitor (peak 1, 19.7 kDa) and the BvVI1 protein (peak 3, 42.4 kDa). The other maxima consist of inhibitor dimers and probably one or two invertase molecules, as it can be concluded from the molecular weights in table 3.3. Although it was shown for NtCIF and AtPMEI1, the best characterized members of the PMEI-RP gene family, that the formation of two disulfide bridges is essential for the function of the inhibitor, it is intriguing that the BvC/VIF1-dimer, which posses at least one malformed disulfide bridge between the two molecules, is also able to form complexes with one or two BvVI1 molecules. After SEC, the invertase activity profile closely followed

the protein content of the fractions (Fig. 3.33). This indicated, that although the complex is stable during SEC, part of the complex dissociates after the separation, leading to returning invertase activity.

When recombinant NtCIF-protein (only monomeric) was combined with BvVI1, only one invertase-inhibitor complex peak was observed (Fig. 4.1), probably representing the native 1:1 complex found *in planta*. This is corroborated by the observed elution of invertase-inhibitor complexes extracted from wounded taproot tissues (Fig. 3.26.B) where both proteins elute together between 72 and 80 ml, corresponding to peak number 4 in Fig. 3.30.

Figure 4.1: Complex formation between recombinant BvVI1 and NtCIF during size exclusion chromatography
A: Elution profile of recombinant BvVI1 and NtCIF proteins when run combined over a Superdex200 column. The elution profile of BvVI1 alone is shown in red (Peak 3). Protein content was detected photometrically at 280 nm. NtCIF was added in excess, leading to a complex peak (2) and a peak of free inhibitor (1). The elution volumes and molecular masses of the used standard proteins are also given (A, β-Amylase; B, Alcohol Dehydrogenase; C, BSA; D, Carbonic Anhydrase; E, Cytochrome C).
B: Samples from the SEC run with BvVI1 and NtCIF were analyzed via SDS-PAGE and Coomassie-staining. Peak 1 at 94 ml consists of only NtCIF, whereas peak 2 at 78 ml contains BvVI1 and NtCIF protein.

In tobacco, CWI is protected from inhibition by NtCIF through the presence of sucrose, whereas VI is not (Sander et al., 1996). For the complex of BvVI1 and BvC/VIF1, the addition of sucrose during SEC has no influence on complex formation (Fig. 3.30.B). Therefore, if the two proteins are co-localized in the vacuoles of sugar beet taproots, the high sucrose concentration present should not circumvent complex formation. Also no significant effect on invertase inhibition was observed, when sucrose was present during complex formation between BvVI1 and BvC/VIF1 (data not shown).

Invertase-inhibitor complexes can be purified from plant extracts

The presence of stable invertase-inhibitor complexes was also shown for proteins extracted from sugar beet taproots and suspension cultures. During the cultivation of the sugar beet

suspension culture, BvC/VIF1 is expressed strongly in parallel with a CWI isoform. At least in 8 d old cells, a complex between the CWI and the invertase inhibitor is present and can be isolated from the cells by non-invasive salt-elution (Fig. 3.27). In extracts from wounded taproots, the two inhibitor proteins elute together during size exclusion chromatography. In contrast, in unwounded taproots the inhibitor is, due to the lack of target protein, predominantly present in monomeric form (Fig. 3.26). This shows, that at least after extraction of the taproot proteins under the given conditions (extraction and chromatography was carried out at pH 5.5), the major part of the present inhibitor is bound to invertases. However, the obtained fractions still possessed significant invertase activity, indicating that either the invertase is present in surplus of the inhibitor, the association in the complex is not tight enough to silence all invertase activity and dissociation occurs after the chromatography.

4.2.3 Presence of multiple inhibitor isoforms in taproot tissues

BvC/VIF1 is strongly expressed in taproots

The BvC/VIF1 cDNA shows an increasing expression level during taproot development. It is already present in 6 week old taproots and its expression increases with a maximum expression in mature taproots at harvest (Fig. 3.19). In cross-sections, the gene is slightly higher expressed in the cortex, but shows a strong expression throughout the taproot. Also during wounding, the expression stays at a very high level, with a slight increase three and five days after wounding. It is also expressed strongly in the *Beta vulgaris* suspension culture cells and in floral tissues. Compared to the expression of its probable target enzymes, the acid invertases, BvC/VIF1 shows an inverse regulation during taproot development (compare Fig. 3.2 and 3.19).

Only during wounding and in flowers, acid invertases and BvC/VIF1 are expressed in parallel. The increase of both CWI and VI activity after wounding appears despite the strong expression of BvC/VIF1 observed on RNA level. The question arises, why no inhibition of the invertase activity is observed. One explanation could be, that during wounding pH values in either the cell wall or the vacuole change, which impede an inhibition of the invertases. Also, the amount of newly formed invertase could be above the amount of inhibitor present and only part of the invertase can be inhibited.

In taproots, two inhibitor related proteins are detected

For a closer characterization of the expression of the sugar beet inhibitor, an antiserum was generated. To further increase the specificity of the antiserum, it was purified against the recombinant BvC/VIF1 protein, which reduced the presence of unspecific bands detected in taproot extracts (Fig. 3.24).

The detection of a 17 kDa protein, the expected molecular weight of BvC/VIF1, hinted, that this signal is formed by the BvC/VIF1 gene product. Also the signal was detected in

taproots and suspension culture, but not leaf tissue, where, according to Northern blots, the gene is not expressed. A closer inspection of samples from taproots on higher resolving gels (Fig. 3.25.A) revealed, that in taproots two proteins with a small size difference are detected. In suspension culture cells, only the smaller of the two was detected and this protein was strongly enriched when only a salt-eluted cell wall fraction of the cells was analyzed. During differential extraction of the soluble and the cell wall bound proteins from taproot tissues, a differential localization of the two proteins was observed (Fig. 3.25.B). In the soluble fraction, containing proteins localized in either the cytosolic compartments including the vacuole and proteins only loosely attached to the cell wall matrix, both proteins appeared. By contrast, in the fraction containing proteins, which maintain bound to the cell wall during the applied washing steps, only the smaller protein was detected.

Taken together, these data indicated the presence of at least two related inhibitor proteins in taproots. The larger protein was found exclusively in the soluble fraction. The smaller protein however, was detected in both fractions in taproots.

Purification of BvC/VIF1 from the cell wall of suspension culture cells

In the salt-eluted cell wall fraction from suspension culture cells, which can be obtained without prior disruption of the cells, only the smaller protein was detected. This indicates, that the protein is at least in these cells secreted into the apoplast. During lectin and size-exclusion chromatography, the cell wall localized inhibitor is found partially bound to a CWI. The suspension culture cells grow in a sucrose rich medium and here a CWI-isoform is strongly expressed, leading to an apoplastic cleavage of sucrose prior to the uptake into the cells. The situation resembles the data obtained from transformed tobacco suspension culture cells, where it was shown, that NtCIF forms a tight complex with a CWI (Weil et al., 1994).

To test, whether the cell wall localized protein in suspension culture cells corresponds to the BvC/VIF1 sequence or represents a second inhibitor isoform, the protein was purified from the cells and partially sequenced in a diploma thesis (Claussen, 2005). Two out of four obtained peptides matched with the known sequence, whereas the other peptides did not show homology to other invertase inhibitors or any sequences found in public databases (Tab. 3.2 on page 60). Due to the general low sequence conservation in the PMEI-RP gene family (Rausch & Greiner, 2004), the detection of peptides with a perfect match to the known sequence strongly indicated, that the protein recognized by the antiserum in the cell wall of the *Beta vulgaris* suspension culture cells is indeed encoded by the BvC/VIF1 gene.

Partial sequencing of both taproot expressed proteins

To elucidate the nature of the two observed signals found in Western blots of unwounded taproot tissues, a new approach was developed to purify and sequence both proteins. To

achieve this, the recombinant expressed vacuolar invertase BvVI1 was coupled to a Sepharose resin and used as an affinity matrix. Both proteins bound to the recombinant invertase at acidic pH, albeit with a relatively low efficiency (Fig. 3.28). By increasing the pH on the affinity column, the inhibitors could be eluted from the invertase. The resulting purified fraction was analyzed by Western blot and the bands corresponding to the inhibitor signals were subjected to tryptic digestion and partial sequencing using mass spectroscopy methods (carried out by Dr. Th. Ruppert, ZMBH Heidelberg). The results (Tab. 3.2) revealed, that for both protein bands two identical peptides matching the BvC/VIF1 sequence were identified. For the upper band, three additional peptides with no homology were identified, which probably are contamination with unrelated proteins. It has however to be kept in mind, that the sequenced peptides arose from bands with only a small size difference and that cross-contamination of the two bands can not be ruled out completely. The separation capacity of SDS-PAGE should however be large enough to separate proteins with only a small differences in size (Th. Ruppert, personal communication).

The finding, that both proteins contained peptides matching the known sequence opens some new interesting possibilities. Either the taproot contains two proteins, coded by closely related genes or the two observed signals represent differentially processed proteins of a single gene. If the proteins arise from different genes, these genes seem to show an unusual high sequence conservation, leading to for both proteins to the recognition by the antiserum against BvC/VIF1 and the identification of identical peptide sequences. A strong indication for the proteins being the product of a single gene comes from the results obtained from the analysis of sugar beet plants transformed with a BvC/VIF1 overexpression construct. In the leaves of transgenic plants, two proteins of a similar size than the proteins observed in untransformed taproots were expressed. This indicates, that the BvC/VIF1 gene may encode both inhibitor proteins (see. 4.3.2).

BvC/VIF1:GFP fusion proteins are localized in central vacuoles and show a specific processing

To test where the BvC/VIF1 protein is localized, constructs for the expression of the inhibitor in fusion with GFP at the C-terminus were generated and used for transient transformation of onion epidermal cells (Fig. 3.21 and 3.22) and sugar beet protoplasts. In both cases, GFP fluorescence appeared in the central vacuole. In some cases, especially in protoplasts from sugar beet, the endoplasmic reticulum was labeled in part of the cells, which could be due to aggregation of the proteins due to the high expression level in the transformed cells. These results showed, that the inhibitor:GFP fusion contains a functional signal peptide, leading to co-translational entry in the ER. Furthermore, the BvC/VIF1 sequence seems to contain a vacuolar sorting signal (VSS), as can be deduced from the observed GFP-fluorescence in vacuoles. In plants, three types of VSS have been described. The signals are found either as a N-terminal or C-terminal propeptide or as a physical structural motif inside

the protein's sequence (Neuhaus & Rogers, 1998; Matsuoka & Neuhaus, 1999). However, none of the described motifs of known VSSs shows a noticeable homology to the BvC/VIF1 sequence and, apart from motifs found in some storage proteins, the motifs are generally not very conserved (Matsuoka & Neuhaus, 1999), rendering a prediction of vacuolar localization difficult. A analysis of the vacuolar proteome of *A. thaliana* also revealed, that several of the soluble proteins found in leaf vacuoles contain no recognizable sorting signal (Carter et al., 2004), indicating the presence of additional pathways leading to vacuolar localization.

GFP-fluorescence in the cell wall was not detected, even when the tissue was bathed in neutral medium to prevent rapid degradation of GFP (Scott et al., 1999). A degradation of GFP could also explain the missing fluorescence inside the vacuole observed in some cases. For *A. thaliana* it is known, that GFP is subjected to specific degradation processes in the vacuole, which show a light and pH dependent regulation (Tamura et al., 2003). The authors also showed, that degradation of GFP is tissue dependent and they observed stronger degradation of GFP in leaf epidermal cells than in root cells. Variations in the degradation activities of the transformed cells could also be responsible for the lack of vacuolar fluorescence observed in some cells.

When tobacco leaves were transiently transformed with the inhibitor:GFP fusion construct, a different localization was observed (Fig. 3.23). GFP fluorescence was restricted to large vesicle-like structures. From these vesicles, the fusion protein could only be extracted by addition of SDS and was detected by antisera against GFP and BvC/VIF1. It is known from other studies, that strong, transient overexpression can lead to an unspecific aggregation of the overexpressed proteins, while the proteins are only slowly degraded (Pedrazzini et al., 1997; Brandizzi et al., 2003). The BvC/VIF1:GFP fusion protein could only be re-solubilized by addition of a strong detergent indicating that it formed tight aggregates inside the cell. Additional to the BvC/VIF1:GFP fusion protein, the inhibitor alone could also be detected in the transformed tissue. The size of the protein was comparable to the size observed in taproots. Due to the strength of the signal detected during the Western blots, a distinction whether this signal was formed by two different proteins was not possible. However, preliminary data from stable *A. thaliana* plants transformed with the same construct indicate, that this construct also forms a double band in the soluble fraction (data not shown).

In summary, these data indicate that the fusion construct is processed inside the cells. The cleavage must occur at the C-terminal end of the BvC/VIF1 part, or at the N-terminus of the GFP part of the fusion protein, since the cleaved signal is in size comparable to the native inhibitor observed in sugar beets. Whether this processing at the C-terminus also occurs in sugar beet and is responsible for the observed double signal, or occurs only in conjunction with the GFP-fusion has to be further investigated. The observations of Tamura et al. (2003) of a specific proteolytic degradation of recombinant GFP at its N-terminus in a light and pH-dependent manner indicate however, that the processing of the fusion protein might occur at the GFP part of the protein. After cleavage of the fusion protein, the GFP-part is probably

further degraded and neither fluorescence nor the protein in Western blots was detected. The inhibitor part of the fusion protein seems to be processed in a similar manner than the native protein, since two signals were detected in the *A. thaliana* transformants (see above).

4.3 Regulation of sucrose cleavage by ectopic overexpression of invertase inhibitors

Analysis of the mechanism of sucrose degradation in wounded and stored sugar beet taproots indicated, that the cleavage of stored sucrose by a vacuolar invertase is a key step for post-harvest sucrose losses occurring in sugar beet. An increase in invertase activity, which is paralleled by a rise in the content of reducing sugars is observed during storage of sugar beets (Wyse, 1974; Berghall et al., 1997). This increase is more pronounced during wounding of taproots, were a specific induction of a vacuolar and cell wall invertase is observed (this work, Rosenkranz et al., 2001). The increase in reducing sugars is paralleled with the induction of the BvVI1 protein (Fig. 3.4), emphasizing the importance of this enzyme for the cleavage of sucrose stored inside the vacuoles. A strong accumulation of glucose and fructose is observed after wounding. Also during storage, a continuous increase in reducing sugar content has been reported (Wyse, 1974). Since the formed hexoses are not consumed by the cell's metabolism, a large proportion of this cleavage seems to be dispensable.

From a biotechnological perspective, a minimization of these sucrose losses is desirable. On the one hand, because the implementation of longer storage periods after harvest will increase the loss of the extractable sucrose, on the other hand, because the accumulation of reducing sugars is a severe disturbance during the process of large-scale sucrose extraction (Burba, 1976; Milford, 2006). Invertase inhibitors pose a powerful biotechnological tool to modulate invertase activity in plants (Rausch & Greiner, 2004). They have been used successfully to influence senescence in tobacco leaves (Balibrea Lara et al., 2004) and to reduce cold-induced hexose accumulation in potato tubers (Greiner et al., 1999). In a first approach to silence invertase activity in wounded and stored taproots, NtVIF, the vacuolar invertase inhibitor from tobacco, was transformed into sugar beet under the control of the constitutive CaMV 35S promotor.

4.3.1 Overexpression of the tobacco vacuolar inhibitor NtVIF in sugar beet

In taproots, NtVIF overexpression does not affect sucrose loss after wounding

Altogether 19 individual transgenic lines overexpressing NtVIF were analyzed. From these, part of the lines from one genotype showed a reduction of VI activity after wounding and three lines from this genotype were analyzed in more detail.

