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Modifying post-harvest sucrose loss in sugar beet:

Assessment of transgenic approaches

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1. Summary/ Zusammenfassung

1.1 Summary

Sugar beet (*Beta vulgaris* L.) is one of the economically most important plants storing high levels of sucrose. Sucrose is accumulated in the taproot inside the vacuoles of parenchyma cells, with up to nearly 20 % of fresh weight. Harvesting of sugar beet includes wounding of taproots, leading to induction of invertases and consequently to sucrose loss.

In the present study, the interaction of the sugar beet invertase inhibitor BvC/VIF (*Beta vulgaris* cell wall and/or vacuolar inhibitor of ß-fructosidase) and its putative target enzymes was characterized and transgenic approaches, aiming at the modification of post-harvest sucrose metabolism investigated.

Analysis of the inhibitor-invertase-interaction during wounding uncovered that complex formation between BvC/VIF and invertases does not necessarily lead to an inhibition of invertase activity. During the elucidation of prerequisites, needed for the down-regulation of invertase activity by proteinaceous inhibitors, site directed mutagenesis of BvVI1 (*Beta vulgaris* vacuolar invertase 1) demonstrated a putative involvement of the C-terminus of the invertase in the inhibition process.

Characterization of BvC/VIF revealed two BvC/VIF protein species, differing slightly in molecular size. The analysis of transgenic lines confirmed that both species are encoded by the same gene. Further elucidation of the origin of the observed difference-in-size revealed that (i) a proteolytical cleavage of BvC/VIF can be excluded and that (ii) the postulated post-translational modification appears to be limited to the homologous system (i.e. sugar beet).

In order to intervene in post-harvest sucrose metabolism, sugar beet has been engineered to overexpress the endogenous invertase inhibitor BvC/VIF. Alternatively, the expression of endogenous BvC/VIF was silenced, with the purpose to determine the role of BvC/VIF within post-harvest sucrose-metabolism. The heterologous expression of BvC/VIF under control of the taproot specific 2-1-48 promoter did not lead to high expression levels, whereas under the control of the duplicated 35S-promoter, BvC/VIF was highly expressed and silenced via an RNAi construct, respectively. By overexpressing BvC/VIF, wound induced cell wall (CWI) as well as vacuolar (VI) invertase activity were strongly reduced. Notably, the extra-cellular localization of BvC/VIF was proven by a non-invasive approach and via immunolocalization, whereas no further evidence for an additional (earlier postulated) vacuolar localization was gained thus far.

Unexpectedly, sucrose loss was not altered in BvC/VIF overexpressing lines, despite strongly reduced invertase activity. This observation led to the hypothesis of a demand-driven sucrose

metabolism in wounded sugar beet taproots, based on altered activities of other sucrose hydrolytic enzymes in the case of hampered invertase activity.

The determination of wound induced invertase activities in individual plants of BvC/VIF RNAi lines implicated an important role of BvC/VIF in regulating invertase activity after wounding, thus in limiting sucrose loss.

1.2 Zusammenfassung

Die Zuckerrübe (*Beta vulgaris* L.) ist eine der, wirtschaftlich gesehen, wichtigsten Nutzpflanzen, die hohe Konzentrationen an Saccharose speichern. Die Saccharosespeicherung geschieht in Rübenkörpern, innerhalb der Vakuolen der Parenchymzellen. Heutige Zuchtformen der Zuckerrübe akkumulieren bis zu 20% des Rübengewichtes an Saccharose. Während der Zuckerrübenernte kommt es zur Verwundung des Rübenkörpers. Durch diese Verwundung werden Invertasen induziert, was wiederum zum Abbau der Saccharose und somit zu ungewollten Zuckerverlusten führt.

Innerhalb der vorliegenden Arbeit wurde die Interaktion zwischen dem Invertaseinhibitor der Zuckerrübe, BvC/VIF (*Beta vulgaris* cell wall and/or vacuolar inhibitor of ß-fructodsidase) und seiner potentiellen Zielenzyme charakterisiert sowie transgene Ansätze, die darauf zielen in den Zuckermetabolismus nach der Ernte einzugreifen, geprüft.

Die Analyse der Inhibitor-Invertase-Interaktion während der Verwundung deckte auf, dass die Komplexbildung zwischen Inhibitor und Invertase nicht zwangsläufig zu einer Inhibierung der Invertaseaktivität führt. Die Charakterisierung nötiger Voraussetzungen für die Regulierung der Invertase durch Inhibitorproteine, implizierte eine Rolle des C-Terminus der Invertase innerhalb des Inhibierungsprozesses.

Während der Analyse von BvC/VIF wurden zwei Proteingattungen, die sich um circa 1 kDa unterscheiden, identifiziert. Die Charakterisierung transgener BvC/VIF Linien bestätigte, dass beide Gattungen von demselben Gen kodiert werden. Weitere Experimente, zur Bestimmung der Herkunft des Größenunterschiedes, zeigten, dass (i) eine proteolytische Prozessierung ausgeschlossen werden kann und dass (ii) die postulierte post-translationale Modifikation speziell im homologen System auftritt.

Durch Überexpression von BvC/VIF in Zuckerrüben sollten Zuckerverluste nach der Ernte reduziert werden. Darüber hinaus wurde ein RNAi-Ansatz gewählt, um die Rolle von BvC/VIF innerhalb des Zuckermetabolismus nach Verwundung zu identifizieren. Die heterologe Expression von BvC/VIF unter der Kontrolle des Rübenkörper spezifischen Promotors p2-1-48 führte zu keiner starken Expression, wobei BvC/VIF unter der Kontrolle des doppelten p35S Promotors sehr stark exprimiert, beziehungsweise die Expression durch ein RNAi-Konstrukt, deutlich verringert wurde.

Die Überexpression von BvC/VIF resultierte in einer deutlichen Runterregulierung der wundinduzierten Zellwand- sowie der vakuolären Invertaseaktivität im Speicherorgan der Zuckerrübe. Während die extra-zelluläre Lokalisation von BvC/VIF mittels eines nicht- invasiven

Ansatzes sowie einer Immunolokalisierung gezeigt wurde, konnte allerdings bisher keine zusätzliche intra-zelluläre Lokalisation bestätigt werden.

Unerwarteter Weise wurde der Saccharoseabbau in den BvC/VIF- Überexpressionslinien trotz deutlich reduzierter Invertaseaktivitäten nicht beeinflusst. Diese Beobachtung führte zu der Hypothese eines, über die Nachfrage gesteuerten Zuckermetabolismus in der verwundeten Zuckerrübe, basierend auf veränderten Aktivitäten anderer Saccharose spaltender Enzyme bei eingeschränkter Invertaseaktivität.

Wundinduzierte Invertaseaktivitäten in einzelnen Pflanzen einer BvC/VIF RNAi- Linie implizierten zudem eine wichtige Rolle von BvC/VIF bei der Regulierung der Invertaseaktivität nach Verwundung und somit bei der Limitierung von Saccharoseverlusten.

2. Introduction

2.1 Sugar beet

2.1.1 Sugar beet, an important crop for industrial sugar production

Sugar beet (*B. vulgaris* L.) is a species of high agricultural importance, belonging to the family of Chenopodiaceae, including sugar beets, fodder beets and mangels. Several members of the family are common arable weeds. Cultivated forms are thought to derive from sea-coast plants of Europe and Asia, which are very variable in habit and duration (Körber-Grohne, 1995). Blockade of Continental ports during the Napoleonic wars cut off the supply of sugar cane from the West Indies and favored development of an alternative source of sugar. Original forms of sugar beet, developed in Europe in the eighteenth century, contained only about 4% sugar but selection and breeding have raised this to a today's maximum of 20%.

Culture

Sugar beet is a temperate climate biennial root crop. During the first year the plant develops an expanding storage root, which accumulates high amounts of sucrose. In the second year, after vernalization, sucrose is utilized for shoot, flower and seed production (Elliot, 1993). In commercial beet production, the root is harvested after the first growing season, when the taproot accumulates the maximal sucrose content. In most temperate climates, beets are planted in spring and harvested in autumn. In warmer climates sugar beet is a winter crop, being planted in autumn.

Harvesting

In central Europe sugar beet taproots are harvested in autumn and early winter. During harvesting, which is by now entirely mechanical, sugar beet taproots get decapitated, since they have to be separated from beet leaves and the crown before processing. Afterwards, taproots are left in piles at the side of the field for several weeks and get then delivered to the factory. Harvesting and processing of the beet is referred to as "the campaign," reflecting the amount required to deliver crop to the factories for the duration of harvest and processing. In Germany the campaign lasts approximately four months.

Post-harvest sucrose loss in sugar beet

After harvest of sugar beet taproots, sucrose is lost (Jaggard et al., 1997) due to the enzymatically breakdown of sucrose, which provides energy for wound reactions, and mainly after prolonged storage, due to respiration of the tissue (Wyse et al., 1978; Campbell, 2006). During sucrose mobilization, hexoses like glucose and fructose accumulate, which leads to a lower quality of sugar beet taproots. Many attempts have been made to further understand the molecular backgrounds for sucrose breakdown in sugar beet, in order to identify candidate genes for biotechnological approaches aiming at the reduction of post-harvest sucrose loss.

2.1.2 Sugar beet, a target crop for biotechnological approaches but also a model for vegetative storage tissues

In sugar beet, one of the primary biotechnological goals is the reduction of post-harvest sucrose loss (Bosemark, 1993). The physiology of sugar beets during storage has been studied extensively in the past (Ibrahim et al., 2001). Recently, much effort was made to enlighten the molecular background. Since taproots get decapitated during harvest, wounding experiments were performed, aiming on the identification of sucrolytic enzymes which are responsible for post-harvest sucrose loss (Rosenkranz et al., 2001). By this approach two wound-induced invertases in sugar beet taproots, being responsible for post-harvest sucrose loss, were identified. Furthermore, the impact of stresses encountered during sugar beet post-harvest storage, like wounding, anoxia and cold, on sucrose synthase expression and activity has been investigated (Klotz and Haagenson, 2008). Also, post-harvest gene-expression in general has been addressed recently (Rotthues et al., 2008), whereas previous gene expression studies in sugar beet concentrated on the taproot and on leaves (Bellin et al., 2002; Kloos et al., 2002; Oltmanns et al., 2006; Bellin et al., 2007).

The further characterization of the response to post-harvest stress-conditions in sugar beet has a high potential for agriculture. Nevertheless, basic research in sugar beet taproots is challenging. For instance, the generation time is quite long, since sugar beet is a biennial plant. On the other hand, stable transformation of sugar beet is well established (Lindsey and Gallois, 1990; Hall et al., 1996; Yang et al., 2005; Liu et al., 2008). However, every transgenic approach has to be considered carefully; since it takes quite long until analysis of transgenic seed grown taproots can be performed. In order to circumvent such time loss, adventitious sugar beet roots can be chosen for first characterizations (Liu et al., 2008). Therefore, from transformed explants regenerated shoots are transferred first to a rooting medium and subsequently rooted plants are transferred to soil. These plants develop a storage organ which looks in principle like a sugar

beet taproot. Like sugar beet taproots, adventitious roots display a particular growth with repeated concentric rings of cambium producing secondary phloem, xylem and parenchymous cells. Inside the parenchyma cells, sucrose accumulates to concentrations comparable to taproot. Nevertheless, adventitious roots are physiologically quite different from seed grown taproots. For instance, taproots in contrast to adventitious roots are mainly formed by root tissue and only to a smaller extent of hypocotyl (Elliot, 1993). Adventitious roots are not adequate with regard to developmental studies in transgenic lines; still analysis of adventitious roots provides an efficient tool especially for characterization of the post-harvest situation.

2.2 The role of sugar in plants; by far more than providing energy

2.2.1 Sugar signaling

Sugar signaling during plant development

Plants comprise sugar exporting (source) tissue and sugar importing (sink) tissue. Low sugar levels lead to increased source activities like photosynthesis, nutrient mobilization and export. Under high sucrose levels, sink activities like growth and storage are upregulated (Rolland et al., 2006). Sucrose as well as its hydrolytic products, glucose and fructose, might act as signal molecules. In general, sugar allocation and generation of sugar signals are determined by sucrose transport and hydrolysis. Thus, sucrolytic enzymes, like invertases and sucrose synthases are assumed to be key enzymes with respect to sugar signaling. Several processes during plant development are postulated to be sugar regulated. One example is the so called "sugar-switch" model of embryo development. During early seed development a high cell wall invertase activity leads to high hexose levels, which promote embryo growth driven by cell division. In the following transition phase, the embryo switches from a mitotic growth to differentiation and growth driven by cell expansion. This switch is accompanied by decreasing CWI activity and hexose levels, whereas storage products, like starch are accumulated (Weber et al., 2005; Morley-Smith et al., 2008). Sugar signaling is also involved in seed germination, since it was shown that glucose leads to delayed seed germination in Arabidopsis (Price et al., 2003; Dekkers et al., 2004). Moreover, high levels of external sugars during Arabidopsis early seedling development repressed normal growth (Rolland et al., 2006). During all studies it became apparent that the sugar signaling pathway is closely connected to the hormone signaling pathway. Especially, a central role of ABA in plant sugar signaling was determined (Rolland et al., 2006). Moreover, it was shown that glucose and ethylene signaling are closely linked together (Yanagisawa et al., 2003). During senescence sugars and cytokinins work

antagonistically (Moore et al., 2003). Leaf senescence is induced by sugars and can be delayed by cytokinin. It was shown that this delay is based on cytokinin-induced CWI expression (Balibrea Lara et al., 2004).

Sugar signaling in response to stress

Carbohydrates seem to be essential for successful plant defense (Essmann et al., 2008). Defense responses presumably are supported by accumulation of high hexose levels (Roitsch et al., 2003; Biemelt and Sonnewald, 2006; Swarbrick et al., 2006; Berger et al., 2007). It was shown, that invertases as well as pathogenesis- related (PR) genes are co-induced by soluble sugars (Roitsch et al., 1995; Herbers et al., 1996; Thibaud et al., 2004). Presumably, a high invertase activity in response to pathogen attack leads to high accumulation of hexoses and causes reprogramming of the mesophyll from source to sink. This in turn, would enable the plant to reimport sugars in the infected mesophyll cells, which then support plant defense (Essmann et al., 2008). Recently, sugars were suggested to be effective candidates for the oxidative burst in response to various environmental stresses (Van den Ende and Valluru, 2009). Moreover, remodeling of carbon metabolism in *Arabidopsis* is interpreted as an emergency strategy under oxidative stress (Scarpeci and Valle, 2008).

2.2.2 Sugar transport

The primary product of photosynthetic CO_2 fixation in higher plants is sucrose. The biosynthesis of sucrose takes place in the mesophyll of leaves, representing the source-tissue. 80% of the photoassimilates are subsequently transported to import-dependent, heterotrophic tissue, representing the sink tissue. The non-reducing disaccharide sucrose is the predominant form of carbon transported to sink tissues. However, some plant families have only low sucrose concentrations in their phloem sap and high concentrations of raffinose-family oligosaccharides (RFOs) or sugar alcohols (Sauer, 2007). Recently, it was even postulated that hexose translocation has to be regarded as commonly used mode of carbohydrate transfer via the phloem in certain plant families (van Bel and Hess, 2008). The long-distance sugar transport is managed via the phloem, in which hydrostatic power drives phloem sap movement toward sink tissue according to the model of Münch (Münch, 1930). The high hydrostatic pressure is established by the difference in solute concentrations caused by assimilate loading in the source tissue and unloading in the sink tissue. Two types of phloem-loading exist, the symplastic loading and the apoplastic loading. Plant families which transport RFOs are symplastic loaders; they possess an "open minor vein configuration" with many plasmodesmata connecting the SE-

CC (sieve-element-companion cell) complex with the adjacent cells. In these plants, sucrose concentrations in the phloem are only slightly higher than in the mesophyll cells (Knop et al., 2001). Nevertheless, a part of sucrose loading seems also to be performed by transporter (Knop et al., 2004). Plant families transporting sucrose only, represent the apoplastic loaders. They have only few plasmodesmata between their SE-CC complexes and their adjacent cells (= closed minor vein configuration) (Sauer, 2007). The sucrose concentration in the phloem of those families is much higher than in the mesophyll. The release of sucrose in the apoplast, followed by an energy dependent uptake into the phloem helps to overcome the concentration gradient. The uptake is mediated by H+-sucrose symporting sucrose transporters (named SUC or SUT proteins) (Lalonde et al., 2004; Reinders et al., 2006; Sauer, 2007). Moreover, SUTs are also responsible for the release of sucrose in apoplastic sinks, which are, in contrast to symplastic sinks, not symplastically connected to the phloem (Kuhn et al., 2003). Sucrose released in the apoplast is either immediately taken up from sink cells by sinks specific SUTs or hydrolyzed by apoplastic, acid invertase followed by the uptake of resulting hexoses by monosaccharide transporters. Whether the one or the other mechanism is preferred, depends on the sink tissue. Hydrolysis of sucrose by CWI occurs mainly in rapidly, growing, dividing cells, while direct sucrose uptake seems to be typical for non-dividing storage sink tissues (Weber et al., 1997; Sauer, 2007). Interestingly, the expression of hexose transporters strongly increases in response to wounding and pathogen attack (Truernit et al., 1996). Moreover, a coordinated expression of AtSTP4 (a high-affinity hexose transporter from *A. thaliana*) and AtCWI1 (cell wall invertase from *A. thaliana*) was shown in response to fungal attack (Fotopoulos et al., 2003). These results support the assumption that sugar transport proteins (SPTs) and cell wall invertases interact in order to supply sink tissues with hexoses (Büttner, 2007). After uptake in sink tissues, sugar is either further metabolized or converted into osmotically inactive storage compounds. Furthermore, sucrose can be transported in the vacuolar lumen by recently characterized tonoplast localized transporters (Endler et al., 2006).

Sugar beet represents a typical apoplastic loader. The sucrose symporter from sugar beet (BvSUT1) is regulated by the availability of sucrose in the source tissue (Chiou and Bush, 1998). The transcription of BvSUT1 was down-regulated by external sucrose feeding (Vaughn et al., 2002). Concerning phloem unloading, a transition between apoplastic and symplastic unloading seems to occur during taproot development (Godt and Roitsch, 2006). In the early stages, a high CWI activity is responsible for the apoplastic pathway for sugar supply, whereas symplastic phloem unloading in mature taproots is driven by sucrose synthase activity.

Sucrose import and export from the vacuole

High concentrations of the disaccharide sucrose can be stored in vacuoles. The import of sucrose in vacuoles from leaves of C3- and CAM species is not energy dependent and simply driven by the concentration gradient between the cytosol and the vacuolar lumen (Neuhaus, 2007). Only recently, two vacuolar SUTs (sucrose transporter), located at the tonoplast, were identified, the barley carrier HvSUT2 and the Arabidopsis carrier AtSUT4 (Endler et al., 2006). It was shown, that AtSUT4 is expressed in the mesophyll cells. Since both SUTs do represent H+-sucrose symporter and due to an existing proton gradient, it is assumed that they are involved in sucrose export rather than import from the vacuole into the cytosol (Neuhaus, 2007). Concerning mobilization of sucrose from the vacuole, it is suggested that sucrose gets enzymatically cleaved prior to mobilization (Leigh et al., 1979; Martinoia et al., 2007). Echeverria and Gonzalez (Echeverria and Gonzalez, 2000) suggested different pathways for mobilization of sucrose. The authors reported on a sucrose export in red beet by an ATP-dependent sucrose transporter, which channels sucrose directly to a tonoplast located SuSy. Moreover, a vesicle-mediated system for long-distance sucrose transport from the vacuole from storage cells to the apoplast in red beet is assumed (Echeverria, 2000). Recently, a vesicle-mediated transport model for the movement of vacuolar fructans was described, which is assumed to enable an efficient transport of fructans and sucrose from the vacuole to the apoplast (Valluru et al., 2008).

In sugar beet taproots, sucrose is probably imported via an H+- antiport against the concentration gradient (Getz et al., 1987). Furthermore, a sugar transporter could be isolated from taproot vacuoles (Chiou and Bush, 1996). However, the exact transport activity of this carrier is yet not clear.

2.3 Sucrose hydrolyzing enzymes

In plants, two enzymatic pathways of sucrose cleavage are known. One is catalyzed by invertases (sucrose+ H_2O → glucose and fructose) and the other by sucrose synthase (sucrose + UDP ← → fructose + UDP-glucose). Although both enzymes have the same substrate, their reaction products are quite different (Winter and Huber, 2000). SuSy produces UDP-glucose and invertases free glucose. Therefore, invertases produce twice as many hexoses as sucrose synthases and thus are assumed to fulfill important function with respect to sugar signaling (Koch, 2004; Gibson, 2005). On the other hand, the reversible cleavage of sucrose via SuSy in contrast to the hydrolysis via invertases preserves much of the energy stored in the chemical bond of glucose and fructose.

2.3.1 Sucrose Synthase (SuSy)

SuSy plays an important role for the entry of sucrose into the cellular metabolism in nonphotosynthetic cells and is assumed to be involved in determining sink strength (Zrenner et al., 1995), for instance in rice grain filling (Tang et al., 2009). SuSy associated with vascular tissue, supplies energy for phloem loading by providing the substrate for respiration (Fu, 1995; Hänggi and Fleming, 2001). Different SuSy isoforms exist, which are assumed to be mainly localized in the cytosol. However, an association of SuSy with the plasmalemma (Amor et al., 1995; Carlson and Chourey, 1996) was shown, too. Moreover, a tonoplast-associated form in red beet was described by Echeverria and Gonzalez (Echeverria and Gonzalez, 2000). Individual SuSy isoforms are needed for normal development in several plant organs, as shown for potato tubers (Zrenner et al., 1995), tomato fruits (D'Aoust et al., 1999), cotton fibers (Ruan et al., 2003) and maize seeds (Chourey et al., 1998). In addition, a predominant role of SuSy in cellulose synthesis is assumed (Koch, 2004). It is described that SuSy is associated with the cellulose synthase complex, thus UDP-glucose can easily be supplied for cellulose synthesis (Haigler et al., 2001; Ruan et al., 2003). However, SuSy activity is not essential for cellulose synthesis in all plants as shown recently by a study in *A. thaliana* (Barratt et al., 2009). Since transgenic potato plants in which SuSy activity has been altered displayed different starch levels in leaves, SuSy is also believed to participate in the conversion of sucrose to starch, (Munoz et al., 2005). In contrast to invertases, SuSy is likely to be important under low-oxygen conditions (Koch, 2004), since less ATP is needed for entry of the cleavage products into glycolysis after sucrose degradation via SuSy. Whereas invertases are rapidly repressed under low-oxygen conditions (Zeng et al., 1999), SuSy is still able to support biosynthesis of cellulose and callose

under hypoxia (Albrecht and Mustroph, 2003; Subbaiah and Sachs, 2003) and is thought to provide substrates for the increased glycolytic demand under anaerobic or osmotic stresses (Ricard et al., 1998).

Most plant species contain two or more SuSy genes (Sturm and Tang, 1999). For instance in *A. thaliana* (Barratt et al., 2001; Baud et al., 2004) and rice (Hirose et al., 2008), six genes encoding SuSy have been characterized, respectively.

In sugar beet, two isoforms were isolated so far (Hesse and Willmitzer, 1996; Klotz et al., 2003). The expression of both SuSy isoforms is highly organ specific, showing both a sink associated expression in roots and only a low one in leaves (Haagenson et al., 2006). SuSy, not invertases, represents the key sink enzyme in sugar beet taproots in the later stages of development, showing a high expression level in mature taproots. Since the increase in SuSy activity correlates with sucrose accumulation, the conclusion is supported that SuSy is responsible for supplying sucrose for storage processes in taproots (Godt and Roitsch, 2006). Still, further research has to be performed to clarify the contribution of each SuSy isoform to carbon partitioning in sugar beet.

Regulation of sucrose synthase

Diverse expression studies confirmed an enhanced SuSy expression level in sink tissues. In response to various treatments like anoxia, dehydration, osmotic stress and cold exposure, a stress induced transcript accumulation has been reported (Hesse and Willmitzer, 1996; Déjardin, 1999; Kleines et al., 1999). Additionally, expression in response to sugar supply was investigated (Baud et al., 2004; Koch, 2004) and only recently it was shown that SuSy expression and activity seems to be regulated by the sugar status *in vivo* (Tang et al., 2009). As shown by Ciereszko and Kleczkowski, there is strong evidence that sugars regulate SuSy via a hexokinase-modulated sugar sensing mechanism (Ciereszko and Kleczkowski, 2002). Moreover, several studies describe cases of both post-transcriptional and post-translational regulation of SuSy (McElfresh and Chourey, 1988; Chourey and Taliercio, 1994; Zeng et al., 1998). Amongst others, SuSy is regulated by changes in subcellular localization and by phosphorylation (Koch, 2004).

As mentioned before, also sugar beet SuSys display a developmental and organ specific expression. In general, the protein expression of SuSy corresponds to transcript levels; still changes on protein level have been observed strongly delayed compared to transcription (Haagenson et al., 2006). Especially in sugar beet taproots exposed to stress like wounding, anoxia and cold, discrepancies between transcript levels, protein abundance and activity strongly suggest a post-transcriptional regulation (Klotz and Haagenson, 2008). In contrast to

findings in others plants, in which an induction of SuSy expression and activity in response to stress indicates a role for this enzyme in stress responses (Ricard et al., 1998; Déjardin, 1999), the sugar beet SuSy isoforms known so far, seem not to be important in stressed sugar beet taproots.

2.3.2 Invertases

Invertases (EC 3.2.1.26) split sucrose into glucose and fructose by the irreversible hydrolytic cleavage of the α1-β2-glycosidic bond. Invertases play an important role in carbon metabolism and sugar signaling. Different invertase isoforms exist, which fulfill distinct roles during various developmental stages. They can be classified according to (i) their subcellular localization into cytosolic, vacuolar and cell wall invertases or to (ii) their pH optimum into neutral/alkaline and acid invertases, respectively. Vacuolar and cell wall invertases, which are both representing acid invertases belong to the same glycoside hydrolase family (GH 32), whereas neutral/alkaline invertases share no similarity with acid invertases with respect to primary amino acid sequence, and thus are allocated to another GH family (GH100) (Sturm and Tang, 1999; Lammens et al., 2008).

Alkaline/ neutral invertases (A/NI)

A/NIs display a pH optimum close to 6.5 and 8, respectively. Sucrose is their only substrate (Sturm, 1999). The members of this class, which might have originated from cyanobacteria by endosymbiosis (Vargas et al., 2003) are highly homologous among each other, but differ distinctly from acid invertases. For instance a different active site is assumed for A/NIs compared to acid invertases, as they are not affected by heavy metals (Koch, 2004). Since A/NIs are extremely labile and the enzyme activity is rapidly lost upon extraction (Sturm, 1999; Roitsch and Gonzalez, 2004), they have been investigated to a much lower extent than acid invertases, thus only limited information on the physiological role of this group of invertases is present. Only recently research focused on the physiological characterization of A/NIS and several studies revealed that cytosolic invertase activity is essential for normal plant growth and development (Jia et al., 2008; Barratt et al., 2009; Welham et al., 2009). Qi et al. (Qi et al., 2007) showed that cytosolic invertases are moreover involved in stress responses. So far A/NIs were believed to be exclusively located in the cytosol (Chen and Black, 1992; Van den Ende, 1995), but recently also localization to chloroplasts and mitochondria was shown (Murayama and Handa, 2007). It is assumed that sucrose hydrolysis in chloroplasts represents a further possible step in controlling carbon translocation between chloroplast and cytosol (Vargas et al., 2007).

Evidence exists that cytosolic invertases represent a target for signaling pathways, coordinating carbohydrate availability with plant development and growth (Barratt et al., 2009). In *Arabidopsis thaliana* and in *Oryza sativa* multigene families of putative A/NIs were found (Vargas et al., 2003; Ji et al., 2005) and differential expression of A/NIs isoforms, displaying different pH optima, suggest distinct physiological roles of individual members (Vargas et al., 2007).

Acid invertases

As indicated by their appellation, the pH optimum of acid invertases is between 3.5 and 5.5. Cell wall located as well as vacuolar isoforms belong to this group of isoenzymes. Acid invertases preferably hydrolyze sucrose, but in contrast to A/NIs degradation of other substrates, displaying a sucrose-backbone is also possible (De Connick et al., 2005; Verhaest et al., 2007). Acid invertases belong to the glycoside hydrolase family 32 (GH32), which comprises amongst others plant fructan exohydrolases (FEHs) and plant fructan biosynthetic enzymes (FBEs). It is assumed that plant FEHs originated from cell wall invertases by only few mutations, whereas FBEs evolved from vacuolar invertases (Van den Ende et al., 2002; Lammens et al., 2009). Only by heterologous expression and protein purification it was shown that two predicted invertases from *A. thaliana* do represent FEHs (De Connick et al., 2005). Recently, a hydrogen bonding network in a conserved motif was determined in VIs, which is absent in fructosyltransferases (FTs), a member of FBEs. Site-directed mutagenesis of VI, disrupting the bond, resulted in a formation of a high-affinity FT (Schroeven et al., 2008).

Alberto et al. (Alberto et al., 2004) published the first GH32 structure. It represented the structure from an extracellular ß-fructosidase from *Thermotoga maritima*. The structure of a FEH from *Cichorium intybus* (CiFEHIIa) was the first reported GH32 structure from plants (Verhaest et al., 2005a). The elucidation of the structure of a cell wall invertase from *A. thaliana* (AtCWI1) succeeded shortly after (Verhaest et al., 2005b; Verhaest et al., 2006). Except the described bacterial isoform, all GH32 structures display glycosyl chains. It was shown that glycosylation protects cell wall invertase from rapid degradation (Pagny et al., 2003). The structure of AtCWI1 contains an N-terminal fivefold ß-propeller domain, compromising the active site, followed by a C-terminal domain formed by two ß-sheets. Interestingly, AtCWI1 in contrast to CiFEHIIa displays a glycosylation chain occluding the cleft formed between ß-propeller and the ß-sheet domain. It was assumed that this fact may play an important role in substrate specificity. However analysis of corresponding mutants did not confirm this suggestion (Verhaest et al., 2006). The active side of invertases comprises three amino acids which are conserved among the GH32 family, corresponding to Asp23, Asp149 and Glu203 in AtCWI1. These residues belong to the conserved motifs NDPNG, FRDP and WECPD, playing a particular role in the

hydrolysis of the glycosidic bond (Reddy and Maley, 1996; Sturm, 1999). An exchange of one amino acid within the WEC-P/V-D box was shown to be responsible for the different pH optimum of cell wall and vacuolar invertases. Cell wall invertases, which are slightly more acidic (pH 3.5 – 5.0) carry a proline residue at the fourth position, while vacuolar invertases (pH optimum between pH 5.0 and 5.5) display a valine residue (Goetz and Roitsch, 1999; Roitsch and Gonzalez, 2004). As mentioned before, GH32 family enzymes typically comprise a second domain, located at the C-terminus. For AtCWI1 a disulfide-bridge, located within the second domain was revealed (Verhaest et al., 2006). The particular function of it could not be determined so far (Lammens et al., 2009). Acid invertases are synthesized as prepropeptides, containing an N-terminal signal peptide for the entry into the secretory pathway and a propeptide, the function of which is not yet clarified (Sturm and Chrispeels, 1990; Unger et al., 1994). Presumably, CWIs reach the apoplast without further sequence signal; while for VIs it was assumed that a short C-terminal extension may lead to the vacuolar localization (Unger et al., 1994). But in no other VI described so far such a sequence motif was observed (Matsuoka and Neuhaus, 1999; Vitale and Raikhel, 1999).

2.4 Physiological roles of acid invertases

2.4.1 Roles of invertases during plant development

Plants as photosynthetic organisms contain source (sugar exporting) and sink (sugar importing) tissues. The major carbon transport form in plants is sucrose. Sucrose can be imported into cells in sink tissues directly via plasmodesmata (symplastic transport) or via the cell wall by invertases (apoplastic transport). Intracellular sucrose is either cleaved by neutral, cytosolic invertases or imported and stored in the vacuole.

The cleavage of vacuolar stored sucrose by VIs represents the major intracellular source of hexoses in expanding tissues (Rolland et al., 2006). During this process, osmotically active solutes are transported into the vacuole, in which VI activity leads to an increasing osmotic pressure of cells needed for expansion (Koch, 2004). VIs are assumed to have an important role during sink initiation and the initial expansion growth of several sink tissues (Klann et al., 1996; Sturm and Tang, 1999; Andersen et al., 2002). Antisense suppression of a soluble acid invertase in muskmelon altered plant growth and fruit development (Yu et al., 2008).

Cell wall invertases are responsible for sucrose cleavage in the apoplast. CWI activity is representing the major driving force, determining sink strength via enabling sugar unloading and maintaining sucrose gradients in sink tissues. Apoplastic hexoses can subsequently be taken up

into sink cells by hexose-transporters, which are co-expressed with CWIs (Roitsch and Gonzalez, 2004). Therefore, CWIs feature an essential role in regulating phloem unloading and sink strength, as shown for instance in transgenic carrots (Tang et al., 1999) and in tomato (Zanor et al., 2009). Moreover, CWIs are essential in sink tissues, in which plasmodesmatal connections are missing, such as developing seeds and pollen (Wobus and Weber, 1999; Patrick and Offler, 2001; Weschke et al., 2003; Koch, 2004). Additionally, high maternal CWI activity during early seed development promotes embryo growth by cell division. In the subsequent transition phase the CWI activity declines and the embryo switches from a mainly mitotic growth to differentiation and growth driven by expansion. The decrease of CWI activity is accompanied by an increase in SuSy activity (Rolland et al., 2006). It is observed in general that during development a high invertase activity is associated with active growth processes, while a high SuSy activity is associated with storage processes and differentiation. The same is true in sugar beet. The analysis of sucrolytic enzyme expression and activity during plant development revealed an inverse regulation of invertases and SuSy (Godt and Roitsch, 2006). Only in the early phase high extracellular and vacuolar invertase activity is present in the sugar beet taproots. CWI activity is responsible for the supply of carbohydrates for maintaining sink metabolism in developing taproots, whereas in mature taproots, SuSy represents the key sink enzyme for sucrose uptake and cleavage.

2.4.2 Acid invertase activity in response to wounding and pathogen attack

Sucrose cleaving enzymes are not only important for carbon partitioning during plant development but they are also important in mediating stress responses (Roitsch et al., 2003; Rolland et al., 2006; Essmann et al., 2008). Wounding and pathogen attack represent severe environmental stress factors. In response to pathogen attack, several cellular reactions like generation of reactive oxygen species (ROS), synthesis of pathogenesis-related (PR) proteins, cell wall fortification and hypersensitive reaction (HR) are induced (Garcia et al., 1986). The induction of defense responses is accompanied by a strong increase of sink metabolism (Berger et al., 2007). The defense-related callose deposition and generation of ROS are assumed to represent the strongest sink reaction in plants (Maor and Shirasu, 2005; Essmann et al., 2008). Accordingly, invertases play an important role in stress responses (Roitsch and Gonzalez, 2004). CWI is regarded as PR protein and displays an induction in response to both abiotic stress and pathogen attack (Roitsch et al., 2003; Rolland et al., 2006). The induction of CWI was shown to be one of the early defense related reactions in tobacco (Scharte et al., 2005) and barley (Swarbrick et al., 2006) after fungal infection. CWI was found to be responsible for the

generation of hexoses and reprogramming of the mesophyll cells from source to sink metabolism in response to wounding and pathogen attack, respectively. Presumably, CWI activity is needed to ensure carbohydrate supply required for a successful plant defense (Essmann et al., 2008).

Wounding of sugar beet taproots resulted in induction of both vacuolar and cell wall invertase, accompanied by severe sucrose loss (Rosenkranz et al., 2001; Eufinger, 2006). Recently, it was shown that BvCWI (BIN35) is also induced in wounded source leaves, thus CWI in sugar beet is part of the defense response in adult plants as described before for other species, like tomato (Proels and Roitsch, 2009) and tobacco (Herbers et al., 2000; Essmann et al., 2008).

2. 5 Regulation of invertases

2.5.1 Transcriptional regulation of invertases

Several regulatory mechanisms control plant invertase activities. A key role plays the transcriptional regulation of invertases, observed in response to various environmental changes (Koch, 2004; Roitsch and Gonzalez, 2004). Several cell wall invertases are induced in response to stress, such as starvation (Contento et al., 2004), senescence (Balibrea Lara et al., 2004), wounding (Rosenkranz et al., 2001; Eufinger, 2006) and infection (Essmann et al., 2008). For instance, in wheat pollen invertase expression is inhibited by water stress. Notably, only particular members of a family, encoding the same isoform are influenced depending on the cell type (Koonjul et al., 2005). Also vacuolar invertase expression is regulated by abiotic stresses like drought, hypoxia and cold (Roitsch and Gonzalez, 2004). Moreover, a feed-back/forward regulation of invertases by sugar was described, thus providing a very sensitive self- regulatory system (Rolland et al., 2006). In most of the cases, invertases are sugar-induced, however some invertase genes are sugar repressed (Huang et al., 2007). Commonly, individual members of one family coding for the same isoform (CWI/ VI), are conversely regulated. Already in 1996, it was shown by Xu et al. that in maize invertase Ivr2 is upregulated by increasing carbohydrate supply, while Ivr1 is repressed by sugars and upregulated by carbohydrate depletion (Xu et al., 1996). Invertase expression is furthermore regulated by several phytohormones (Roitsch and Gonzalez, 2004). The most prominent example is the induction of CWI by cytokinin, since it was shown that delayed senescence in transgenic tobacco with upregulated cytokinin production correlates with an increased CWI activity (Balibrea Lara et al., 2004).

The enhanced transcription of invertase seems to be affected by kinases (Huang et al., 2007), so far only an effect on vacuolar invertase could be determined (Kohorn et al., 2006).

In sugar beet, invertase expression is strongly regulated developmentally (Eufinger, 2006; Godt and Roitsch, 2006). A carbohydrate response could not be determined so far (Godt and Roitsch, 2006). The expression of a vacuolar isoform in sugar beet petioles shows circadian oscillation (Gonzalez et al., 2005). As mentioned in the preceding chapter the expression of both cell wall and vacuolar invertase in mature taproots is induced by wounding (Rosenkranz et al., 2001; Eufinger, 2006).

2.5.2 Post-transcriptional regulation of invertases

For two sugar repressed invertases from rice and Arabidopsis, respectively, apparent downstream (DST) elements were identified, which are presumably leading to rapid turnover of plant mRNAs. For AtvacINV2, a vacuolar invertase from *A. thaliana*, a rapid repression by sugars was observed, which is probably due to a glucose-based destabilization of mRNAs (Huang et al., 2007). Moreover, a post-transcriptional regulation via exon-skipping of a CWI gene in potato has been described by Bournaey et al. (Bournay et al., 1996).

2.5.3 Post-translational regulation of invertases

For vacuolar invertase, a regulation on protein level, including compartmentalization and degradation was described in *A. thaliana* (Rojo et al., 2003; Koch, 2004; Huang et al., 2007). According to these authors, newly translated VI enters precursor protease vesicles (PPV). PPVs are plant specific ER-bodies, surrounded by ribosomes. PPVs in turn surround the large, central vacuole, as well as smaller protein storage vacuoles (Chrispeels and Herman, 2000; Hayashi et al., 2001; Rojo et al., 2004). In general, PPVs are known as storage sites for precursor cysteine proteases (VPEγ) which maturate after their release into the acidic vacuole (Schmid et al., 2001). Moreover, a function as transfer- and protective storage side for VI is assumed (Koch, 2004). The contents of PPVs enter the vacuole by a fusion of the PPVs with the tonoplast (Chrispeels and Herman, 2000). Only after entering the acidic vacuole, both vacuolar invertase and the protease are active. Although invertase is a target of VPEγ after entering the acidic vacuole, only a moderate proteolytic degradation occurs (Huang et al., 2007). The PPV and VPEγ system represents a potential mechanism for the fine control of timing and duration of VI activity. So far it is not determined to which extent VIs enter this system and whether the amount is varying under certain conditions (Rojo et al., 2003). Fusion of PPVs with acid vacuoles was observed during senescence (Schmid et al., 2001; Rojo et al., 2003; Rojo et al., 2004), salt stress (Hayashi et al., 2001) and may contribute to pathogen response (Rojo et al., 2004).

Moreover, invertase activity can be regulated via proteinaceous inhibitors, addressed within the following section.

2.6 Proteinaceous inhibitors of plant invertases

The first biochemical characterization of invertase inhibitors was performed in the 1960s. It was observed that invertase preparations from potato tubers displayed lower activities due to a low molecular weight protein, which was bound to the endogenous invertase (Schwimmer et al., 1961; Pressey, 1966). Subsequently, the occurrence of such proteins was also described for the storage tissues of sweet potato, red beet and sugar beet (Pressey, 1968) and in the endosperm of maize kernels (Jaynes and Nelson, 1971). The first sequence data were obtained in the 1990s via the purification and N-terminal sequencing of inhibitors in tomato (Pressey, 1994) and tobacco (Weil et al., 1994). The first cDNA encoding a cell wall invertase inhibitor from tobacco was cloned by Greiner et al.(Greiner et al., 1998). Since then, cDNAs encoding putative inhibitor proteins were isolated from several plant species, such as maize (Bate et al., 2004), Arabidopsis (Link et al., 2004) and tomato (Reca et al., 2008; Jin et al., 2009) as well as from sugar beet (Eufinger, 2006).

2.6.1 Structural features of invertase inhibitor proteins

Invertase inhibitors are low-molecular, non-glycosylated proteins, displaying an enormous stability against heat and acidic treatment (Rausch and Greiner, 2004). According to Hothorn et al., invertase inhibitor proteins are members of the sequence family named PMEI-RPs (pectin methylesterase inhibitor-related proteins) (Hothorn et al., 2004b). It was shown that proteins, related to invertase inhibitors can be ineffective against invertases and instead inhibit a family of cell-wall modifying enzymes, called PMEs (pectin methylesterases). Camardella et al. isolated a PME inhibiting protein from kiwi fruit, showing homologies to a cell wall invertase inhibitor (NtCIF) from tobacco (Camardella et al., 2000). Hence, all genes showing homologies to PMEIs or invertase inhibitors, respectively, are grouped into the PMEI-RP family.

This gene family only displays a sequence homology between 20 and 35%. Due to the low general sequence conservation within this family, it is not possible to predict whether the target enzyme of the inhibitor protein is an invertase or a PME (Hothorn et al., 2004b). Despite the low sequence homology, all members possess four cysteine residues at conserved positions. These residues are forming two disulfide bridges. As shown by crystallization of NtCIF (Hothorn et al., 2004a) and AtPMEI (Hothorn et al., 2004b), a principal similarity exists between these two

proteins. The structure revealed that despite the low conservation of amino acids, both proteins display a very similar overall fold. Both, NtCIF and AtPMEI are consisting of a four-helix bundle and an uncommon N-terminal extension. The N-terminal extension plays an important role with respect to the overall protein structure. Furthermore, the protein is stabilized by its two disulfide-bridges (Hothorn et al., 2004a). Therefore, inhibitor proteins are sensitive to treatment with reducing agents (Pressey, 1967; Ovalle et al., 1995). A difference between both proteins became apparent, while regarding the orientation of the N-terminal extension. In NtCIF, the extension is directed towards the bundle core, while in AtPMEI it contacts a second inhibitor molecule, which in turn leads to the formation of a dimer (Hothorn et al., 2004b). However, the crystallographic structure of a complex between AcPMEI from kiwi (*Actinidia chinensis*) and a PME from tomato revealed that PMEI is contacting the active site of PME via the four helix-bundle, whereas the N-terminal extension seems not to be involved, since it points away from the PME (Di Matteo et al., 2005). Moreover no PMEI dimers were observed.

Nevertheless, no structure is yet available for the complex between invertase inhibitor and its target enzyme invertase. Since invertases and PMEs display completely different structures, no conclusions from the described PME-PMEI complex can be drawn.

2.6.2 Expression and physiological roles of invertase inhibitors

Like their target enzymes, invertase inhibitor proteins are assumed to be localized either to the vacuole or to the cell wall (Greiner et al., 1998; Krausgrill et al., 1998; Greiner et al., 2000). Recently, the extracellular localization of invertase inhibitors from tomato was shown (Reca et al., 2008; Jin et al., 2009).

The invertase inhibitor from sugar beet, BvC/VIF, is expressed in taproots and floral tissues. BvC/VIF is only weakly expressed in young taproots and shows an increasing expression during development, reaching its maximum in mature taproots. BvC/VIF is transcriptionally regulated in response to stress, displaying an increased expression in response to wounding (Eufinger, 2006).

In *A. thaliana,* a differential expression of the two isolated invertase inhibitors AtC/VIF 1 and 2 during plant development was observed. AtC/VIF1 is expressed strongly in the vascular tissues of flowers, roots and senescence leaves, whereas AtC/VIF2 a weaker but broader expression in all analyzed tissues (Link et al., 2004). Similar to the expression pattern of AtC/VIF1, a high expression of the tomato invertase inhibitor INVINH1 was observed in roots, fruits and old leaves. An increasing mRNA was observed as leaves progressed from sink to source and as fruit developed from flowering. Moreover, it was shown that INVINH colocalizes with the cell wall

invertase LIN5 in the phloem parenchyma of the placenta vasculature and fruit pericarp (Jin et al., 2009). This is in accordance with the observations made by S. Bayer (personal communication) for NtCIF. NtCIF expression is found in source and senescent leaves, but additionally displays a high expression in floral tissues, in which NtCIF is co-expressed with a CWI isoform during later stages of floral development.

Silencing of INVINH1 from tomato resulted in a delayed ABA- induced leaf senescence. Via this transgenic approach it was shown that the decrease of CWI activity in old tomato leaves is mainly due to post-translational regulation by its proteinaceous invertase inhibitor (Jin et al., 2009). As already determined for NtCIF, the expression of invertase inhibitors is increased in response to ABA and PEG treatment, which stimulate conditions of senescence and drought stress (Rausch and Greiner, 2004). Balibrea Lara et al. could show that CWI activity determines leaf senescence, which can be delayed by increased CWI activity (Balibrea Lara et al., 2004). By now, it is assumed that the ABA induced leaf senescence is dependent on the expression of the inhibitor gene and independent of cytokinin, as shown in tomato. Moreover, it is postulated that the inhibitor may be required for the induction of senescence-associated genes (Jin et al., 2009). Beside delayed senescence, silencing of INVINH1 in tomato resulted in increased seed weight and fruit hexose levels, which is presumably due to enhanced apoplastic sucrose hydrolysis, phloem unloading and hexose accumulation. Moreover, heterologous overexpression of the inhibitor in *A. thaliana* resulted in infertile seeds. A joint action of the invertase inhibitor and the CWI is suggested to determine sucrose cleavage in cell walls for delivering hexoses to the developing seeds within the placenta phloem parenchyma cells (Jin et al., 2009).

2.6.3 Regulation of invertase inhibitors

The characteristics and the functionality of invertase inhibitors have been largely determined by *in vitro* assays; including analysis of their recombinant proteins (Greiner et al., 1998; Bate et al., 2004; Eufinger, 2006). It was shown that plant invertase inhibitor proteins exclusively inhibit plant invertases and no invertases from fungal sources (Pressey, 1967; Greiner, 1999). It is suggested that cell wall invertase inhibitor proteins inhibit CWI and VI activity, whereas vacuolar localized are only inhibiting VI activity (Huang et al., 2007 and references therein). A much faster complex formation was observed between NtCIF and NtCWI, compared to NtCIF and VI from tomato (Sander et al., 1996).

For NtCIF and NtCWI, a complex formation was shown in tobacco suspension culture. Although the complex was stable over the whole culture period, a different invertase activity was measured (Krausgrill et al., 1998). The inhibition of activity might be dependent on sucrose

concentrations, since substrate protection was described for NtCWI (Weil et al., 1994). However, this represents not a general regulatory mechanism and is presumably an intrinsic quality of particular invertases (Sander et al., 1996; Greiner, 1999).

The regulation of invertases by proteinaceous inhibitors is strongly pH dependent (Rausch and Greiner, 2004). The strongest inhibition occurs at pH 4.5, whereas no inhibition is observed at pH 6 (Weil et al., 1994; Eufinger, 2006). This pH dependency might indicate an *in vivo* modulation of the inhibition by pH.

2.7 Redox regulation in plants

In the present study, the post-harvest sucrose metabolism in wounded sugar beet taproots is reflected. Since wounding leads to the generation of ROS (reactive oxygen species) and sucrose metabolism might be influenced by changing redox states, redox regulation in plants is addressed in this chapter.

2.7.1 Redox signaling

In plants low molecular weight antioxidants, like ascorbat, glutathione and tocopherol are redox-buffers, which interact with several cellular components. On the one side, antioxidants provide information on cellular redox state; on the other side, they influence gene expression associated with stress responses (Foyer and Noctor, 2005). Antioxidants determine the duration and effect of ROS (reactive oxygen species) signals. Large pools of mainly glutathione and ascorbat absorb and buffer reductants and oxidants and thus ensure redox homeostasis. The cytoplasm possesses a low thiol-disulfide redox potential, due to the high concentrations of glutathione. Moreover, high amounts of ascorbat are accumulated within the cytoplasm. Ascorbat buffers are known to protect very efficiently against oxidative challenges (Foyer and Noctor, 2005).

The adjustment of ROS- antioxidant interactions plays a role in many processes during plant development and within responses to environmental changes, such as stress and wounding (Shao et al., 2008 and references therein). For instance, decreasing ascorbat levels or changes of the glutathione pool are inducing defense- related genes, including pathogenesis-related (PR) proteins (Pastori et al., 2003; Barth et al., 2004).

The best characterized redox signal transduction system in plants is the stromal ferredoxin-thioredoxin system, which is involved in the regulation of photosynthetic carbon metabolism. The signal-transmission is mediated by a disulfide-thiol conversion in the corresponding enzymes. Presumably the conversion is enabled by a light induced increase in the thioredoxin redox

potential (Setterdahl et al., 2003). In general, thiol-based regulation is suggested to be very important in plants, since thiol-containing domains are oxidized by ROS, resulting in relatively stable oxidation products with modified physical confirmation or biochemical activities (Foyer and Noctor, 2005).

Several sulfur species might play a role in redox controls, such as disulfides, sulfenic acid, sulfenyl amide groups or glutathionylated cysteines. Sulfur species can be classified according to the connected cysteines. For instance, intra- as well as intermolecular disulfide-bridges exist, whereas glutathionylation represents a mixed disulfide bond between specific cysteines and glutathione. This post-translational modification occurs in response to oxidative stress and is presumably protecting the protein. Furthermore, glutathionylation can modulate enzyme activity. Two enzymes, belonging to the carbon metabolism are known to be regulated by this system, namely aldolase and triose phosphate isomerase (Ito et al., 2003).

2.7.2 Antioxidant status of the apoplast

In the apoplast, flavonoides and polyamine are present as antioxidants, whereas neither NAD(P)H nor glutathione are found. The redox buffering capacity of the apoplast is much weaker than inside the cell (Horemans et al., 2000; Pignocchi and Foyer, 2003). Since the apoplast possesses a very active ascorbat oxidase, a much higher amount of ascorbat than in the cytoplasm is oxidized. Furthermore, it was shown that the pathway for ascorbat degradation is located in the apoplast (Green and Fry, 2005).

As electron-acceptor oxygen and 3,4 dihydroxyphenolic compounds as chlorogenic acid, caffeic acid, quercetin and catechin do act in the apoplast. All named molecules regulate the production of reduced and oxidized forms of ascorbate, this in turn is assumed to control ROS mediated signal transmission and cell expansion. In response to pathogen attack, the hypersensitive response is induced, which is accompanied by an accumulation of ROS in the apoplast (Dangl and Jones, 2001). This pathogen- induced apoplastic burst is suggested to be involved in triggering the default death pathway (PCD) (Mur et al., 2005).

It is assumed that redox-sensitive signal transduction can occur in the apoplast, whereas the threshold for ROS signals is much higher in highly buffered compartments, such as the cytosol (Foyer and Noctor, 2005).

2.7.3 Potential role of the vacuole within redox signalling

Thus far, not much is known about the participation of the vacuole in redox signalling. However, it is suggested that the vacuole plays an unanticipated essential role in the control of ROS metabolism in plants (Mittler et al., 2004; Van den Ende and Valluru, 2009). The vacuole occupies the major part of the cell and accumulates a mixture of strong antioxidant compounds, such as anthocyanins, phenolics and malate (Kytridis and Manetas, 2006).

Moreover, ROS-scavenging capacity of sucrose shown *in vitro* is suggested to occur *in planta* (Van den Ende and Valluru, 2009). At low concentrations, sucrose is assumed to serve as signal for stress-induced responses (Rolland et al., 2006 and references therein), whereas at high concentrations, sucrose can function directly as a protective agent (Uemura and Steponkus, 2003). Especially in plants, storing extremely high concentrations of sucrose, like sugar beet and sugar cane, sucrose might play a particular role as antioxidant (Van den Ende and Valluru, 2009).

2.8 Research objectives

Previous studies revealed the presence of an invertase inhibitor in sugar beet. Recently, the inhibitor was isolated and first molecular analysis showed that the inhibitor BvC/VIF strongly inhibits vacuolar and cell wall invertase activity *in vitro*, in a pH dependent manner.

The aim of the present study was to establish BvC/VIF as a potential candidate for biotechnological approaches in order to reduce post-harvest sucrose-loss in sugar beet, caused by acid invertases.

Notably, in sugar beet taproots, invertase activity is induced in response to wounding despite parallel expression of BvC/VIF. It was hypothesized that particular conditions are required for the invertase-inhibitor interaction, leading presumably to a regulation of invertase activity after prolonged wounding. Thus, the present study aimed at

i) the determination of BvC/VIF and invertase expression, as well as invertase activity during late wound response,

ii) the identification of the subcellular localization of BvC/VIF,

iii) the elucidation of the dynamics of complex formation between BvC/VIF and invertases,

iv) the clarification of BvC/VIF's impact on sucrose metabolism during late wound response.