In contrast to these preliminary results, when a larger number of individual plants from these three lines were analyzed, neither in adventitious roots of the T_0 generation, nor in seed-grown taproots of the T_1 generation, a significant reduction of VI activity, sucrose loss and hexose content after wounding was observed (Fig. 3.10 and 3.11). Also no significant reduction of VI activity in leaves was observed (data not shown). This is in contrast to the results obtained, when the same inhibitor was ectopically expressed in potato tubers. Here a specific reduction of soluble invertase activity was observed in leaves (Greiner et al., 1999) and tubers after wounding (Pfister, 2003). The missing effect of NtVIF on wound induced VI activity in sugar beet can have several causes.

Concerning their activity against soluble invertase preparations from wounded sugar beet taproots, the activity of recombinant NtVIF protein was weaker than the activity of the endogenous sugar beet inhibitor BvC/VIF1. Although NtVIF is able to inhibit the invertase, much larger amounts of recombinant protein are needed to reach a significant inhibition (Fig. 3.17.E and F). At the same time, the recombinant NtVIF protein was able to inhibit soluble invertase from tobacco leaves, its putative native target, effectively. The minor effect of NtVIF overexpression on the wound induced invertase activity might therefore be due to a weak affinity of the tobacco inhibitor for the sugar beet invertase, since tobacco and sugar beet are not closely related. In contrast to this, potato and tobacco belong to the same plant family (Solanaceae) and therefore the target invertases are more closely related leading to the successful inhibition of VI during transgenic overexpression of NtVIF in potato (Greiner et al., 1999).

Another explanation could be, that the amount of the ectopically expressed inhibitor is not sufficient for the large amount of invertase protein produced after wound induction. Additionally, the conditions inside the vacuole could reduce the affinity of the inhibitor for the invertase, e.g. through an increase in vacuolar pH during wounding. This is supported by the fact, that untransformed taproots show a strong induction of both cell wall and vacuolar localized invertase activity after wounding, although BvC/VIF1, the endogenous sugar beet inhibitor, is strongly expressed at the same time. The amount of BvC/VIF1 protein is therefore either not sufficient to inhibit all of the massively induced invertases, or the ambient conditions, most likely the pH, prevent inhibition. Several of the above factors probably contribute to the missing reduction of invertase activity after wounding.

Both, adventitious roots and seed-grown taproots, showed a reduced root weight compared to untransformed plants of the same age (Fig. 3.10.A and 3.11.A). Although the difference was due to large variations in the plant material not significant, the repeated appearance of this observation indicated an effect of the transgene on overall taproot development. A reduction of root size has also been observed in *A. thaliana*, when a VI isoform was knocked out (Sergeeva et al., 2006). This could indicate, that the overexpression of NtVIF in the taproot could also reduce the size of taproots by inhibition of VI present in early root development.

The creation of plants, which overexpress the endogenous inhibitor BvC/VIF1 (see below) will hopefully also add to the understanding of metabolic reactions caused by ectopic expression of invertase inhibitors.

4.3.2 Transgenic approaches using the endogenous inhibitor BvC/VIF1

The characterization of the endogenous sugar beet invertase inhibitor BvC/VIF1 showed, that this protein shows a stronger affinity for sugar beet vacuolar invertases than NtVIF. Therefore, the generation of new transgenic sugar beet plants with an overexpression or down-regulation of this gene was initiated. After cloning of the constructs, the transformation was carried out by KWS. Due to the considerable time needed for the generation of transformed sugar beets, only *in vitro* grown plantlets of these plants were available for analysis during the preparation of this thesis.

Constitutive Overexpression

In vitro grown leaves from plants overexpressing BvC/VIF1 under the control of the constitutive CaMV 35S promotor show a strong expression of the transgene, as can be deduced from the detection of the transcript in Northern blots and the inhibitor protein with the BvC/VIF-antiserum (Fig. 3.34). No expression was detected in WT or RNAi plants. In Western blots, the same protein bands as in extracts from taproots were observed (compare Fig. 3.25 and Fig. 3.34). Two proteins were detected in the soluble fraction, whereas in the cell wall fraction only the smaller protein was found. Together with the result, that both proteins purified from taproots contained peptides of the BvC/VIF1 sequence (see 4.2.3), this provides further evidence, that both proteins are products of one gene. The larger protein is again only detected in the soluble fraction, but the signal is compared to the smaller fragment weaker in the transformants. It might therefore be possible that the larger protein represents a propeptide of the inhibitor that is not fully processed.

The overexpression of BvC/VIF1 led to significant inhibition of the soluble invertase activity, whereas the CWI activity remained unchanged compared to the wildtype (Fig. 3.35). Since the smaller, presumably fully processed inhibitor protein was found both in the soluble and in the cell wall bound fraction, these data emphasize the specificity of BvC/VIF1 for vacuolar invertases, since the CWI activity in the insoluble fraction was not affected. When the inhibitor was produced recombinantly in bacteria, it also showed a stronger inhibition of VIs from leaves compared to CWIs (Fig. 3.17.A). CWI prepared from *Beta vulgaris* suspension culture cells however was inhibited completely, indicating distinct affinities of the inhibitor for different CWI isoforms.

Whether the marked inhibition of the soluble invertase activity in the transformed plants is due to the co-localization of the overexpressed inhibitor protein with the VI in the vacuole

in planta, or the inhibition is rather due to the binding of the inhibitor during the extraction can at this moment not be decided. However, when NtCIF, the cell wall localized inhibitor from tobacco, was overexpressed in tobacco and potato, a specific reduction of only CWI was observed, whereas VI activity, which is also inhibited by recombinant NtCIF, was not affected (Greiner, 1999). Similarly, the overexpression of NtVIF in potato only led to a specific inhibition of vacuolar invertase in leaves (Greiner et al., 1999) and tubers (Pfister, 2003) and CWI activity was not affected.

Taproot-specific Overexpression

Down-regulation of either CWI or VI activity in the whole plant can lead to severe distortions of plant development, as has been observed during repression of invertases by an antisense approach in carrot (Tang et al., 1999). Similar effects might arise if a strong invertase inhibitor is expressed constitutively in the whole plant. Therefore, in parallel with the plants with a constitutive overexpression of BvC/VIF1, a second strategy was used to overexpress the inhibitor under control of a taproot specific promotor.

In some lines expressing the inhibitor under control of the presumably root specific 2-1-48 promotor, a strong expression in leaves is detected. The used fragment of the 2-1-48 promotor, which normally controls the expression of the *Mll* gene, is described to deliver a strong expression in young and old sugar beet roots and only very little activity in leaves (Oltmanns et al., 2006). Although the detected BvC/VIF1 transcripts seem to contradict these results, the leaves analyzed here were not from soil-grown plants but from tissue culture, which might severely influence gene expression in the plant. A selection of plants showing the desired strong expression in roots, but not in leaves, therefore can only be done based on results obtained from seed-grown plants.

Downregulation of BvC/VIF1 Expression

The knock-down of the BvC/VIF1 gene via an RNAi approach is aimed at the generation of plants with a decreased expression of the inhibitor. Silencing obtained by overexpression of hairpin RNAs, which include an intron inside the linker of the sense and antisense part of the target gene, has proved to be a very efficient method for the targeted reduction of the expression of a certain gene (Wesley et al., 2001). However, the reduction of the targeted mRNA can vary between individual transgenic lines, giving rise to lines with different degrees of gene-silencing (Waterhouse & Helliwell, 2003). Therefore several transgenic lines have to be analyzed concerning the expression of the BvC/VIF1-gene and to be compared with the wildtype. Since the gene is strongly expressed only in taproots, this experiments can only be carried out when such material will be available. Lines with a strong reduction in BvC/VIF1 expression will then be further characterized in order to elucidate the physiological role of the inhibitor during taproot development. A first focus of the analyses will be the development of

invertase activity and hexose content during taproot development. An increase of both would be indicative of an important function of the inhibitor in maintaining minimal invertase activity during taproot development.

4.4 BvC/VIF1: CIF of VIF, one or two genes?

At present, some important questions concerning the function of the newly identified and characterized invertase inhibitor from sugar beet still lack a decisive answer.

First, the identification of two proteins in taproot, separated by a small difference in molecular weight, indicated the presence of a second, closely related isoform to BvC/VIF1. However, following research brought up new results indicating that both proteins may be encoded by the same gene and represent differently processed variants.

Second, investigations about the subcellular localization of the BvC/VIF1 gene product(s) yielded partially conflicting results. Transiently overexpressed fusion proteins with the fluorescent marker GFP yielded different results depending on the transformed plant material. Furthermore, partial sequencing of proteins from *Beta vulgaris* suspension culture cells and taproots, yielded sequences of the BvC/VIF1 protein, although the purified proteins are differentially localized.

In tobacco, two isoforms of invertase inhibitors have been found, which are either localized in the cell wall (CIF) or in the vacuole (VIF), whereas in *A. thaliana*, the subcellular localization of two described invertase inhibitors has not yet been determined (Link et al., 2004).

4.4.1 Detection of two inhibitor proteins in taproots and BvC/VIF1 overexpressing plants

Several observations provide evidence, that the two observed proteins in the soluble fraction of taproots are both encoded by the BvC/VIF1 gene. The overexpression of the BvC/VIF1 cDNA in sugar beet plants led to the detection of two proteins in the soluble fraction. These proteins are of sizes similar to those observed in taproots. Again the protein with the smaller molecular weight is also found in the cell wall bound fraction.

Also the partial sequencing of both bands from untransformed taproots yielded for both protein bands peptide sequences fitting to the predicted BvC/VIF1 protein sequence. The latter finding could also be explained by the existence of two highly homologous genes, that are expressed in parallel and show stretches of identical amino acids. However, the sequence conservation of members of the PMEI-RP gene family is low and the characterized members show only a moderate sequence conservation (Rausch & Greiner, 2004) and up to now, no closely related ESTs from sugar beet have been found in available databases. Also Southern blot analysis did not indicate the existence of sequences closely related to the BvC/VIF1

sequence (Fig. 3.18).

Given that the above assumption is correct, the question arises what modification leads to the appearance of the two protein signals. One possibility could be a post-translational processing event, occurring either at the N- or C-terminus of the protein. When the predicted BvC/VIF1 protein sequence is compared to other PMEI-RP sequences (Fig. 3.15), a relatively high sequence conservation at the C-terminus is observed, making a major processing here unlikely. The N-terminus of the BvC/VIF1 however, contains variations to the other members leaving open the possibility for post-translational cleavage. For the best characterized member of the gene family, NtCIF, the true *in planta* N-terminus was identified (Weil et al., 1994) and the 3D-structure of the protein has been determined (Hothorn et al., 2004a). The homology between the BvC/VIF1 and the NtCIF sequence begins with the first α-helix of the mature NtCIF protein containing the first cysteine residue. Compared to the NtCIF sequence, the BvC/VIF1 protein contains further amino acids between the end of the predicted signal peptide and this first α-helix. A cleavage of these amino acids could lead to the size difference observed between the two proteins (approx. 1 kDa or less). Also the tobacco vacuolar inhibitor NtVIF contains further amino acids here, which might lead to the vacuolar localization of the protein, although this has not been shown yet. Furthermore, the end of the signal peptide is not unambiguously predicted (Fig. 3.20), leaving open the presence of additional amino acids that could be part of the cleaved propeptide.

In the *Beta vulgaris* suspension culture, only the smaller of the two proteins was found in the cell wall fraction and the larger protein was not observed. Peptide sequences of this protein matched the BvC/VIF1 sequence. If both proteins arise from one gene, this would indicate, that the processing or the stability of the protein is also tissue dependent.

In order to investigate potential processing events further, the N-terminus of both proteins has to be identified by N-terminal sequencing. Since purification of larger amounts of the inhibitor proteins from taproots proved to be rather difficult, the newly generated plants overexpressing BvC/VIF1 will be a valuable tool. The high expression in these plants should simplify the purification of higher amounts of pure protein needed for Edman sequencing. With this method, the native N-terminus of both proteins can be identified, which would also elucidate, whether a differential processing at the N-terminus is the reason for the size difference between the two proteins.

4.4.2 Localization of the inhibitor proteins

Only the smaller of the two inhibitor proteins was found in soluble and salt-eluted fractions containing proteins ionically bound to the cell-wall. The larger protein is only found in the soluble fraction (Fig. 3.25). This indicated, that the two proteins, irrespective if they are encoded by one or two genes, have a different subcellular localization. In suspension culture cells, the smaller protein was shown to be localized in the cell wall. Since the smaller of the two proteins is also found in the cell wall fraction of taproots and the BvC/VIF1

overexpressing plants, a cell wall localization of this protein can be concluded.

However, several other findings support a vacuolar localization of at least the larger of the two proteins. In transient transformation experiments, BvC/VIF1:GFP fusion proteins were targeted to the vacuole (Fig. 3.21 and 3.22), or, after proteolytic cleavage of GFP, led to a reduction of soluble invertase activity (Fig. 3.23). In sugar beet leaves overexpressing BvC/VIF1, again only soluble invertase activity was inhibited, although the protein was found in the soluble and the cell-wall fraction (Fig. 3.34 and 3.35). The missing inhibition of CWI in the overexpressing plants can likewise be explained by the weaker affinity of the protein for CWIs, however, it highlights vacuolar invertases as the most likely target for at least the larger of the two proteins.

A dual targeting of one gene product is only common in plants for proteins found in parallel in mitochondria and plastids (Small et al., 1998; Mackenzie, 2005), which have a related protein import machinery. A dual targeting of a soluble protein to the cell wall and the vacuole has only been observed during transient overexpression of vacuolar proteins, leading to a saturation of the vacuolar sorting system followed by partial secretion of the protein (Neuhaus et al., 1994; Frigerio et al., 1998).

Further efforts have to be undertaken to elucidate the true *in vivo* localization of the inhibitor protein(s). One approach could be immuno-localization experiments using the BvC/VIF1 antiserum. However, this antiserum does not discriminate the two inhibitor proteins, which could complicate interpretation of the results. Also a close monitoring of the changes in the activities of acid invertases in the inhibitor overexpressing sugar beet plants will help to find the target invertases of the BvC/VIF1 gene.

4.5 Outlook

Sugar beet breeders are trying to identify sugar beet lines with differences in post-harvest sucrose losses in order to find genetic markers and target genes correlating with this trait. The identified genes regulating respiration efficiency in sugar beet, which show in the case of alternative oxidase a strong response to wounding and storage of taproots, are potential target genes for this trait. The obtained data about these genes can be used for the comparison of sugar beet lines with extreme phenotypes in post-harvest respiration. The question to address will be, whether these lines differ in the expression and activity of UCPs and AOX, which could influence differences in the overall respiration rates. However, both activities could also be indispensable for the plant as a protection mechanism against reactive oxygen species.

The role of acid invertases for sucrose losses in wounded and stored taproot has been analyzed in detail. The endogenous invertase inhibitor BvC/VIF1 is a possible key regulator of invertases during taproot development. The high expression in the developing taproot most likely ensures the silencing of residual invertase activity. This assumption will be tested in

transgenic sugar beet plants with a down regulation of BvC/VIF1 expression by testing whether these plants show alterations of the invertase activities during plant development. The overexpression of the gene in sugar beet will help to elucidate, why after wounding of taproots the invertase activity dramatically increases, albeit the strong expression of the inhibitor. Also the effects of constitutive overexpression of invertase inhibitors on plant development have to be further analyzed.

The presence of two inhibitor proteins in taproots and BvC/VIF1 overexpressing plants has raised further questions about a potential processing of the inhibitor and its subcellular localization. Identification of the *in vivo* N-terminus of both proteins can provide new evidence for processing events. Likewise, the impact of BvC/VIF1 overexpression and down-regulation on invertase activities in transgenic plants will help to understand the localization and the role of the gene.

Although NtVIF overexpression did not yield the expected strong reduction of invertase activity after wounding, changes in root weight were observed. A more detailed analysis of the impact of NtVIF overexpression during all stages of plant development might provide helpful hints, how the inhibitor overexpression influences taproot development. These data can then be compared with similar analyses of transgenic plants with altered BvC/VIF1 expression.

The establishment of overexpression of active plant invertases in *E. coli* opens many new approaches for the in-depth characterization of the enzyme and its interaction with inhibitor proteins. Both partners can now be produced in significant amounts and are accessible for further molecular studies, e.g. for mutational approaches aiming at the identification of important domains for the interaction.