The present project was performed together with KWS Saat AG (Einbeck, Germany) and Südzucker AG (Mannheim/ Ochsenfurt, Germany). The cooperation provided the opportunity to establish transgenic sugar beet lines either overexpressing BvC/VIF or silencing BvC/VIF. Therefore, it was possible to analyze the impact of BvC/VIF on the regulation of wound-induced invertase activity, closely connected to sucrose loss, in the homologous system. This is of particular interest, since sugar beet accumulates extremely high sucrose contents and represents the most important crop for industrial sugar production.

3. Results

3.1 BvC/VIF and its putative target enzymes are expressed in parallel upon wounding

Recently, an invertase inhibitor was isolated from sugar beet (Eufinger, 2006). BvC/VIF (*Beta vulgaris* cell wall and/ or vacuolar inhibitor of ß-fructosidase) inhibits various invertases in a pH dependent manner, typical for the invertase inhibitor protein family.

3.1.1 Constant high expression levels of BvC/VIF and invertases in wounded taproots

As shown by Eufinger, BvC/VIF is expressed throughout the entire cross-section of the storage taproot (Eufinger, 2006). Transcript analysis revealed that BvC/VIF is weakly expressed in young taproots and that high expression levels are only detected in mature storage taproots. Interestingly, BvC/VIF transcript levels are enhanced upon wounding. Accordingly, during this time the inhibitor is expressed in parallel with its putative target enzymes, namely cell wall invertase and vacuolar invertase.

Thus far, the expression of BvC/VIF after wounding had been analyzed only until five days after wounding (Eufinger, 2006). In the present study, an extended time of wounding has been analyzed, including inhibitor and invertase expression. It was shown that the enhanced protein levels of both BvC/VIF and invertases stayed high during the investigated period of time (Figure 1A). In young taproots, enhanced BvC/VIF transcript levels correlated with protein levels. Only nine days after wounding, transcript levels as well as protein levels slightly decreased. In old taproots, displaying already high BvC/VIF expression levels in unwounded tissue, the elevated transcript levels in response to wounding did not correlate with protein levels, showing no further increase.

In mature storage taproots, no invertase transcript is detectable throughout the entire cross section except for the cortex (Eufinger, 2006). The transcript level of one isoform of both invertases (CWI1 and VI1) was elevated upon wounding (Rosenkranz et al., 2001). CWI1 transcript in wounded taproots is already detectable 10h after wounding, whereas VI1 transcript is only detectable 24h after wounding. Like shown in Figure 1B, the detected transcript levels of the vacuolar invertase (VI1) remained stable after induction and were slightly lower nine days after wounding. Whereas the wound-induced transcript levels of CWI were already lower on day five after wounding.

On protein level, typical cleavage products of the invertase proteins are detectable by immunoblot, as also shown in previous studies (Rosenkranz et al., 2001; Eufinger, 2006). The cell wall invertase protein was already detected one day after wounding, whereas the typical cleavage products of the vacuolar invertase (Eufinger, 2006) were only detected three days after wounding. During the monitored period, the decreased invertase transcript levels after prolonged wounding did not result in reduced protein amount, presumably due to the high stability of the glycosylated invertases (Pagny et al., 2003).
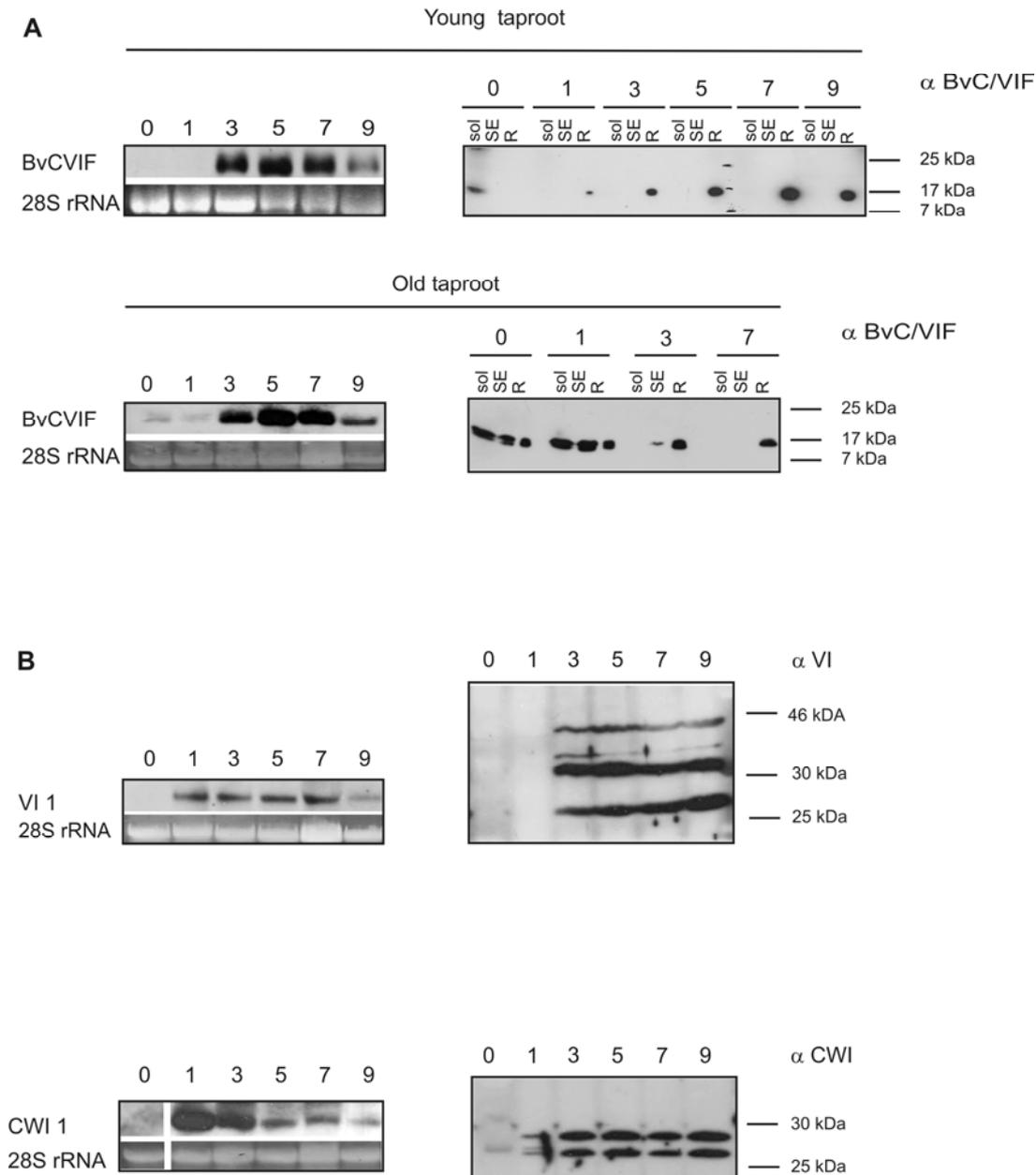
Figure 1: Wound-induced expression of BvC/VIF and its putative target enzymes VI (vacuolar invertase) and CWI (cell wall invertase) in sugar beet taproots.
A: Expression of BvC/VIF after wounding in young taproots (approximately eight weeks old, harvested in June) and old taproots (approximately twenty weeks old, harvested in September).
B: Expression of vacuolar (VI) and cell wall invertase (CWI) in old taproots, after wounding.
Left hand side: Transcript analysis; 10 µg of total RNA isolated from taproots were loaded and hybridized with probes against the coding regions of BvC/VIF, VI1 and CWI1 respectively. Ethidium bromide stained 28S rRNA band is shown as loading control.
Right hand side: Western blot analysis, 50mg fresh weight equivalents were loaded. BvC/VIF protein was detected via immunoblot with an antiserum directed against recombinant BvC/VIF protein. VI and CWI protein expression was detected with antisera raised against the N-terminal part of each protein.
0-9: days after wounding, sol: soluble proteins (10,000 g), SE: cell wall associated proteins, eluted by high salt, R: residual 10,000 g cell wall pellet after salt elution

3.1.2 During the late wound response, invertase activity decreases despite stable protein amount

Wounding of taproot tissues leads to an induction of acid invertases as shown in Figure 1B and also described by Rosenkranz et al. (Rosenkranz et al., 2001). In response to wounding, the cell wall invertase activity was induced, reaching its maximum three days after wounding (Figure 2). In previous studies, invertase activity was analyzed only until five days after wounding (Eufinger, 2006). In the present study, we were especially interested in invertase activity and regulation after prolonged wounding. Interestingly, the invertase activity decreased after prolonged wounding. Nine days after wounding, only a remaining cell wall invertase activity of approximately 60% was measured, although the protein level stayed high (Figure 1B). Besides this, it was observed that wound-induced cell wall invertase activity in young taproots was slightly lower than in old taproots. However, the invertase activity course upon wounding was comparable in both taproot stages (Figure 2). A down-regulation of vacuolar invertase activity was detectable five days after wounding. In young taproots, vacuolar invertase activity increased until three days after wounding, stayed on the same level until five days after wounding and was subsequently strongly down-regulated. In old taproots, wound-induced vacuolar invertase activity increased until five days after wounding. Subsequently, also a down-regulation of the vacuolar invertase activity was detected despite stable protein amount.

The induction of invertase activity correlated with the breakdown of sucrose. In young taproots, accumulating only approximately 5% of the sucrose concentration observed in old taproots, nearly all sucrose was degraded nine days after wounding.

In old taproots, storing more than 400 µmol sucrose/ g FW, approximately 50% of sucrose were degraded during nine days after wounding. Correlating with decreasing invertase activity after prolonged wounding, the sucrose breakdown between day seven and day nine was not as high as in the initial wounding phase.
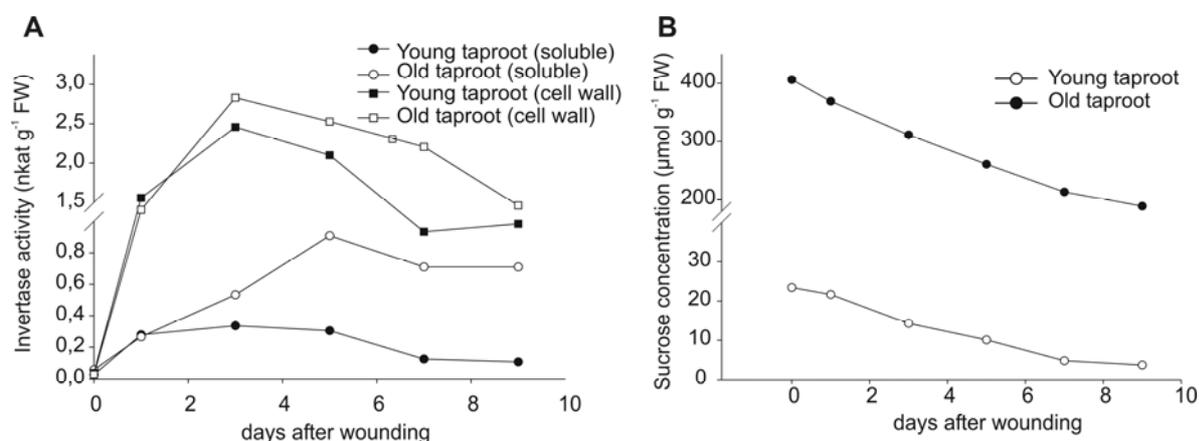
Figure 2: Wound-induced invertase activity and sucrose breakdown upon wounding in *Beta vulgaris* taproots.
A: Cell wall and soluble invertase activity in response to wounding (0-9 days after wounding)
B: Sucrose breakdown after wounding
Young taproot: approximately 8 weeks old, harvested in June; old taproot: approximately 20 weeks old, harvested in September
The mean value of two taproots, harvested at the same time is shown

3.1.3 In wounded sugar beet taproots, a cell-wall associated localization of BvC/VIF is detected

With the purpose to get first hints for the subcellular localization of the invertase inhibitor protein in wounded sugar beet taproots, a sequential protein extraction as described by Eufinger was performed (Eufinger, 2006). Taproot extracts were centrifuged at 10,000 g to separate soluble from insoluble proteins. Subsequently, proteins, ionically bound to the cell wall, were eluted from the 10,000 g cell wall pellet by incubation in high salt buffer (0.5 M NaCl). The residual cell wall pellet (after salt elution) was boiled in SDS buffer. Using this method, Eufinger detected in unwounded sugar beet taproots two protein signals of BvC/VIF in the soluble protein fraction, reflecting intra-cellular proteins. The lower molecular weight species was additionally detected in the cell wall fraction, thus assumed to be extra-cellular localized. In the present study, BvC/VIF was exclusively detected in the soluble fraction in young, unwounded taproots (Figure 1A). In old taproots, BvC/VIF was observed in all fractions, namely soluble, from the cell wall salt elutable and residual cell wall pellet. The analyzed taproot stage by Eufinger most likely represents an intermediate stage between the analyzed taproot stages in the present study.

After wounding, BvC/VIF was exclusively detected in the residual cell wall pellet. Notably, an intracellular localization was not detected anymore, but BvC/VIF could not be eluted from the cell wall.

3.2 BvC/VIF is localized in the apoplast

3.2.1 Non-invasive salt-elution of BvC/VIF

In order to exclude the possibility that putative intra-cellular BvC/VIF might bind to the cell wall invertase or to the cell wall during extraction and thus is detectable in the cell wall fraction after wounding, a non-invasive method was chosen to confirm the extra-cellular localization of BvC/VIF. Like described previously for the sequential protein extraction from disrupted cells, proteins ionically bound to the cell wall, are eluted by incubation in high-salt buffer (0.5 M NaCl). The same approach is suitable for the elution of extra-cellular proteins from intact cells. Via such a non-invasive salt-elution, the extracellular localization of cell wall invertase in tobacco suspension cultures was determined (Weil & Rausch, 1990).

First experiments were performed with *B. vulgaris* hairy roots. As BvC/VIF was detected in the fraction eluted from intact cells via incubation in a high-salt buffer, the extracellular localization of BvC/VIF in *B. vulgaris* hairy roots was confirmed by this approach (Figure 3A). In order to compare the results obtained by the non-invasive approach with the results obtained from disrupted cells, aliquots from hairy roots were taken before and after incubation in high salt buffer. Analyzing the sample without prior non-invasive salt-elution revealed that in hairy roots exclusively an extracellular localization was observed with the standard protein extraction. To some extent the inhibitor was salt elutable from the cell wall after disruption, but BvC/VIF was also detected in the residual cell wall pellet, probably bound to cell wall invertase, which is highly expressed in this tissue. In the sample taken after incubation in high-salt buffer, a BvC/VIF signal was only detected in the residual cell wall pellet, representing the part of proteins, which are not salt-elutable. Hence, with respect to the subcellular localization of BvC/VIF, the non-invasive approach confirmed the observations, previously shown by the commonly used sequential protein extraction from disrupted cells.

The same approach was used to verify the extracellular localization of BvC/VIF in sugar beet taproots. In Figure 3B the immunological analysis of two unwounded taproots, displaying a different subcellular localization of the two detected BvC/VIF protein species, is presented. In taproots 1 the same pattern as described by Eufinger (Eufinger, 2006) was observed (two species in the soluble fraction of unwounded tissue, the smaller species additionally detectable in the cell wall fraction) after sequential protein extraction from disrupted cells. Via the described non-invasive approach, the extra-cellular localization of the smaller protein species was confirmed.

Taproot 2 displayed another pattern. In taproot 2 the smaller as well as the larger species were detected in the cell wall fraction, when following the described protocol for sequential protein

extraction from disrupted cells. The extra-cellular localization of both species was also shown by salt elution from intact cells. Again, the non-invasive approach confirmed the observations made by sequential protein extraction from disrupted cells.

The extra-cellular localization of BvC/VIF was confirmed, whereas the origin of the additional putative intra-cellular signal is not known. Thus far, it can not be distinguished between BvC/VIF observed *in transit* and an alternative intra-cellular localization of BvC/VIF.

To a minor extent BvC/VIF could be recovered by salt elution also from wounded taproots (data not shown). However, as it was already observed previously, while using the sequential protein extraction from disrupted cells, nearly no inhibitor protein was salt-elutable from the cell wall after wounding (Figure 1).

Glucose-6-phosphat dehydrogenase activity was determined from the different fractions to exclude a cytosolic contamination. Nearly no activity could be determined in the protein solution extracted by high salt (table 1).
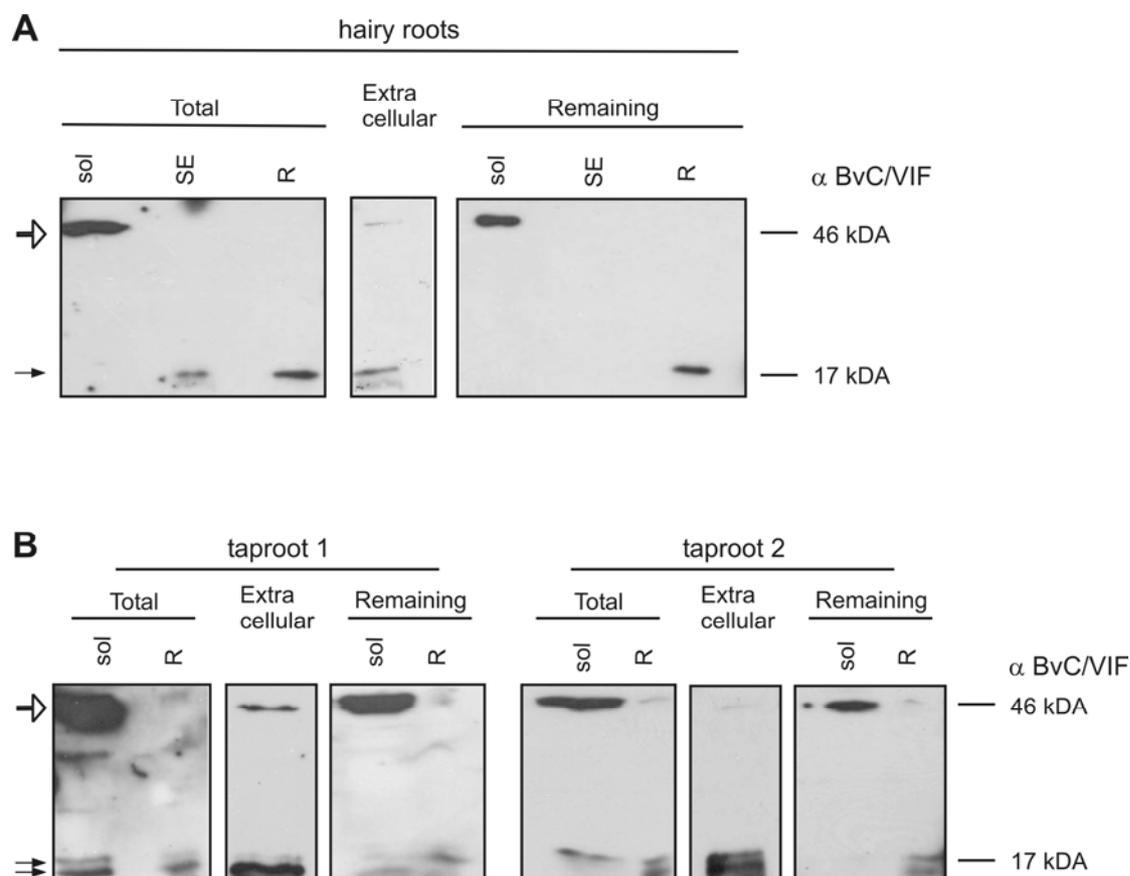
Figure 3: Non-invasive salt-elution of cell wall associated BvC/VIF in comparison to disruptive protein extraction of BvC/VIF from *Beta vulgaris* hairy roots and taproots
A: Immunological detection of BvC/VIF in *Beta vulgaris* hairy roots
B: Immunological detection of BvC/VIF in two different *Beta vulgaris* taproots
Beta vulgaris hairy roots and washed taproot slices have been incubated in high salt buffer. Before and after this treatment an aliquot of each sample has been analyzed after sequential protein extraction from disrupted cells as described before. The immunoblot was detected with an antiserum directed against recombinant BvC/VIF. With this antiserum the specific inhibitor bands are detected at about 17 kDa (denoted by a closed arrow), furthermore an unspecific protein band is detected only in the soluble fraction at about 45 kDa (denoted by an open arrow).
Total: analysis of disrupted cells (frozen and grinded) without prior salt elution from intact cells
Extra cellular: isolated cell wall proteins from non-disrupted cells via incubation of hairy roots/ taproot slices in high salt buffer.
Remaining: analysis of disrupted cells (frozen and grinded) after extracellular proteins were eluted from intact cells
Disruptive protein extraction from hairy roots: Sol: soluble proteins (after 10,000 g centrifugation step, from disrupted cells), SE: salt eluted proteins from 10,000 g pellet. R: residual 10,000 g pellet after salt elution; disruptive protein extraction from tap roots: Sol: soluble proteins (after 10,000 g centrifugation step, from disrupted cells), CW 10,000 g cell wall pellet

Table1: Glucose-6-phosphat dehydrogenase activity

B. vulgaris taproots	Protein (mg/ g FW)	Glc6PDH (nkatal/ g FW)	Glc6PDH (pkatal/ mg protein)
Extract before salt elution	1,6	5,24	3,18
Salt elution fraction	0,03	0,003	0,20
Extract after salt elution	0,9	3,06	3,31

3.2.2 Extra-cellular localization of BvC/VIF, shown by immuno-localization

An additional approach to determine the subcellular localization of BvC/VIF is the immunolocalization with an antiserum directed against recombinant BvC/VIF.

First attempts to obtain a labeling of BvC/VIF protein expression in wildtype taproots were not successful (data not shown). Therefore, transgenic adventitious roots, overexpressing the invertase inhibitor BvC/VIF under the control of the duplicated 35S (p70S) promoter, were analyzed. A strong fluorescence was observed in the BvC/VIF overexpressing lines. The pattern of the fluorescence resembled typical cell wall labeling. Thus, the immunolocalization study confirmed the extra-cellular localization of BvC/VIF.

An additional vacuolar localization of BvC/VIF could not be shown, but due to known difficulties concerning labeling of soluble proteins in the vacuole it can not be excluded.

As a negative control a BvC/VIF RNAi line, showing a strong down-regulation of BvC/VIF was used. In samples of the BvC/VIF-RNAi line, only a very weak fluorescence was detectable.
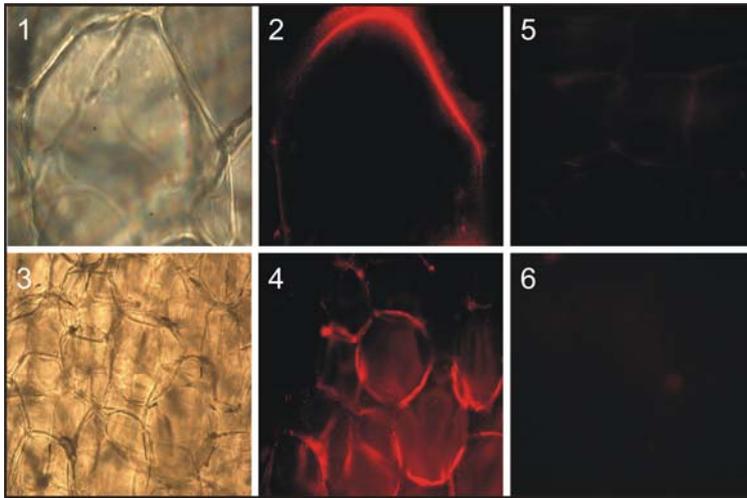
Figure 4: Immunolocalization of BvC/VIF in parenchyma cells of adventitious roots
A transgenic line overexpressing BvC/VIF under the control of the 35S promoter and an RNAi line which shows a strong reduction of BvC/VIF have been analyzed. Very thin adventitious root slices (0.2 mm) were fixed and subsequently incubated with affinity purified antiserum, directed against BvC/VIF. The used second antibody was biotinylated, thus a binding of the streptavidin labeled fluorescence dye Cy3 could be detected.
1+3: DIC images of 2+4
2+4: Fluorescence detection of Cy3 in BvC/VIF overexpressing lines
5+6: No signal in BvC/VIF- RNAi lines

3.3 Two immuno-signals for BvC/VIF as a result of post-translational modification?

3.3.1 Overexpression of the BvC/VIF sequence leads to the expression of both protein species in sugar beet adventitious roots

When overexpressing BvC/VIF cDNA in *Beta vulgaris,* two strong immunological signals were detected by immunoblot analysis with an antiserum directed against recombinant BvC/VIF (Figure 5). The immunological signals corresponded in size to the observed signals in untransformed taproots. Both signals were strongly reduced in BvC/VIF- RNAi lines. This observation supports the idea that both signals arise from one gene.

As also shown in untransformed taproots, a different subcellular localization of BvC/VIF upon wounding can be observed in wounded transgenic lines, when performing a sequential protein extraction from disrupted cells.
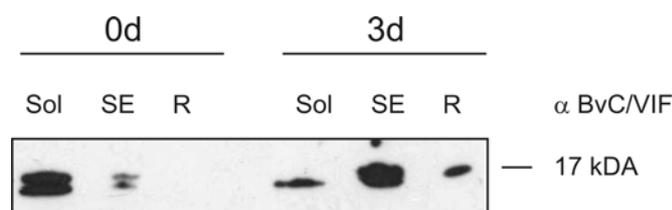
Figure 5: Overexpression of BvC/VIF in *Beta vulgaris* leads to two strong immuno-signals, as observed in untransformed taproots.
Western blot analysis of BvC/VIF expression in transgenic *Beta vulgaris* adventitious roots overexpressing BvC/VIF under the control of the duplicated 35S promoter after sequential protein extraction. The immunoblot was detected with an antiserum directed against recombinant BvC/VIF.
0d: unwounded adventitious roots, 3d: adventitious roots, wounded for three days
Sol: soluble proteins (10,000 g), SE: cell wall associated proteins, eluted by high salt, R: residual 10,000 g cell wall pellet after salt elution

3.3.2 No evidence for a proteolytical cleavage, occurring at the C-terminus of BvC/VIF

During BvC/VIF localization studies via fusion to GFP (green fluorescent protein) (Eufinger, 2006), it was shown that the ectopic expression of the fusion construct resulted in a proteolytic processing event, separating the GFP-tag from the inhibitor. It was assumed that the cleavage occurs either at the C-Terminus of BvC/VIF, or at the N-terminus of the GFP part of the fusion protein, since the cleaved BvC/VIF signal is in size comparable to the native inhibitor observed in sugar beets.