5 Materials and Methods

5.1 Plant Material

5.1.1 Sugar Beet

A diploid inbred line (Partie-Nr. VV-I/ZR 10738, KWS SAAT AG) of sugar beet plants (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima* DÖLL) were either field-grown between march and october on the trial-field of the Heidelberg Institute for Plant Sciences or grown in the green-house in special rose-pots (20 cm high) with 13 h of supplementing light.

Procedure for wounding of sugar beet taproots

Wounding of sugar beet taproots was carried out according to Rosenkranz et al. (2001) by removing cylinders (2 cm in diameter) from the taproot interior with a cork borer and cutting the cylinders into 2 mm thick slices with a set of fixed razor blades. The slices were incubated in a moist atmosphere for up to six days at room temperature in the dark.

***Beta vulgaris* suspension culture**

The heterotrophic *Beta vulgaris* suspension culture was grown in the following medium:
3.17 g/l Gamborg B5 Medium (Serva) pH 5.5, 60 g/l sucrose, 0.5 mg/l Kinetin, 1 µg/l NAA (1-Naphthaleneacetic acid) . The medium was sterilized by autoclaving.
Approximately 5 ml of a densely grown culture were transferred weekly to 50 ml fresh medium and cultivated in the dark at 25 °C with shaking at 90 rpm.

5.1.2 Tobacco

For transient, *Agrobacterium*-mediated, transformation of leaves (see 5.6.2), *Nicotiana tabacum* L. SNN and *Nicotiana benthamiana* L. plants were used. The plants were grown in soil in the greenhouse and only young plants (5 to 10 expanded leaves) were used for the transformation.

5.2 Microbiological techniques

5.2.1 *Escherichia coli*

For cloning procedures the *E. coli* strain XL1-Blue (Stratagene) was used.
Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI^q Z*Δ*M15* Tn*10* (Tet^r)]

For the expression of recombinant proteins the strain Rosetta-gami (Novagen) was used. The strain carries an additional plasmid (pRARE, Chloramphenicol resistance), coding for six tRNAs seldom used in *E. coli*, and therefore supports the expression of eukaryotic genes. Mutations in the thioredoxin (*trxB*) and glutathione (*gor*) reductase enzymes promote the formation of disulfide bonds in the *E. coli* cytoplasm.

Genotype: Δara-$leu7697$ $\Delta lacX74$ $\Delta phoAPvu$ II $phoR$ $araD139$ $ahpC$ $galE$ $galK$ $rpsL$
F'[lac$^+$(lacI^q)pro] $gor522$::$Tn10$ $trxB$::kan pRARE

Media and antibiotics

Bacteria were either grown in LB-medium for cloning purposes or in TB-medium for bacterial overexpression (both prepared according to Sambrook et al., 1989). Selection was carried out with the following concentrations of the antibiotics:
Ampicillin 100 µg/ml, Chloramphenicol 34 µg/ml, Kanamycin 50 µg/ml, Spectinomycin 100 µg/ml, Tetracyclin 12.5 µg/ml, Zeocin 25 µg/ml (low salt LB (5g NaCl/l))

Preparation of electrocompentent *E. coli* cells and transformation by electroporation

One liter of low salt LB containing the appropriate antibiotics was inoculated with 20 ml of an over night bacterial culture and incubated until OD_{600nm} reached 0.7. Then the culture was cooled to 4℃ and cells were collected by centrifugation. The pellet was washed twice with 500 ml of dd-H_2O, then with 40 ml 10% glycerol and finally resuspended in 4 ml 10% glycerol, frozen in 50 µl aliquots in liquid nitrogen and stored at -80℃.
The electrocompetent cells were transformed by electroporation with a GenePulserII (Bio-Rad) set to 200 Ω, 1.8 kV, 25 µF and incubated in 1 ml SOC-medium for 1 h at 37℃ before plating variable volumes on selective LB-plates.
SOC-medium: 20 g/l tryptone; 0.5 g/l yeast extract; 0.5 g/l NaCl; 0.186 g/l KCl; 2.03 g/l $MgCl_2$; 3.96 g/l glucose-monohydrate; pH 7.0

5.2.2 *Agrobacterium tumefaciens*

For stable transformation of *Arabidopsis thaliana* and transient transformation of *Nicotiana tabacum*, the binary plasmids were transformed into the Agrobacterium strain C58C1 carrying the T_i-plasmid pGV2260. The plasmids for the transformation of sugar beet were transformed into the strain GV3101.
Agrobacteria were grown in YEB-medium (1 g/l yeast extract, 5 g/l beef extract, 5 g/l peptone, 5 g/l sucrose, 0.493 g/l $MgSO_4$ x 7 H_2O, pH 7.5) supplemented with 100 µg/ml rifampicin (genomic resistance) and either 50 µg/ml carbenicillin (C58C1) or 25 µg/ml gentamycin (pGV2260) and depending on the transformed plasmid with 50 µg/ml kanamycin or 100 µg/ml spectinomycin.
Electrocompetent Agrobacteria were prepared by inoculating 200 ml YEB-medium supplemented with the appropriate antibiotics with 3 ml of an overnight culture and grown until OD_{600nm} reached 0.95. Cells were collected (4℃ , 2,000 g, 5 min) and washed twice with 10 % glycerol, 1 mM Hepes, pH 7. The cells were finally resuspended in 2 ml of the same solution and frozen in liquid nitrogen as 50 µl aliquots.
Agrobacteria were transformed as described for *E. coli*, except that after transformation the cells were incubated for 2 h at 28℃ in SOC-medium and allowed to grow on selective plates for 2 days at 28℃.

5.2.3 *Pichia pastoris*

For the purification of the IbVI2 protein, the *Pichia pastoris* strain X-33 carrying the plasmid pYIT2-M was used, which was kindly provided by Prof. Hsien-Yi Sung, National Taiwan

University (Wang et al., 2005). Media and plates were prepared according to the Pichia Expression Kit Manual provided by Invitrogen (Invitrogen, 2002).

5.3 DNA techniques

5.3.1 Separation of DNA molecules by electrophoresis

Agarose gels

For separation of purified DNA, 0.8 to 1.5 % agarose gels were prepared in 1xTAE-buffer (Sambrook et al., 1989). DNA samples were prepared by adding a suitable volume of 5x loading buffer (50 % glycerol, 5x TAE-buffer, 1 % Orange G (w/v)). As molecular weight markers, either SmartLadder (Eurogentec) or the 2-log ladder (NEB) were used. After the gel run, DNA was stained using a solution of 0.1 µg/ml Ethidium bromide in water.

Polyacrylamide gels

For the separation of smaller DNA fragments (< 800 bp) and to detect minor size differences, DNA was separated in 11.25 % polyacrylamide gels. Gels were prepared using 3 ml dd-H_2O, 2 ml native separating buffer (1.5 M Tris, pH 8.8), 3 ml acrylamide (29.2 % (w/v) acrylamide, 0.8 % N,N'-Methylene bisacrylamide (37.5:1)), 45 µl APS (10 % ammonium peroxodisulfate) and 15 µl TEMED (N,N,N,N'-Tetramethyl-ethylenediamine)). The gel run was carried out in native electrophoresis buffer (3.6 g/l Tris, 14.4 g/l glycine, pH 8.6) at 200 V and the gels were stained as described above.

5.3.2 Oligonucleotides

All oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). The lyophilized primers were dissolved in TE-buffer (10 mM Tris, 1 mM EDTA) at a concentration of 100 pmol/µl. In the following list, the oligonucleotides are sorted according to the experiments they were used for. A systematic name, the primer name and the primer sequence in 5' to 3' direction are given.

Overexpression of BvC/VIF1 (pQE30/pETM-20)		
-	Bv-inh_l	TCTAGTAGATGGTACCTATTCTCGCAAGACCACCAAC
-	Bv-inh_r	TTAGAACATTCTGCAGTCATTCCAAACTCTTAATCATAG
JE2	Bv_INH_forw2	TCACGATGTACATGTTCACCACCTCTACTTTAGCG
JE3	Bv_INH_rev	TTAGAACATTGCGGCCGCTCATTCCAAACTCTTAATCATAG
RACE BvC/VIF1		
JE4	Bv-INHH-Race1	GGCCTCATTACCCGCATCCACCATA
JE5	BV-INHH-Race-Nested	ACACTCTTGCAGGGCCCGGATTACA
JE109	5'RACE-nested	CGGAGCGAAGCTTCGCAAAGAATCGGGTCGGGT
JE110	5'RACE	CCGGGTCAAGTCCTCTACATATCGAAA
JE6	Bv-INHH-RT-left 3'RACE	GCGTATTCTCGCAAGACCACCAACG
CC9	spez-forw 3'RACE nested	AACACCCGACCCGATTCTTTGCGAAGCT
RACE BvAOX		
JE29	5'Race	TGCACGGCAACCATAGCGCCTCTTA
JE30	5'Race_nested	CGTGATGTTTGCTCAGATCAATGGA
JE31	3'Race	GCCGAAATGGTACGAGAGACTATTGG
JE32	3'Race_nested	TCCCCTAAGTTGGCACACAGAGT

Overexpression of BvVI1 (pET-G vectors/pPICZαA)		
JE75	12attB1TEV_BvVIwit	TATTTTCAGGGCGGAGAAAGTGGTATTTCG
JE76	12attB2_BvVIwit_rev	AGAAAGCTGGGTTCAAAAAATGTAGGGAG
JE81	pic_VIwit_sgis_KpnI	ACTGCAGGGTACCAGTGGTATTTCGTTTGCG
JE82	pic_VIwit_reverse	TAGCATCCCGCGGCAAAAATGTAGGGAGAAAG
Gateway 2-step PCR		
JE77	attB1_TEV_adapt	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTGAGAATCTTTATTTTCAGGGC
JE78	attB2adapter	GGGGACCACTTTGTACAAGAAAGCTGGGT
Construction of BvC/VIF1-GFP fusion constructs (pK7FWG2/pFF19-GFP)		
JE33	Bv_full_forw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACCATGACAACTCTAAACACCTCTTTACC
JE34	Bv_full_rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATTCCAAACTCTTAATCATAG
JE44	BvC/VIF_NruI_forw	TACTATATTCGCGACCACCATGACAACTCTAAACACC
JE42	BvCVIF_full_KpnI	TACTATATGGTACCTTCCAAACTCTTAATC
Construction of BvC/VIF1 plant transformation constructs (see p. 101)		
Construction of p2-1-48-BvC/VIF1		
JE56	2-1-48_HindIII_XmaI_forw	CATCGGAAGCTTCCCGGGCTGAACTGTTAATTATTTCAACTA
JE57	2-1-48_AgeIAscISacI_rev	CATCGGGAGCTCTTGGCGCGCCAAACCGGTGGCTTTTGAAAATTTTGAAACGC
JE58	BvCVIF_AgeI_forw	CATCGGAAGCTTCCCGGGCTGAACTGTTAATTATTTCAACTA
JE59	BvCVIF_AscI_rev	CATCGGGGCGCGCCTCATTCCAAACTCTTAATCATAG
Construction of p70S-BvC/VIF1		
JE63	p35d_XmaI_L	CATCGGACTCCCGGGATGCCTGCAGGTCTCAGAAG
JE64	p35d_AgeI_R	CATCGGACTACCGGTGTGGCCACTCGAGGTCCTC
Construction of p70S-RNAi-BvC/VIF1		
JE47	BvCVIF_anti_fo	CATCGGGGTACCCTCGAGTCATTACCCGCATCCACCAT
JE48	BvCVIF_anti_SacII_rev	CATCGGCCGCGGAAGGCTGCTGACTCTGAAGGTT
JE49	BvCVIF_sense_SpeI_forw	CATCGGACTAGTAAGGCTGCTGACTCTGAAGGTT
JE50	BvCVIF_sense_SacI_rev	CATCGGGAGCTCTCATTACCCGCATCCACCAT
JE51	BvCVIF_intron_SacII_for	CATCGGCCGCGGTCTAGGTCAGATTCGCTATCTAT
JE52	BvCVIF_intron_SpeI_rev	CATCGGACTAGTCTTCCTCCTGTTTATCTTTTAAC
Generation of labeled probes		
-	vC175L	TACTATATGGATCCAACACGACCTGCAGAGCCAC
-	v558R	TACACAATGTCGACTCAATTCATATCAGAAACTGGAG
JE7	lBv_AOX	TCCGGCTGATTTGTCCATTG
JE8	rBv_AOX	TGCCAACTTAGGGGAAAGCA
JE15	lBV_UCP1_UTR	CGTTTTCTTAATCATCAGGCATC
JE16	rBV_UCP1_UTR	GCAAAATTACAAGGCAATCCT
JE17	lBv_UCP2_UTR	AAGGCCTTGCTGTCTCAGTC
JE18	rBV_UCP2_UTR	TGGCGACAGGTACATTTTCA
JE37	CWI1_wit_forw	GGCTAACGAGTCTTCAAGTGTGGA
JE38	CWI1_wit_rev	AGAGAAGACCTGCTTTGGTCACTG
JE39	CWI2_nit_forw	CCAGCCTCTCAAGAACTGGATGAA
JE40	CWI2_nit_rev	TTGGTACAGATACGCCATTCCTCG
-	bvi31/L	ATAAATGGTGTTTGGACAGGGTCCGC
-	bvi31/R	ACTCACCAACCCAACCCCACAAAATCC
JE71	BvVInit_Sonde_l	TCAATCGATCAATCTCCAAAAC
JE72	BvVInit_Sonde_r	GGACCGTTTGGATCATTCAT
JE92	SBSS1_UTR_f	GTGATCTGGCCAACTCTGTT
JE93	SBSS1_UTR_r	TGATCACTGAAGAATCCGTT
JE94	SBSS2_UTR_f	ACTTAGAAATGTTCTACGCC
JE95	SBSS2_UTR_r	CTCCACAATCAAAAGACAAT

5.3.3 PCR techniques

For most PCR applications Taq Polymerase from Invitrogen was used with the supplied buffers. A standard sample consisted of 1 µl template (various concentrations of cDNA or plasmid), 1 µl dNTPs (10 mM each), 2 µl of each primer (10 pmol/µl), 5 µl 5x PCR-buffer,

1.5 µl MgCl$_2$ (50 mM), 0.2 µl Taq (5 U/µl) and was adjusted to 50 µl with water. PCR was carried out with in a Biometra Personal cycler with the following program:

Initial denaturation	95 ℃	5 min	1 cycle
Denaturation	95 ℃	45 sec	
Annealing	48 - 60 ℃	45 sec	35 cycles
Extension	72 ℃	0.5-3 min	
Final extension	72 ℃	5-10 min	1 cycle

The extension time and the annealing temperature were adjusted according to the length of the amplified product and the used primers respectively.

For cloning of larger PCR products, the proofreading Vent DNA polymerase (NEB) or AccuPrime Pfx DNA Polymerase (Invitrogen) was used according to the manufacturers instructions.

Production of biotinylated probes

Biotinylated probes were generated by adding (instead of normal dNTPs) 8 µl of a mixture containing 0.25 mM Biotin-16-dUTP (Roche), 0.75 mM dTTP and 1 mM each of dATP, dGTP and dCTP to a 100 µl PCR sample. Success of biotinylation was monitored by running the PCR-generated probe on a polyacrylamide gel next to a PCR reaction carried out with standard dNTPs. Biotinylated PCR products run, due to the incorporation of biotinylated dUTP, at a slightly increased molecular weight compared to the unlabeled products.

Production of digoxigenin-labeled probes

For Southern blots, either digoxigenin (DIG) or biotin labeled probes were used. For the generation of DIG-labeled probes, 10 µl of a mix containing 0.1 mM DIG-11-dUTP (alkali-labile, Roche), 1.9 mM dTTP, and 2 mM each of dATP, dCTP, dGTP were added to a 100 µl PCR reaction. PCR was performed as described for biotinylated probes.