In the present study, it was investigated whether a processing at the C-terminus of BvC/VIF is responsible for the observed double signal in sugar beet. Thus, C-terminal deletion constructs of BvC/VIF, lacking four, eight and twenty amino acids respectively were cloned and ectopically expressed in *N. tabacum* leaves, in order to find out whether the proteolytical cleavage of GFP can be prevented. The expression and localization of the BvC/VIF deletion constructs fused to GFP were compared to BvC/VIF expression under the control of the 35S promoter without GFP-tag and with GFP-tag. When overexpressing BvC/VIF alone without GFP-tag, immunoblot analysis of sequentially extracted proteins with an antibody directed against BvC/VIF showed an immunosignal at 17 kDA in the soluble and in the salt elution fraction from the cell wall pellet. Interestingly, only one BvC/VIF species was detected and not both protein species as observed in sugar beet. Overexpression of BvC/VIF fused to GFP resulted in the via immunoblot detectable cleavage of the fusion protein, as described by Eufinger (Eufinger, 2006). Cleaved BvC/VIF (17 kDa) was detected in the same fractions as shown for the expression of the inhibitor alone (soluble and salt elutable from the cell wall). Additionally, the fusion protein was detected in both cell wall fractions (salt elutable and not elutable by high salt treatment). When

overexpressing the deletion constructs, the immunological analysis showed an altered distribution of BvC/VIF immunosignals. When overexpressing the BvC/VIF C-terminal deletion construct lacking four amino acids, cleaved BvC/VIF was not detected anymore. Only the fusion protein BvC/VIF-GFP was detectable in both cell wall fractions. Notably, the missing immunosignal corresponding to cleaved BvC/VI, was not reflected in a stronger signal correlating to the molecular size of the fusion protein.

Immunoblot analysis of the same extracts with an antiserum directed against GFP revealed that GFP was still cleaved from the C-terminal deletion constructs (Figure 6B). As shown with BvC/VIF- antiserum, also with antiserum directed against GFP, the localization of the fusion protein in the cell wall fractions was detected. But furthermore, free GFP protein (27 kDa) was observed in the soluble fraction of both extracts (entire BvC/VIF-GFP and C-terminal deletion construct fused to GFP). Therefore, it seems unlikely that a cleavage of the fusion protein was prevented by deleting putative cleavage sites at the C-terminus. The fact, that no free inhibitor could be detected anymore when deleting amino acids at the C-terminus is rather due to degradation of the truncated protein.

Since all three deletion constructs showed the same pattern, only the deletion construct missing four amino acids is shown here.
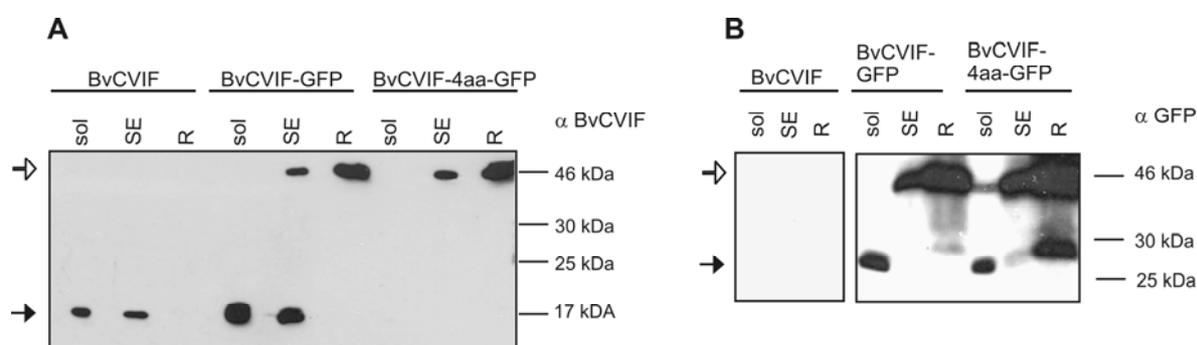
Figure 6: Western Blot analysis of the ectopic expression of BvC/VIF, BvC/VIF-GFP and a C-terminal deletion construct of BvC/VIF fused to GFP in *Nicotiana tabacum* leaves under the control of the duplicated 35S (p70S) promoter.
A: BvC/VIF antiserum, B: GFP- antiserum
25 mg fresh weight equivalents have been loaded. The BvC/VIF fusion protein is marked with an opened arrow, cleaved BvC/VIF with a closed arrow.
BvC/VIF-4aa-GFP: BvC/VIF, lacking four amino acids at the C-terminal part, fused to GFP
Sol: soluble proteins (10,000 g), SE: cell wall associated proteins (10,000 g pellet), eluted by salt, R: residual 10,000 g cell wall pellet after salt elution.

3.3.3 In the heterologous system, only the smaller species of BvC/VIF is detected

In order to determine whether a proteolytical cleavage event occurs *in planta*, being responsible for the detection of two immunological signals for BvC/VIF, it was analyzed whether recombinant BvC/VIF protein, purified from *E. coli*, represents the larger protein species detected in sugar beet taproots. For this purpose, recombinant BvC/VIF protein and extracts from sugar beet taproots were mixed in different proportions and analyzed subsequently by immunoblot analysis. This spiking experiment revealed that the recombinant protein correlates in size to the smaller protein form detected in sugar beet taproots (Figure 7). Since bacteria do not possess appropriate proteases, this experiment revealed that a post-translational modification of BvC/VIF is more likely to occur than a processing event like cleavage of the C- or N-terminal part.

Interestingly, both protein species of BvC/VIF were not detected in all plants. The immunological analysis of ectopic expression of BvC/VIF in tobacco revealed only one immuno-signal in contrast to the double signal observed in sugar beet taproots (Figure 7). Also in *A. thaliana*, the ectopic expression of BvC/VIF resulted in the detection of only one protein signal (data not shown). A spiking experiment, like described previously, revealed that in tobacco only the smaller protein is detected.
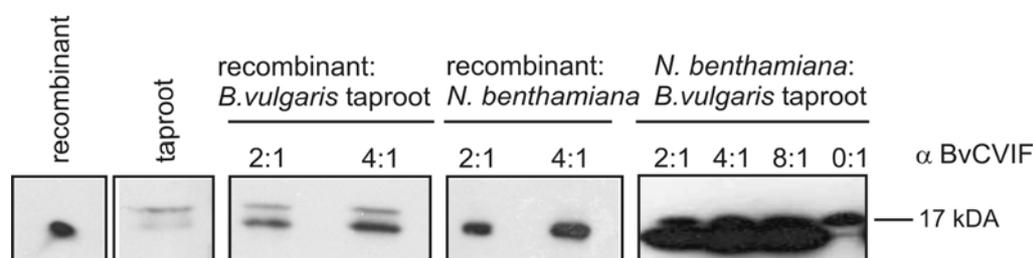
Figure 7: Analysis of the immunologically detected double band of BvC/VIF
Spiking of BvC/VIF protein from *B. vulgaris* taproots with recombinant BvC/VIF and extracts from *N. benthamiana*, overexpressing BvC/VIF under the control of the duplicated 35S (p70S) promoter. Immunological analysis via immunoblot with an antiserum against BvC/VIF.
Recombinant: Recombinant BvC/VIF protein purified from *E.coli*, taproot: BvC/VIF expression in *Beta vulgaris* taproots, *N. benthamiana*: ectopic expression of BvC/VIF in *N. benthamiana* leaves

3.4 Complex formation of inhibitor and invertase is not sufficient for inhibition

3.4.1 Increasing invertase activity although the inhibitor is bound to the invertase

Wounding of sugar beet taproots led to an induction of both vacuolar and cell wall invertase (see 2.1). After induction, the expression level of both enzymes remained stable but the activity decreased three to five days after wounding. During wounding, the inhibitor is expressed in parallel with its putative target enzymes cell wall and vacuolar invertases. In young taproots, only weakly expressing BvC/VIF, wounding resulted in a higher expression level. In older taproots, a high expression level of BvC/VIF was already detected in unwounded tissue. However, the parallel expression of inhibitor and invertase did not lead to an instant inhibition of invertase activity. The delayed inhibition could be correlated to the expression level of BvC/VIF, being enhanced after wounding. But nevertheless, in old taproots, displaying a very high BvC/VIF expression level in unwounded taproots, the wound-induced invertase activity course is the same as observed in young taproots. Therefore, it was analyzed whether complex formation of invertase and inhibitor occurs only after prolonged wounding.

For this purpose, protein extracts from wounded sugar beet taproots, including soluble and cell wall salt-eluted proteins, have been purified, using Concanavalin A chromatography. Only with high mannose chains glycosylated proteins such as invertases bind to the ConA matrix. The invertase inhibitor BvC/VIF is not glycosylated with high mannose chains itself; therefore BvC/VIF can only be detected in the ConA bound fraction in complex with the invertase. Immunoblot analysis after ConA purification clearly revealed an invertase-inhibitor complex formation before down-regulation of invertase activity occurred (Figure 8A). Vacuolar invertase and BvC/VIF were both found in the ConA bound fraction already two days upon wounding.

As described before (2.1), cell wall invertase as well as much of BVC/VIF protein remained in the cell wall pellet of wounded tissues (Figure 8B). Since only soluble and cell wall elutable proteins can be applied to the ConA matrix, the cell wall invertase and bound BvC/VIF do not appear in the ConA fractions. Considering that after prolonged wounding more BvC/VIF was detected in the residual cell wall pellet, it is conceivable that less BvC/VIF was detected in the ConA bound fraction after prolonged wounding.

A

B
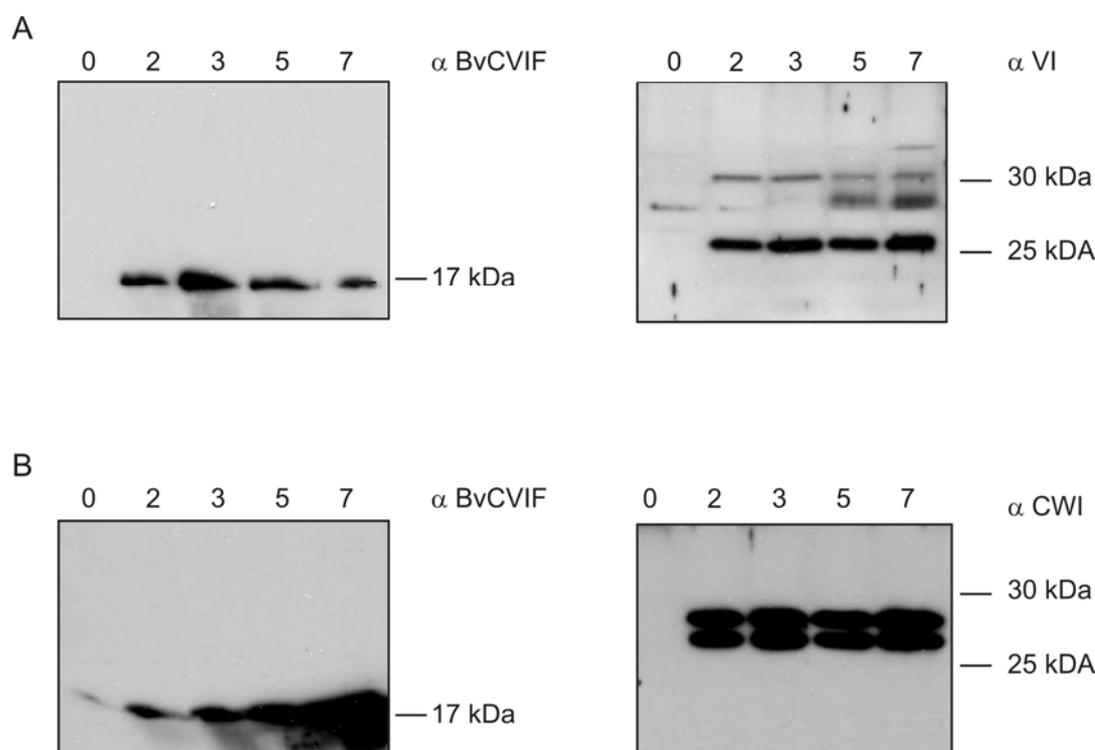
Figure 8: Complex formation of BvC/VIF and invertase already two days after wounding.
Immunoblot analysis of ConA purification of wounded taproot extracts. Proteins have been extracted with ConA buffer pH 5, including 500 mM NaCl. The inhibitor BvC/VIF can only be detected in the ConA bound fraction when bound to invertase, which is glycosylated with high mannose chains, in contrast to the inhibitor.
A: ConA bound fraction, B: Residual cell wall pellet, not elutable by high salt.
A+B: Left hand side detected with an antiserum directed against BvC/VIF, right hand side against vacuolar (VI)/ cell wall invertase (CWI).
0-7: days upon wounding; 50 mg fresh weight equivalents have been loaded

3.4.2 Complex dissociation during extraction leads to a different invertase activity course after prolonged wounding

As described previously (Weil et al., 1994), the inhibition of invertases through inhibitor proteins is strongly pH dependent. Eufinger (Eufinger, 2006) could show via Size Exclusion Chromatography that recombinant vacuolar invertase (BvVI1) and recombinant BvC/VIF do not form a complex at basic pH (pH 7.5). Based on this finding, it was elucidated whether different pHs during extraction show an effect on the regulation of invertase activity. Therefore, wounded taproots were extracted at pH 5 (complex formation) and at pH 8 (complexes get disrupted). Subsequently, immunoblot analysis revealed that when extraction was performed at pH 8 most of the inhibitor was detected in the soluble fraction and not in the cell wall fraction anymore, whereas the cell wall invertase remained in the cell wall associated fraction, no matter at which pH the extraction was performed (Figure 7). Surprisingly, more cell wall invertase protein was

detected in the samples extracted at pH 8. Nevertheless, at both pH values the cell wall invertase protein amount was seven days after wounding as high as three days after wounding but the invertase activity course was different, depending on pH value during extraction (Figure 7B). The higher CWI protein amount detectable in samples extracted at pH 8 was reflected in a higher invertase activity determined in these samples compared to extraction at pH 5. However, the invertase activity course was completely identical up to day three after wounding, but altered after prolonged wounding. When extraction took place at pH 5 the down-regulation of invertase activity after prolonged wounding was observed as shown before (2.1). In contrast to this, in samples extracted at pH 8, in which only a minor part of BvC/VIF was detected in the cell wall fraction, no down-regulation of invertase activity occurred.
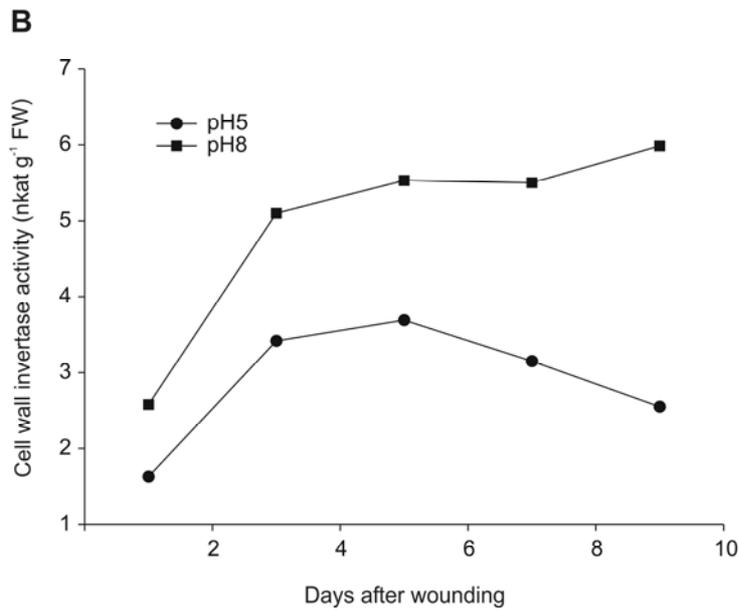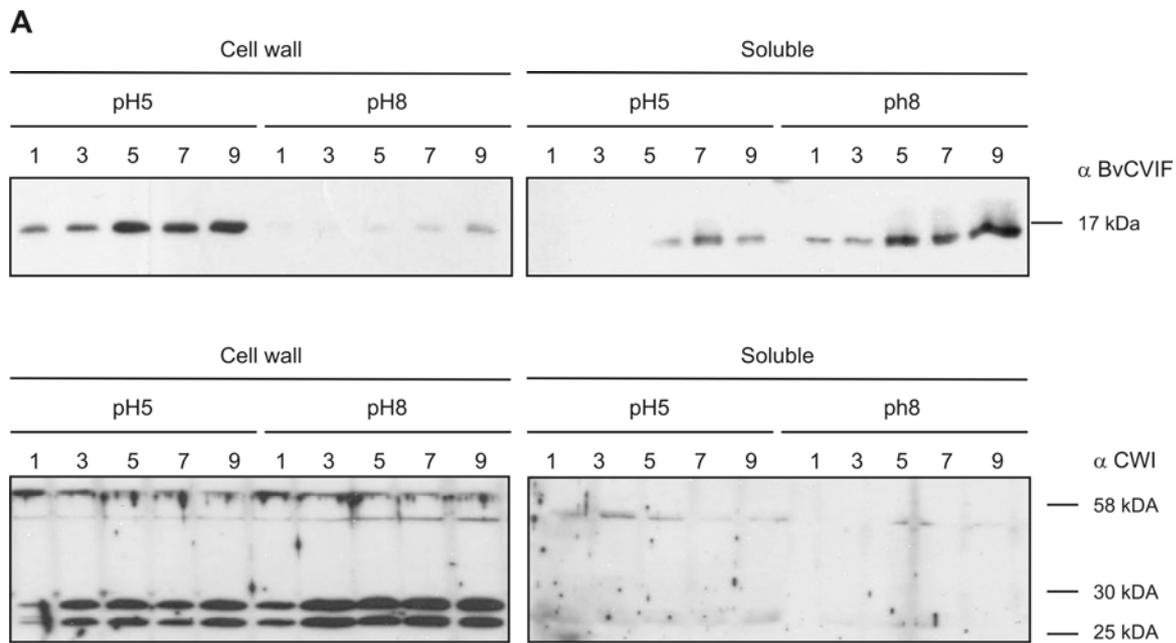
Figure 7: Influence of different pH values during extraction on invertase activity in wounded taproots. BvC/VIF was found in the soluble fraction after protein extraction at pH 8, whereas the cell wall invertase remained in the cell wall. Subsequently the invertase activity in the cell wall extracts was tested at pH 5 (optimal for invertase activity and inhibition).
A: Immunological analysis of cell wall invertase and BvC/VIF after different extraction in the soluble fraction and the cell wall pellet (after centrifugation at 10,000 g)
1-9: days after wounding
B: Cell wall invertase activity upon wounding depending on pH value of the extraction buffer (pH 5/ pH 8). Invertase activity assay was performed at pH 5. The experiment was repeated four times and one representative is shown (the same as depicted in the immunoblot).

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3.5 Modification of the C-terminus of BvVI1 has an effect on inhibition, but does not alter pH dependency

As described previously for the tobacco suspension culture system (Krausgrill et al., 1998), also in wounded sugar beet taproots the complex formation of invertases and inhibitor seems not sufficient for the inhibition of invertase activity (see 2.4.1). Interestingly, the crystallographic structure of a cell wall invertase from *Arabidopsis thaliana* (Verhaest et al., 2006) revealed a disulfide bridge in the C-terminal domain. The involved cysteines are conserved within the acid invertase family. With the aim to investigate a possible redox dependent regulation of invertase activity inhibition through inhibitor proteins, the conserved cysteines Cys399 and Cys448 of *Beta vulgaris* vacuolar invertase 1 (BvVI1) were mutated into Serine (diploma thesis Lindner, H., 2006). The mutation of the cysteines involved in the disulfide bridge did not influence the invertase activity or the stability of the recombinant protein purified from *E.coli*, whereas as the Cys399Ser mutant, which was further tested in inhibition assays, was inhibited more effective by recombinant BvC/VIF. Thus far, it could not be verified whether this increased inhibition of the Cys399Ser mutant is due to structural changes or if redoxregulation is involved in the regulation of invertase activity by inhibitor proteins.

3.5.1 Disulfide bridge mutant of BvVI1 still displays pH dependency

With the objective to verify whether the disulfide bridge is involved in the regulation of invertase activity through inhibitor proteins, the pH dependency of the disulfide bridge deficient mutant compared to the wild type invertase was tested. It was shown that the activities of both proteins, wildtype vacuolar invertase and Cys399Ser mutant, are pH dependent in the same manner (Figure 8B). As observed previously, the Cys399Ser mutant is inhibited more effectively through the inhibitor compared to the wild type invertase (diploma thesis Lindner, H., 2006 and Figure 8). However, the inhibition of the wildtype as well as the one of the disulfide bridge deficient mutant is strongly pH dependent. The disulfide bridge has no influence on pH dependency neither of invertase activity itself nor of inhibition through inhibitor proteins.

The optimal pH range of inhibition seemed to be slightly narrower for the BvVI1 mutant Cys399Ser compared to wildtype BvVI1.
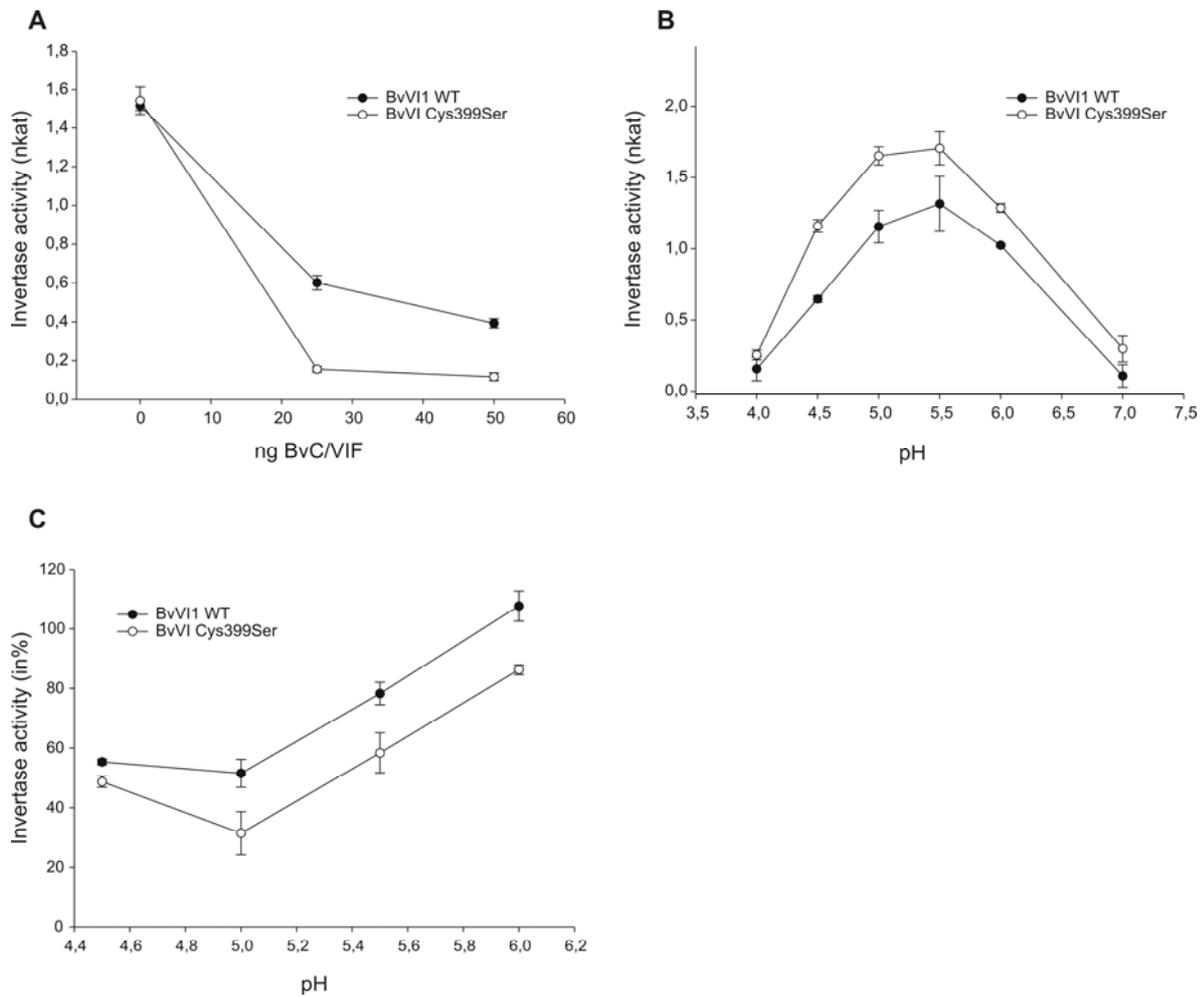
Figure 8: Activity of disulfide-bridge deficient BvVI1 (*Beta vulgaris* vacuolar invertase 1) mutant and inhibition through BvC/VIF at different pH values.
A: Inhibition of vacuolar invertase (BvVI) wild type and Cys399Ser mutant through BvC/VIF
B: Invertase activity against different pH values (100ng BvVI1)
C: Inhibition through BvC/VIF against different pH values (40ng BvVI1 and 20ng BvC/VIF)
The error bars represent standard deviation of three experiments (except for A: only one representative experiment out of five is shown, error bars denote standard deviation of three technical replicates).

3.6 Overexpression of BvC/VIF in sugar beet leads to reduced invertase activity upon wounding

As described in Eufinger (Eufinger, 2006), transgenic sugar beet plants were generated in cooperation with KWS, with the aim to decrease post-harvest sucrose loss on one hand and to verify the involvement of the inhibitor during wounding on the other hand. *Beta vulgaris* (genotype 3TC4174) was transformed with the following constructs:

- **p70S-BVC/VIF:** BvC/VIF cDNA under the control of the constitutive CaMV 35S promoter, the sequence of which was duplicated (= p70S promoter)
- **p2-1-48-BvC/VIF:** BvC/VIF cDNA under the control of the primarily taproot specific 2-1-48 promoter
- **p70S-RNAi-BvC/VIF:** approximately 260 bp of the BvC/VIF coding region first in antisense then in sense direction, linked by intron 2 of the *A. thaliana* gene AtAAP6 (at5g49630), under the control of the duplicated CaMV 35S promoter (= p70S)
- **Transgenic control:** luciferase under the of the constitutive CaMV 35S promoter, the sequence of which was duplicated (= p70S promoter)

The transformation of *Beta vulgaris* and regeneration of adventitious roots were carried out by PLANTA Angewandte Pflanzengenetik und Biotechnologie GmbH (KWS Saat AG, Einbeck).

3.6.1 Identification of transgenic lines, showing a strongly increased and reduced expression of the invertase inhibitor BvC/VIF, respectively.

In order to determine altered expression levels of BvC/VIF in the transgenic lines, the expression levels of BvC/VIF in sugar beet adventitious roots were analyzed by northern and immunoblots (Figure 9). Under the control of the duplicated 35S promoter high expression levels of BvC/VIF were detected in several lines, whereas the expression of BvC/VIF in untransformed adventitious roots and in transgenic controls was hardly detectable. The low expression levels are probably correlated to the developmental stage of the young adventitious roots, as previously shown for wildtype taproots. In the lines expressing BvC/VIF under the control of the 2-1-48 promoter, only expression levels similar to the controls were detected. Since BvC/VIF expression levels in the analyzed adventitious roots were too low, it was not possible to identify

BvC/VIF-RNAi lines showing strongly decreased BvC/VIF expression levels. Based on the findings that BvC/VIF expression levels are enhanced upon wounding (see 2.1.1), the expression levels of BvC/VIF in wounded adventitious roots were analyzed. As observed in taproots, BvC/VIF expression levels are elevated upon wounding. Also in transgenic lines, expressing BvC/VIF under the control of the duplicated 35S promoter distinct higher expression levels of the invertase inhibitor were detected upon wounding (Figure 9B). Moreover, it was possible to identify RNAi lines showing strongly decreased BvC/VIF expression levels in adventitious roots, wounded for three days (Figure 9C).