Probes for Northern and Southern Blotting

The following table gives the primer combinations used for the production of Biotin- or DIG-labeled probes. (For the sequences of the individual primers see 5.3.2). The probes covered either part of the open reading frame (ORF), or the more variable untranslated regions of the mRNAs to allow distinction of closely related isoforms.

Gene	Primer combination	Length of generated probe
BvAOX	JE7/JE8	391 bp ORF
BvC/VIF1	JE2/JE3	491 bp ORF
BvCWI1	JE37/JE38	468 bp ORF
BvCWI2	JE39/JE40	468 bp ORF
BvUCP1	JE15/JE16	250 bp 3' UTR
BvUCP2	JE17/JE18	360 bp 3' UTR
BvVI1	bvi31L/bvi31R	490 bp ORF
BvVI2	JE71/JE72	57 bp 5' UTR + 482 bp ORF
NtVIF	vC175L/v558R	384 bp ORF
SBSS1	JE92/JE93	83 bp ORF + 213 bp 3' UTR
SBSS2	JE94/JE95	77 bp ORF + 272 bp 3' UTR

2-step PCR for addition of Gateway-compatible overhangs

For the creation of PCR products with ends compatible for Gateway cloning, a two step PCR protocol was used[1]. For the first PCR step, template specific primers were used with the following bases added to the specific sequence:

left: 5'-TATTTTCAGGGC-(template specific sequence)-3'
right: 5'-AGAAAGCTGGGTN-(template specific sequence)-3'

A first PCR was carried out, which consisted of only the initial denaturation and ten PCR cycles. Only 1 µl of each primer (10 pmol/µl) was included in a 50 µl reaction.
In a second PCR, 4 µl of the following primers, containing the complete Gateway overhangs and the TEV protease recognition site (amino acids: ENLYFQG, underlined bases), were added:

left: JE77   5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCTGAGAATCTTTATTTTCAGGGC-3'
right: JE78   5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'

The reaction included 10 µl of the first PCR as template and 4 µl of the two primers (10 pmol/µl). The PCR program consisted of an initial denaturation step, followed by 5 cycles with an annealing temperature of 45 °C, 20 cycles at 52 °C and 10 min of final extension. Denaturation and extension was carried out as described for standard PCRs (see above).

RACE-technique: Determination of cDNA-ends

For the determination of the full length sequence of partially known cDNAs, the Generacer Kit (Invitrogen) was used. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen). The preparation of the RACE-cDNA was done according to the Generacer Kit manual. First, incomplete mRNAs were 5' dephosphorylated with calf intestine phosphatase (CIP), then the 5' cap structure of intact mRNAs was removed by tobacco acid pyrophosphatase (TAP). At the resulting free 5' phosphate, the Generacer RNA oligo was ligated with T4 RNA ligase.
The reverse transcription using Superscript III reverse transcriptase and the Generacer Oligo dT primer results in the RACE-cDNA containing full length cDNAs with known sequences at the 5' and 3' end. By combining primers directed against either the Generacer oligo or the overhang of the Generacer Oligo dT primer and an adequate gene-specific primer, the 5' or the 3' end of the cDNA can be amplified by PCR. In order to increase the specificity of the reaction, a nested PCR was carried out using the first PCR as template. The PCR products were subcloned, and several clones from independent PCR reactions were sequenced.

5.3.4 Cloning procedures

Gel extraction and PCR purification

For the purification of DNA fragments from agarose gels or the clean-up of PCR products the NucleoSpin Extract II Kit (Macherey-Nagel) was used according to the manufacturers instructions.

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[1] http://www.embl-hamburg.de/~geerlof/webPP/protocoldb/Cloning/gateway_2step_PCR.html

Cloning using restriction digestion

All restriction enzymes were purchased from New England Biolabs (NEB) and used with the supplied buffers. For analytical digestions 1 µg and for preparative digestion 5 to 20 µg of plasmid DNA were used. Incubation was performed for 2 to 16 h at recommend temperature. Usually 3 U of enzyme were added per µg of DNA.
Ligation of digested DNA fragments was carried out using T4-DNA Ligase (NEB). To 100 ng of vector, the digested insert was added in 3 to 10 times molar excess. Ligation was carried out in a thermal cycler using a program according to Lund et al. (1996), consisting of 100 alternating, 30 sec long incubations at 10 ℃ and 30 ℃. Finally the ligase was denatured for 20 min at 65 ℃.
For subcloning of PCR fragments, the pGEM-T vector system (Promega) or the TA Cloning Kit (Invitrogen) was used according to the manufacturers instructions.

Cloning using the Gateway system

For the recombination reactions used in Gateway technology, all enzymes were purchased from Invitrogen. To 100 ng of pDONR201 vector, 1 to 3 µl purified PCR products with Gateway-compatible attB-overhangs, 1 µl of 5xBP clonase reaction buffer and 1 µl of BP Clonase mix was added and incubated for at least 1 h at 25 ℃. The reaction was stopped by adding of 0.5 µl Proteinase K (2 µg/µl) and incubating for 10 min a 37 ℃.
The recombination into a destination vector was carried out similarly, using approximately equal molar ratios of entry and destination vector and LR clonase mix and buffer.

5.3.5 Construction of the sugar beet transformation constructs

pBinAR-NtVIF

For the generation of sugar beet plants overexpressing NtVIF, the construct described in Greiner et al. (1999), featuring the NtVIF cDNA in the vector pBinAR was used.

p70S-BvC/VIF1

In this construct, the expression of the BvC/VIF1 gene is driven by a double CaMV 35S ("p70S") promoter. The promoter was amplified from the p70S-luc-kan plasmid (provided by D. Stahl, Planta) using primers JE63 and JE64 and subcloned in pGEM-T (Promega). The promoter was released from the pGEM-T plasmid by digestion with XmaI and AgeI and introduced into the p2-1-48-BvC/VIF1 vector (see below), from which the 2-1-48 promoter had been removed by digestion with the same enzymes to yield the p70S-BvC/VIF1 construct.

p2-1-48-BvC/VIF1

The 2-1-48 promoter was first amplified from the plasmid pGUS3-2-1-48 (provided by D. Stahl, Planta) using primers JE56 and JE57 and digested with HindIII and SacI. By digestion of p70S-luc-kan with the same enzymes, the p70S promoter and the luciferase gene was removed and subsequently replaced by the 2-1-48 promoter by ligating the digested PCR product yielding p2-1-48. Thereafter, the BvC/VIF1 cDNA was amplified using primers JE58 and JE59, which carried AgeI and AscI restriction sites respectively. The PCR product and the p2-1-48 plasmid were digested with the corresponding enzymes and ligated, to produce the p2-1-48-BvC/VIF1 construct.

p70S-RNAi-BvC/VIF1

The BvC/VIF1-RNAi cassette was first assembled in the pBK-CMV vector (Stratagene). First the antisense part of the construct was amplified using primers JE47 and JE48 and ligated into the pBK-CMV vector using KpnI / SacII digested PCR product (*pBK-CMV-anti*). Then intron 2 of the *Arabidopsis thaliana* AtAAP6 gene (at5g49630) was amplified using primers JE51 and JE52 and subcloned into pGEM-T. The intron was released from pGEM-T by digestion with SacII and SpeI and ligated into the equally digested *pBK-CMV-anti* plasmid, giving rise to *pBK-CMV-anti-intron*. This construct was then digested with SacI and SpeI and the sense part of the RNAi cassette, which had been amplified using primers JE49 and JE50 and equally digested, was ligated to yield *pBK-CMV-anti-intron-sense*. The complete BvC/VIF1-RNAi cassette was then released from the pBK-CMV vector by digestion with XhoI and SacI and ligated into the equally digested p70S-luc-kan vector.

5.3.6 Isolation of plant genomic DNA

To 100 mg of grinded leaf material 550 µl of extraction buffer (200 mM Tris-HCl, 400 mM LiCl, 25 mM EDTA, 1 % SDS, pH 9) and 550 µl of PCI (phenol:chloroform:isoamyl alcohol 25:24:1 (v:v:v)) were added and vortexed for 20 sec. After centrifugation (5 min, 15,000 g, 4 ℃), the supernatant was again vortexed with 550 µl of PCI and centrifuged. DNA was precipitated by adding one volume of isopropanol to the supernatant and, after 15 min incubation at RT, collected by centrifugation (10 min, 15,000 g, 4 ℃). After removing the supernatant, the pellet was air dried and resuspended in 500 µl TNE (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8) plus 20 µg RNaseA (from a 10 mg/ml stock) and incubated for 10 min at 37 ℃ to allow RNA digestion. RNase was removed by shaking out with 550 µl PCI and centrifuging as before. The supernatant (approx. 475 µl) was precipitated with 750 µl isopropanol and centrifuged (10 min, 15,000 g, 4 ℃). The pellet was washed once with 70 % ethanol and, after drying, resuspended in 50 µl TE-buffer (10 mM Tris, 1 mM EDTA, pH 8).

5.3.7 Southern Blotting

Restriction digestion

Usually, 10 to 15 µg of the genomic DNA were digested with suitable restriction enzymes (10 U/µg DNA) for 4 h or overnight. The DNA was precipitated by adding 1/10 vol. of 3 M sodium acetate and 2.5x vol. of ethanol and incubation for at least 1 h at -20 ℃. After centrifugation (15 min, 15,000 g, 4 ℃) the pellet was washed with 500 µl of 70 % ethanol and resuspended in 20 µl TE-buffer. Completion of the restriction digestion was monitored on an agarose gel.

Gel electrophoresis and transfer

The digested DNA was separated on a TAE-agarose gel (0.7% agarose) and stained with EtBr and photographed. The gel was then incubated in depurination solution (0.2 M HCl) for 10 min. Thereafter the gel was incubated twice for 10 min in denaturation solution (1.5 M NaCl, 0.5 M NaOH) and then for 15 min neutralized with 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.4. Between each step, the gel was washed in water for 5 min.
Before the transfer, the gel was incubated for 10 min in 10x SSC (1.5 M NaCl, 0.3 M sodium citrate, pH 7) and the DNA was transferred by capillary blotting over night onto a nylon membrane (Duralon-UV, Stratagene). On the next day, the membrane was incubated for

5 min in 10x SSC and dried. The DNA was then crosslinked to the membrane using UV-light (Stratalinker, Stratagene, setting=Auto).

Hybridization and detection using biotinylated probes

The hybridization and detection steps were carried out in glass tubes inside a hybridiser (HB-1D, Techne). The membrane was bathed in LSW (low stringency wash, 2x SSC, 0.5 % SDS) and inserted into a tube containing prehybridization solution (30 % formamide, 1 % SDS, 1 M NaCl, 6 % polyethylene glycol 6000, 250 µg/ml DNA from salmon testes), which was heated beforehand to 90 °C. The biotinylated PCR product was denatured for 10 min at 95 °C in 0.5 ml of prehybridization solution and added to further 20 ml of the prehybridization solution. After incubating the membrane for 45 to 60 min at 42 °C in prehybridization solution, the hybridization solution was added. Hybridization was carried out at 42 °C over night. The membrane was then washed twice for 15 min with LSW at RT and then for 40 min with HSW (high stringency wash, 0.2x SSC, 0.5 % SDS) at 55 °C.

All following steps were carried out at RT. The membrane was incubated in blocking buffer (1x PBS, 0.5 % SDS, 0.2 % I-Block (Tropix)) twice for 5 min and 1 time for 30 to 60 min and then for 40 min in conjugate buffer (1:6,000 alkaline phosphatase (AP) streptavidin conjugate (Avid X, Tropix) in blocking buffer). The membrane was washed again 3 times for 5 min with blocking buffer, followed by three 5 min washes in wash buffer (1x PBS, 0.5 % SDS). Finally the membrane was incubated twice for 5 min in assay buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5) and, after removal of excess buffer, sprayed with AP substrate (CDP-Star, ready to use (Tropix)) and incubated for 10 min inside a plastic bag. The membrane was sealed in a new plastic bag and chemiluminescence was detected on photographic film (Hyperfilm ECL, Amersham).

Hybridization and detection using DIG-labeled probes

For the detection using DIG-labeled probes, prehybridization was carried out with DIG Easy Hyb (Roche) for 1 to 2 h at 42 °C. For the hybridization, the DIG-labeled PCR product was denatured in 500 µl prehybridization solution for 10 min at 95 °C and added to 25 ml of the same solution. The DIG Easy Hyb solutions were stored at -20 °C and preheated to 65 °C before use. The LSW and HSW washes were carried out as described above for biotinylated probes. Subsequently the membrane was washed for 5 min in wash buffer (Maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, autoclaved) + 0.3 % Tween20) and then incubated for 1 h in blocking buffer (1 % Blocking Reagent (Roche) in Maleic acid buffer). Then the membrane was incubated for 30 to 60 min in conjugate buffer (1:10,000 anti-DIG-alkaline phosphatase (0.75 U/µl, Fab fragments from sheep, Roche) in blocking buffer), washed 4 times for 5 min in wash buffer and finally incubated twice for 5 min in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl pH 9.5). The incubation with the CDP-Star substrate and further steps were carried out as described for biotinylated probes.

5.4 RNA techniques

5.4.1 Isolation of total RNA

For cDNA synthesis, total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. For Northern blotting, total RNA was isolated with a modified protocol according to Logemann et al. (1987). All described solutions

were prepared using DEPC-treated water. This was produced by addition of 0.1 % DEPC (diethylpyrocarbonate) to dd-H_2O, stirring over night and subsequent two cycles of auto-claving.

Plant material was grinded in deep frozen state using a ball mill (Retsch Mixer Mill MM 200) and to each 500 mg of plant material, 1 ml of extraction buffer was added (8 M guanidine-HCl, 20 mM MES, 20 mM EDTA, pH 7; before use 8 µl of β-mercaptoethanol per ml buffer was added). For leaf material, 500 mg of tissue were sufficient, whereas for taproot samples 2 g had to be used.

After thawing, 1 ml PCI (phenol:chloroform:isoamyl alcohol 25:24:1 (v:v:v)) were added, vortexed and centrifuged (10 min, 15,000 g, RT). The aqueous supernatant was shaken out with 1 ml of CI (chloroform:isoamyl alcohol 24:1). The resulting supernatant was precipitated with 0.2x vol. of 1 M acetic acid and 0.7x vol. ethanol (over night -20 ℃) and on the next day centrifuged (15 min, 4 ℃, 15,000 g). The resulting pellet was washed first with 1x vol. of 3 M sodium acetate (pH 5.2) and then with 1x vol. of 70 % ethanol and finally resuspended in 50 to 100 µl DEPC-treated water, depending on pellet size. After incubation for 15 min at 65 ℃, residual insoluble material was removed by centrifugation.

Determination of RNA concentration

Concentration of RNA was determined photometrically at 260 nm (ε=25 µl x µg^{-1} x cm^{-1}), using appropriate dilutions of the RNA sample (usually 1:200). The OD at 230 nm and 280 nm was used to estimate contamination with polysaccharides or proteins, respectively (Good quality RNA should have an $OD_{260\,nm}/OD_{280\,nm}$ ratio of 1.8 to 2.0 and an $OD_{260\,nm}/OD_{230\,nm}$ ratio greater than 1.8).

5.4.2 Northern Blotting

For each gel lane, 15 µg RNA were used and the volume was adjusted to 16.6 µl with formamide for all samples. To each sample, 8.4 µl sample mix (consisting of 4.15 µl 37 % formaldehyde, 1.25 µl 20x MOPS (0.4 M MOPS, 0.1 M sodium acetate, 20 mM EDTA, pH 7), 2.5 µl RNA loading buffer (50 % glycerol, 5 % 20xMOPS, 1 % bromphenol blue), 0.5 µl EtBr (0.5 mg/ml)) were added. Before loading, the RNA was denatured for 10 min at 65 ℃ and cooled on ice. The samples were loaded on a denaturing agarose gel (1.4 % agarose, 1x MOPS, 5.5 % formaldehyde(37 %)) and run at 70 V in 1x MOPS buffer.

After the run completed, the gel was photographed and washed twice for 10 min each in 10x SSC (1.5 M NaCl, 0.3 M sodium citrate, pH 7). The RNA was transferred overnight by capillary blotting with 10x SSC as transfer buffer onto a nylon membrane (Duralon-UV, Stratagene). Completion of transfer was confirmed by inspecting the membrane under UV-light. After drying of the membrane, RNA was crosslinked using a UV stratalinker (Stratagene, setting=Auto).

The hybridization and detection steps were performed as described for Southern blotting (see 5.3.7) with biotin-labeled probes, except that the detection was carried out with the more sensitive horseradish peroxidase (HRP) instead of alkaline phosphatase. The washing steps were identical to the above protocol, with the exception that the conjugate buffer contained the Streptavidin-HRP conjugate (1:20,000 Streptavidin-HRP conjugate (1mg/ml, Pierce) in blocking buffer) and that the washing in assay buffer was omitted. The membrane was incubated with the chemiluminescent HRP substrate (North2South Chemiluminescent Substrate for HRP, Pierce) as described for AP detection.

5.5 Protein techniques

5.5.1 General protein techniques

SDS-PAGE

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was done according to Sambrook et al. (1989), using resolving gels containing 12 to 15 % polyacrylamide and stacking gels with 5 %. Gels were either directly stained with Coomassie Brilliant Blue or used for Western blotting. Samples for SDS-PAGE were denatured by addition of a 4x concentrated SDS-sample buffer containing a reducing agent (Roti-Load1, Roth) and boiling for 5 min at 95 ℃. For the conservation of disulfide bridges, the non-reducing Roti-Load2 was used.

Coomassie staining

Gels were stained in 0.25 % Coomassie Brilliant Blue G250, 45 % methanol, 10 % acetic acid, destained with 45 % ethanol, 10 % acetic acid and stored in 45 % methanol, 2.5 % glycerol. For more sensitive detection, gels were stained using colloidal Coomassie staining. Staining was carried out in 20 % ethanol, 1.6 % phosphoric acid, 8 % ammonium sulfate, 0.08 % Coomassie Brilliant Blue G-250 over night. After destaining with water the gels were stored in 0.1 % acetic acid.

Silver staining

For silver staining, protein gels were fixed in 50 % ethanol, 10 % acetic acid, 0.05 % formaldehyde (37 %) for at least 1 h, washed twice for 25 min in 50 % ethanol. The gel was sensitized for 1 to 2 min in 0.02 % sodium thiosulfate ($Na_2S_2O_3$x5H_2O), followed by rinsing the gel in water twice. The gel was stained for 20 min in staining solution (0.2 % $AgNO_3$, 0.075 % formaldehyde (37 %)), rinsed three times in water and developed until bands were visible with developing solution (6 % Na_2CO_3, 0.0004 % $Na_2S_2O_3$x5H_2O, 0.05 % formaldehyde). Development was stopped by incubating the gel in 12 % acetic acid.

5.5.2 Immunological techniques

Western Blot

After SDS-PAGE, the resolving gel was incubated in transfer buffer (48 mM Tris-base, 39 mM glycine, 20 % methanol (v/v), 0.0375 % SDS) for 10 min. The protein transfer was accomplished via a "semi-dry" electro transfer using the Trans-Blot SD apparatus (Bio-Rad). On the anode, 3 layers of blotting paper (Whatman 3 MM), moistened in transfer buffer, the membrane (Immobilon-P (Millipore), first incubated in methanol and then in transfer buffer), the gel and three further moistened paper were assembled, taking care to avoid trapped air bubbles. Then the cathode was placed on top and the transfer was carried out for 45 min at 15 V and 3.5 A per cm^2 membrane.

After the blot, the membrane was blocked by incubation in 5% skim milk powder (w/v) in TBST (20 mM Tris, 150 mM NaCl, 0.05 % Tween 20, pH 7.4) for 1 h at ambient temperature. The primary antibody solution was prepared in TBST + 1% skim milk powder at the dilutions indicated below. (For conservation purposes, 0.02 % NaN_3 were added.) The primary antibody solution was usually incubated over night at 4 ℃. Thereafter the membrane was washed four times with TBST for 5 min each under vigorous shaking, followed

by a one hour incubation in the secondary antibody solution (for rabbit primary antibody: horseradish peroxidase-conjugated goat anti-rabbit antibody (Pierce) 1:20,000 in TBST + 1 % skim milk powder) at room temperature and a repetition of the washing procedure.

The membrane was incubated for 5 min in the substrate solution (Super Signal Dura, Pierce) and chemiluminescence was detected by putting the membrane under photographic film (Hyperfilm ECL, Amersham). Exposure times were adjusted according to signal strength, usually between 30 sec and 30 min.

Finally the proteins on the membrane were stained in Amido Black (0.1 % Amido Black, 45 % ethanol, 10 % acetic acid) for 15 min and, after background destaining in water, dried.

Production of the BvC/VIF antiserum

The production of the antiserum was carried out by Eurogentec (Belgium). Initially preimmune bleedings of twenty rabbits were tested by Western blotting for the presence of unspecific antibodies against *Beta vulgaris* proteins. The immunization was carried out using the rabbit with the lowest amount of cross-reacting proteins detected. The immunization protocol included four injections of the antigen, each time using 120 µg of recombinant BvC/VIF1 protein expressed in soluble form. The obtained bleedings were tested individually (see Fig. 8.1) and for further Western blots, an affinity purified fraction (see below) of the final bleeding was used.

Affinity purification of antisera

Due to the presence of multiple immuno-signals in Western blots with plant extracts from sugar beet, the BvC/VIF-antiserum was affinity purified against recombinant BvC/VIF1-protein (see Fig. 3.24). 2 mg of the recombinant protein (dialyzed in coupling buffer (0.1 M NaHCO$_3$, 0.5 M NaCl pH 8.3)) was incubated with 0.5 g of CNBr-activated Sepharose 4B (preswollen in 100 ml 1 mM HCl and equilibrated in coupling buffer) for 1 h at ambient temperature. The matrix was poured into a column (Econo-Pac, Bio-Rad) and the buffer was allowed to drain by gravity flow.

The column was first washed with 50 ml of coupling buffer and residual amine binding sites were blocked by incubation in 10 ml 100 mM Tris-HCl, pH 8 for 2 h at ambient temperature. For washing, the column was incubated with 10 ml 100 mM sodium acetate, 500 mM NaCl, pH 4.5 and 10 ml 100 mM Tris-HCl, 500 mM NaCl, pH 8 and this procedure was repeated three times. Finally the affinity matrix was incubated in 1xTBS (20 mM Tris, 150 mM NaCl, pH 7.4).

The following steps were carried out at 4 °C. For binding of the antibodies, 500 µl of 10xTBS (200 mM Tris, 1.5 M NaCl, pH 7.4) were added to 5 ml of the final bleed and incubated with the CNBr-coupled BvC/VIF1 protein overnight. Then the unbound antibodies were allowed to drain from the column. After washing with 50 ml of 1xTBS, bound antibodies were eluted with 0.2 M glycine, 1 mM EGTA, pH 2.8, first as 500 µl then as 1 ml aliquots (5 aliquots each). The eluted antibodies were immediately neutralized by addition of 1/10 volume of 2 M Tris, pH 8.5. For stability reasons, 0.1 mg/ml BSA and 0.02 % NaN$_3$ were added to the eluted antibodies. The protein content of the produced fractions was tested and the fractions with the highest protein content were combined. The purified serum was tested using different dilutions. A dilution of 1:500 to 1:1000 was found suitable for Western blotting.

Detection of acid invertases

For the detection of cell wall invertases, an antiserum raised against a tobacco CWI (Genbank accession X81834), for the detection of vacuolar invertases an antiserum against the BvVI1 protein (AJ277457) was used. The production of the antisera is described in Rosenkranz et al. (2001). Both antisera were used in a dilution of 1:5,000 of the crude final bleeding.

Detection of Alternative Oxidases

For the detection of the AOX proteins, a monoclonal antiserum raised against the AOX proteins from *Sauromatum guttatum* was used diluted 1:100. This antiserum is described to detect all three AOX-isoforms of this species (Elthon et al., 1989). As secondary antibody a horseradish peroxidase-conjugated goat anti-mouse antibody (Pierce) was used.

Detection of green fluorescent protein

For the detection of GFP-fusion proteins, a polyclonal GFP antiserum from Invitrogen was used in a 1:5000 dilution.

5.5.3 Purification of recombinant inhibitor and invertase proteins

Purification of recombinant BvC/VIF1 from *E. coli*

The BvC/VIF1-coding sequence without the predicted signal peptide was cloned into the pQE30-vector (Qiagen, Primers Bv-inh_l and Bv-inh_r), which leads to the expression of the protein in fusion with a N-terminal 6xHis-Tag. The vector was transformed into the *E. coli* strain Rosetta-gami (Novagen).

In a typical purification of the recombinant BvC/VIF1 protein, 3 l TB-medium were inoculated with 100 ml overnight culture in LB-medium. Both, LB and TB medium, were supplemented with 100 µg/ml Ampicillin, 34 µg/ml Chloramphenicol and 1% glucose (w/v) in order to decrease leaky expression of the protein. Bacteria were grown at 37 °C until $OD_{600\,nm}$ reached 0.8 to 1.0. After cooling the culture to 18 °C, expression was induced by addition of 0.2 mM IPTG.

Bacterial cells were harvested after 18 to 22 hours at 18 °C and 200 rpm by centrifugation, resuspended in 200 ml wash buffer (500mM NaCl, 50mM NaPO$_4$, 25 mM imidazole, 10 % glycerol, pH 7.5) and lysed with an Emulsifier (EmulsiFlex-C5, Avestin) at 70 to 100 MPa. Insoluble protein was removed by centrifugation (22,000 g, 45 min) and the supernatant was applied to an IMAC-column filled with 2 to 3 ml Ni-NTA agarose matrix (Qiagen). The column was washed with 300 ml wash buffer and the purified protein was eluted in six 2 ml fractions with elution buffer (500mM NaCl, 50mM NaPO$_4$ pH 7.5, 10% Glycerol, 250 mM Imidazole).

As an alternative to the Ni-NTA matrix, a silica-based Ni-TED matrix (Protino Ni-TED, Macherey-Nagel) was used. For this matrix the imidazole was omitted from the wash buffer. BvC/VIF1-containing fractions were usually dialyzed into an acidic buffer for activity testing (P1 buffer: 20 mM Triethanolamine, 7 mM Citric Acid, pH 4.6 or 50 mM sodium acetate, 300 mM NaCl, pH 5). Proteins precipitated during dialysis were removed by centrifugation. For longer storage ($>$ 3 days), the purified protein was frozen in liquid nitrogen and stored at -80 °C without substantial loss of activity.

Purification of recombinant NtCIF and NtVIF from *E. coli*

For the purification of NtCIF, the pQE30 construct described in Greiner et al. (1998) was used. The construct was transformed into Rosetta-gami cells, and expression was carried out as described for BvC/VIF1 (see above) with the exception, that no glucose was added to the TB-medium.

NtVIF was only expressed in insoluble form in inclusion bodies. Therefore the protein was first resolubilized using a 8 M urea solution. The protein was then bound to a Nickel matrix and refolded in renaturing buffer (25 mM HEPES, 150 mM NaCl, 100 mM KCl, 2 mM MgCl$_2$, 0.005 % Tween20, 1 mM PMSF, 0.66 mM GSSG, 0.33 mM GSH pH 7.5) over night. After refolding, the protein was eluted from the Nickel-column with renaturing buffer + 250 mM imidazole. The pQE30-construct and the refolding method was kindly provided by Katja Lauer (Lauer, 2006).

Purification of recombinant BvVI1 from *E. coli*

The BvVI1 protein was amplified from *Beta vulgaris* cDNA using the primers JE81 and JE82 and first cloned into pPICZαA (Invitrogen) using KpnI and SacII restriction sites. This plasmid was used for the two-step amplification with primers JE75 and 76 (step 1) and JE77 and JE78 (step 2) to add Gateway compatible overhangs and the TEV-protease-cleavage site (see 5.3.3).
After recombining the PCR product into the Gateway entry-vector pDONR201 (Invitrogen) and sequence verification, it was introduced into the pETG vector series (EMBL, Heidelberg, see table 3.1 on page 41). Best yields of soluble BvVI1 protein were achieved using the pETG-30 vector (providing a N-terminal 6xHis- and GST-tag), which was used for the following purifications.
The expression and purification using Nickel-resins was carried out as described above for BvC/VIF1, except that 2.5 % glucose were added to the TB-medium and after elution from the Nickel-matrix, the protein was dialyzed in a buffer for TEV-protease-cleavage (50 mM NaPO$_4$, 200 mM NaCl, pH 7.5). Recombinant 6xHis-tagged TEV protease and 5 mM DTT were added to the dialyzed protein and incubated for 3 h at 30 °C or over night at 4 °C. Subsequently 1x vol. of wash buffer (500mM NaCl, 50mM NaPO$_4$, 25 mM imidazole, 10 % glycerol, pH 7.5) was added and the sample was passed over 1 ml of Ni-NTA matrix. Cleaved BvVI1-protein was collected in the flow-through (FT) of this second column, whereas the TEV-protease and the cleaved GST-tag bound to the Ni-NTA matrix due to the presence of 6xHis-Tags. Further BvVI1 protein was collected by washing the column with 2 ml fractions of wash buffer and after washing with another 25 ml of wash buffer, TEV-protease and the GST-tag were eluted with elution buffer. Wash fractions containing BvVI1-protein (determined by SDS-PAGE and Coomassie staining) were combined with the FT and dialyzed against a buffer of choice (usually 50 mM sodium acetate, 300 mM NaCl, pH 5). See Fig. 3.12 on page 42 for a typical BvVI1 purification.

Purification of recombinant IbVI2 from *Pichia pastoris*

For the purification of the recombinant IbVI2 protein, the *Pichia pastoris* strain X-33 carrying the pPICZαA vector with the open reading frame of the *Ipomoeas batatas* soluble acid invertase FRUCT2 (Wang et al., 2005 , Acc. number AAK71504) was used. A single colony was used to inoculate 100 ml BMGY-medium with 100 µg/ml Zeocin and was grown overnight at 30 °C. To induce expression from the alcohol oxidase promoter, the culture was

centrifuged (5 min, 1300 g), resuspended in 1.5 l BMMY medium in a 5 l erlenmeyer flask and grown overnight at 30 ℃ with vigorous shaking.

To harvest the IbVI2 protein secreted into the medium, the culture was centrifuged (30 min, 4,000 g, 4 ℃) and the medium precipitated by adding 430 g of ammonium sulfate per liter medium (70% saturation) under constant stirring at 4 ℃. The precipitated protein was collected by centrifugation (30 min, 9,800 g, 4 ℃), resuspended in 50 ml of $NaPO_4$ buffer (50 mM $NaPO_4$, 200 mM NaCl, pH 7.5) and residual ammonium sulfate was removed by dialysis against 10 l of $NaPO_4$ buffer.

Then 500 µl of Ni-NTA (Qiagen) was added to this solution and incubated for 1 h at 4 ℃. The matrix was poured in a Econo-Pac column (Bio-Rad) and washed with 200 ml wash buffer (500 mM NaCl, 50 mM $NaPO_4$, 10 mM imidazole, 10 % glycerol, pH 7.5). Finally the IbVI2 protein was eluted in five 1 ml fractions with elution buffer (500 mM NaCl, 50 mM $NaPO_4$, 250 mM Imidazole, 10 % glycerol, pH 7.5) and dialyzed against 50 mM sodium acetate, 300 mM NaCl, pH 5.