The altered transcript levels were reflected in protein amounts as shown for several transgenic lines by immunoblots (Figure 9D).
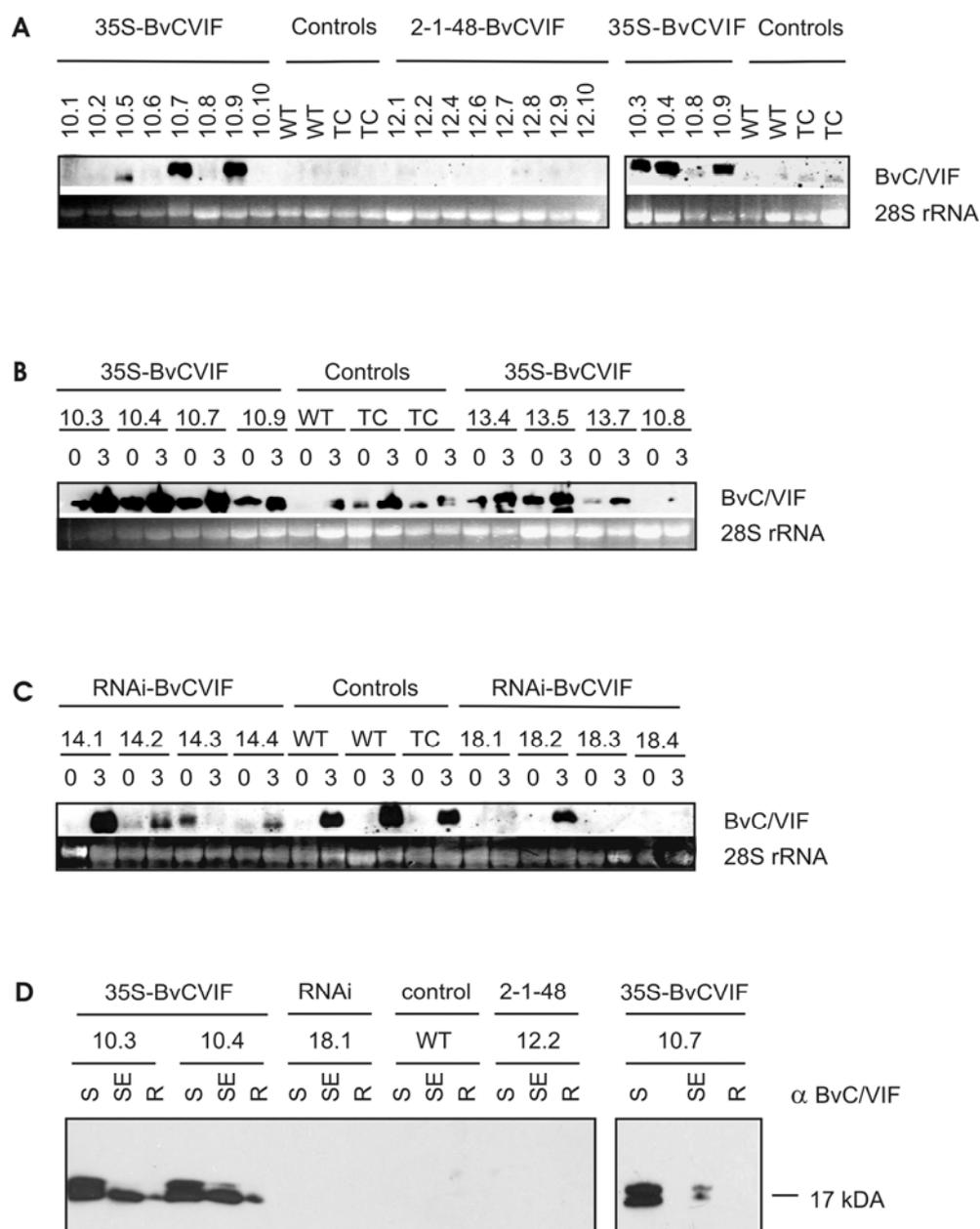
Figure 9: Screening of sugar beet transformants (adventitious roots)
A: Transcript analysis of BvC/VIF expression levels in transformed adventitious roots via Northern Blot with a probe against the coding region of BvC/VIF
B+C: Transcript analysis of BvC/VIF in unwounded (0d) and three days (3d) wounded transgenic adventitious roots with a probe against the coding region of BvC/VIF (B: controls in comparison to BvC/VIF- overexpressing lines, C: controls in comparison to BvC/VIF- RNAi lines).
10 µg total RNA were loaded. Ethidium bromide stained 28S rRNA is shown as loading control.
D: Immunoblot analysis of BvC/VIF expression levels in adventitious roots of selected lines.
25 mg fresh weight equivalents were loaded. The antiserum was raised against recombinant BvC/VIF protein.
Sol: soluble proteins (10,000 g), SE: cell wall associated proteins (10,000 g pellet), eluted by salt, R: residual 10,000 g pellet after salt elution. WT: wild type, TC: transgenic control, 35S-BvC/VIF: lines overexpressing BvC/VIF under the control of the duplicated 35S (p70S) -promoter, 2-1-48-BvC/VIF: lines expressing BvC/VIF under the control of the taproot specific promoter 2-1-48), RNAi-BvC/VIF: lines, transformed with an RNAi construct of BvC/VIF. Each number (X.X) represents one independent line.

3.6.2 Single integration lines display strong overexpression of BvC/VIF

Via Southern Blot it was determined how many copies of the transgene each line is carrying. Therefore genomic DNA was isolated, digested with restriction enzymes and hybridized with a probe against the 35S promoter (Figure 10). As no clearly elevated BvC/VIF expression levels were obtained under the control of the 2-1-48 promoter, these lines were not further analyzed. Interestingly, nearly all lines showing high expression levels of BvC/VIF under the control of the duplicated 35S promoter are single integration lines. Among the RNAi lines, showing a knock-down of BvC/VIF expression, only multi copy lines were identified.

For further characterization of the transformants, two single integration lines, displaying the highest BvC/VIF expression levels and two multi-copy BvC/VIF- RNAi lines, showing clearly decreased BvC/VIF expression levels, were chosen, namely:

- **p70S- BvC/VIF:** lines 10.3 and 10.7
- **RNAi- BvC/VIF:** lines 18.1, 18.3

Additionally, a third putative BvC/VIF RNAi line, showing no clear knock-down of BvC/VIF, was chosen for further characterization.
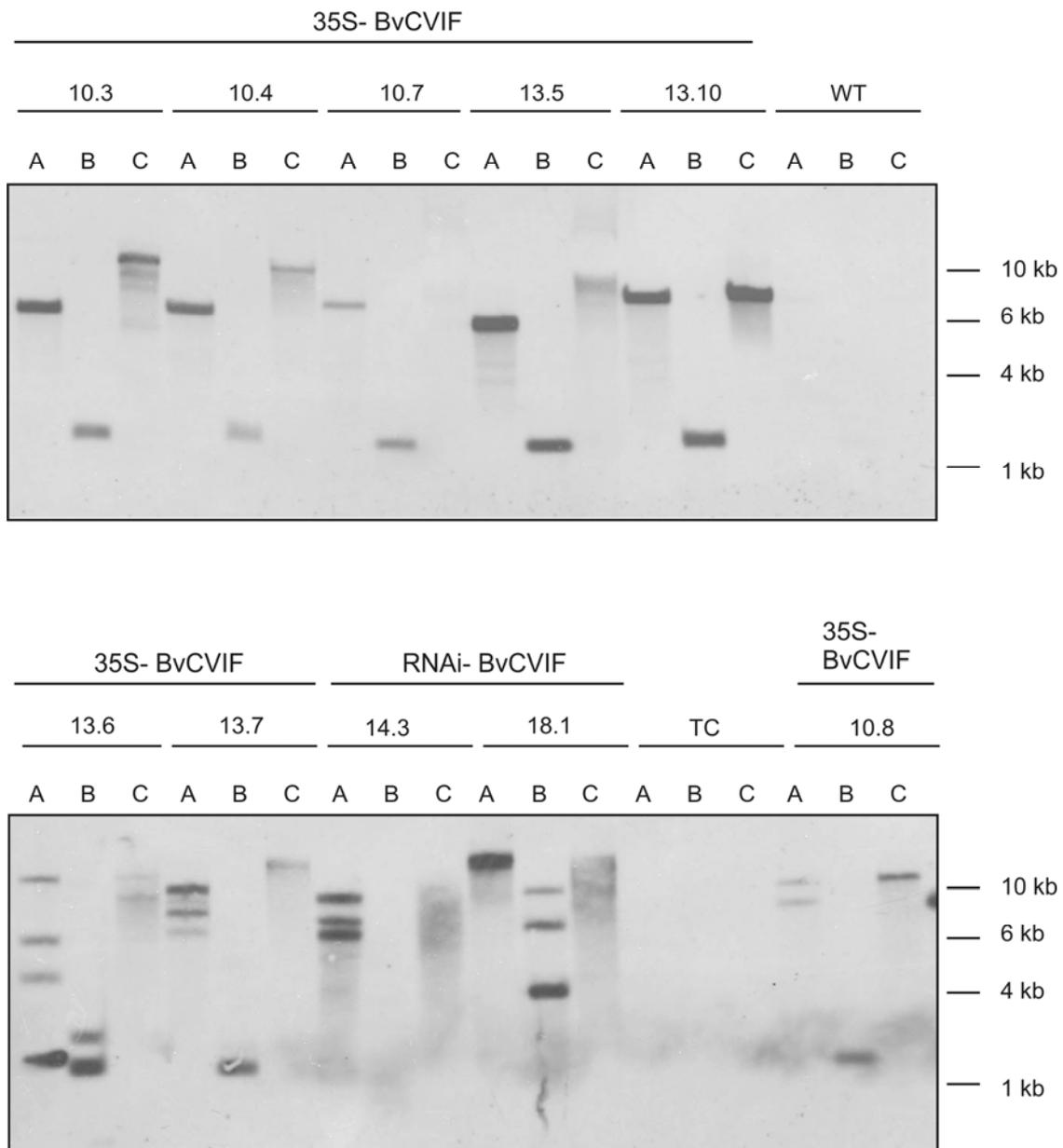
Figure 10: Southern Blot analysis of *Beta vulgaris* transformants
In each lane 20 µg genomic DNA, digested with the indicated restriction enzymes, were loaded and hybridized with a DIG-labeled probe against the 35S-promoter.
A: EcoRI, B: HindIII, C: XbaI
WT: wild type, TC: transgenic control, each number (X.X) represents one independent transgenic line (PLANTA GmbH, Einbeck)

3.6.3 Analysis of BvC/VIF expression in transgenic lines after wounding

Two single integration lines, expressing BvC/VIF under the control of the duplicated 35S promoter, showing a high expression of BvC/VIF (lines 10.3 and 10.7) and two RNAi lines, showing an efficient knock-down of BvC/VIF (lines 18.1 and 18.3), were chosen for further characterization. Additionally, a third putative RNAi line (14.3) was also characterized, but a down-regulation of BvC/VIF could not be confirmed (Figure 11).

Transgenic adventitious roots were characterized until nine days after wounding. As described before (2.6.1), only a weak BvC/VIF expression was detected in control adventitious roots (wild type and transgenic control), but after wounding the expression of BvC/VIF was increased. As shown for young sugar beet taproots (2.1.1), the increased expression from day to day over a long wounding period was also detected in the analyzed adventitious roots. As mentioned before, the putative RNAi line 14.3 displayed a BvC/VIF expression, comparable to the controls, whereas an efficient down-regulation of BvC/VIF occurred the whole wounding period in the BvC/VIF- RNAi lines 18.1 and 18.3. A stronger knock-down of BvC/VIF expression was determined in the RNAi line 18.3 compared to line 18.1. Regarding BvC/VIF overexpressing lines, a stronger BvC/VIF expression was detected in line 10.3 compared to line 10.7. However, both transgenic lines displayed much higher expression levels than the controls. Interestingly, although BvC/VIF is expressed under the control of the constitutive CaMV duplicated 35S promoter, the expression levels of BvC/VIF were enhanced after wounding. Elevated expression levels of an invertase inhibitor in response to wounding, despite expression under the control of the 35S- promoter, were already observed previously (Eufinger, 2006).

As observed in sugar beet taproots, BvC/VIF protein was detected in the cell wall fraction after wounding. In contrast to this, in the BvC/VIF overexpressing lines 10.3 and 10.7 a high BvC/VIF protein amount was additionally detected in the soluble fraction of wounded tissues (Figure 11B). Thus far, it cannot be distinguished whether the intracellular localization of BvC/VIF is due to mistargeting of the protein or only observed after extraction.
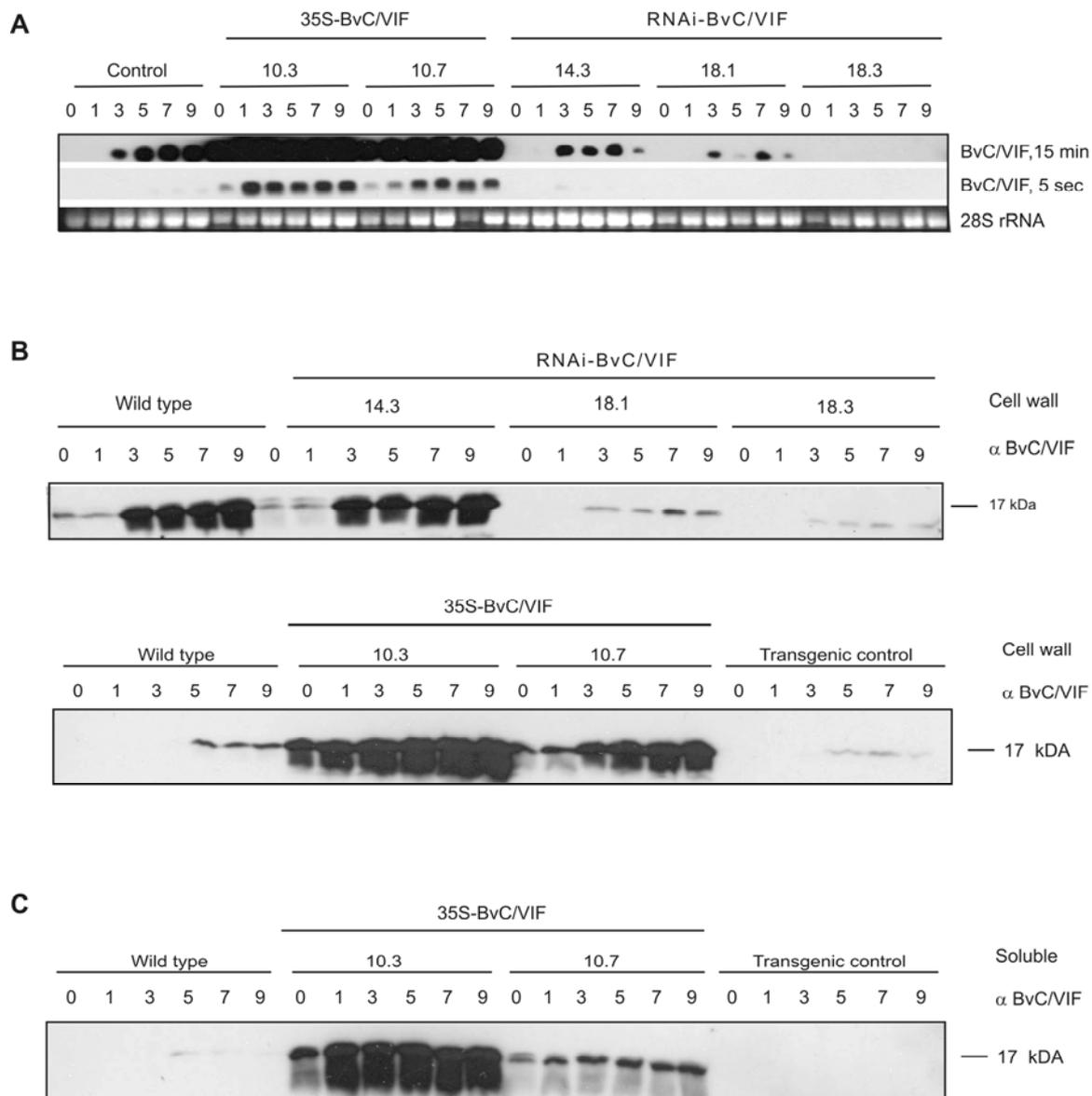
Figure 11: Expression of BvC/VIF in wounded adventitious roots.
A: Northern blot analysis of BvC/VIF transcript levels. 10 µg of total RNA isolated from adventitious roots were loaded and hybridized with a probe against the coding regions of BvC/VIF. Ethidium bromide stained 28S rRNA band is shown as loading control. Two different exposure times are shown (BvC/VIF 10 min: film exposure for ten minutes, BvC/VIF, 5sec: film exposure for five seconds)
B+C Western blot analysis of extracts with an antiserum directed against recombinant BvC/VIF, 50 mg fresh weight equivalents were loaded.
B: Cell wall fraction (Please note: upper film panel after prolonged exposure time than the lower one),
C: soluble fraction, 0-9: Zero to nine days wounding
35S-BvC/VIF: transgenic lines, expressing BvC/VIF under the duplicated 35S promoter
RNAi-BvC/VIF: BvC/VIF RNAi lines

3.6.4 The expression of cell wall and vacuolar invertase is influenced neither by overexpression nor by knock-down of BvC/VIF

It was verified, whether the expression of cell wall and vacuolar invertase is influenced in the transgenic lines, depending on the expression of BvC/VIF. Western blot analysis revealed that there was no difference in invertase protein expression between BvC/VIF overexpressing lines and BvC/VIF-RNAi lines (Figure 12, shown for cell wall protein expression). Interestingly, the ratio of the detected cleavage products of cell wall invertase was different in the controls as compared to transgenic lines. In the controls, the ratio between the larger cleavage product and the smaller one was equal, whereas in the transgenic lines the larger cleavage product was more prominent (Figure 12A). Concerning vacuolar invertase expression, again there was no obvious difference between the expression in the BvC/VIF overexpressing lines and the BvC/VIF-RNAi lines; however, the detected immunological signals for vacuolar invertase were stronger in the controls (data not shown).
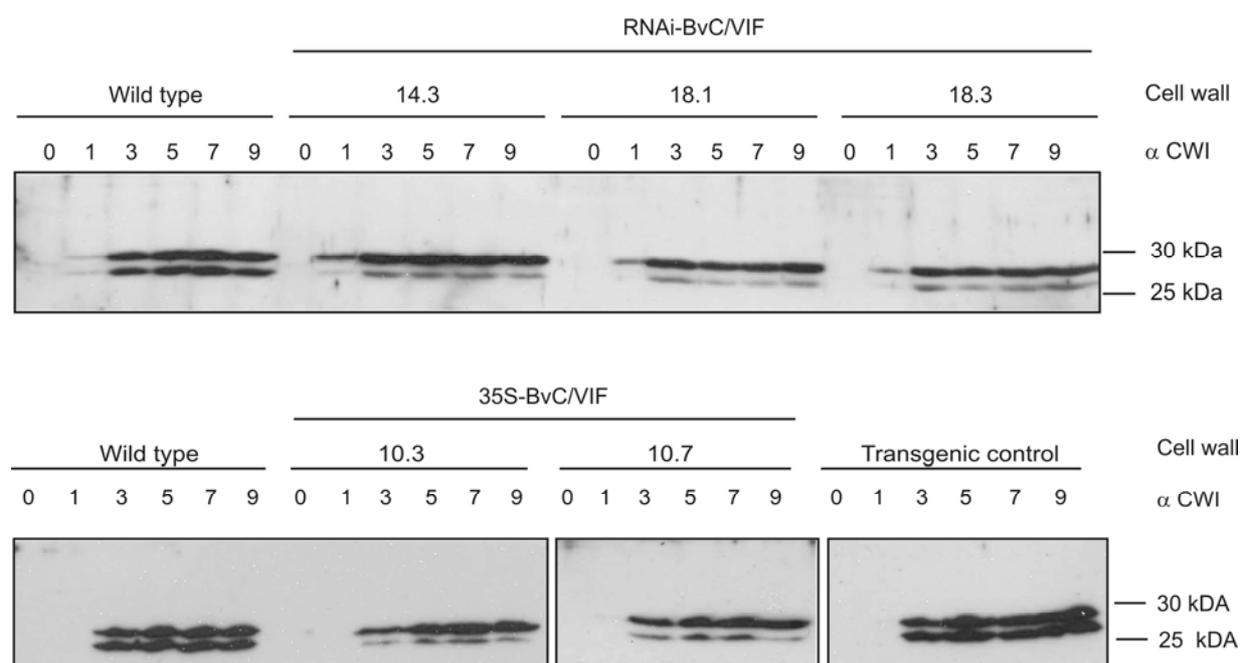
Figure 12: Invertase expression is not influenced by BvC/VIF expression. Immunoblot analysis of adventitious root extracts with (A) an antiserum against the N-terminal part of cell wall invertase. 50 mg fresh weight equivalents of the cell wall fraction were loaded. 0-9: zero to nine days wounding, 35S-BvC/VIF: BvC/VIF under the control of the duplicated 35S promoter (line 10.3), RNAi-BvC/VIF: RNAi lines of BvC/VIF (line 14.3 showing no knock-down of BvC/VIF expression, line 18.3: efficient down-regulation of BvC/VIF)

3.6.5 Wound-induced cell wall and vacuolar invertase activities are reduced in BvC/VIF overexpressing lines

Samples from wounded adventitious roots, used for immunological analysis, were also taken for quantification of invertase activity. Invertase activity was measured from cell wall extracts (= cell wall invertase activity) and from the soluble fraction (= vacuolar invertase activity). Overexpression of BvC/VIF resulted in a much lower induction of cell wall and vacuolar invertase activity compared to the controls and RNAi lines (Figure 13). Nine days after wounding, cell wall and vacuolar invertase displayed only an activity of approximately 20 to 30% compared to the controls and to the RNAi lines, whereas invertase activity was slightly stronger down-regulated in the BvC/VIF overexpression line 10.3, showing a higher BvC/VIF expression level compared to line 10.7 (Figure 11).

An RNAi effect on cell wall invertase activity was determinable neither on cell wall invertase nor on vacuolar invertase. The invertase activity course in all analyzed adventitious roots was different than observed in sugar beet taproots (see 2.1.1). In contrast to taproots, no down-regulation of invertase activity after prolonged wounding was observed in adventitious roots during the investigated wounding period of nine days.
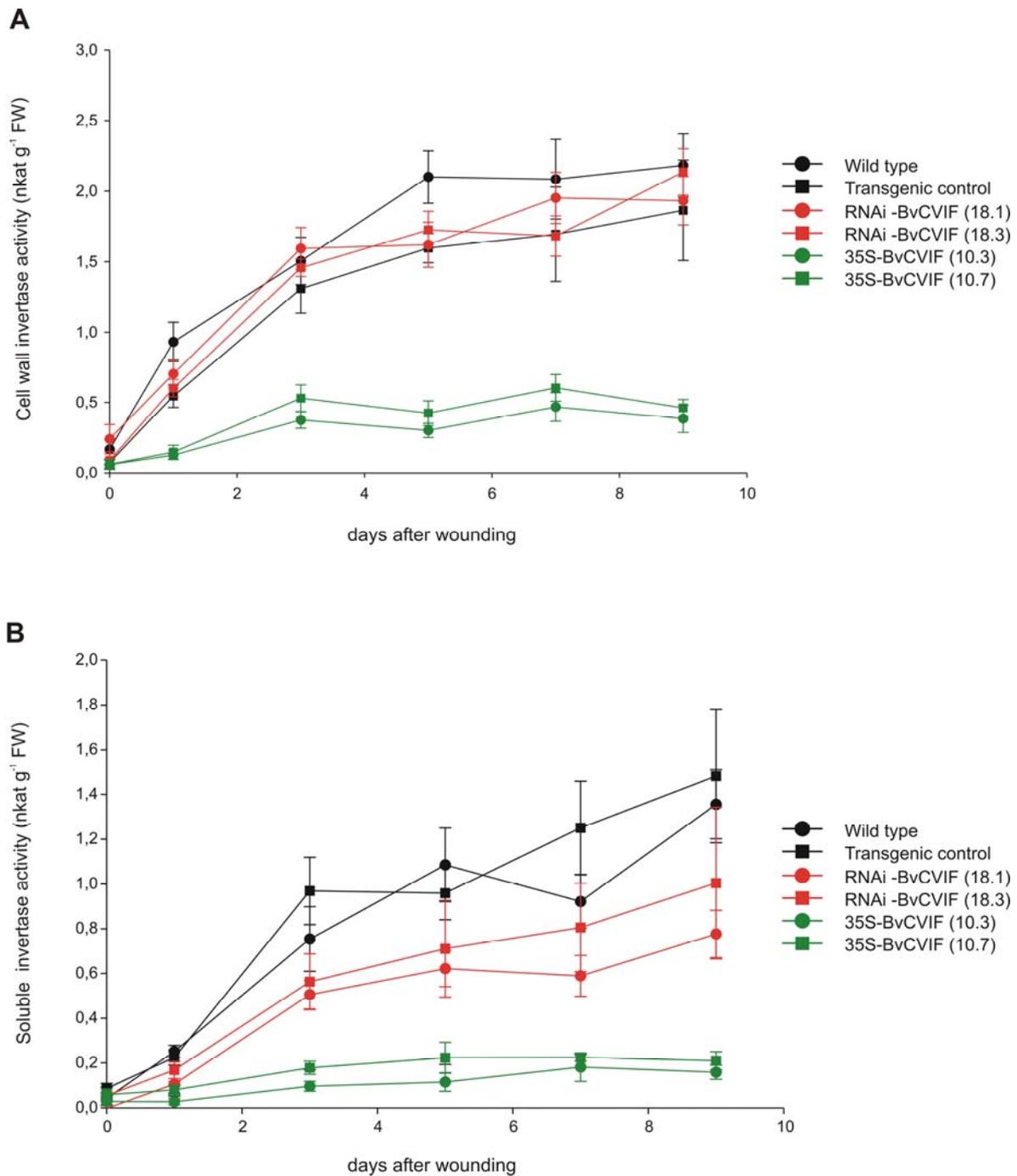
Figure 13: Invertase activity in response to wounding in sugar beet adventitious roots. Invertase activity was measured from extracts divided into (A) cell wall fraction and (B) soluble fraction. Error bars indicate standard error of six to seven adventitious roots of each line.
A: Cell wall invertase, B: Vacuolar invertase