5.5.4 Extraction of soluble and cell wall proteins

Approximately 300 mg of grinded leaf or taproot material was resuspended in 600 µl of extraction buffer (30 mM MOPS, 250 mM sorbitol, 10 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, pH 6) and after thorough vortexing centrifuged at 15,000 g in a table top centrifuge. All steps were carried out at 4 ℃. The supernatant, containing the soluble proteins, was removed and the pellet resuspended in extraction buffer plus 1% triton X-100, and, after vortexing, centrifuged as before. The supernatant from this step is referred to as Triton-Wash. The residual pellet was washed twice in extraction buffer without Triton, the supernatants from these steps were discarded. The residual cell wall pellet was resuspended in 100 µl of 2x SDS-sample buffer (Roti-Load1, Roth). After boiling for 5 minutes and sequential centrifugation, the supernatant contained the cell wall fraction.

Alternatively, the residual cell wall pellet was incubated in extraction buffer+500 mM NaCl for 30 min at 4 ℃ using an overhead shaker. The salt-eluted fraction was separated from the residual pellet by centrifugation. During this step, proteins ionically bound to the cell wall matrix are solubilized and removed from the cell wall material.

The soluble, the Triton-Wash and the salt-eluted fractions were precipitated by the addition of 1600 µl of ice-cold acetone to 400 µl of each fraction. After incubation for 20 minutes at -20 ℃ the sample was centrifuged and the protein pellet was resuspended in 100 µl of 2x SDS-sample buffer. For leaf samples, the soluble fraction was not precipitated, but used directly after denaturation in SDS-sample buffer.

Salt-elution of the cell wall fraction from *Beta vulgaris* suspension culture cells

For the elution of ionically bound proteins from the cell walls of intact suspension culture cells, the medium was removed from cells 8 days after transfer to fresh medium by filtration. The cells were transferred to a double volume of extraction buffer (see above) plus 500 mM NaCl and gently stirred at 4 ℃ for 1 h. The cells were removed by centrifugation and the supernatant was acetone precipitated and the resulting pellet was taken up in SDS-sample buffer.

5.5.5 Lectin chromatography

For the purification of glycosylated proteins, lectin chromatography was carried out using a Concanavalin A (ConA) sepharose conjugate. Plant material was extracted in 1x ConA

buffer (50 mM sodium acetate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM PMSF (added freshly), pH 6.3) and after centrifugation added to ConA-sepharose (equilibrated in the same buffer) and incubated for 1 h at RT. The suspension was loaded onto a column and non-glycosylated proteins were collected in the flow-through (ConA$^-$-fraction). The column was washed with 50 ml of ConA buffer and the bound proteins were eluted by addition of ConA-buffer + 15 % methyl-α-D-glucopyranoside, giving rise to the ConA$^+$-fraction.

5.5.6 Size exclusion chromatography

For size exclusion chromatography (SEC) of plant extracts and recombinant proteins, a Pharmacia FPLC system at ambient temperature was used. The system consisted of a P-500 Pump, a Superdex 200 Hiload 16/60 column (120 ml matrix volume), a UV-MII UV-meter and the fraction collector Frac-100 (all from Pharmacia). The system was controlled and the results were processed using the software "FPLC-director version 1.1". For buffer changes, the column was equilibrated with at least 2 column volumes of the new buffer. All buffers were filtrated using 0.2 µm sterile filters. Samples were loaded using a 1 ml sample loop.
Plant extracts were separated in MES-buffer (50 mM MES pH 5.5, 250 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$), recombinant proteins in either sodium acetate (50 mM sodium acetate, 300 mM NaCl pH 5) or sodium phosphate buffer (50 mM $NaPO_4$, 300 mM NaCl, pH 7.5).
For inspection via SDS-PAGE and Western blotting, the collected fractions were precipitated by the addition of 4x vol ice-cold acetone. The resulting pellet was taken up in SDS sample buffer directly.

5.5.7 Measurement of soluble and cell-wall bound invertase activity

To each 100 mg of grinded plant material (portioned in deep-frozen state into 1.5 ml reaction tubes) 250 µl of extraction buffer (see 5.5.4) were added and the sample was vortexed vigorously. The soluble proteins were collected by centrifugation at 8,500 g at 4 °C. The pellet was washed once with extraction buffer + 1 % Triton X-100 and twice with extraction buffer without Triton. Then the cell-wall pellet was resuspended in 250 µl extraction buffer and used directly for the determination of invertase activity.
For the measurement of soluble invertase activity from sugar beet taproots, endogenous sucrose was removed by acetone precipitation of the soluble fraction with 4 vol. of ice-cold acetone and incubation for 20 min at -20 °C. After centrifugation (15,000 g, 10 min, 4 °C) the pellet was resuspended in 500 µl P1 buffer (20 mM triethanolamine, 7 mM citric acid, pH 4.6). For the determination of acid invertase activity, 30 to 100 µl (depending on activity of sample) of the obtained preparations were incubated with 100 µl of substrate (100 mM sucrose in P1 buffer) and P1 buffer up to 300 µl. After 1 h at 37 °C the reaction was stopped by the addition of 30 µl 1 M sodium phosphate, pH 7.5 and heating to 95 °C for 5 min.
Liberated glucose was measured in a coupled enzymatic-optical assay. 10 to 100 µl of the reaction, 20 µl 30 mM ATP, 20 µl 30 mM NADP, 2 µl Hexokinase/Glucose-6-Phosphate De-hydrogenase suspension (340 U/ml HK, 170 U/ml G6P-DH, Roche) and up to 1 ml buffer (40 mM Triethanolamine, 8 mM $MgSO_4$ pH 7.5) were mixed and incubated for 5 min at room temperature. Formation of NADPH was measured photometrically at 340 nm and the liberated glucose was calculated using Lambert-Beer law ($\varepsilon_{340nm}^{NADPH}$=6,23 l x mmoles^{-1} x cm^{-1}). Invertase activity was expressed in nkat per g fresh weight (1nkat=1 nmole Glc liberated / sec).

5.5.8 Functional assay of recombinant invertase inhibitors

To test recombinant inhibitor proteins for inhibitory activity against invertases, variable amounts of the recombinant inhibitor proteins were added to a suitable invertase preparation (recombinant or extracted from plant tissues) in P1 buffer (20 mM triethanolamine, 7 mM citric acid, pH 4.6) or sodium acetate buffer (50 mM sodium acetate, 300 mM NaCl pH 5) in a total amount of 200 µl and incubated for 30 min at 37 °C to allow complex formation. Then, 100 µl 100 mM sucrose in the same buffer were added and incubated for 60 min at 37 °C. The reaction was stopped by neutralization with 30 µl 1 M $NaPO_4$ and boiling for 5 min at 95 °C. The amount of glucose released was measured as described in 5.5.7. In each experiment samples without inhibitor proteins were included. For every combination of invertase and inhibitor, 4 replicates were prepared, of which one was neutralized and boiled immediately after sucrose addition. This value was subtracted from the others as background absorption.

5.5.9 Purification of BvC/VIF proteins from sugar beet taproots

Preparation of invertase-column

For the coupling of recombinant BvVI1-protein to CNBr-matrix, 0.5 g CNBr-activated sepharose 4B (Amersham) was swollen by washing with 120 ml 1 mM HCl and equilibrated in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). Then 15 mg of recombinant BvVI1 protein (dialyzed against coupling buffer) was added and allowed to bind for 1.5 h at RT with constant agitation. The matrix was poured into an Econo-Pac column (Bio-Rad), the flow-through was collected and the column washed with 25 ml of coupling buffer. Blocking was done according to the manufacturer's instructions for CNBr-sepharose over night with 0.1 M Tris (pH 8) at 4 °C. Then washing was carried out with 4 alternating washes with 0.1 M sodium acetate buffer (pH 4) and 0.1 M Tris (pH 8), each buffer supplemented with 0.5 M NaCl. The column was stored in a buffer containing 0.02 % NaN_3 to prevent microbial growth.

Affinity-purification of inhibitors

For the purification of both BvC/VIF proteins from *Beta vulgaris* taproots, 250 g of cut taproot material was homogenized in a Waring blender with 250 ml of ice cold MES-buffer (50 mM MES, 250 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, pH 5) and filtered through Miracloth (Calbiochem). All steps were carried out at 4 °C. After centrifugation (3,840 g, 20 min), the supernatant was precipitated by adding solid ammonium sulfate to 80 % saturation (561 g/l). The ammonium sulfate was added and stirred over night. The pellet was collected (15,000 g, 30 min), resuspended in 5 ml of acetate buffer (50 mM sodium acetate, 300 mM NaCl pH 5) and dialyzed against the same buffer to remove residual ammonium sulfate. After dialysis, the sample was cleared by high speed centrifugation (267,000 g, 30 min) and passed repeatedly over the invertase-column equilibrated with the same buffer for 90 min at 37 °C. The flow-through was collected and the column was washed with 60 ml acetate buffer. The inhibitor proteins were eluted from the invertase by incubating the matrix in 4 ml of 100 mM Tris, 500 mM NaCl, pH 8.5 for 20 min at RT. A second elution was carried out for 60 min at RT.

Protein sequence determination by mass spectroscopy

The eluted fractions were acetone precipitated and resuspended in 60 µl of SDS-sample buffer (Roti-Load1, Roth). Proteins were separated by SDS-PAGE and the inhibitor bands were identified on the gel stained with colloidal Coomassie by carrying out a Western blot with

the BvC/VIF antiserum with the second half of the gel in parallel.

Tryptic digestion and protein sequence determination by nanoESI QTOF was carried out by Dr. Th. Ruppert at the ZMBH, Heidelberg in the department of Biomolecular Chemistry.

5.5.10 Purification of mitochondria for Western blots

Sugar beet mitochondria were isolated according to Schwitzguebel & Siegenthaler (1984) and Vanlerberghe & McIntosh (1992). 100 g tap root or 70 g leaf material were cut and disrupted in 400 ml of ice-cold extraction buffer (350 mM mannitol, 30 mM MOPS, 1mM EDTA, 4 mM cysteine, 0.2 % BSA, 0.6 % polyvinylpolypyrolidone, pH 7.5) in a Waring blender and filtered through three layers of Miracloth (Calbiochem).

The filtrate was centrifuged at 5,500 g for 2 min to eliminate most of the plastids and nuclei and the supernatant was then centrifuged for 5 min at 23,000 g. The pellet was resuspended in 250 ml wash buffer (300 mM mannitol, 20 mM MOPS, 1 mM EDTA, 0.2 % BSA, pH 7.2) and centrifuged again for 2 min at 5,500 g. Finally the mitochondria were sedimented from the supernatant (5 min, 18,000 g), resuspended in wash buffer and the protein content determined (BSA contained in the wash buffer was subtracted).

A suitable amount of mitochondria were collected by centrifugation and the pellet taken up in 2x SDS sample buffer (Roti-Load1, Roth) at a concentration of 2 µg/µl and denatured for 5 min at 95 °C.

5.6 Plant Transformation methods

5.6.1 Transient ballistic transformation

Ballistic transformation was carried out with the Biolistic PDS-1000/He particle gun (Bio-Rad) according to the manufacturer's instructions. With this device, DNA-coated particles are delivered with high pressure into plant tissues. When particles are delivered to cell nuclei, the genes are expressed inside these cells.

Preparation of particles

30 mg tungsten-paricles ("M-20 Microcarriers" Ø 1.318 µm, Bio-Rad) were vortexed in 1 ml 70 % ethanol for 20 sec. After 10 min incubation, particles were centrifuged for 30 sec at 4,000 g. The supernatant was removed and the pellet vortexed for 10 sec in 500 µl sterile water and incubated for 10 min. After centrifugation as before, particles were resuspended in sterile 50 % glycerol and stored at -20 °C until use.

DNA-coating of particles

For each shot, a total of 1 µg plasmid-DNA was added to 10 µl of resuspended particles and incubated for 15 min on ice. Thereafter, 10 µl 2.5 M $CaCl_2$, 4 µl 1.2 mM spermidine and 23 µl 100 % ethanol were added subsequently with short vortexing in between. After 15 min incubation on ice, particles were collected by centrifugation (10 sec, 1,000 g) and resuspended in 7 µl 100 % ethanol per shot.

Bombardment of onion epidermal cells

Onions were purchased at local markets. Before the bombardment, the onion was quartered and the individual leaves were separated and put into a 6 cm petri dish on moist filter paper. The DNA-coated particles were sonicated for 6 sec (Labsonic, U Braun), spread on a macrocarrier (Bio-Rad) and the ethanol was allowed to evaporate. Setup of the particle gun was performed according to the manufacturer, using 650 PSI rupture disks and applying a vacuum of 25 inches Hg. Each onion leaf was bombarded for three times targeting the inner epidermis and incubated at room temperature in the dark for 24 to 48 h.
In order to visualize GFP-fluorescence in the apoplast, the epidermis was removed and incubated for 12 to 24 h in 20 mM PIPES (piperazine-*N,N*'-bis (2-ethanesulfonic acid), pH 7.0 as described by Scott et al. (1999).

5.6.2 Transient *Agrobacterium*-mediated transformation

The transient transformation of *Nicotiana tabacum* or *Nicotiana benthamiana* was done according to Wroblewski et al. (2005). A liquid culture (5 ml YEB with antibiotics in a 50 ml tube) of a single colony containing the binary vector in the *Agrobacterium* strain C58C1 was started and incubated over night at 28 °C with shaking. From this starter culture, 2 ml were used to inoculate a new over night culture of 50 ml YEB medium. After growing over night, the bacteria were centrifuged (2,000 g, 15 min, RT) and resuspended in sterile water. The OD_{600nm} of the suspension was adjusted to 1. Young leaves of tobacco plants were infiltrated by injecting the suspension through the lower side of the leaves using a 10 ml syringe without a cannula. Samples were taken between 36 and 48 h post infiltration.

5.7 Microscopy

Fluorescence microscopy
Microscopic analysis of the plant cells transformed with fluorescent reporter protein constructs was carried out using an inverse light microscope (DMIL, Leica). For detection of GFP fluorescence, a FITC filter (excitation 450-490 nm, emission 515 nm longpass) and for RFP-fluorescence the filter XF 137-2 (excitation 540 +/- 30 nm, emission 585 nm longpass) was used. Results were documented using a digital camera and the analySIS software (Soft Imaging System).

Confocal laser scanning microscopy
Further microscopic analyses were carried out using a confocal laser scanning microscope (LSM510 Meta, Zeiss). The following excitation and detection wavelength were used:
GFP: excitation: 488 nm; detection: bandpass 505-530 nm
RFP: excitation: 543 nm; detection: bandpass 560-615 nm
Chlorophyll autofluorescence: excitation: 488 nm; detection: longpass 650 nm

5.8 Determination of soluble sugars

For the extraction of soluble sugars, taproot tissue was grinded in deep-frozen state. To 100 mg of tissue, 500 µl of ethanolic extraction buffer (80 % ethanol, 10 mM HEPES, pH 7.5) were added and incubated for 40 min at 80 °C. After centrifugation (3 min, 15,000 g, RT) the extraction was repeated and both supernatants were combined and stored at -20 °C.

For the determination of sucrose, taproot extracts were usually diluted 1:40 with ethanolic extraction buffer. The measurement was carried out in 96-well plates (Greiner Nr. 655101) using a 96-well plate-reader (Fluostar Optima, BMG Labtech) at 340 nm. From each plant sample, three independent extracts were prepared and every extract was measured in triplicates.

Measurement of sucrose

In each well to 10 µl of the diluted extract, 160 µl of master mix were added. Per well, the master mix contained 2 µl 30 mM NADP, 2 µl 30 mM ATP, 0.4 µl glucose-6-phosphate-dehydrogenase (700 U/ml, Roche), 0.4 µl hexokinase (1500 U/ml, Roche) and 155.2 µl reaction buffer (100 mM imidazole, 3 mM $MgCl_2$, pH 6.9). The plate was inserted into the platereader, shaken vigorously and after 15 min background absorption (abs1) from hexoses present was measured. Then, 10 µl of invertase (2 mg/ml in reaction buffer, Sigma) were added to each well, mixed and incubated for 60 min (until absorption was constant) followed by determination of absorbance (abs2). For the calculation of the extract's sucrose concentrations, a standard curve was generated using sucrose solutions in ethanol between 0.1 mg/ml and 0.8 mg/ml. Absorption caused by present hexoses was removed by subtracting abs1 from abs2.

Measurement of hexoses

For the measurement of glucose and fructose from wounded taproot tissue, 2 µl of undiluted extract were added to 160 µl master mix containing 2 µl 30 mM NADP, 2 µl 30 mM ATP, 0.4 µl glucose-6-phosphate-dehydrogenase (700 U/ml, Roche) and 155.6 µl reaction buffer (100 mM imidazole, 3 mM $MgCl_2$, pH 6.9). After determination of background absorption (abs1), 4 µl of hexokinase (62,5 U/ml, diluted in reaction buffer) were added to each well. After mixing and incubating for 15 min, absorption (abs2) was measured. For the determination of fructose, 4 µl of phosphoglucoisomerase (Roche, 44 U/ml, diluted in reaction buffer) was added and absorption (abs3) was determined after mixing and incubation for 30 min. For the calculation of glucose, abs1 was subtracted from abs2, and for fructose, abs2 was subtracted from abs3. The standard curve was prepared from measurements of solutions containing between 0.1 and 0.8 mg/ml glucose and fructose and concentrations of the extracts were calculated according to the standard curve.

5.9 Electrochemical measurement of oxygen consumption

Oxygen consumption of sugar beet disks was measured using a Clark-type O_2 electrode according to Walker (1990). For a review of the application see Hunt (2003). An O_2 electrode is a specialized form of electrochemical cell, consisting of a platinum cathode and silver anode. The cathode and anode are connected by an electrolyte bridge (3 M KCl) and an polarizing voltage of around 700 mV is applied. At the cathode, electrons are transferred to oxygen and concomitantly at the anode silver is oxidized and silver chloride formed. The electrolyte solution mediates the current flow, which is directly related to the amount of oxygen present. It is converted to a voltage output signal by the electrode control box and recorded on a pen recorder. In the oxygen electrode units, the sample is put inside an airtight chamber separated from the cathode only by an air-permeable teflon membrane.

For the measurements, LD1/2 oxygen electrode units (Hansatech Instruments Ltd., King's Lynn, UK) connected to CB1-D manual control units (also from Hansatech) were used. The

voltage signals were recorded on chart recorders (Kipp+Zonen). Calibration of the instrument was done by injecting 1 ml of air into the sample chamber and recording the occurring change in current flow, which is stoichiometrically related to the amount of oxygen injected. By using the law of Charles and Gay-Lussac for ideal gases, the volume of one mole of air at the ambient temperature of 22 °C can be calculated as 24.22 l. One ml of air therefore equals 41.3 µmoles and, given an O_2 content of 20.9 %, contains 8.63 µmoles of oxygen. The calibration was carried out 3 times before and after each measurement.

For the measurement of oxygen consumption by sugar beet disks, 7 disks (1 cm in diameter, 4 mm thick) were inserted into the closed sample chamber and O_2 consumption was monitored in the dark for 30 to 45 min. Respiration was expressed as nmoles O_2 used per minute. For measurement of the respiration rates during wounding, the disks were incubated in petri dishes in which a moist filter paper was inserted into the lid and measurements were carried out at the indicated points in time (see Fig. 3.5).

6 Abbreviations

6xHis	hexa-histidine tag
acc	accession
AG	Aktiengesellschaft
AOX	alternative oxidase
AP	alkaline phosphatase
A. thaliana	*Arabidopsis thaliana*
AU	absorption unit
bp	base pairs
BSA	bovine serum albumin
Bv	*Beta vulgaris*
CaMV	Cauliflower mosaic virus
cc	cell culture
ch	chapter
CI	cytosolic invertase
CIF	cell wall inhibitor of β-fructosidase
CLSM	confocal laser scanning microscopy
C/VIF	cell wall or vacuolar inhibitor of β-fructosidase
ConA	Concanavalin A
CWI	cell wall invertase
dd-H_2O	double distilled water
DEPC	diethylpyrocarbonate
DTT	dithiothreitol
f.w.	fresh weight
ER	endoplasmic reticulum
EST	expressed sequence tag
EtBr	Ethidium bromide
GFP	green fluorescent protein
GH	glycosyl hydrolase
HRP	horseradish peroxidase
IMAC	immobilized metal ion affinity chromatography
Inv	invertase
IPTG	Isopropyl-*beta*-D-thiogalactopyranosid
kb	kilo base pairs
M	mol/l
mRNA	messenger RNA
MW	molecular weight
Nt	*Nicotiana tabacum*
OD	optical density
o/n	over night
ORF	open reading frame
p	page
PAGE	polyacrylamide gel electrophoresis
PME	pectin methylesterase
PMEI	pectin methylesterase inhibitor
PMEI-RP	pectin methylesterase inhibitor-related protein
PP_i	pyrophosphate

RACE	Rapid Amplification of cDNA Ends
ref	references
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
SPS	sucrose-phosphate synthase
SuSy	sucrose synthase
tr	taproot
UCP	uncoupling protein
VI	vacuolar invertase
VIF	vacuolar inhibitor of β-fructosidase
vol	volume
WT	wildtype
w/v	weight per volume
w/w	weight per weight

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8 Appendix

8.1 Sequence Data

All sugar beet genes analyzed were identified in EST-collections of KWS or sugar beet EST-collections provided by the GABI primary database (GABI-PD[1]). The corresponding bacterial clones (in the vector pCMV-SPORT6) were provided by KWS and the containing cDNAs were sequenced using the T7 and SP6 promoter sequencing primers and internal primers if necessary.

8.1.1 BvC/VIF1

The cDNA clone containing the BvC/VIF1 sequence carried the number KWS3_190_f04 and contained the complete 3' UTR of the cDNA but only 5 bp of 5' UTR. The 5' UTR was completed by RACE and additional 29 bp could be added to the sequence. A shorter EST-clone, which contains a fragment of the BvC/VIF1 cDNA sequence is annotated in Genbank under the accession number BQ593123.

```
        ---------|---------|-------- ==> cDNA clone -----|---------|---------|---------|
     1  GTTTTGTAGTAAATATACATTATACACACTAATCatgacaactctaaacacctctttaccacatcttcacctcctcttca  80
     1                                     M  T  T  L  N  T  S  L  P  H  L  H  L  L  F  I  16
        ---------|---------|---------|---------|---------|---------|---------|---------|
    81  ttacccttcttacactattcaccacctctacttttagcgtattctcgcaagaccaccaacgaccttgtaaccaccacgtgc  160
    17    T  L  L  T  L  F  T  T  S  T  L  A  Y  S  R  K  T  T  N  D  L  V  T  T  T  C   42
        ---------|---------|---------|---------|---------|---------|---------|---------|
   161  aagcaaacacccgacccgattctttgcgaagcttcgctccgatcagactctcggagctccaaggctgctgactctgaagg  240
    43  K  Q  T  P  D  P  I  L  C  E  A  S  L  R  S  D  S  R  S  S  K  A  A  D  S  E  G   69
        ---------|---------|---------|---------|---------|---------|---------|---------|
   241  tttaatcctgatcatgatcgacgttgtcaaaactcggttttcggactcgtttcgatatgtagaggacttgacccggaaga  320
    70  L  I  L  I  M  I  D  V  V  K  T  R  F  S  D  S  F  R  Y  V  E  D  L  T  R  K  T  96
        ---------|---------|---------|---------|---------|---------|---------|---------|
   321  cccatgacccggatgtaatccgggccctgcaagagtgtaagcaactctatcgggttgtgttagatgtaagtgtaggttta  400
    97    H  D  P  D  V  I  R  A  L  Q  E  C  K  Q  L  Y  R  V  V  L  D  V  S  V  G  L   122
        ---------|---------|---------|---------|---------|---------|---------|---------|
   401  gcagtgagagcagtaaagcaaggggatccgaaattcggggagcaagctatggtggatgcgggtaatgaggccgaggggtg  480
   123  A  V  R  A  V  K  Q  G  D  P  K  F  G  E  Q  A  M  V  D  A  G  N  E  A  E  G  C  149
        ---------|---------|---------|---------|---------|---------|---------|---------|
   481  tcggatggcgttcccggaaggtaaggttccgggtcggatcgtgggtcgaacacggatgctccatggagtatctaatgtgg  560
   150  R  M  A  F  P  E  G  K  V  P  G  R  I  V  G  R  T  R  M  L  H  G  V  S  N  V  A  176
        ---------|---------|---------|---------|---------|---------|---------|---------|
   561  ctgcttctatgattaagagtttggaatgaTGTTTTTTGTGATTTATGGTTAGCAAAGTATACAAGTATTTGACTATATGT  640
   177    A  S  M  I  K  S  L  E  *                                                        184
        ---------|---------|---------|---------|---------|---------|---------|---------|
   641  CAAGGTATGTTGACATGTTTCCTTGAAAAATAAACGTCTCAGGAGCCTTGTCTATACTCTATAAAAAAAAAAAAAAAAAAA  720
        ---------|---------|-
   721  AAAAAAAAAAAAAAAAAAAAA  741
```

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[1]http://gabi.rzpd.de/

8.1.2 BvAOX1

The BvAOX1 sequence was initially identified by a homology search using protein sequences of AOX isoforms from *A. thaliana* and *Sauromatum guttatum* as a query sequence and EST-collections from *B. vulgaris* as input sequences (carried out by KWS, Einbeck). The EST-clone with the highest homology carried the identification number kws3_003_b02_ZR_PLT_DS and the available sequence of this EST is marked in the sequence below. In order to obtain the full sequence of the cDNA, the corresponding plasmids were isolated and sequenced. However several bacterial clones were analyzed, which did not carry the expected AOX related sequence, probably due to wrong labeling in the cDNA collection. The full length sequence of the clone was therefore determined using 5' and 3' RACE.

```
       ---------|---------|---------|---------|---------|---------|---------|---------|
   1   AAAAATAAAAGTAAAAAAAATAATCAATCATCATATATAATTCCTACATACAAGTATACAACCGAAACAACTCACTGAGT 80
       ---------|---------|---------|---------|---------|---------|---------|---------|
  81   CAACTCACCGAGTCACCGAGGAAGAAAGatgaatcgtctggtagcaaactcgtcgttgttgaagctttctctctcctccg 160
   1                               M  N  R  L  V  A  N  S  S  L  L  K  L  S  L  S  S  V 18
       ---------|---------|---------|---------|---------|---------|---------|---------|
 161   ttaatggcggacgtcggattctctccggaaacggcgtaattacgccggagtttcacacgtggaggtttggtgtgacggat 240
  19    N  G  G  R  R  I  L  S  G  N  G  V  I  T  P  E  F  H  T  W  R  F  G  V  T  D 44
       ---------|---------|---------|---------|---------|---------|---------|---------|
 241   ttgcgccgggatatgacgtcatattcgccgaagtcggaggagaaagcggttgaggagagagaaaagaaggagttgaagaa 320
  45   L  R  R  D  M  T  S  Y  S  P  K  S  E  E  K  A  V  E  E  R  E  K  K  E  L  K  K 71
       ---------|---------|---------|---------|---------|---------|---------|---------|
 321   gatgaaggatggtttgccgatggtgtctagctattgggcggtttctaggccggaagttatgaaagaggatggtagtaaat 400
  72   M  K  D  G  L  P  M  V  S  S  Y  W  A  V  S  R  P  E  V  M  K  E  D  G  S  K  W 98
       ---------|---------|---------|-------==>  EST-sequence ----|---------|---------|
 401   ggaattggaactgttttatgccatgggagacttatgaggctgatttgtccattgatctgagcaaacatcacgtaccaaag 480
  99    N  W  N  C  F  M  P  W  E  T  Y  E  A  D  L  S  I  D  L  S  K  H  H  V  P  K 124
       ---------|---------|---------|---------|---------|---------|---------|---------|
 481   acatttgtggacaaagttgccttcagaacagtgaaactcttaaggatcccaactgatatcttctttaagaggcgctatgg 560
 125   T  F  V  D  K  V  A  F  R  T  V  K  L  L  R  I  P  T  D  I  F  F  K  R  R  Y  G 151
       ---------|---------|---------|---------|---------|---------|---------|---------|
 561   ttgccgtgcaatgatgcttgaaacagtggctgctgttcctggtatggttggcgggctactgctccatttgaggtcactcc 640
 152   C  R  A  M  M  L  E  T  V  A  A  V  P  G  M  V  G  G  L  L  L  H  L  R  S  L  R 178
       ---------|---------|---------|---------|---------|---------|---------|---------|
 641   gtaggttcgagcagagtggtgggtggatcaaagctttgctggaagaagctgagaatgagcggatgcacctaatgacattg 720
 179    R  F  E  Q  S  G  G  W  I  K  A  L  L  E  E  A  E  N  E  R  M  H  L  M  T  L 204
       ---------|---------|---------|---------|---------|---------|---------|---------|
 721   gtggagcttgtgcagccgaaatggtacgagagactattggttctagttgtgcagggagttttctttaactgttatttttgt 800
 205   V  E  L  V  Q  P  K  W  Y  E  R  L  L  V  L  V  V  Q  G  V  F  F  N  C  Y  F  V 231
       ---------|---           EST-sequence  <==  -|---------|---------|---------|---------|
 801   gctttatctgctttccccctaagttggcacacagagtggttggttacttggaagaggaggcaatttattcatacacagagt 880
 232   L  Y  L  L  S  P  K  L  A  H  R  V  V  G  Y  L  E  E  E  A  I  Y  S  Y  T  E  Y 258
       ---------|---------|---------|---------|---------|---------|---------|---------|
 881   atcttaaggacatagacagtggcgcgatcgaaaatgtacctgctcctgctattgctatcgactactggaggctgccaaag 960
 259    L  K  D  I  D  S  G  A  I  E  N  V  P  A  P  A  I  A  I  D  Y  W  R  L  P  K 284
       ---------|---------|---------|---------|---------|---------|---------|---------|
 961   gatgccaatctgaaggatgtttataactgtcattcgtgctgatgaagctcatcatcgtgatgtcaatcactttgcttccga 1040
 285   D  A  N  L  K  D  V  I  T  V  I  R  A  D  E  A  H  H  R  D  V  N  H  F  A  S  D 311
       ---------|---------|---------|---------|---------|---------|---------|---------|
1041   tgtccatttccaggacaaaaaactgaaagaagcaccagctccaattggttatcattagATACGTTACATTTTGAGAAGTT 1120
 312   V  H  F  Q  D  K  K  L  K  E  A  P  A  P  I  G  Y  H  *                         329
       ---------|---------|---------|---------|---------|---------|---------|---------|
1121   CTATTTCATTCTTAACTCTATTAGATTGGGTACACGTATTAGTACAGCGCTACATGCATGTAAATCCTTCCGAACTTCTT 1200
       ---------|---------|---------|---------|---------|---------|---------|---------|
1201   GTATCCTGGAAGAGTGATTACTACTCATAAGTCATAACATCCATTTATGGACACCGATTTATTTCAAAAAAAAAAAAAAA 1280
       --------
1281   AAAAAAAA 1288
```

8.1.3 Uncoupling proteins

For the identification of sugar beet cDNAs coding for uncoupling proteins, a BLAST search was carried out using the sequences of the AtPUMP1 (At3g54110) and AtUCP2 (At5g58970) proteins from *Arabidopsis thaliana* and the uncoupling protein from *Solanum tuberosum* (AJ002586). Below the sequences with the predicted protein sequences of the cDNA-clones for BvUCP1 and BvUCP2 are shown.