3.6.6 In individual BvC/VIF- RNAi plants, the degree of silenced BvC/VIF expression is correlated to a higher wound-induced invertase activity

It was not possible to determine any RNAi effect on invertase activity, with respect to mean values of different plants of each line. However, the analysis of individual plants of the BvC/VIF RNAi line 18.3, representing the RNAi line with the strongest knock-down of BvC/VIF expression, revealed a strong correlation between BvC/VIF knock-down and invertase activity (Figure 14). In adventitious roots 18.3.1 and 18.3.2, showing a stronger knock-down of BvC/VIF expression than the other adventitious roots, the cell wall invertase activity was much higher after wounding. In 18.3.2 (even less BvC/VIF protein detectable than in 18.3.1) the highest cell wall invertase activity was observed. In this plant, even the vacuolar invertase activity was about six times higher than in the other plants. The higher invertase activity is reflected in sucrose breakdown. In adventitious root 18.3.2, the sucrose loss was highest and in 18.3.1 still higher than in 18.3.3 and 18.3.4. However, these findings are only based on individual plants, giving a hint for the physiological role of BvC/VIF during wounding, which has to be confirmed.
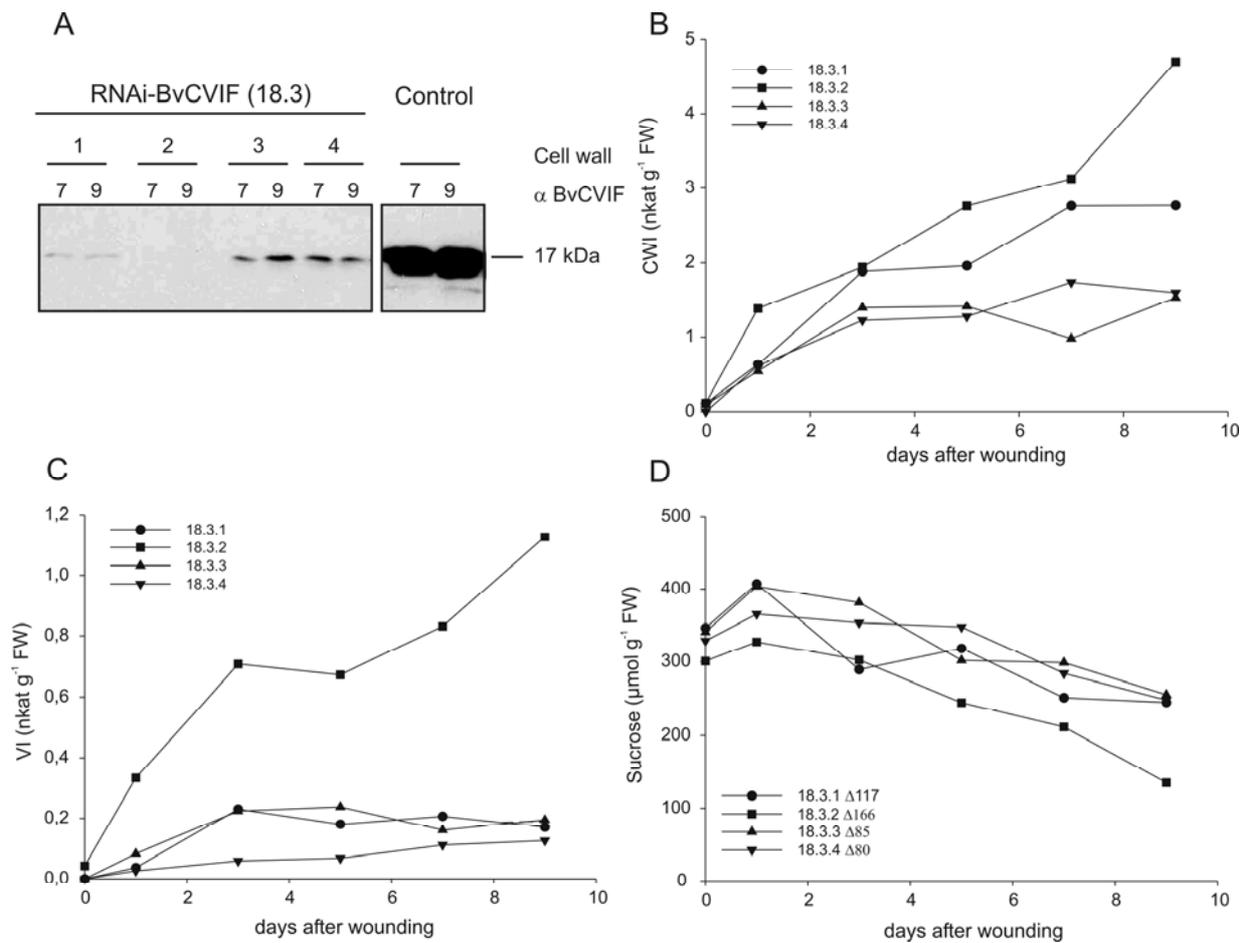
Figure 14: Different expression levels of BvC/VIF are correlated to invertase activity in BvC/VIF-RNAi line 18.3
A: Western blot analysis of BvC/VIF expression levels in adventitious roots of BvC/VIF-RNAi line 18.3, wounded for seven (7) and nine (9) days. 1-4: adventitious roots one to four, from line 18.3
B: Wound-induced cell wall invertase activity in individual adventitious roots
C: Wound-induced vacuolar invertase activity in individual adventitious roots
D: Sucrose breakdown upon wounding, Δx: sucrose loss after nine days wounding (in µmol g^{-1} FW)

3.6.7 Down-regulation of wound-induced invertase activity in BvC/VIF overexpressing lines does not prevent sucrose breakdown upon wounding

Adventitious roots accumulate comparable amounts of sucrose as seed grown taproots. No effect on sucrose accumulation was determined neither in BvC/VIF overexpressing lines nor in BvC/VIF RNAi lines (Figure 15A).

In transgenic lines, strongly overexpressing BvC/VIF, wound-induced invertase activities of only about 20 to 30% compared to the controls and RNAi lines were determined (Figure 13). In these lines (10.3 and 10.7) both cell wall and vacuolar invertase showed a significantly lower activity than the controls (wild type and transgenic control) and the RNAi lines. Unexpectedly, this pronounced difference in invertase activity did not result in an altered sucrose breakdown after wounding (Figure 15). The sucrose was cleaved to the same extent in BvC/VIF overexpressing lines as in the BvC/VIF RNAi lines, regardless of the reduced invertase activity in the BvC/VIF overexpressing lines. Surprisingly, in wild type adventitious roots more sucrose than in all (Figure 15B) transgenic lines was degraded, however the sucrose loss was not significantly higher (p≥ 0, 03).

Figure 15: Sucrose breakdown in adventitious roots upon wounding. Sucrose was determined via an indirect enzymatic assay. The error bars denote the standard error of six adventitious roots from each line.
A: Sucrose concentration, please note: higher sucrose concentration determined at day one after wounding compared to unwounded tissue due to water loss as observed in Rosenkranz et al., (2001).
B: Degraded sucrose (shown only from day one on, since before no sucrose breakdown can be measured).

3.6.8 In BvC/VIF overexpressing lines, less hexoses are accumulated

Even though different wound-induced invertase activities were detected in adventitious roots, this was not reflected in sucrose loss after wounding. Notably, much less hexoses were accumulated in BvC/VIF overexpressing lines than in wild type adventitious roots and BvC/VIF RNAi lines (table 2 and 3). An RNAi effect was again not observable. The slightly higher sucrose loss in wild type adventitious roots compared to all transgenic lines was also reflected in a higher accumulation of hexoses, not only as compared to overexpressing lines but also to RNAi lines. Nevertheless, a distinct difference in hexose accumulation was observed between BvC/VIF overexpressing lines and RNAi lines although the same amount of sucrose was cleaved. This became very apparent, while analyzing hexose to sucrose ratios (table 3).

Table 2: Hexose accumulation in adventitious roots nine days after wounding. Mean values of six to seven adventitious roots from each line are given.

	glucose	fructose
WT	121± 6	34± 4
10.3 (p35S)	53± 2	0
10.7 (p35S)	53± 8	7± 2
18.1 (RNAi)	103± 4	30± 5
18.3 (RNAi)	80± 5	21±2

Table 3: Hexose to sucrose ratio in adventitious roots nine days after wounding.

	Ratio			
	glucose/ sucrose	fructose/ sucrose	hexoses/ sucrose	fructose/ glucose
WT	0.60	0.17	0.77	0.28
10.3 (p35S)	0.36	0.00	0.36	0.00
10.7 (p35S)	0.36	0.05	0.41	0.13
18.3 (RNAi)	0.53	0.14	0.67	0.27
18.1 (RNAi)	0.64	0.19	0.83	0.29

4. Discussion

4.1 Invertase activity and sucrose loss after prolonged wounding

Harvesting of sugar beets goes along with decapitation and wounding of taproots. During the unavoidable, subsequent storage period of taproots in piles on the field, sucrose is constantly lost. This sucrose loss is due to wound-induced invertase activity as shown by Rosenkranz et al. (Rosenkranz et al., 2001) and Eufinger (Eufinger, 2006). In these previous studies invertase activity and sucrose loss have only been analyzed until day five after wounding. In the present study, invertase activity after prolonged wounding was elucidated, in order to verify a potential role of BvC/VIF in the regulation of invertase activity after first wounding reactions.

4.1.1 Down-regulation of wound-induced invertase activity is accompanied by high BvC/VIF expression levels

Wounding of plant cells leads to defense reactions, which are consuming energy, reducing power and carbon skeletons (Garcia-Brugger et al., 2006). Particularly, CWI is suggested to represent a PR protein (Rolland et al., 2006) the activity of which enables the plant to respond to the increased energy demand of affected cells. In wounded sugar beet leaves, CWI activity is induced (Godt and Roitsch, 2006), whereas in sugar beet taproots wounding leads to the induction of both cell wall and vacuolar invertase activity (Rosenkranz et al., 2001; Eufinger, 2006 and the present study). In wounded taproots, cell wall and vacuolar invertase activity reach their maximal activity three and five days after wounding, respectively. Thereafter, invertase activity is down-regulated, although invertase protein amount stays high (Figures 1 and 2).

In the analyzed taproots, neither cell wall nor vacuolar invertase were detected in unwounded tissue. Hence, the analyzed taproots represent rather mature taproots, since only at early stages of root development, high CWI and VI activities are present (Godt and Roitsch, 2006). Upon wounding, expression of both invertases was induced. The transcripts of CWI1 and VI1, the only wound-induced invertase isoforms known in sugar beet taproots (Rosenkranz et al., 2001), were already detectable one day after wounding and decreased after prolonged wounding. Transcriptional regulation of invertases in response to external and internal stimuli is known to be very efficient, comprehensive and fast (Koch, 2004; Huang et al., 2007). However, the lower transcript levels of CWI1 and VI1 were not reflected in protein amount; in contrast to the transcript levels, invertase protein amount stayed high even after prolonged wounding. The high protein stability of acid invertases, gained by glycosylation, is a well known feature (Pagny et al.,

2003), thus invertases are post-translationally regulated by proteinaceous inhibitors (Rausch and Greiner, 2004). In sugar beet, the transcript levels of the invertase inhibitor BvC/VIF is elevated during wounding (Eufinger, 2006). In young taproots only weakly expressing BvC/VIF, the higher transcript levels after wounding were closely correlated with protein amount (Figure 1). In mature taproots, BvC/VIF expression stayed high after wounding. Due to the chosen time frame in previous studies (till five days after wounding, (Rosenkranz et al., 2001; Eufinger, 2006)), only wound-induced invertase activity was observed and unexpectedly elevated invertase inhibitor expression levels were detected during wounding, too. In the present study, after prolonged wounding, a decreasing invertase activity was measured, which did not correlate with the amount of invertase protein.

These findings provide strong evidence for a key role of BvC/VIF with respect to down-regulation of invertase activity after first wounding reactions to limit sucrose breakdown, which in turn is of particular interest in the model organism sugar beet. Wounding of the taproot leads to the induction of vacuolar invertase activity and since sugar beets store extremely high amounts of sucrose within the vacuole of parenchyma cells in their taproot, it is coherent that cleavage of sucrose in the vacuole has to be tightly controlled. Nevertheless, an increase of both cell wall and vacuolar invertase activity after wounding was observed despite strong BvC/VIF expression. Only after prolonged wounding, a decrease of invertase activity was monitored. Thus, it was investigated in subsequent experiments which factors impede the immediate inhibition of invertase activity.

4.2 Interaction of BvC/VIF and invertases during wounding

4.2.1 Complex formation does not necessarily lead to inhibition

As pointed out previously, parallel expression of invertase and inhibitor after wounding is not sufficient for inhibition of invertase activity. Although BvC/VIF is highly expressed in mature taproots already in unwounded tissue, activity of both cell wall and vacuolar invertase increased till day three to five after wounding and was only down-regulated thereafter.

It was shown by Eufinger that invertase and inhibitor form stable complexes in sugar beet taproots at day five after wounding (Eufinger, 2006). In the present study, it was determined whether invertase-inhibitor complex formation only occurs after prolonged wounding and is therefore the limiting step for inhibition. In contrast to the expectations, an invertase inhibitor complex formation was already observed in the early phase of wounding (Figure 8). Since acid invertases are glycosylated (Sturm, 1999; Pagny et al., 2003) and inhibitor proteins are not

(Rausch and Greiner, 2004), it could be shown via Concanavalin A purification that BvC/VIF is bound to the invertase two days after wounding. At this time, invertase activity was not yet down-regulated. A similar situation was shown for NtCIF, a cell wall localized invertase inhibitor from tobacco, in which association of NtCIF with cell wall invertase was independent of its inhibitory effect (Krausgrill et al., 1998).

One potential drawback of the experimental set-up is, that only soluble and cell wall elutable proteins can be analyzed via Concanavalin A purification. As BvC/VIF and CWI remain mainly in the cell wall fraction after prolonged wounding, only a minor part of BvC/VIF is therefore present in the analyzed extract. Thus, it is explainable that after prolonged wounding less BvC/VIF is detectable in the ConA bound fraction, which in turn presumably reflects inhibitor bound to the invertase.

Altered invertase activity can be monitored after complex dissociation *in vitro*

Inhibition of invertase activity by inhibitor proteins is strongly pH dependent (Rausch and Greiner, 2004), with the strongest inhibition being observable at acidic pH. At neutral pH, no complex formation occurs, as shown for recombinant BvVI1 and BvC/VIF (Eufinger, 2006). Based on these findings, protein extraction from wounded sugar beet taproots at different pH (pH 5 versus pH 8) should reveal whether (i) complex dissociation also occurs in plant extracts and (ii) how BvC/VIF is involved in the down-regulation of wound-induced cell wall invertase activity.

Extraction at different pHs suggested a complex dissociation of CWI and BvC/VIF at pH 8 (Figure 7A). While CWI remained ionically bound to the cell wall regardless of pH, much of BvC/VIF was found in the supernatant at pH 8 and only a minor part of BvC/VIF was still determined in the cell wall fraction. In this context, it has to be mentioned that at pH 8 BvC/VIF is expected to be uncharged due to its estimated pI of 8.1, whereas CWI, displaying a pI of 9.3, should still be charged. This enables CWI, in contrast to BvC/VIF, to be still associated with the cell wall at higher pH values. The assumption that BvC/VIF is ionically bound to the cell wall at pH 5 can be excluded, since the major part of BvC/VIF is only detected in the cell wall fraction when its target enzyme, namely cell wall invertase, is also expressed. Therefore, it is much more likely that BvC/VIF and cell wall invertase form a complex when both encounter each other, which in turn dissociates at pH 8.

A down-regulation of invertase activity was determined after prolonged wounding, if extraction took place at pH 5 and the inhibitor was detected in the same extract as the invertase. In contrast to this, cell wall invertase activity stayed on the same level and even increased slightly in samples extracted at pH 8, in which nearly no BvC/VIF was found in the cell wall extract but to

a major part in the soluble fraction. Under both conditions a stable expression level of cell wall invertase was detected after induction in response to wounding. Only in the samples, no BvC/VIF is detected, the invertase activity course did not correlate with protein amount. This observation gave a further hint that BvC/VIF is involved in down-regulation of unwanted invertase activity after wounding. Since a change in invertase activity was only observable after prolonged wounding, this experiment gave further evidence that binding is not sufficient for inhibition and another regulatory mechanism is probably involved in invertase inhibition through proteinaceous inhibitors.

4.2.2 Which regulatory mechanism is involved in fine-tuning of wound-induced invertase activity?

As described for NtCIF, complex formation of inhibitor and cell wall invertase does not necessarily lead to inhibition (Krausgrill et al., 1998). Similar results were obtained for BvC/VIF in the present study. Taken these observations together, it seems likely that another regulatory mechanism is present. As assumed by Weil et al., a transition from the non-inhibited to the inhibited conformation of NtCIF-CWI complex may be due to a change in sucrose concentration (Weil et al., 1994). However, it was shown by Sander et al. that sucrose protects cell wall invertase from tobacco but not vacuolar invertase from tomato against proteinaceous inhibitors (Sander et al., 1996). Therefore, other yet unknown factors may be responsible for the transition of conformation (Krausgrill et al., 1998).

Possible regulation of invertase- inhibitor interaction by pH shift
Invertase inhibitor proteins act in a pH dependent manner (Rausch and Greiner, 2004). For instance *in vitro* BvC/VIF inhibits vacuolar invertase only below pH 5.1, whereas the invertase is still active above this pH (Eufinger, 2006). These findings led to the hypothesis the invertase inhibition can be regulated by changes in vacuolar or apoplastic pH *in planta.*
The apoplastic pH in roots, for example, is normally between 5.1 and 5.6 and is maintained by active regulation (Taylor et al., 1996; Felle, 1998). Still, pH changes in response to external and internal stimuli (Pignocchi and Foyer, 2003). For instance, the apoplast gets acidified during auxin mediated cell growth and expansion (Vreeburg et al., 2005) or in response to light, when photosynthesis is stimulated (Marrè et al., 1989). In response to oxygen stress (Felle, 2006) or fungal attack (Felle et al., 2008) the apoplastic pH increases by up to two pH units.
Plant vacuoles in general are acidic. Like described for the apoplasmic space, pH changes have been reported also for vacuoles, as for instance rising pH in the vacuole in response to salt

stress (Gruwel et al., 2001). These transient changes of pH in subcellular compartments could play a physiological role with regard to invertase inhibition. The determination of the crystallographic structure of NtCIF at different pHs revealed no major rearrangements depending on pH shift (Hothorn and Scheffzek, 2006). Therefore, the pH dependency of inhibition could be due to changes in the surface charge of both interacting proteins as the authors assume but also by conformational changes of the invertase.

Potential redox-control of invertase activity and inhibition via proteinaceous inhibitors

Wounding and pathogen attack can lead to HR (Hypersensitive Response), which results in a fast and strong increase of ROS (Foyer and Noctor, 2005). In the apoplast, accumulation of ROS results in the initiation of various signal transduction cascades, as direct or indirect regulation of enzyme activities (Lamb and Dixon, 1997).

Interestingly, the crystallographic structure of a cell wall invertase from *Arabidopsis thaliana* revealed a disulfide bridge in the C-terminal domain of AtCWI1 (At3g13790, (Verhaest et al., 2006)). Sequence analysis showed that the involved cysteines are highly conserved (diploma thesis, Lindner, 2008) among invertases. Thus, this disulfide bridge could be target for a possible redox regulation of invertase activity itself or the inhibition of invertases through proteinaceous inhibitors during wounding.

With the purpose of elucidating a potential redox-regulation of invertase activity and inhibition through inhibitor proteins, the conserved cysteines of BvVI1 were mutated into serine and the resulting recombinant proteins were purified from *E.coli* according to Eufinger (Eufinger, 2006). Only BvVI was analyzed, since purification of soluble BvCWI did not succeed so far. The used *E. coli* strain possesses an oxidative cytosol and the purified wild type BvVI featured a disulfide-bridge as shown by the quantification of protein thiols via 4,4′-dithiodipyridine (data not shown). Invertase activity itself was not influenced in the disulfide-bridge deficient mutant (diploma thesis Lindner, 2008 and Figure 8), but interestingly the mutant was inhibited more effectively by BvC/VIF compared to wild type BvVI. Approaches to confirm the altered inhibition depending on the redox-state via H_2O_2/ DTT treatment of the wt protein did not succeed so far (diploma thesis Lindner, 2008). Nevertheless, the more efficient inhibition was specific for the disulfide-bridge deficient mutant and was not observed for a control mutant (mutation of another conserved cysteine into serine, not predicted to be involved in disulfide-bridge formation), thus it seemed likely that the loss of the ability to form a disulfide-bridge leads to a conformational change which is responsible for the better inhibition.

The disulfide bridge is located at the C-terminus which is formed of two ß-sheets (Verhaest et al., 2006), whereas the active site is positioned at the N-terminus. Until now, the function of the

C-terminus remains unknown. Therefore, it is highly relevant that BvVI shows an improved inhibition via BvC/VIF after mutational changes within the C-terminus, since these results suggest that the C-terminus of invertases plays a role in the interaction between invertase and its proteinaceous inhibitor.

Unexpectedly, the activity as well as the inhibition of the disulfide-bridge deficient mutant of BvVI is as pH dependent as shown for wildtype BvVI (Figure 8). Therefore, it has to be reconsidered whether a structural change of the C-terminus of invertases can occur due to reduction and oxidization *in planta,* respectively*.* The vacuole and the apoplast belong to the secretory pathway where an oxidizing milieu is present (Hwang at al., 1992). The redox-buffering capacity in the apoplast is very weak (Horemans et al., 2000; Pignocchi and Foyer, 2003), which is assumed to heighten or facilitate redox regulation (Foyer and Noctor, 2005). Still, not much is known about the redox-state of the vacuole. However, vacuoles accumulate several antioxidants and especially in sugar beet, sucrose might play an important role as antioxidant (Mittler et al., 2004; Van den Ende and Valluru, 2009). Altogether, it remains difficult to predict whether invertases might underlie any redox-regulation or rather are always present as the oxidized form *in planta*.

Proteolytic splitting of invertase: Impact on activity?

Typical splitting products of BvVI (Eufinger, 2006) are detected upon wounding. Interestingly, different splitting products for vacuolar invertase are detected after prolonged wounding compared to the early wounding phase in ConA purified extracts (Figure 8). As reported in several studies, vacuolar invertase splitting products do occur in several plant species (Reca et al., 2008 and references therein). It is assumed that proteolysis occurs *in planta* and seems to be under developmental control (Sturm, 1999). Nevertheless, the contribution of this process to the regulation of enzymatic activity still remains to be elucidated. The increasing cleavage products after prolonged wounding probably hint to a regulatory mechanism of invertase activity. However, the immunoblots from wound-induced cell wall invertase, which also displays protein cleavage (Weil et al., 1994; Krausgrill et al., 1996), did not display any difference in splitting products, still CWI activity was down-regulated after prolonged wounding (Figure 2). Thus, regulation of invertase activity by proteinaceous inhibitors presumably represents the more general and comprehensive post-translational process.

4.3 Characterization of BvC/VIF

To examine whether BvC/VIF functions as a cell wall invertase and/ or a vacuolar invertase inhibitor *in vivo,* the subcellular localization was analyzed. Bioinformatic analysis of the BvC/VIF protein sequence revealed an N-terminal transitpeptide for the co-translational entrance into the secretory pathway. Previous investigations on the subcellular localization of BvC/VIF were inconsistent (Eufinger, 2006). For instance, GFP imaging led to different results depending on the transformed plant material. Heterologous expression of BvC/VIF fused to GFP resulted in vacuolar localization in onion epidermis, whereas in tobacco leaves only vesicular structures showed GFP fluorescence (Eufinger, 2006).

4.3.1 Is BvC/VIF exclusively localized in the apoplast?

In the hairy root system, displaying a high extra cellular invertase expression and activity (data not shown), BvC/VIF is exclusively localized in the apoplast (Figure 3), probably co-expressed with its putative target enzyme (CWI) as described for NtCIF and NtCWI in suspension- cultured tobacco cells (Krausgrill et al., 1998). According to this study, NtCIF and its target NtCWI are co-expressed throughout the entire culture period with permanent complex formation.

Via a non-invasive approach, as used for determining the localization of tobacco cell wall invertase NtCWI (Weil and Rausch, 1990), an extracellular localization of BvC/VIF in sugar beet taproots was shown, too (Figure 3). Additionally, the extra-cellular localization of BvC/VIF was detected via immuno-localization in fixed taproot slices (Figure 4).

Notably, the non-invasive approach was not quantitative. Moreover, after protein extraction from disrupted cells, an intracellular localization of BvC/VIF was observed in unwounded sugar beet taproots. However, thus far, it can not be stated whether an alternative intra-cellular localization of BvC/VIF exists or just extra-cellular targeted BvC/VIF *in transit* was detected.

Nevertheless, the ability of BvC/VIF to inhibit cell wall invertase as well as vacuolar invertase activity (Eufinger, 2006), rather implicates that BvC/VIF represents a cell wall inhibitor of ß-fructosidase, since CIFs are broadly active against both CWI and VI, whereas VIF inhibition is specific to VIs (Huang et al., 2007 and references therein).

Does a processing event determine secretion in the apoplast?
Eufinger described the occurrence of two BvC/VIF protein forms, differing in molecular weight (Eufinger, 2006). It is noteworthy that depending on the physiological state of sugar beet taproots the non-invasive approach via salt-elution revealed an extracellular localization of both detected BvC/VIF protein forms. Assuming that different processing events are detected, it is

therefore unlikely that only fully processed BvC/VIF is exported as described for instance for PMEs (Wolf et al., 2009). The physiological relevance of the occurrence of the two protein species could not be clarified so far but possible reasons for the observed difference in molecular weight are addressed in the next chapter.

4.3.2 A proteolytic cleavage of BvC/VIF is unlikely

Immunoblot analysis of sugar beet taproot extracts revealed two BvC/VIF species (Eufinger, 2006). The same was observed in chicory taproots for another invertase inhibitor CiC/VIF (Kusch et al., 2009). At least for BvC/VIF it is most likely, that both species are encoded by one gene, since overexpression of BvC/VIF cDNA in sugar beet led to upregulation of both protein species in sugar beet adventitious roots (Figure 5). The occurrence of both protein species could be due to a post-translational processing-event. In principle, it is possible that such processing takes place at the N- or the C-terminus of the inhibitor protein.

First evidence for a C-terminal processing event gave an immunoblot analysis of BvC/VIF-GFP fusion protein, ectopically expressed in tobacco leaves (Eufinger, 2006). The fusion protein was processed inside the cells. Since cleaved BvC/VIF signal was in size comparable to native BvC/VIF observed in sugar beet taproots, it was assumed that cleavage has to occur at the C-terminal part of BvC/VIF or at the N-terminal part of GFP. Ectopic expression and subsequent analysis of C-terminal deletion constructs of BvC/VIF fused to GFP were performed, in order to reveal putative cleavage sites within the BvC/VIF sequence, with the purpose to investigate whether a processing at the C-terminus is responsible for the appearance of two BvC/VIF species in sugar beet. However, the proteolytic cleavage of the BvC/VIF-GFP fusion protein was not prevented in the BvC/VIF deletion constructs (Figure 6). Therefore, no further evidence was gained for a C-terminal processing of BvC/VIF and it is seems likely that processing takes place at the N-terminal part of GFP due to the acidic pH as described by Tamaru et al. (Tamura et al., 2003). However, this seems not to be a common problem, since it was possible to determine the extracellular localization of the invertase inhibitor INVINH1 from tomato via GFP analysis, although GFP was also cloned downstream of the inhibitor (Jin et al., 2009).

A C-terminal processing of BvC/VIF, leading to the detection of two species was not observable; still it is possible that an N-terminal cleavage of BvC/VIF occurs (Eufinger, 2006). However, both species of inhibitor proteins were only observed in the homologous system, being true for BvC/VIF in sugar beet and CiC/VIF in chicory, respectively. When overexpressing the inhibitor for instance in tobacco leaves, only one species is detectable (Figure 7 and Kusch, 2009). Spiking experiments revealed that the ectopically expressed BvC/VIF species corresponds to

the smaller species observed in sugar beet taproots. The question arose if other plants, like in this case tobacco, possess a more efficient protease leading to the complete proteolytic cleavage. But spiking experiments of taproot extracts expressing BvC/VIF with recombinant BvC/VIF, purified from *E.coli* clarified that proteolytical cleavage is rather unlikely, since the recombinant BvC/VIF, purified from *E.coli,* represents the smaller species. In case, a proteolytical cleavage of BvC/VIF occurs *in planta*, the larger species should be expressed in *E.coli*, since *E. coli* does not express corresponding proteases. Therefore, it is assumed that another post-translational modification leads to the occurrence of both species, which differ in about 1 kDa in size. So far, glycosylation by high mannose chains can be excluded as shown by previous ConA purification (Rausch and Greiner, 2004), whereas other glycosylation forms have to be elucidated. Post-translational modifications, as for instance phosphorylation and ubiquitination, are rather unlikely, since these modifications are known to occur in the cytosol and/or nucleus; whereas BvC/VIF is assumed to enter the secretory pathway.

4.4 Post harvest situation in BvC/VIF transgenic sugar beet lines

Rosenkranz at al. (2001) reported on the induction of cell wall and vacuolar invertase activity in sugar beet taproots in response to wounding, which is accompanied by sucrose breakdown, leading to the accumulation of hexoses. Wound-induced invertase activity correlated to elevated invertase expression and is therefore not due to inhibitor inactivation but due to *de novo* synthesis. In addition, the transcript levels of the SuSy isoforms known in sugar beet accumulate in wounded taproots. However, the accumulation of transcripts of SuSy isoforms is not reflected on protein level and activity, therefore these SuSy isoforms are not involved in sucrose-breakdown or stress-responses in sugar beet taproots (Klotz and Haagenson, 2008). Interestingly, invertase expression and activity only correlated in the early wounding period, but not prolonged after extended wounding (Figures 1+2). After first wounding reactions, invertase activity decreased despite a stable protein expression, giving first evidence for a putative physiological role of BvC/VIF concerning fine-tuning of remaining invertase activity, as discussed in 4.1. Based on these results, BvC/VIF is suggested as a promising candidate for biotechnological approaches in order to limit undesired sucrose loss by reducing wound-induced invertase activity.

Overexpression of invertase inhibitors represents an interesting approach to alter invertase activity in plants (Rausch and Greiner, 2004; Roitsch and Gonzalez, 2004). For instance, in transgenic potato tubers, overexpressing NtVIF, cold induced hexose accumulation was reduced by up to 75%, without any effect on potato tuber yield (Greiner et al., 1999). First attempts to

reduce wound-induced vacuolar invertase activity in sugar beet taproots by overexpressing NtVIF did not succeed (Eufinger, 2006). Subsequently, the endogenous inhibitor BvC/VIF was characterized and transformed into sugar beet under the control of a duplicated CaMV 35S-promoter and the taproot specific promoter 2-1-48 (Oltmanns et al., 2006) respectively, with the objective to minimize post-harvest sucrose-loss. Additionally, a BvC/VIF RNAi construct was transformed in sugar beet in order to further elucidate the physiological role of BvC/VIF during wounding.

4.4.1 BvC/VIF expression in transgenic adventitious roots

Under the control of the duplicated 35S promoter, BvC/VIF was strongly expressed in sugar beet adventitious roots as shown by Northern- and Western Blots (Figure 9), whereas BvC/VIF expression was hardly detectable in control adventitious roots. According to the weak expression of BvC/VIF in the analyzed adventitious roots, their developmental state is presumably corresponding to young taproots (Figure 1, (Eufinger, 2006)). Due to the low expression of BvC/VIF, silencing of BvC/VIF in RNAi lines could only be identified in wounded adventitious roots, since adventitious roots displayed a wound enhanced BvC/VIF expression (Figure 9) as already observed for sugar beet taproots. In control adventitious roots, BvC/VIF is detected in the cell wall, as already described previously for wounded taproots (Figure 1). Immunoblot analysis of wounded adventitious roots, expressing BvC/VIF under the control of the 35S promoter, detected a strong expression of BvC/VIF not only in the cell wall fraction but also to a large extent in the soluble fraction. Two possibilities may explain these results. On one side the high expression level of BvC/VIF leads possibly to mistargeting. On the other side it may be that BvC/VIF is exclusively extracellular targeted but exceeds present CWI and therefore cannot form a complex with CWI. Thus, free BvC/VIF is found in the soluble fraction. Still, via immuno-localization only the cell wall localized expression could be confirmed in BvC/VIF overexpressing lines (Figure 4).

4.4.2 Efficient down-regulation of wound-induced invertase activity in adventitious roots by ectopic overexpression of BvC/VIF

Overexpression of BvC/VIF in sugar beet influenced wound-induced cell wall as well as vacuolar invertase activity (Figure 13). Both, CWI and VI displayed only 25% of wound-induced activity in comparison to the controls. A dose response concerning BvC/VIF expression and activity (Figure 12) was observed in two independent BvC/VIF overexpressing lines, differing in BvC/VIF

expression. Regarding immunoblots of the invertases, it became apparent that the different invertase activities are due to post-translational regulation, since the expression of CWI and VI was the same irrespective if BvC/VIF is silenced or highly expressed (Figure 12). The reduction of both invertases, CWI as well as VI, is in contrast to the effect on invertase activities observed in sugar beet leaves. In leaves overexpression of BvC/VIF led only to an inhibition of vacuolar invertase activity of 50%, whereas CWI activity was not influenced at all (Eufinger, 2006). These results support the assumption that BvC/VIF displays distinct affinities for different CWI isoforms. Apoplastic invertase isoforms show a specific expression pattern in a development- and organ-specific manner (Schaarschmidt et al., 2007). Since BvC/VIF is expressed to a major part in tap-/adventitious roots and not in leaves, it is coherent that a high affinity of BvC/VIF for a wound-induced and in the tap-/adventitious root localized CWI is observed.

As mentioned above, it cannot be stated whether BvC/VIF co-localizes with vacuolar invertase *in planta* or if BvC/VIF detection in the soluble fraction represents a post-extractional artifact. This in turn would mean that an inhibition of vacuolar invertase was only determined, since BvC/VIF bound to VI during extraction. However, overexpression of INVINH, a cell wall localized invertase inhibitor from tomato, in *A. thaliana* led specifically to a reduced cell wall invertase activity, whereas soluble invertase activity was not influenced at all (Jin et al., 2009). Moreover, overexpression of NtCIF and NtVIF resulted in a specific reduction of their respective target enzymes (Greiner, 1999).

Already during the early wounding phase invertase activity was altered in BvC/VIF overexpressing lines compared to the controls. Thus, further regulatory mechanisms, hampering the prompt inhibition of invertase activity, as discussed for wild type taproots, seem not do be relevant under these circumstances, in which the inhibitor is expressed in a great surplus.

4.4.3 Indications for putative *in vivo* function of BvC/VIF?

A significant effect on wound-induced invertase activity by silencing BvC/VIF expression could not be determined. The mean values of wound-induced CWI and VI activities in BvC/VIF RNAi lines are comparable to those determined for the controls.

It is noteworthy that the expression of CWI and VI was not influenced by altering expression of BvC/VIF, still invertase immunosignals in all BvC/VIF transgenic lines differed from those observed in the controls (wild type and transgenic control, Figure 12) and in taproots (Figure 1). Previous immunoblot analysis of wound-induced CWI revealed two cleavage products, which were present in one to one ratio. In transgenic BvC/VIF lines, both cleavage products were detected, too but the smaller one only to a much lower extent. VI expression seemed to be even

lower in the analyzed transgenic BvC/VIF lines. It is assumed that proteolytic fragmentation of invertases is under developmental control (Unger et al., 1994; Sturm, 1999; Greiner et al., 2000), and that the process might regulate invertase activity. It should be excluded that a putative BvC/VIF RNAi effect is missed, since variable invertase activities might occur irrespective of BvC/VIF expression. Thus, individual adventitious roots from one BvC/VIF-RNAi line, displaying all the same invertase expression and cleavage pattern but different invertase activity, were again analyzed in detail. Unexpectedly, a different degree of BvC/VIF silencing was observed in individual adventitious roots from one line (Figure 14). High variations with respect to level of reduction of target RNA among multi copy RNAi lines were also reported previously (Kerschen et al., 2004). The analyzed BvC/VIF- RNAi lines in this study were multi copy lines, since no single integration RNAi line could be identified (Figure 10). In contrast to BvC/VIF RNAi lines, analyzed BvC/VIF overexpressing lines were single integration lines.

Interestingly, the degree of BvC/VIF silencing correlated exactly with wound-induced invertase activity in individual adventitious roots. In all individuals, a strong BvC/VIF silencing was determined. However, in two adventitious roots, displaying the strongest silencing effect, wound-induced cell wall invertase activity was much higher. Moreover, a strongly increased vacuolar invertase activity occurred in the one adventitious root, in which no BvC/VIF expression was detectable via immunoblot (Figure 14). This might lead to the assumption that an effect on the post-translational regulation of invertase activity can only be determined if BvC/VIF expression is below a certain threshold, which in turn is different for particular invertases. On the other side, it is possible that increased VI activity is only indirectly linked to BvC/VIF silencing and rather mediated by sugar signaling based on a feed-forward mechanism (Koch, 2004; Huang et al., 2007). Based on this hypothesis, a higher CWI activity in the BvC/VIF RNAi line is leading to increasing amounts of hexoses, which in turn would act as sugar signals, leading to the induction of VI. Thus, it is still hard to predict whether both cell wall and vacuolar invertases are target enzymes of BvC/VIF *in planta*.

In both adventitious roots, displaying the strongest BvC/VIF silencing, higher invertase activities are reflected in an increased sucrose loss (Figure 14D). A slightly higher sucrose loss was observed in adventitious roots showing only a BvC/VIF-mediated decrease in cell wall invertase activity, without effecting vacuolar invertase activity. This observation provides an indication for the involvement of cell wall invertase in sucrose breakdown in response to wounding in the apoplast, representing an important site of energy demand in case of wounding and pathogen attack (Essmann et al., 2008). The observation that CWI activity is correlated to sucrose breakdown would support the assumption that sucrose hydrolysis in storage tissues does not only occur in the vacuole but a vesicular sucrose transport to the apoplast exists (Echeverria,

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2000; Valluru et al., 2008). Nevertheless, the additional increase of vacuolar invertase in one adventitious root resulted in a distinct higher sucrose loss. Taken together these observations, a role of BvC/VIF with respect to fine-tuning of invertase activity during wounding and therefore preventing unwanted sucrose loss appears reasonable. Nevertheless, these interesting results were only obtained with individual BvC/VIF RNAi plants and have therefore to be further elucidated on large-scale. The reduction of wound-induced invertase activity by overexpressing BvC/VIF on the other hand was very distinct in all analyzed plants.

4.4.4 Demand-driven sucrose breakdown in BvC/VIF transgenic sugar beet lines?

Overexpression of BvC/VIF prevented wound-induced cell wall as well as vacuolar invertase activity to large extents (4.4.1). As wound-induced invertase activity is responsible for sucrose loss in sugar beet taproots (Rosenkranz et al., 2001; Eufinger, 2006), it was assumed that a reduction of invertase activity would lead to less sucrose loss. Against all expectations, the much lower invertase activity in BvC/VIF overexpressing lines was not reflected in sucrose breakdown at all (Figure 15). Even though, wound-induced invertase activity was inhibited to about 25% compared to the controls and BvC/VIF RNAi lines, sucrose loss was not influenced. This might be explained by different hypotheses.

First it is possible, that the achieved reduction of CWI and VI activity is not sufficient to influence sucrose hydrolysis. For instance, in maize mutants it was shown that a residual invertase activity of <1% in developing endosperm compared to wildtype is sufficient to support seed development, even though resulting in a loss of >70% of seed weight (Miller and Chourey, 1992; Chourey et al., 2006). Secondly, the endogenous BvC/VIF expression might be already adequate to execute the maximal post-translational control of sucrose breakdown, thus no gain of function can be achieved by overexpressing BvC/VIF. A third hypothesis would be that BvC/VIF *in vivo* is only repressing CWI activity and the effect on VI activity is just due to a post-extractional artifact. This hypothesis would imply that VI is the main responsible enzyme for sucrose mobilization.

In another model, it is suggested that other sucrolytic enzymes fulfill sucrose break-down, if invertase activity is decreased. Analysis of hexose accumulation in BvC/VIF- transgenic lines supported the theory of an alternative way of sucrose cleavage (table 2). Controls and BvC/VIF-RNAi lines accumulated much more hexoses as BvC/VIF overexpressing lines. Such high concentrations of hexoses as determined in the controls can presumably only be accumulated in the vacuole, since hexoses would be soon phosphorylated in the cytosol and further

metabolized. On the contrary, in BvC/VIF overexpressing lines, hydrolyzing the same amount of sucrose, much lower hexose concentrations were measured. Hence, it seems reasonable that cleavage of sucrose into glucose and fructose occurs in the cytosol, in which generated hexoses are metabolized immediately.

If this assumption is correct, it is most likely that either cytosolic invertases or sucrose synthases are involved therein (Figure 16). If sucrose breakdown can not be mediated by acidic invertases, sucrose instead of the hexoses has to be delivered to the cytosol, either from the vacuole, if VI is responsible for sucrose hydrolysis, or from the apoplast, if sucrose is transported in vesicles to the apoplast before cleavage, as postulated by Echeverria (Echeverria, 2000). Higher sucrose efflux from the vacuole into the cytosol could be realized by a higher expression of vacuolar sucrose transporter. Although vacuolar compartmentalization of sucrose is of high importance, only recently first evidence on the molecular nature of a vacuolar sucrose carrier was gained (Endler et al., 2006). This carrier is assumed to be responsible rather for sucrose export from the vacuole to the cytosol than sucrose import (Neuhaus, 2007). From sugar beet taproots, a sugar transporter localized at the vacuolar membrane has been reported, the exact transport activity of which is yet not clear (Chiou and Bush, 1996). It would be very interesting to isolate the SUT homologue in sugar beet, responsible for sucrose export from the vacuole in tap-/ adventitious roots parenchyma cells, in order to identify putative alterations in sucrose export, depending on invertase activity in transgenic lines.
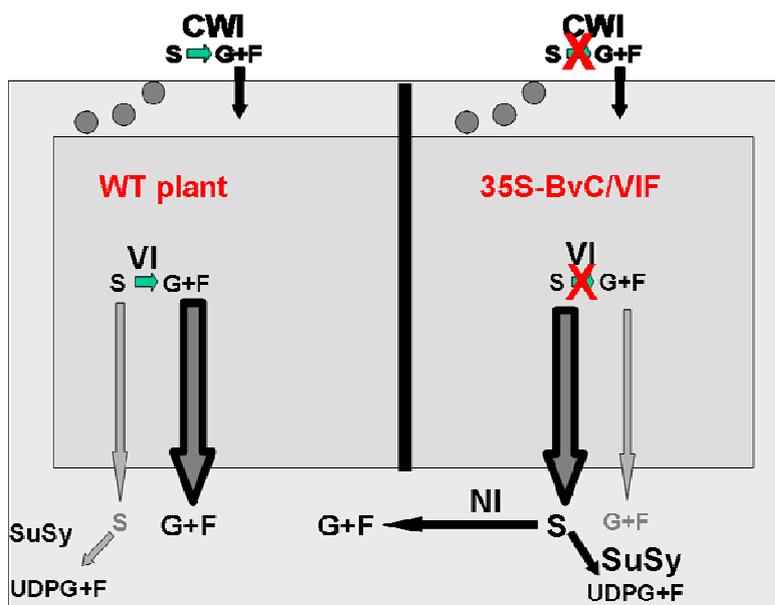
Figure 16: Model of demand - driven sucrose metabolism in wounded sugar beet tap-/ adventitious roots. In the wild type (WT plant) situation, wound-induced vacuolar and cell wall invertase activity lead to sucrose breakdown either in the vacuole or in the cell wall and hexoses are released into the cytosol. Inhibition of CWI (Cell wall invertase) and VI (Vacuolar invertase) activity in BvC/VIF overexpressing lines (35S-BvCVIF) may be compensated by enhanced release of sucrose into the cytoplasm for metabolic conversion via NI (neutral invertase) and/ or SuSy (sucrose synthase). S: sucrose, G: glucose, F: fructose

4.5 Outlook

In future experiments the focus will be on the post-harvest situation in BvC/VIF transgenic sugar beet plants. Since a distinct reduction of VI and CWI in BvC/VIF overexpressing lines did not lead to a decreased sucrose loss, alternative ways of sucrose breakdown have to be evaluated. In order to identify a putative demand-driven sucrose metabolism, the expression of the most likely involved enzymes should be analyzed. It is suggested that a different expression and/ or activity of other sucrose degrading enzymes besides acid invertases can be determined in BvC/VIF overexpressing plants. Furthermore, it needs to be addressed if a change in sucrose efflux either from the vacuole or from the apoplast is involved in plants in which acid invertase activity is hampered.

In case no further hints for alternative pathways of sucrose cleavage are gained in BvC/VIF overexpressing lines, showing a strongly reduced acid invertase activity without effect on sucrose loss, it should be taken into account that the reduction of both acid invertases (vacuolar and cell wall) does not necessarily reflect the situation *in planta*. Therefore, it has to be ensured that the invertase(s), causing the sucrose breakdown upon wounding and BvC/VIF co-localize. Thus far, it can not be stated whether BvC/VIF is exclusively located into the cell wall. Hence, the most straight-forward experiment would be to target BvC/VIF into the vacuole by fusing BvC/VIF with a target motif of a vacuolar protein, in order to determine any putative differences in sucrose breakdown upon wounding.

Besides this, the analysis of interaction of BvC/VIF and its target enzymes should be extended. For the molecular analysis of the interaction between BvC/VIF and acid invertases it is quite helpful that BvC/VIF and BvVI can be purified from *E.coli* in adequate amounts. In the mean time, it would be interesting to purify also BvCWI, since cell wall and vacuolar invertases show differences in interaction with the inhibitor like, for instance substrate protection could only be shown for CWI and not for VI (Sander et al., 1996).

The active centre of invertases is localized in the N-terminal part and so far no physiological role for the C-terminus of invertases could be determined (Verhaest et al., 2006). Nevertheless, mutation of a conserved cysteine of BvVI, localized in the C-terminal part resulted in a better inhibition of invertase activity by BvC/VIF. This finding gave first evidence for an involvement of the C-terminal part in the interaction between invertase and inhibitor. One approach to clarify the relevance of the C-terminal part is the separate purification of the N-terminus and the C-terminus of BvVI, with subsequent, potential reassembly.

5. Material and methods

5.1 Plant material

5.1.1 Sugar Beet (*Beta vulgaris* L.)

Sugar beet plants (*Beta vulgaris* L. ssp. vulgaris var. altissima DÖLL) of a diploid inbred line (Partie-Nr. VV-I/ZR 10738, KWS SAAT AG) were field-grown between April and October on the trial field of the Heidelberg Institute of Plant Sciences. Adventitious roots, received from KWS SAAT AG, Einbeck, were grown in the greenhouse in special rose-pots (20 cm high) with 16 h of supplementing light.

Beta vulgaris *hairy roots*

Beta vulgaris hairy root cultures were provided by the RooTec AG (Witterswill). Cultures were grown in 3.2 g/l Gamborg B5 medium (Serva) with 3 % sucrose and, in the case of plate culture, 0.8 % plant agar (Duchefa), pH 5.8. Plate cultures were incubated in the dark at 22 °C and liquid cultures at 25 °C and 90 rpm shaking. Hairy root cultures were transferred to new plates once per month or grown for three weeks in liquid culture.

Procedure for wounding of sugar beet taproots

Wounding of sugar beet taproots was carried out according to Rosenkranz et al., 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 (Rosenkranz et al., 2001). The slices were incubated in a moist atmosphere for up to ten days at room temperature in the dark.

5.1.2 Tobacco

For *Agrobacterium tumefaciens* leaf infiltration, 8-12 week old *Nicotiana benthamiana* plants grown in a growth chamber under 16 hours light period (300 µ E), were used.

5.2 Microbiological techniques

5.2.1 Bacterial strains

For cloning procedures, *E. coli* strain DH5α (Invitrogen) was used.

Genotype: *supE44, Δ lacU169 (phi 80 lacZ Δ M15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1*.

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 genes promote the formation of disulfide bonds in the *E. coli* cytoplasm. Genotype: *Δara-leu7697 ΔlacX74 Δ phoAPvu II phoR araD139 ahpC galE galK rpsL F'[lac+(lacIq)pro] gor522 ::Tn10 trxB ::kan pRARE.*

For transient transformation of *N. benthamiana* leaves, the bacterial strain *Agrobacterium tumefaciens* C58C1 (Rifampicin resistance) carrying the Ti plasmid pGV2260 (Carbenicillin resistance) was used.

5.2.2 Media and antibiotics

E. coli bacteria were either grown in low salt LB-medium (5 g/L NaCl, 5 g/L Yeast Extract, 10 g/L Tryptone/Peptone) for cloning purposes or in TB-medium for bacterial overexpression (prepared according to Sambrook et al., 1989). Selection was carried out with the following concentrations of 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

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 MgSO4 x 7 H_2O, pH 7.5) supplemented with 100 µg/ml Rifampicin (genomic resistance) and 50 µg/ml Carbenicillin or 100 µg/L Ampicillin and depending on the transformed plasmid with 50 µg/ml Kanamycin, 50 µg/ml Spectinomycin or 50 µg/ml streptomycin.

5.2.3 Preparation of electrocompentent *E. coli* cells and transformation

One litre 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 – 1.0. The culture was cooled to 4 °C 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°C.

The electrocompetent cells were transformed by electroporation with a GenePulserII (Bio-Rad) set to 200 W, 1.8 kV, 25 µF and incubated in 1 ml 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) for 1 h at 37 °C before plating variable volumes on selective LB-plates.

5.2.4 Transformation of *Agrobacterium tumefaciens*

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 0.7- 1. Cells were collected (4 °C, 2.000 x 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 °C in SOC-medium and allowed to grow on selective plates for two days at 28°C.

5.3 Nucleic acid techniques

5.3.1 Agarose gels

For separation of purified DNA, 0.7 to 2% 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 marker, either SmartLadder (Eurogentec) or the 2-log ladder (NEB) was used. After the gel run, DNA was stained using a solution of 0.1 µg/ml Ethidium bromide in water.

5.3.2 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, 2ml native separating buffer (1.5M 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.

5.3.3 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. Primer name, internal primer number and primer sequence in 5' to 3' direction are given.

Overexpression of BvC/VIF in pQE30

Name	Internal #	sequence
Bv-inh_l	-	tctagtagatggtacctattctcgcaagaccaccaac
Bv-inh_r	-	ttagaacattctgcagtcattccaaactcttaatcatag

Overexpression of BvVI1 in pET-G30

Name	Internal #	sequence
12attB1TEV_BvVIwit_fw	75	tattttcagggcggagaaagtggtatttcg
12attB2_BvVIwit_rev	76	agaaagctgggttcaaaaaatgtagggag

Gateway 2-step PCR

Name	Internal #	sequence
attb1_TEV_adapter	77	ggggacaagtttgtacaaaaaagcaggctctgagaatctttattttcagg gc
attB2_adapter	78	ggggaccactttgtacaagaaagctgggt

Construction of BvC/VIF-GFP fusion constructs (incl. C-terminal deletion constructs) (pK7FWG2)

Name	Internal #	sequence
BvC/VIF_fw	113	ggggacaagtttgtacaaaaaagcaggctgtagtaaatatacattatac aca
BvC/VIF_rev	AJ19	ggggaccactttgtacaagaaagctgggttttccaaactcttaatcatag
BvC/VIF-4aa_rev	AJ20	ggggaccactttgtacaagaaagctgggttaatcatagaagcagccac
BvC/VIF-9aa_rev	AJ21	ggggaccactttgtacaagaaagctgggttcacattagatactccatgg
BvC/VIF-20aa_rev	AJ22	ggggaccactttgtacaagaaagctgggttacccacgatccgacccg

Construction of BvVI disulfide bridge deficient mutants via side directed mutagenesis (pET-G30)

Name	Internal #	sequence
C399S_SacII_N	-	ggggacaagtttgtacaaaaaagcaggctctgagaatctttattttcagg gc
C399S_SacII_C	-	ggggaccactttgtacaagaaagctgggt
C494S_XhoI_N	-	atcgatactcgaggttatggatgtacgccctccttgaccaaaag
C494_XhoI_C	-	aatcgtactcgagaatatatccaacaaaggcaatc

Generation of labelled probes

Name	Internal #	sequence
BvINH_fw	2	tcacgatgtacatgttcaccacctctactttagcg
BvINH_rev	3	ttagaacattgcggccgctcattccaaactcttaatcatag
CWI1_fw	37	ggctaacgagtcttcaagtgtgga
CWI1_rev	38	agagaagacctgctttggtcactg
BvVI1_fw	Bvi31_L	tattttcagggcggagaaagtggtatttcg
BvVI1_rev	Bvi31_R	agaaagctgggttcaaaaaatgtagggag

5.3.4 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 in a Biometra Personal cycler with the following program:

Initial denaturation	94 °C	5 min	1 repeat
Denaturation	94 °C	30 sec	35 repeats
Annealing	52 °C	30 sec	
Extension	72 °C	1 min/1 kb	
Final extension	72 °C	10 min	1 repeat

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 PCR products, the proofreading Vent DNA polymerase (NEB) or AccuPrimePfx 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 Blotting digoxigenin (DIG) 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 and analysis were 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 5.3.3). 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	Length of generated probe	Primer combination
BvC/VIF	491 bp ORF	2/3
BvCWI1	468 bp ORF	37/38
BvVI1	490 bp ORF	bvi31L/bvi31R

2-step PCR for addition of Gateway-compatible overhangs including TEV cleavage site

For the creation of PCR products with ends compatible for Gateway cloning, a two step
PCR protocol was used. 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), were added:

Left primer (# 77):

5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCTGAGAATCTTTATTTTCAGGGC-3'

right primer (# 78): 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 45ºC, 20 cycles at 52 ºC and 10 min of final extension. Denaturation and extension was carried out as described for standard PCRs (see above).

5.3.5 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. If a PCR product or digestion product was destined for Gateway recombination reactions, a PEG/ $MgCl_2$ precipitation was carried out as described in the Gateway manual instead. This step eliminated primer dimers and increased the cloning efficiency dramatically.

5.3.6 Isolation of plant genomic DNA

Before isolation of gDNA, the 5 x extraction buffer (0.5 M Tris pH8, 1.75 M sorbitol, 0.125 M EDTA, 0.05% Triton X-100) was diluted with sterile H_2O-bidest (1:5) and $NaHSO_3$ was added (40 mM final concentration). The plant material was thoroughly homogenized on ice with 5ml/g fresh weight ice cold extraction buffer. Then, the extract was filtered through 80 µm pore sized nylon net. The filtrate was centrifuged for 20 min at 750 x g and 4°C. The pellet was resuspended carefully in extraction buffer without $NaHSO_3$ (1 ml extraction buffer/ 5g fresh weight). One volume of lyses buffer (0.2 M Tris pH 8.0, 2.0 M NaCl, 50 mM EDTA, 2% CTAB, prewarmed to 65°) and 0.5 volumes 5% N-Laroylsarcosin solution were added to the suspension. The sample was mixed by inverting the tube 10 times and incubated for 15 minutes at 65 °C. The suspension was cooled down on ice and 1 volume of CI (Chloroform/ Isoamylalkohol 24:1) was added, followed by incubation for 15 min at room temperature with mild agitation. Afterwards, the suspension was centrifuged for 15 min at 5,000 g for phase separation. If necessary the upper, aqueous phase was centrifuged again.

In order to precipitate the DNA, the upper phase was transferred to a Falcon tube and mixed carefully with 1/10 volumes of a 3 M Na-Acetate solution (pH 7.2) and 1 volume of Isopropanol.

After incubation for 15 minutes at room temperature, the DNA was sedimented by centrifugation at 10,000 g for 15 minutes. The pellet was washed with 70% EtOH followed by centrifugation for 5 minutes at 10,000 g. The dried pellet was dissolved in T low E buffer (10 mM Tris, 0.1 mM EDTA, pH 8) and stored at 4 °C.

5.3.7 Southern Blotting

Restriction digestion

Usually, 20 µg genomic DNA (treated with RNaseA) were digested with suitable restriction enzymes (10 U/µg DNA) overnight. The DNA was precipitated by adding 1/10 volume of 3 M sodium acetate and 2.5 x volume of ethanol and incubation for at least 1 h at -20°C. After centrifugation (15 min, 10,000 g, 4°C) the pellet was washed with 500 µl of 70% ethanol and resuspended in 20 µl T low E buffer (10 mM Tris, 0.1 mM EDTA, pH 8). 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), 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, 0.5M 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 Roti-Nylon (0.2 µm) transfer membrane (Roth). 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 DIG-labeled probes

For the detection using DIG-labeled probes, prehybridization was carried out with DIG Easy Hyb (Roche) for 1h at 42°C. For hybridization, the DIG-labeled PCR product was denatured in 500 µl prehybridization solution for 10 min at 95 ºC 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. Hybridization was carried out at 42 °C over night. The membrane was then washed twice for 15 min with LSW (low stringency wash, 2x SSC, 0.5% SDS) 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 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 6 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) and, after removal of excess buffer, sprayed with AP substrate (CDPStar, 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 Fuji Medical X-Ray film (FUJIFILM Europe, Düsseldorf) developed with an Optimax TR automatic developing machine (MS-Laborgeraete).

5.3.8 Isolation of total RNA

For Northern Blotting, total RNA was isolated with a modified protocol according to Logemann et al. (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 autoclaving.

Plant material was grinded in deep frozen state using a ball mill (Retsch Mixer Mill MM200) 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).

After thawing, 1 ml PCI (phenol:chloroform:isoamyl alcohol 25:24:1 (v:v:v)) was added, vortexed and centrifuged (10 min, 10,000 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 70% ethanol (over night -20 °C) and on the next day centrifuged (15 min, 4 ºC, 10,000 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 °C, residual insoluble material was removed by centrifugation.

Determination of RNA concentration

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

5.3.9 Northern Blotting

For each gel lane, 10 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% 20x MOPS, 1% bromphenol blue), 0.5 µl EtBr (0.5 mg/ml)) were added. Before loading, the RNA was denatured for 10 min at 65 °C 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 70V in 1x MOPS buffer. After the run was 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 Roti-Nylon (0.2 µm) transfer membrane (Roth). Completion of transfer was confirmed by inspecting the membrane under UV-light. After air drying the membrane, RNA was covalently bound to the membrane by UV crosslinking ("Autocrosslink", UV stratalinker 1800, Stratagene) and subsequently incubated 5 min with low stringency wash buffer (2x SSC, 0.5% SDS). All following steps (except the application of chemiluminescent substrate) were carried out in a Hybridiser HB-1D (Techne) hybridisation incubator. After 60 min prehybridization in prehybridization solution (30% formamide, 1% SDS, 1M NaCl, 6% polyethylene glycol 6000, 250 µg/ml DNA from salmon testes, which was heated beforehand to 90°C) at 42°C, membranes were hybridized with gene specific probes (biotinylated PCR reactions denatured in prehybridization solution for 10 min at 95°C) at 42°C overnight. The membrane was washed twice with low stringency wash buffer (2x SSC, 0.5% SDS) for 15 min at RT and once with high stringency wash buffer (0.2x SSC, 0.5% SDS) for 1 h at 55°C, blocked for at least 40 min in blocking buffer at room temperature, incubated for 40 min with conjugate buffer (Immunopure Streptavidine HRP Conjugate, Pierce; 1:20,000 in blocking buffer) at room temperature and subsequently washed at least 6 times for 5 min with wash buffer. Subsequently, the membrane was incubated 5 min with North2South® Chemiluminescent Substrate (Pierce). Chemiluminescent signals were detected on Fuji Medical X-Ray film (FUJIFILM Europe, Düsseldorf) developed with an Optimax TR automatic developing machine (MS-Laborgeraete).

5.4 Cloning techniques

5.4.1 T/A cloning of PCR products

Cloning of PCR fragments via PCR derived poly-A overhangs was carried out with the Invitrogen Original TA cloning kit (pCR2.1 vector) or the Promega pGEM-T vector System (pGEM-T vector), according to the respective manufacturer's instructions. PCR products were purified in advance with the Nucleospin Extract II kit (Macherey-Nagel) according to the manufacturer's instructions.

5.4.2 Cloning via restriction enzyme digestion

Restriction enzymes were purchased from New England Biolabs (NEB) and used according to the manufacturer's instructions. Usually 4 U per µg plasmid DNA were used, for control digestions for 1 h in a 10 µl volume, for cloning purposes in accordingly up scaled reactions.

Ligation of digested DNA fragments was carried out using T4-DNA-Ligase (NEB). To 100 ng of vector, the digested insert was added in 10 times molar excess, Ligation was carried out in a thermal cycler using aprogram according to Lund et al., consisting of 100 alternating, 30 sec long incubations at 10 ºC and 30 ºC (Lund et al., 1996). Finally, the ligase was denatured for 20 min at 65ºC.

For subcloning of PCR fragments, the TA Cloning Kit (Invitrogen) was used.

5.4.3 Gateway cloning

Gateway cloning was carried out via Gateway compatible attB-PCR products which were purified before BP reactions via precipitation with PEG/MgCl$_2$ solution according to instructions of the Gateway BP Clonase Enzyme Mix (Invitrogen). For BP reactions, 100 ng entry vector and 50 ng purified PCR product were incubated overnight at 25°C with 1 µl 5x BP Clonase Reaction Buffer and 1 µl BP Clonase Enzyme Mix in a total volume of 5 µl (adjusted with TE buffer). BP reactions were stopped by incubation for 10 min at 37°C with 0.5 µl Proteinase K.

LR reactions were carried out analogous with 100 ng destination vector, 100 ng entry clone, 1 µl 5x LR Clonase Reaction Buffer and 1 µl LR Clonase Enzyme Mix.

5.4.4 Cloning of BvC/VIF C-terminal deletion constructs (via Gateway)

BvC/VIF was amplified from the plasmid "p70S-BvC/VIF-luc-can" (provided by J. Eufinger) using the primers 113 and AJ 19. Likewise, the BvC/VIF sequence, missing four, nine or twenty amino acids at the C-terminus was amplified using primer 113 and AJ20, 21 and 22, respectively. The PCR fragments were introduced into pDONR201 and subsequently into pK7FWG2 according to the manufacture's instructions except that recombination reactions were scaled down to one fourth of the recommended volumes.

5.4.5 Cloning of BvVI1 mutants (via Gateway)

BvVI1 was cloned according to Eufinger (Eufinger, 2006). The desired mutation was incorporated into one of the internal primers and a unique restriction site was introduced via silent mutagenesis in both internal primers (primer list, see 5.3.3). After digestion of the fragments with the appropriate restriction enzyme the two fragments were ligated and introduced into pDONR201. Generation of the binary vector pET-G30 was performed as described above.

5.5 Protein techniques

5.5.1 SDS-Polyacrylamide gel electrophoresis

Before SDS polyacrylamide gel electrophoresis (SDS-PAGE), samples were boiled in SDS sample buffer containing a reducing agent (Roti-Load1, Roth) for 5 min at 95°C. Samples were separated together with molecular weight markers (LMW calibration kit, GE Healthcare or Prestained Protein Marker, New England Biolabs) on self-cast SDS polyacrylamide gels. 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 %. The gels were run at 100 V until the samples reached the resolving gel and completed by running at 200 V.

5.5.2 Coomassie staining

For visualization of proteins on SDS polyacrylamide gels via Coomassie staining, gels were incubated after the gel run in Coomassie staining solution (0.2 % Coomasie Brilliant Blue G-250 in 45% (v/v) ethanol and 10% (v/v) acetic acid) for 1 h at room temperature, destained in destaining solution (45 % ethanol, 10 % acetic acid) for 20 min at room temperature and

destained completely overnight in ddH_2O with several facial tissues added to take up excess Coomassie Blue G250.

5.5.3 Immunoblotting

After SDS-PAGE, the resolving gel was incubated in transfer buffer (48mM Tris-base, 39mM glycine, 20% methanol (v/v), 0.0375 % SDS) for 10 min. The protein transfer was accomplished through a "semi-dry" electro transfer, using a conventional semi-dry transfer chamber (Peqlab). On the anode, 3 layers of blotting paper (Whatman 3 MM), moistened in transfer buffer, the membrane (Immobilon-P, Millipore, pre-incubated in methanol and then in transfer buffer), the gel and three additional moistened paper were assembled, taking care to remove trapped air bubbles with an decapped 15 ml Falcon tube. Subsequently, the cathode was placed on top and the transfer was carried out for 43 min at 15 V and 350 mA. After blotting, 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 room 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 was added.) The primary antibody solution was usually incubated over night at 4°C. After incubation in primary antibody solution, the membrane was washed eight times with TBST for 5 min each under vigorous shaking, followed by one hour incubation in the secondary antibody solution (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 10 min in the substrate solution (Super Signal Dura, Pierce), and chemiluminescence was detected by putting the membrane under photographic film (Fuji). Exposure times were adjusted according to signal strength, usually between 30 sec and 30 min. After film exposure, the proteins on the membrane were stained in Amido Black (0.1% Amido Black, 45% ethanol, 10% acetic acid) to analyze protein loading.

Detection of BvC/VIF

For the detection of BvC/VIF affinity purified antiserum raised against recombinant BvC/VIF protein was used (see Eufinger, 2006). The affinity purification was paerformed as described below. The purified antiserum was used in a dilution of 1:1000.

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). The antisera were used in a dilution of 1:20,000.

Detection of green fluorescent protein

For the detection of GFP-fusion proteins, GFP antiserum (Molecular Probes A6465) was used in a 1:10,000 dilution.

Affinity purification of antisera

Due to the presence of multiple immunosignals in immunoblots with plant extracts from sugar beet, the BvC/VIF-antiserum was affinity purified against recombinant BvC/VIF protein.

500 µg of recombinant protein was loaded on a SDS gel and transferred on a membrane via "semi-dry" Western Blotting, as described previously. The membrane was colored with Ponceau S Staining Solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid).The colored protein band was cut off. After a TBST washing step the membrane piece with the transferred protein 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 room temperature. 2 ml antiserum against BvC/VIF were mixed with 13 ml blocking solution. In this solution the membrane was incubated overnight at 4ºC. After 5 times washing with TBST bound antibodies were eluted by pipetting 1ml elution buffer (500 mM NaCl, 5 mM Glycine, pH 2, 8) onto the membrane. The eluted antibodies were immediately neutralized by addition of 1/10 volume of 2 M Tris, pH 8.5. The elution was repeated. For stability reasons, 0.1 mg/ml BSA and 0.02% NaN$_3$ were added to the eluted antibodies.

5.5.4 Purification of recombinant inhibitor and invertase proteins

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

The BvC/VIF-coding sequence without the predicted signal peptide was cloned by Eufinger (2006) into the pQE30-vector (Qiagen) which leads to the expression of the protein in fusion with an N-terminal 6xHis-Tag. The vector was transformed into the *E.coli* strain Rosetta-gami (Novagen). In a typical purification of the recombinant BvC/VIF 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 37ºC until OD_{600nm} reached 0.8 to 1.0. After cooling the culture to 18 ºC expression was induced by addition of 0.5 mM IPTG. Bacterial cells were harvested after 18 to 22 hours at 18 ºC and 180 rpm by centrifugation, resuspended in 200 ml wash buffer (500 mM NaCl, 50 mMNaPO$_4$, 10% glycerol, pH 7.5) containing 10 µg/ml DNAseI and lysed with an Emulsifier (EmulsiFlex-C5, Avestin) at 70 to 100 MPa. Insoluble protein was removed by centrifugation (22.000 g, 45 min, 4ºC) and the supernatant was applied to an IMAC-column filled with 2 to 3 ml Ni-TED matrix (Protino Ni-TED, Macherey- Nagel). The column was washed with 300 ml wash buffer and the purified protein was eluted in six 2 ml fractions with elution buffer (500 mM NaCl, 50 mM NaPO$_4$ pH 7.5, 10% Glycerol, 250 mM Imidazole). BvC/VIF-containing fractions were usually dialyzed into an acidic buffer for activity testing (50 mM citric acid, 300 mM NaCl, pH 5). Proteins precipitated during dialysis were removed by centrifugation. For prolonged storage (> 3 days), the purified protein was frozen in liquid nitrogen and stored at –80 ºC without substantial loss of activity.

Purification of recombinant BvVI1 from *E. coli*

As described in Eufinger (2006), the BvVI1 protein was amplified from *Beta vulgaris* cDNA and introduced into the pETG30 vector (providing a N-terminal 6xHis- and GST-tag, EMBL, Heidelberg). The expression and purification using Nickel-resins was carried out as described above for BvC/VIF, 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 3 mM GSH+ 0,3 mM GSSG were added to the dialyzed protein and incubated for 4 h at 30ºC. Subsequently 1x vol. of wash buffer (500 mM NaCl, 50 mM NaPO$_4$, 10% glycerol, pH 7.5) was added and the sample was passed over 1 ml of Ni-TED 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. 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 citric acid, 300 mM NaCl, pH 5).

5.5.5 Extraction of soluble and cell wall proteins

Approximately 500 mg of grinded leaf or taproot material were resuspended in 1000 µl of extraction buffer (50 mM citric acid, 250 mM sorbitol, 10 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, pH 4) and after thorough vortexing, centrifuged at 10,000 g in a table top centrifuge. All steps were carried out at 4ºC. 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 was discarded and 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 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 1 hour at 4 ºC 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 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 ºC the sample was centrifuged and the protein pellet was resuspended in 2x SDS sample buffer.

5.5.6 Non-invasive salt-elution from *Beta vulgaris* hairy roots and taproot slices

Hairy roots

For the elution of ionically bound proteins from the cell walls of intact hairy root cells, the medium was removed 21 days after transfer to fresh medium by filtration. After washing hairy roots (approximately 2 g) were transferred to 50 ml citric acid buffer (50 mM citric acid, pH 5) plus 500 mM NaCl and gently stirred at 4ºC for 1 h. The hairy roots were removed and the supernatant was acetone precipitated. The resulting pellet was taken up in SDS-sample buffer for immunoblot analysis or in 0.1 M Tris-HCl, pH 8.0, followed by dialysis against the same buffer, for subsequent G6PDH activity measurement.

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Taproot slices

As described in "wounding of sugar beet taproots" cylinders (2 cm in diameter) from the taproot interior were removed with a cork borer. Subsequently, very thin slices (0.2 mm) were cut with a razor blade. After washing, the slices were incubated in citric acid buffer plus 500 mM NaCl and the extracts treated as described for hairy roots.

5.5.7 Immunofluorescence localization of BvC/VIF

As described in 5.5.6, very thin slices were cut from *Beta vulgaris* wild type and transgenic adventitious roots. The hand cut slices were fixed in 4% paraformaldehyde in PBS (pH 7,2) containing 300 mM mannitol, for 1 h and rinsed six times for 5 minutes each in PBS buffer pH 7,2. Subsequently, they were incubated in blocking solution (2% BSA in PBS, pH 7.2, sterile filtrated) for 1h. The slices were then incubated with affinity purified antibody directed against BvC/VIF diluted 1:20 in PBS (+ 0.05% Tween) overnight at 4 °C. After six 5 min washing steps with PBS (+0.05% Tween), the slices were incubated with biotin labeled goat anti-rabbit antibody (Sigma) diluted 1:200 in PBS for 2 hours at room temperature. After six rinses in PBS for 5 minutes, the slices were incubated for 30 minutes in streptavidin labeled Cy3 conjugate (Dianova, diluted 1:500 in PBS). After rinsing the slices six times in PBS, they were mounted on glass slides in DABCO (1,4-Diazabicyclo(2,2,2) octan) solution and analyzed by fluorescence microscopy.

5.5.8 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 $MnCl_2$, 500 mM NaCl, 1 mM PMSF (added freshly), pH 5). After vortexing, the extracts were incubated for 1 hour at 4 ºC, using an overhead-shaker. After subsequent centrifugation (4000g, 5 min, 4 ºC), the supernatant was added to ConA-sepharose (equilibrated in the same buffer). The incubation of 1h at RT took place in a 2 ml reaction tube. The suspension was centrifuged and proteins, not glycosylated via high mannose chains were found in the supernatant (ConA minus fraction). The ConA matrix was washed twice with 2 ml of ConA buffer and the bound proteins were eluted by addition of 500 µl ConA buffer + 15% methyl-α -D-glucopyranoside, subsequently representing the ConA plus fraction.

5.6 Enzyme activity assays

5.6.1 Measurement of soluble and cell-wall bound invertase activity

To each 500 mg of grinded plant material 1000 µl of extraction buffer (see 5.5.5) were added and the sample was vortexed vigorously. The soluble proteins were collected by centrifugation at 8.500 g at 4ºC. 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 1000 µl extraction buffer and used directly for the determination of invertase activity.

For the measurement of soluble invertase activity from sugar beet taproots and adventitious roots, endogenous sucrose was removed by acetone precipitation of the soluble fraction with 4 vol. of ice-cold acetone and incubation for 20 min at -20ºC. After centrifugation (10,000 g, 10 min, 4 ºC) the pellet was resuspended in 500 µl extraction buffer.

For the determination of acid invertase activity, 30 to 100 µl (depending on activity of sample) of the obtained preparations were incubated with 100 mM sucrose (solute in citric acid buffer, 50 mM, pH5) and citric acid buffer up to 300 µl. After 1 h at 37 ºC the reaction was stopped by the addition of 30 µl 1 M sodium phosphate, pH 7.5 and heating to 95 ºC for 5 min. For every assay, four replicates were prepared, of which one was neutralized and boiled immediately after sucrose addition. This value was subtracted from the others as background absorption. 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 Dehydrogenase 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 340nm and the liberated glucose was calculated using Lambert-Beer law ("NADPH 340nm =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).

5.6.2 Functional assay of recombinant invertase and inhibition through BvC/VIF

Invertase activity

The invertase activity of different protein amounts of recombinant BvVI1 wild type and Cys399Ser mutant were tested in 300 µl citric acid buffer (50 mM, pH 5), containing 100 mM sucrose as substrate. The assay was incubated for 1h at 37ºC. The reaction was stopped by adding 30µl 1 M sodium phosphate, pH 7.5 and heating to 95 ºC for 5 min. Liberated glucose was calculated as described in 5.6.1.

Inhibition through BvC/VIF

To test the inhibition of BvVI wild type and Cys399Ser mutant through BvC/VIF, variable amounts of recombinant BvC/VIF were added to the recombinant invertases in citric acid buffer (50 mM, pH 5) in a total amount of 200 µl and incubated for 30 min at RT to allow complex formation. Then, 100µl of 300 mM sucrose in the same buffer were added and incubated for 60 min at 37ºC. The action was stopped by neutralization with 30 µl 1 M $NaPO_4$ and boiling for 5 min at 95 ºC. The amount of glucose released was measured as described in 5.6.1.
In each experiment samples without inhibitor proteins were included.

Invertase activity and inhibition at different pHs

All assays were performed as described before, but with citric acid buffer ranging from pH 4.5 to pH 6.5.

5.6.3 Glucose-6-Phosphat-Dehydrogenase activity assay

900 µl G6PDH-assay buffer (0.1 M Tris-HCl, pH 8, 0.2 mM NADP, 2 mM glucose-6-phosphat) were mixed with 100 µl extract and the absorption (340 nm) was measured immediately, every 90 sec. Reactions without substrate (Glc-6-P) and immediately boiled reactions were taken as controls. Transformed Glc-6-P is represented by NADPH, displaying its absorptions maximum at 340 nm. Glc-6-PDH activity can be determined using Lambert-Beer law (as described previously). Before the activity assay, protein extracts were dialysed against 0.1 M Tris-HCl, pH 8.

5.7 Determination of soluble sugars

For the extraction of soluble sugars, taproot and adventitious roots 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 80ºC. After centrifugation (5 min, 10,000 g, RT), the extraction was repeated and both supernatants were combined and stored at -20 ºC.

For the determination of sucrose, extracts were usually diluted 1:20 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 20 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-phosphatedehydrogenase (700 U/ml, Roche), 0.4 µl hexokinase (1500 U/ml, Roche) and 155.2 µl reaction buffer (100mM imidazole, 3 mM $MgCl_2$, pH 6.9). The plate was inserted into the platereader, shaken vigorously and after 15 min background absorption (abs 1) 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 (abs 2). 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 abs 2.

Measurement of hexoses

For the measurement of glucose and fructose from wounded taproot tissue, 20 µl of diluted 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 MgCl2, pH 6.9). After determination of background absorption (abs 1), 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 (abs 2) was measured. For the determination of fructose, 4 µl of phosphoglucoisomerase (Roche, 44 U/ml, diluted in reaction buffer) was added and absorption (abs 3) was determined after mixing and incubation for 30 min. For the calculation of glucose, abs 1 was subtracted from abs 2, and for fructose, abs 2 was subtracted from abs 3. 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.

5.8 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 auto fluorescence: excitation: 488 nm; detection: longpass 650 nm.

5.9 Plant transformation

5.9.1 Transient expression by Agrobacteria leaf infiltration

Agrobacterium tumefaciens cells (strain C58 C1) were grown overnight in 30 ml of YEB-medium supplemented with Carbenicillin (50 µg/ml), Rifampicin (100 µg/ml) and Spectinomycin (50 µg/ml) until stationary phase. After centrifugation at 3.000 g for 30 minutes at room temperature the cells were suspended in 10 - 15 ml of infiltration buffer (10 mM MES, pH 5.9, 150 µM acetosyringone) and incubated with gentle agitation for 2 hours. The cell-suspensions were adjusted to OD 1 with infiltration buffer and infiltrated into the lower epidermis of 8 - 12 week old *Nicotiana benthamiana* leaves with 1 ml syringe. Leaf proteins for analysis were extracted 48h after infiltration if not indicated otherwise.

6. Abbreviation index

6x His	hexa-histidine tag
abs	absorption
Ac	Achinidia chinensis
Acc	Accession
AG	Aktiengesellschaf
A/NI	alkaline/ neutral invertase
APS	ammonium peroxodisulfate
At	*Arabidopsis thaliana*
bp	base pairs
BSA	bovine serum albumin
Bv	*Beta vulgaris*
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
Ci	*Cichorium intybus*
CIF	cell wall inhibitor of ß-fructosidase
CLSM	confocal laser scanning microscopy
C-Terminal	carboxy-terminal
C/VIF	cell wall and/or vacuolar inhibitor of ß-fructosidase
ConA	Concanavalin A
CWI	cell wall invertase
DABCO	1,4- Diazabicyclo (2,2,2) octan
DEPC	diethylpyrocarbonat
DMSO	dimethyl sulfoxide
dNTP	desoxynucleotidetriphosphate
DTT	dithiothreitol
E	Einstein
EtBr	Ethidium bromide
FEH	plant fructan exohydrolase
FEB	plant fructan biosynthetic enzyme
GFP	green fluorescent protein
GH	glycosyl hydrolase
GSH	glutathione
GSSG	oxidized glutathion

GST	glutathione-S-transferase
HR	hypersensitive response
HRP	horse radish peroxidase
IMAC	immobilized metal ion affinity chromatography
Inv	invertase
IPTG	Isopropyl-beta-D-thiogalactopyranosoid
kB	kilo base pairs
kDa	kilo Daltons
M	molar (1 M = 1 mol/l)
mRNA	messenger RNA
MS	Murashigge-Skoog (medium)
MW	molecular weight
nptII	neomycin phosphotransferase II
Nt	*Nicotiana tabacum*
NtCIF	Nicotiana tabacum cell wall inhibitor of β-fructosidase
N-terminal	amino-terminal
ODx	nm optical density at x nm wavelength
PAA	polyacrylamide
PCR	polymerase chain reaction
pH	negative decadic logarithm of [H+]
PH	phloem
pI	isoelectric point
PME	pectin methylesterase
PMEI	pectin methylesterase inhibitor
PMEI-RP	pectin methylesterase inhibitor-related proteins
PPVs	precursor protease vesicles
PR	pathogenesis related
qRT-PCR	quantititative real-time polymerase chain reaction
RFO	Raffinose family oligosaccharide
RFP	red fluorescent protein
ROS	reactive oxygen species
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamidide gel electrophoresis
SE	standard error
SuSy	sucrose synthase

SUT	Sucrose transporter
TEV	tobacco etch virus
tDNA	transfer DNA (of Agrobacterium)
TEMED	N,N,N,N'-Tetramethyl-ethylenediamine
TMV	tobacco mosaic virus
UDP	uridine diphosphate
UTR	untranslated region
VI	vacuolar invertase
VIF	vacuolar inhibitor of ß-fructosidase
Vol	volume
WT	wildtype
w/v	weight per volume

7. Literature

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