BvUCP1

The cDNA-clone, which contained the BvUCP1 sequence is annotated in Genbank under the accession number BQ594533. For the BvUCP1 cDNA the available cDNA sequence contains no start codon, probably due to incompleteness of the 5' region of the cDNA clone.

```
        ---------|---------|---------|---------|---------|---------|---------|---------|
      1 cacgcgtccgcccacgcgtccgcccacgcgtccgctcggtcctcaaaccgagatctcattcgctggaaccttcattagta 80
      1 T  R  P  P  T  R  P  P  T  R  P  L  G  P  Q  T  E  I  S  F  A  G  T  F  I  S  S 27
        ---------|---------|---------|---------|---------|---------|---------|---------|
     81 gcgcaattgctgcttgtttttgccgagtttttgtacgttgccattagacactgctaaagtgaagcttcaacttcaaaagaaa 160
     28  A  I  A  A  C  F  A  E  F  C  T  L  P  L  D  T  A  K  V  K  L  Q  L  Q  K  K 53
        ---------|---------|---------|---------|---------|---------|---------|---------|
    161 gcatcttcagctgatggagctgtttcagtcatatatacaggcatgttgggcactatagttacgattgcaaaggaagaagg 240
     54 A  S  S  A  D  G  A  V  S  V  I  Y  T  G  M  L  G  T  I  V  T  I  A  K  E  E  G 80
        ---------|---------|---------|---------|---------|---------|---------|---------|
    241 tttacctgcgctttggaagggaattgtgccagggttgcatcgccaatgtatctatggtggcttgagaattagcctgtatg 320
     81 L  P  A  L  W  K  G  I  V  P  G  L  H  R  Q  C  I  Y  G  G  L  R  I  S  L  Y  D 107
        ---------|---------|---------|---------|---------|---------|---------|---------|
    321 atcctttcaagatgttctttatgggagcttttcttttttggagattttcctttatttcaaaagatattagctgctctatta 400
    108   P  F  K  M  F  F  M  G  A  F  L  F  G  D  F  P  L  F  Q  K  I  L  A  A  L  L 133
        ---------|---------|---------|---------|---------|---------|---------|---------|
    401 actggtgcaattgcaatacttgttgccaatccaactgatcttgttaaagttcgacttcaagctgaaggaaaattgccacc 480
    134 T  G  A  I  A  I  L  V  A  N  P  T  D  L  V  K  V  R  L  Q  A  E  G  K  L  P  P 160
        ---------|---------|---------|---------|---------|---------|---------|---------|
    481 tggagtgccaaggcgctattcaggagccctacatgcttatagttccataataagacaggaaggactcttggctctttgga 560
    161  G  V  P  R  R  Y  S  G  A  L  H  A  Y  S  S  I  I  R  Q  E  G  L  L  A  L  W  T 187
        ---------|---------|---------|---------|---------|---------|---------|---------|
    561 ctgggctcggacccaacattgccaggaatgcaataataaatgctgctgaactagccagttatgatcaagtgaaactgact 640
    188   G  L  G  P  N  I  A  R  N  A  I  I  N  A  A  E  L  A  S  Y  D  Q  V  K  L  T 213
        ---------|---------|---------|---------|---------|---------|---------|---------|
    641 atattggcgctcccaggattcactgataatgcacttactcacatcctggctggtttaggtgcagggttttttgcagtgtg 720
    214 I  L  A  L  P  G  F  T  D  N  A  L  T  H  I  L  A  G  L  G  A  G  F  F  A  V  C 240
        ---------|---------|---------|---------|---------|---------|---------|---------|
    721 cattggatctcccgtggatgtggtgaaatcaagaatgatgggagatacaacatataaaagcacggttgattgtttcgtca 800
    241  I  G  S  P  V  D  V  V  K  S  R  M  M  G  D  T  T  Y  K  S  T  V  D  C  F  V  K 267
        ---------|---------|---------|---------|---------|---------|---------|---------|
    801 agacattgaagaatgagggaccattggcatttttataaaggatttctcccgaattttggcagattaggatcttggaatgtg 880
    268   T  L  K  N  E  G  P  L  A  F  Y  K  G  F  L  P  N  F  G  R  L  G  S  W  N  V 293
        ---------|---------|---------|---------|---------|---------|---------|---------|
    881 gtcatgttttttgacattggaacaagtcaagaagatgttgcaagggcatgcatagGAGTTCATCCGTTTTCTTAATCATCA 960
    294 V  M  F  L  T  L  E  Q  V  K  K  M  L  Q  G  H  A  *                               310
        ---------|---------|---------|---------|---------|---------|---------|---------|
    961 GGCATCAGCATAGCCATAACAAGAGATGGCACAAACCGACATGTTTTAAGAGTTAAGACCGGCCCCTTGATTTTACATGG 1040
        ---------|---------|---------|---------|---------|---------|---------|---------|
   1041 AGATTCATATGTTTGATTAATTTCGCATATACAGAGCTGCTTCTGAGAAGTTGGAAGGGAAAATGACAACATTGACGAAG 1120
        ---------|---------|---------|---------|---------|---------|---------|---------|
   1121 TTCCAGTAGTATATATATCGATTCATTGTGTTGTAACAAGTTCCATTAGTATAGGATTGCCTTGTAATTTTGCACCATAT 1200
        ---------|---------|---------|---------|---------|---------|---------|---------|
   1201 AGGTAAATTGTACACCATATTCTTTCTATTTGTATTGGTATTGAATTCTCAAGGAATGTCACACTATATATTATATCCTT 1280
```

C

```
          ----------|----------|----------|----------|-----
    1281  TATTCTTGAAGAGCATATGATACTTTTAACAAAAAAAAAAAAAAAA 1325
```

BvUCP2

Genbank accession: BQ593889

```
          ----------|----------|----------|----------|----------|----------|----------|----------|
       1  CTCTGTCTCTGTCTCTCTCCATTATAATCCCTTCGACCCTGCCTTGatgtctccatcgcccgatccaaagtctaacatct 80
       1                                                               M  S  P  S  P  D  P  K  S  N  I  S 12
          ----------|----------|----------|----------|----------|----------|----------|----------|
      81  cctttcctggaactttagctagcagtgcttttgctgcttgctttgctgagatttgtacaatccccttagacactgccaaa 160
      13   F  P  G  T  L  A  S  S  A  F  A  A  C  F  A  E  I  C  T  I  P  L  D  T  A  K  38
          ----------|----------|----------|----------|----------|----------|----------|----------|
     161  gtcaggctacagcttcagaaaaaagtggttggagatgcaataccacaatatcgaggtatgttaggtgcagtggccaccat 240
      39  V  R  L  Q  L  Q  K  K  V  V  G  D  A  I  P  Q  Y  R  G  M  L  G  A  V  A  T  I  65
          ----------|----------|----------|----------|----------|----------|----------|----------|
     241  tggcagagaagaagggatagcatcactctggaaaggaattgttccaggattgcatcgtcaatgcttgtttggtggtctac 320
      66   G  R  E  E  G  I  A  S  L  W  K  G  I  V  P  G  L  H  R  Q  C  L  F  G  G  L  R  92
          ----------|----------|----------|----------|----------|----------|----------|----------|
     321  gaatagggttgtatgaacctgttaagacattctatgtgggtaaagaccatgttggtgacgttccccttgtccaagaaaata 400
      93    I  G  L  Y  E  P  V  K  T  F  Y  V  G  K  D  H  V  G  D  V  P  L  S  K  K  I  118
          ----------|----------|----------|----------|----------|----------|----------|----------|
     401  cttgctgcacttacaactggcgccgttggaatcgcaattgctaatccaactgatcttggaaaagttcggcttcaagctga 480
     119  L  A  A  L  T  T  G  A  V  G  I  A  I  A  N  P  T  D  L  G  K  V  R  L  Q  A  E  145
          ----------|----------|----------|----------|----------|----------|----------|----------|
     481  ggggaaattgcctgctggtgtgcctaggcgctattctggagcattaaatgcctattcaactatagtcaagcaggaaggac 560
     146   G  K  L  P  A  G  V  P  R  R  Y  S  G  A  L  N  A  Y  S  T  I  V  K  Q  E  G  L 172
          ----------|----------|----------|----------|----------|----------|----------|----------|
     561  ttaaggccttatggactggccttgggccaaacgtagcacgcaatgctattataaatgctgctgaactggctagttatgat 640
     173   K  A  L  W  T  G  L  G  P  N  V  A  R  N  A  I  I  N  A  A  E  L  A  S  Y  D  198
          ----------|----------|----------|----------|----------|----------|----------|----------|
     641  caagtgaagcagtccattttaaggatacctggattcacagacaatgttgtcacccatatgttagctggtcttggagcggg 720
     199  Q  V  K  Q  S  I  L  R  I  P  G  F  T  D  N  V  V  T  H  M  L  A  G  L  G  A  G  225
          ----------|----------|----------|----------|----------|----------|----------|----------|
     721  attctttgctgtctgcattggctcacctgttgatgtggttaaatccagaatgatgggagattctacatacaagagtacgc 800
     226   F  F  A  V  C  I  G  S  P  V  D  V  V  K  S  R  M  M  G  D  S  T  Y  K  S  T  L 252
          ----------|----------|----------|----------|----------|----------|----------|----------|
     801  tggattgtttttatcaagaccttaaagaatgatggtcctcttgcattttacaagggatttatcccaaattttggacgtctt 880
     253    D  C  F  I  K  T  L  K  N  D  G  P  L  A  F  Y  K  G  F  I  P  N  F  G  R  L  278
          ----------|----------|----------|----------|----------|----------|----------|----------|
     881  gggtcatggaatgtcattatgttcttgactttggaacaggcaaagaaggttgtgcgaaatttggagtcatcttgaGTTAA 960
     279  G  S  W  N  V  I  M  F  L  T  L  E  Q  A  K  K  V  V  R  N  L  E  S  S  *         302
          ----------|----------|----------|----------|----------|----------|----------|----------|
     961  GGATTTTGATAACATTCTCACTTGCAATCTCCTGAAAACTTATTAAAGAGAAATAAGGCCTTGCTGTCTCAGTCTGAAAG 1040
          ----------|----------|----------|----------|----------|----------|----------|----------|
    1041  ATGACTTAACTGCAATATATCACATGGCATACATCTGAAGGCTGAAGTAGTAATGAACTATACGCAGATGAAGAGCTTAT 1120
          ----------|----------|----------|----------|----------|----------|----------|----------|
    1121  AAATTGTAAAGACAGTCACAAGTAGGATCTTATTACTTCATAATGGTTCTTCTTCATCTTGCCTAGCTACTGAATCCATT 1200
          ----------|----------|----------|----------|----------|----------|----------|----------|
    1201  ATTTTGAGTTCTAGCAAATCACTTTTGCTTCATATTATAACTATGTAANCAATATTGATATTTCTGTAAACTGGGTTAGT 1280
          ----------|----------|----------|----------|----------|----------|----------|----------|
    1281  TGGTGTACCCCATATTATGGATATTTGATCTTGGGCAAACCAAGTGAAATTACCCATAATCGTTCGATTATAATTGAAAA 1360
          ----------|----------|----------|----------|-------
    1361  TGTACCTGTCGCCATTGCTGCAAAAAAAAAAAAAAAAA 1397
```
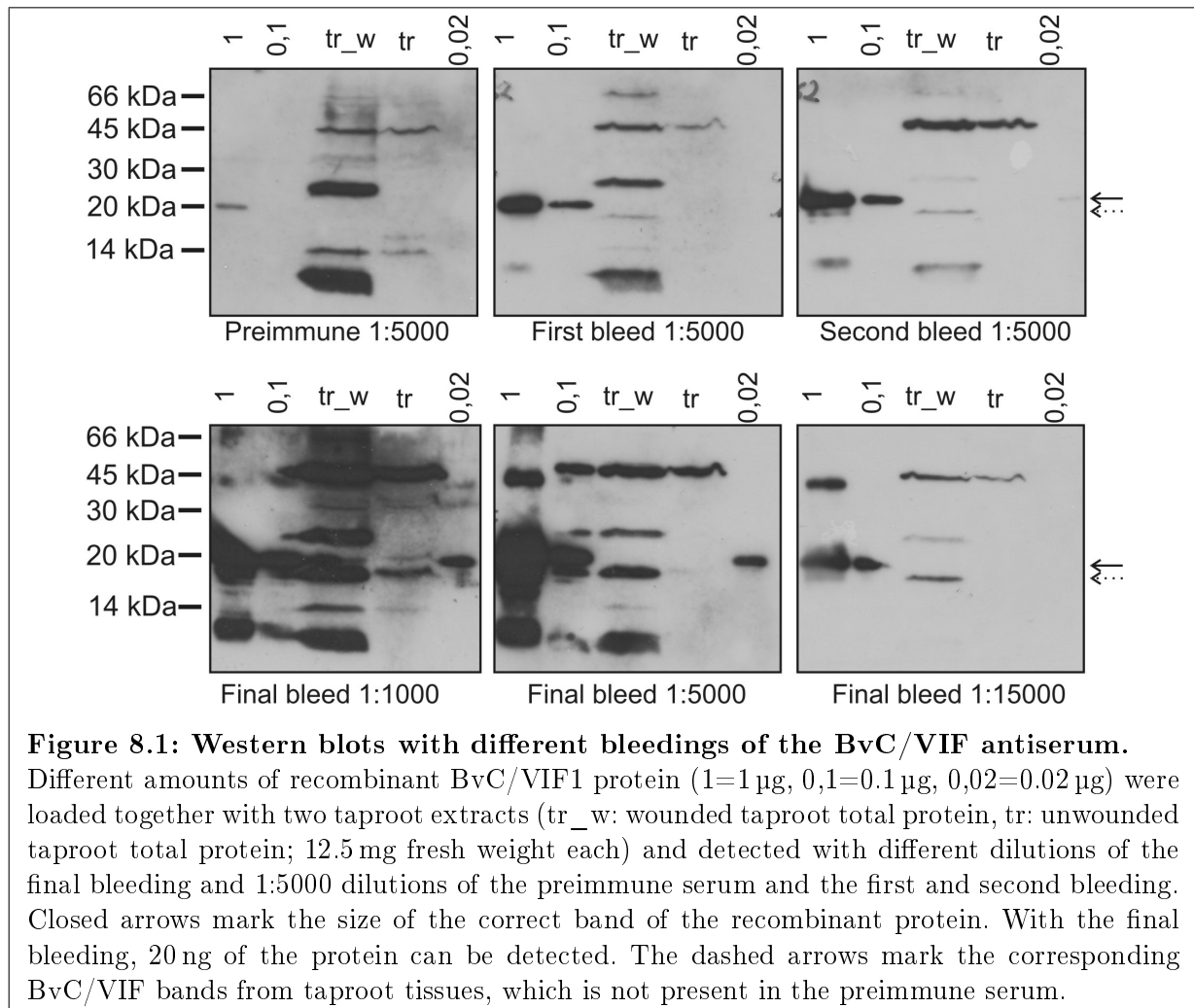
D

8.2 Characterization of the raised BvC/VIF antiserum

Figure 8.1 shows a comparison of the different bleedings obtained from the immunization procedure (Eurogentec, Belgium). Dilutions of the different bleedings were tested by Western blotting with samples of the recombinant proteins and extracts from taproots. Compared to the first and second bleeding, the final bleeding shows a higher sensitivity towards the recombinant BvC/VIF1 protein (closed arrow). In the tested taproot extracts several prominent bands appear, of which most are already present in the preimmune serum, indicating the presence of unspecific antibodies against sugar beet proteins. However, in plant extracts an additional band of the expected size is only present after immunization (dashed arrow). This band of approximately 18 kDa is especially pronounced in the extract from wounded taproots.

Figure 8.1: Western blots with different bleedings of the BvC/VIF antiserum.
Different amounts of recombinant BvC/VIF1 protein (1=1 µg, 0,1=0.1 µg, 0,02=0.02 µg) were loaded together with two taproot extracts (tr_w: wounded taproot total protein, tr: unwounded taproot total protein; 12.5 mg fresh weight each) and detected with different dilutions of the final bleeding and 1:5000 dilutions of the preimmune serum and the first and second bleeding. Closed arrows mark the size of the correct band of the recombinant protein. With the final bleeding, 20 ng of the protein can be detected. The dashed arrows mark the corresponding BvC/VIF bands from taproot tissues, which is not present in the preimmune serum.

F

9 Danksagung

Vielen Menschen gebührt mein Dank für ihre Unterstützung und Mithilfe zum Gelingen meiner Promotion. Den Folgenden möchte ich persönlich danken: