Subcellular Distribution, Regulation of the Synthesis and Functions of Raffinose-Oligosaccharides in *Ajuga reptans* (Lamiaceae)

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This work is dedicated to my husband, who always believed in me
and supported me all time!

Content

1 Introduction

Plants are able to synthesize carbohydrates and oxygen from carbon dioxide and water. This process is called photosynthesis and takes place in the green leaves of a plant. In the photosynthesizing mesophyll cells these carbohydrates are then converted to different compounds necessary for metabolic processes in different parts of the plant.

To those carbohydrates belong mono-, di- and oligosaccharides as well as sugar alcohols. Sugars are polyvalent alcohols with a reactive carbonyl function and are therefore hydroxylaldehydes (aldoses) or hydroxylketones (ketoses). This carbonyl group is either a stable acetal or hemiacetal. Mono-, di- and oligosaccharides are water soluble sugars and are able to form ring structures. Glucose, fructose and galactose are monosaccharides with the general formula $C_6H_{12}O_6$. They have five hydroxyl groups (−OH) and a carbonyl group (C=O). Sucrose, maltose, and lactose are all compound sugars, disaccharides, with the general formula $C_{12}H_{22}O_{11}$. They are formed by the combination of two monosaccharide molecules with the exclusion of a molecule of water. Oligosaccharides are saccharide polymers containing a small number of monosaccharides. One important group of oligosaccharides in plants is those of the raffinose family (RFO) (see 1.1).

As response to different abiotic stress factors, like temperature, light and drought, many plants accumulate those soluble sugars (Klotke et al. 2004, Schneider and Keller 2009). *Ajuga reptans*, member of the Lamiaceae, is such a plant. It translocates RFOs during the whole year and accumulates raffinose, stachyose and verbascose in the winter months (Bachmann et al. 1995).

1.1 Raffinose family Oligosaccharides

1.1.1 Synthesis of RFOs

Raffinose oligosaccharides are α-1,6-galactosyl$_n$ extensions of sucrose which differ in the number of galactosyl moieties (n). Galactinol (1L-1-*O*-(α-D-galactosyl)-myo-

Inositol) serves as a donor of galactosyl moiety in the reactions of raffinose and stachyose synthesis. Galactinol is synthesized by galactinol synthase (UDP-galactose:myo-Inositol galactosyltransferase, EC 2.4.1.123) from UDP-galactose and myo-Inositol. The first member of RFOs, raffinose (degree of polymerization (DP) =3) is synthesized via galactosylation of sucrose by raffinose synthase (galactinol:sucrose 6-α-D-galactosyltransferase, EC 2.4.1.82; RS). The second member of RFOs, stachyose is formed via galactosylation of raffinose by stachyose synthase (galactinol:raffinose 6-α-D-galactosyltransferase, 2.4.1.67; STS) (Peterbauer et al. 2002) (see Figure 1.4). Further extensions of the chain of galactosyls lead to the formation of other members of RFOs and can polymerize to a grade of 15 (Haab and Keller 2002). Probably, the biosynthesis of RFOs takes place in the cytoplasm, since all precursors and enzymes are cytosolic (Keller 1992, Bachmann and Keller 1995, Schneider and Keller 2009).

Like sucrose, RFOs are non-reducing sugars, which are less reactive and therefore more suitable for translocation in the phloem sap. But they are also used for carbon storage and abiotic stress tolerance (Nishizawa et al. 2008) (see 1.1.4).

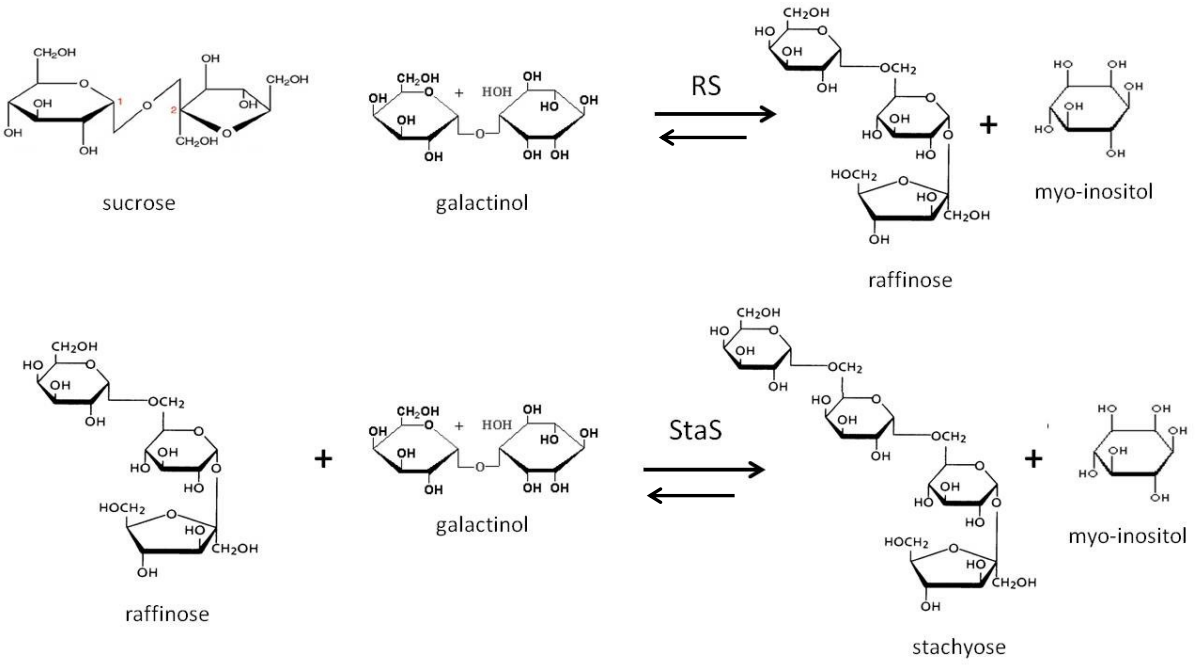


Figure 1.1 Biosynthesis of raffinose and stachyose. RS = Raffinose Synthase; STS = Stachyose Synthase (S.Findling)

1.1.2 Stachyose-Synthase

The biosynthesis of RFOs is a stepwise extension. Every reactions step requires a particular enzyme. The first synthesis step, the formation of raffinose and myo-inositol from sucrose and galactinol, is catalyzed by the raffinose-synthase, the next step is the synthesis of stachyose and myo-inositol from raffinose and galactinol by the enzyme stachyose-synthase (EC 2.4.1.67) (Gaudreault and Webb 1981). Further extensions take place in the vacuole conducted by a galactan:galactan galactosyl transferase (GGT) that transfers galactosyl residues from one oligosaccharide to another, resulting in a lower DP of RFO and in a higher DP of RFO (Bachmann and Keller 1995, Sprenger and Keller 2000) (see 1.1.4). GGT is localized as a soluble enzyme only in the vacuole (Bachmann and Keller 1995, Haab and Keller 2002).

It is assumed that only mature leaves are able to synthesize and export stachyose and young, developing leaves do not show stachyose-synthase (STS) activity, what lead to the supposition that STS-activity depends on the plant developing state (Holthaus and Schmitz 1991). Stachyose synthesis is separated and found at two sites in the leave: in the ICs of the minor vein phloem (Holthaus and Schmitz 1991, Beebe and Turgeon 1992) and in the cytosol of leaf mesophyll cells (Beebe and Turgeon 1992). These two synthesis locations are associated with the function and the ‘place of action’ of stachyose, the transport pool (ICs) on the one hand and the storage pool (mesophyll) on the other hand (Bachmann and Keller 1995). So far, STS has a similar pH-optimum of 7.0 (Tanner and Kandler 1966, Peterbauer and Richter 1998, Hoch et al. 1998) or 6.8 (Holthaus and Schmitz 1991) in all analyzed plants. In addition to raffinose STS can use other metabolites like D-pinitol (Hoch et al. 1998) as substrate for a transferred galactosyl residue.

1.1.3 Subcellular distribution of RFOs

To obtain a subcellular localization of sugars several methods have been applied. For analyzing single compartments the isolation of vacuoles, chloroplasts and protoplasts is very common (Bachmann and Keller 1995, Schneider and Keller 2009). The isolation of protoplasts is accomplished by enzymatic dissolving of the cell wall. This process can take several hours. Vacuoles in turn, are obtained from isolated protoplasts by selective rupture of the plasma membrane (Bachmann and Keller

1995). Chloroplasts are also obtained from isolated mesophyll protoplasts by mechanical fracturing of the plasma membrane (Schneider and Keller 2009). The disadvantage of these processes is that it takes a few hours until metabolites can be measured and during that time metabolite transport at the membranes cannot be excluded, e.g. hexoses could be released from vacuole.

Gerhardt and Heldt (1984) developed a method where plant material is shock-frozen in liquid nitrogen and solved in a nonaqueous medium. In such a medium metabolites are attached to the membrane of the different organelles and can be separated using the different densities of these compartments and by making a density gradient. This method makes the analysis of the three compartments vacuole, chloroplast and cytosol possible and has the big advantage that all metabolic processes are stopped in seconds at the steady state of photosynthesis.

The accumulation of water-soluble carbohydrates is one of the most commonly observed responses of plants to cold or freezing conditions. Changes in the subcellular concentration and distribution of sugars might provide a mechanism to protect specific compartments. Subcellular metabolite partitioning is already done for a number of plants, e.g. spinach (Riens et al. 1991), barley (Winter et al. 1992), tobacco (Heineke et al. 1994), *Plantago* (Nadwodnik and Lohaus 2008), or *Arabidopsis* (Knaupp et al. 2011; Nägele and Heyer 2013), but subcellular localization of raffinose was mainly done for *Arabidopsis* (Iftime et al. 2011; Knaupp et al. 2011; Nägele and Heyer 2013) and only very few reports described the subcellular localization of stachyose (Voitsekhovskaja et al. 2006; Iftime et al. 2011) and probably none the localization of verbascose. *Ajuga reptans* has been object to such studies as well, but only on isolated chloroplasts (Schneider and Keller 2009), vacuoles (Bachmann et al. 1995) or protoplasts (Schneider and Keller 2009).

1.1.4 Functions of RFOs

1.1.4.1 Transport and storage

Plants have the ability to transport and store carbon. Classically, they do this by using sucrose for translocation and starch for storage. Among plants alternative carbohydrates for those purposes exist, with RFOs being the most prominent ones

(Kandler and Hopf 1982, Keller and Pharr 1996). RFOs being used for both, one distinguishes between a transport and a storage pool (Sprenger and Keller 2000). Symplastic loading plants such as members of the Cucurbitaceae, Lamiaceae and Scrophulariaceae mainly translocate raffinose and stachyose in addition to sucrose (Turgeon and Gowan 1992). They have specialized companion cells, so called intermediary cells (ICs), with numerous branched plasmodesmata between bundle sheath cells (BSCs) and ICs (Fisher 1986; Turgeon and Medville 2004). Furthermore ICs are large and densely cytoplasmic with a lot of vacuoles (Turgeon et al. 1993). Figure 1.2 shows that sucrose, a precursor of RFOs, is produced in the mesophyll cells and diffuses from the BSCs through plasmodesmata to the ICs. In these cells the RFO-synthesis of the transport pool takes place. RFOs are larger in size and are thereby probably unable to diffuse back into the BSCs. Furthermore, the continuous conversion of sucrose into RFOs leads to a concentration gradient in such a way that sucrose is always replenished. This model is known as “polymer trap” (Turgeon and Gowan 1990). The trapped RFOs accumulate in the phloem creating a concentration gradient (Rennie and Turgeon 2009; Slewinski et al. 2013). Symplastic loading is an active but little energy consuming loading mechanism (Gamalei 1989).

Another possibility is a combined phloem loading mechanism. Plants using that way for solute translocation possess ordinary CCs as well ICs (Gamalei 1991). Some sucrose is actively loaded in the CCs from the apoplast via SUTs and some is loaded via plasmodesmata into ICs to be synthesized to RFOs. That mechanism is already described for *A. meridionalis* (Knop et al. 2004, Voitsekhovskaja et al. 2009).

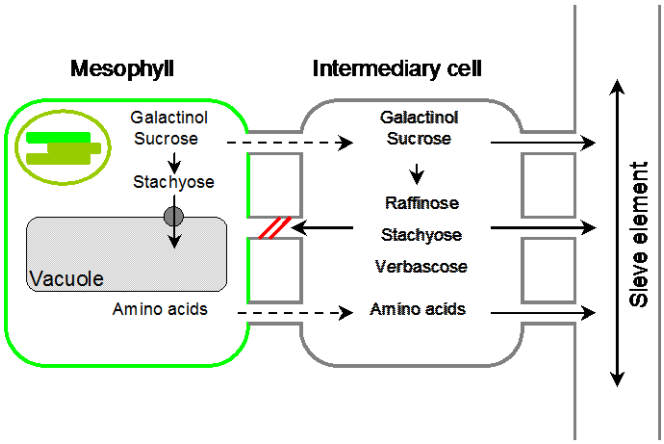


Figure 1.2 symplastic phloem loading (Lohaus)

Furthermore, some plants store RFOs in large amounts in specialized storage organs such as tubers (*Stachys siboldii*), seeds (soybean) or in photosynthesizing leaves (*Ajuga reptans*) (Handley et al. 1983, Keller and Matile 1985, Bachmann et al. 1995). In leaves storage RFOs accumulate in the mesophyll (Bachmann et al. 1995). The storage pool in leaves of *A. reptans* consists additionally to raffinose and stachyose of high-DP RFOs, which are synthesized by GGT (see 1.4.2) (Sprenger and Keller 2000). Although raffinose and stachyose are synthesized in the cytoplasm, stachyose and higher RFOs are stored in the vacuole of mesophyll cells of *A. reptans* (Bachmann et al. 1995). Carbohydrate storage serves for a remobilization to advance growth in spring time (Peters and Keller 2009). From late summer on *A. reptans* stores RFOs (predominantly stachyose and verbascose), but with the end of winter this pool decreases in favor for foliating and growth (Peters and Keller 2009).

1.1.4.2 Stress response

The accumulation of non-reducing water soluble carbohydrates, especially RFOs, is a very widespread response of plants to abiotic stresses (Klotke et al. 2004, Schneider and Keller 2009), like cold temperatures, desiccation or light deficit, for example. In *Arabidopsis thaliana* an increased amount of galactinol and raffinose was observed in cold- or drought-stressed plants (Taji et al. 2002, Zuther et al. 2004, Klotke et al. 2004, Nishizawa et al. 2008). But also for *A. reptans* were increased concentrations of raffinose in conjunction with frost tolerance reported (Bachmann et al. 1994).

Raffinose has the ability to build hydrogen bonds to biomolecules like proteins, and by placing water molecules between the polar head groups of phospholipids in membrane bilayers, it can stabilize membranes directly (Crowe et al. 1996). Raffinose can even serve at low concentrations very effectively as stabilizer for membranes and whole cells (Gaffney et al. 1988, Bachmann and Keller 1995). Furthermore, raffinose found in thylakoid membranes of chloroplasts can reduce the inactivation of the electron- and cyclic photophosphorylation in photosynthesis under freezing, drought and heat stress conditions (Santarius 1973). The ability of a plant to change its metabolism in favor for freezing tolerance is called cold acclimation (see 1.1.4.3).

In various subcellular compartments like chloroplasts and peroxisomes reactive oxygen species (ROS) are generated during an excess of light and increased photosynthesis activity (Asada 2006). RFO sugars have been proposed to fulfill important roles in oxidative stress defense (Nishizawa et al. 2008). Accumulated RFOs in chloroplasts and vacuoles might directly detoxify ROS (van den Emde and Valluru 2009).

Polymerization and disassembling of RFOs can change the osmotic potential of the vacuole and therefore may change the turgor pressure. Thus, RFOs with a high degree of polymerization (e.g. verbascose) may also serve as osmotic regulators (Bachmann and Keller 1995).

Furthermore, it has been observed that during seed maturation RFOs are accumulated concurrently with the reduction of tissue water content and the development of desiccation tolerance of seeds (Peterbauer and Richter 2001). It has been proposed that raffinose and sucrose are involved in cytoplasmic vitrification in dry seeds, thereby stabilizing sensitive macromolecular structures (Zuther et al. 2004). In seeds of cucumber, lupin and soybean, the maturation temperature had little effect on the concentration of RFOs (Widders and Kwantes 1995, Górecki et al. 1996, Obendorf et al. 1998). Even less information is available on the effect of drought stress on the RFO accumulation, but it seems that the effect of environmental stresses on RFO metabolism in seeds is rather small (Peterbauer and Richter 2001). But RFOs also provide easy available energy and substrates to support growth and germination of seeds (Peterbauer and Richter 2001).

1.1.4.3 Effects of temperature and cold acclimation

During low temperatures, photosynthesis is down regulated, implicating reduced rates of phloem transport and an increased accumulation of carbohydrates in the mesophyll cells (Gamalei et al. 1994). Temperatures of 10°C and lower influence the ultrastructure and sugar metabolism of leaf cells and this in turn can have effect on the phloem loading mechanism (Gamalei et al. 1994, Gamalei 1991). Some plants posses a basic freezing tolerance, which is the ability of plants to survive cold without prior acclimation (Stone et al. 1993) and is important to withstand sudden temperature changes (Klotke et al. 2004). Transport of photosynthate out of the

chloroplast and export for phloem loading may be inhibited by chilling temperatures as a result of insufficient energy requiring membrane transport (Kratsch and Wise 2000).

Many temperate and perennial plant species can grow at low temperature and even survive freezing. Exposure to low but nonfreezing temperatures induces a multifaceted and complex process termed cold acclimation by which plants are able to increase their cold tolerance. During cold acclimation, numerous genetic, physiological and biochemical changes occur enabling plants to tolerate temperatures several degrees lower than before cold exposure (Strand et al. 1997; Xin & Browse 2000; Stitt and Hurry 2002; Hannah et al. 2006; Espinoza et al. 2010).
In *Arabidopsis* the RFO pathway is induced during cold acclimation through transcriptional activation of several galactinol synthase (GS) genes. This activation is mediated by the so-called 'CBF family' of transcription factors (C-repeat binding factors), which cause a lot of different low temperature responses that are considered to be important for cold acclimation (Gilmour et al. 2000).

Reprogramming of the central carbohydrate metabolism and concentrations of soluble sugars was shown to play a crucial role during cold acclimation (Scarth and Levitt 1937; Koster and Lynch 1992; Strand et al. 1997). Compatible solutes may act either nonspecifically as osmolytes or as stabilizers for proteins and membranes during freezing (Knaupp et al. 2011). An increase of RFOs, especially raffinose concentration and also sucrose concentration were observed in plants like cabbage, *Ajuga reptans*, saltgrass or *Arabidopsis* exposed to low temperature (Santarius and Milde 1977, Bachmann et al. 1994, Shahba et al. 2003, Klotke et al. 2004). It was demonstrated that exogenous sucrose at high concentrations has a cryoprotective effect on cellular membranes (Uemura and Steponkus 2003) and also raffinose might function in protecting membranes at low temperatures (Schneider and Keller 2009). However, the accumulation of soluble sugars during cold exposure is insufficient to fully explain the process of cold acclimation (Hincha et al. 1996; Zuther et al. 2004).

1.2 Sucrose and Sucrose Uptake Transporters (SUTs)

Together with the accumulation of RFOs is the accumulation of sucrose a common response of plants to abiotic stresses (Santarius 1973, Guy et al. 1992, Nishizawa et al. 2008, van den Emde and Valluru 2009). For example, sucrose has compared to other sugars the strongest antioxidant capability (van den Emde and Valluru 2009). Similar to RFOs, an increased sucrose content should be involved in cold acclimation as already observed in Arabidopsis (Nägele and Heyer 2013), spinach (Guy et al. 1992), ivy (Steponkus and Lanphear 1968) and citrus (Guy et al. 1980). Sucrose accumulates in addition to raffinose in chloroplasts during acclimation, which points toward a direct protection of plastid structures (Knaupp et al. 2011). But the protecting function of sucrose against freeze induced damage is assumed to be less effective than that of the trisaccharide raffinose, because sucrose takes up less space between the polar head groups of the phospholipid bilayers than the larger raffinose (Knaupp et al. 2011). In addition to its direct function in frost protection, sucrose serves also as precursor for RFOs that are proposed to fulfill important roles in stress defense (see 1.4.4.2 and 1.4.4.3). Besides this, sucrose is also part of the carbon storage pool in vegetative parts of the plant or in seeds (Peterbauer and Richter 2001) and it is translocated in the phloem of apoplastic and symplastic loading plants.

For apoplastic phloem loading sucrose, produced in the mesophyll cells, is translocated into the apoplast and then into the minor vein phloem via H^+/sucrose-transporter (Fig. 1.3). This results in a higher sucrose concentration in the phloem than in mesophyll and companion cells. Two types of CCs are accompanying this loading type: ordinary CCs with no specialization and TCs. TCs have numerous cell

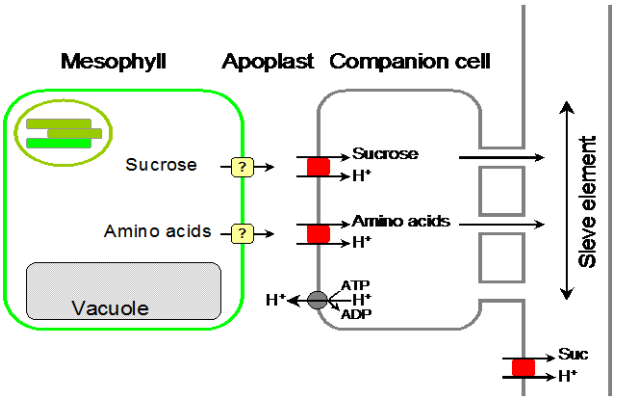


Figure 1.3 Apoplastic phloem loading (Lohaus)

wall ingrowths that increase the surface to accommodate more membrane sitting transport proteins (e.g. sucrose transporter) (Amiard et al. 2005) and only few plasmodesmal conjunctions (Turgeon et al. 1993).

The sucrose transport happens against a concentration gradient and is therefore a thermodynamically active step using the proton gradient as energy source (Slewinski et al. 2013) and involves membrane-localized sucrose transporters (SUTs) (Sauer 2007).

SUT genes are members of the major facilitator superfamily, with 12 membrane spanning domains, the N- and C-termini on the cytoplasmic side of the membrane and a central cytoplasmic loop (Sauer 2007, Ayre 2011). Historically, SUT genes were named in the order in which they were identified; hence, SUT1 in one species was orthologous to SUT5 in another. Furthermore, in Arabidopsis and several other plants, some SUT genes are named SUC for sucrose carriers (Slewinski and Braun 2009). According to Reinders et al. (2012) three different groups of SUTs were here distinguished (Fig. 1.4): type 1 SUTs are only found in eudicots and are essential for phloem loading and unloading (Riesmeier et al. 1994, Gottwald et al. 2000), type 2 SUTs are subdivided into A and B, whereas A is probably an ancestral form of type 2 SUTs that are found in angiosperms (Reinders et al. 2012) and B are monocot specific SUTs that are involved in phloem loading (Slewinski et al. 2009, Reinders et al. 2012), and type 3 SUTs are so far found to be localized at the vacuolar membrane (Endler et al. 2006, Reinders et al. 2008) and might function in sucrose uptake into the cytoplasm from the vacuolar lumen, what has been demonstrated for AtSUT4 from Arabidopsis vacuoles (Reinders et al. 2008, Schulz et al. 2011).

In plants, whole SUT gene families have been detected. The Arabidopsis genome contains nine SUT-like genes including two pseudogenes from three different groups (Sauer et al. 2004) and the rice genome contains five SUT homologues genes, also from different groups (Aoki et al. 2003).

Despite the SE/CC-complex of source leaves, SUTs of group 1 are also expressed in different sink tissues (AtSUC1, Stadler et al. 1999; VfSUT1, Kühn 2003; PmSUC1 Lauterbach et al. 2007). It is assumed that phloem loading SUTs are also responsible for phloem unloading and the retrieval from the extracellular space (Carpaneto et al. 2005, Sauer 2007).

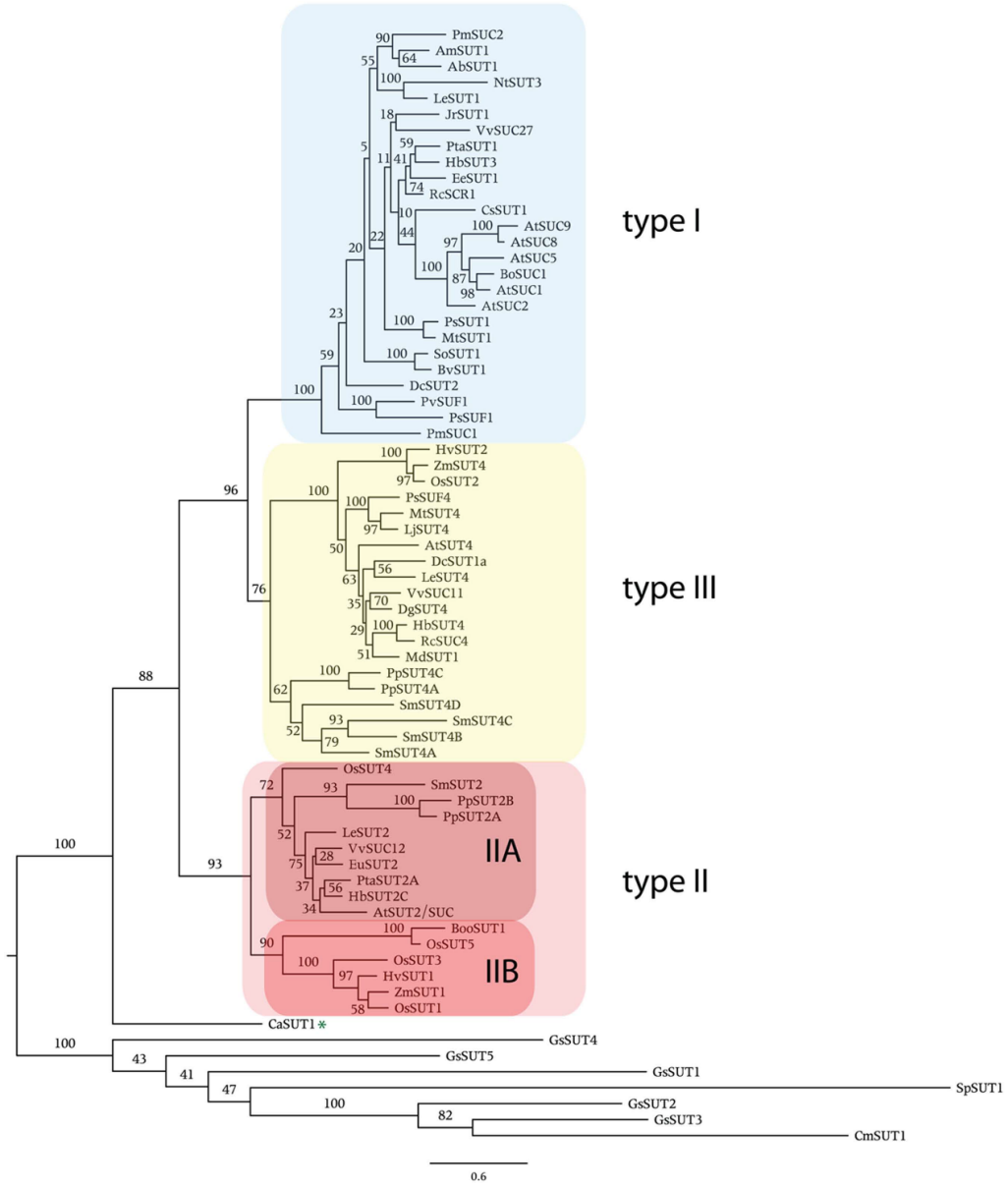


Figure 1.5 Phylogentic analysis of plant sucrose transporters and homologs (from Reinders et al. 2012). Protein alignment was done using Clustal X. The variable length N-and C-terminal regions were trimmed from the alignment. The maximum likelihood tree was generated using PhyML 3.0. Numbers indicate percent of the 100 bootstrap analyses.

Sucrose produced in excess in the light period is transported into the vacuole for temporary storage. The transmembrane distribution of sucrose is catalyzed by a group 3 sucrose transporter (SUT4) (Sauer 2007, Ayre 2011). Several SUT4 transporters are localized in the tonoplast, which is already shown for Arabidopsis, melon, tomato or wheat (Endler et al. 2006, Schneider et al. 2012, Deol et al. 2013). Earlier studies assigned SUT4 transporters also a role phloem loading (Shakya and

Sturm 1998, Weise et al. 2000, Kühn 2003), but recent studies did show that SUT4 transporters only regulate the sucrose uptake from the vacuole into the cytoplasm (Reinders et al. 2008, Schneider et al. 2012, Schulz et al. 2011, Deol et al. 2013). Sucrose is also essential for sink specific metabolism to drive growth and development. That means sinks might retain vacuolar sucrose concentration low and SUT4 transporters might re-export sucrose that had entered the vacuole to the cytoplasm (Schneider et al. 2012).

For several plants it was demonstrated that light has a regulatory effect on SUT-expression: from a reduction to a complete lack of the sucrose-transporter gene expression was observed in shaded mature leaves of tobacco (Sauer 2007) and after 15 – 14 hours dark treatment of tomato and potato leaves (Kühn et al. 1997). Wright (2003) could also show a reduction of SUT expression in leaves grown in shade, an adaption to lower photosynthesis-activity.

1.3 The model plant *Ajuga reptans*

Figure 1.6 *A. reptans* in its natural habitat (from S. Findling)

Ajuga reptans, member of the Lamiaceae, is a perennial evergreen herb (Fig. 1.6) that is distributed in whole Europe through west Asia and parts of northern Africa (Hegi 1964). It has the ability to survive freezing temperatures. *A. reptans* plants grown at low temperatures accumulate and store large amounts of RFOs during cold seasons (Bachmann et al. 1994; Peters and Keller 2009). Having an open minor vein phloem anatomy (Gamalei 1989), it is primarily classified as symplastic loading plant with ICs and ordinary CCs in the minor vein phloem and it translocates mainly stachyose but also raffinose and sucrose (Hoffmann-Thoma et al. 2001, Bachmann et al. 1994). *A. reptans* has already been object to

studies involving the subcellular distribution of sugars, but only on isolated chloroplasts (Schneider and Keller 2009), vacuoles (Bachmann and Keller 1995) or protoplasts (Schneider and Keller 2009). For the study *A. reptans* was chosen because it is a symplastic loading plant, it translocates the highest concentrations of RFOs in comparison with other plant species (own data, Bachmann et al. 1994), it accumulates large amounts of RFOs at cold temperatures and because of its ability to tolerate freezing temperatures. For analyzing the stachyose synthase protein, *A. reptans* was selected because it contains large amounts of stachyose in the leaves (Bachmann et al. 1994).

1.4 The aim of this thesis

This study was focused on several aspects of the raffinose oligosaccharide metabolism in *A. reptans*. The following points had to be studied:

1. Subcellular distribution of various translocated carbohydrates, amino acids and anions in mesophyll cells of plants grown at different temperatures.
2. Different functions of RFOs and sucrose in warm and cold treated *A. reptans* plants.
3. Activity of stachyose synthase in different plant tissues and at different temperatures to better understand the role of stachyose in *A. reptans*
4. Isolation and identification of a full length cDNA of a sucrose transporter from leaves of the symplastic phloem loader *A. reptans.*
5. Analysis of the expression pattern of the stachyose synthase gene and of the sucrose transporter gene *in vivo*.

2 Material and Methods

2.1 Plant material and Preparation

The used plants of *A. reptans* were grown under different conditions, depending on the experiment they were used for. They were either grown in 3 l pots in compost soil in a green house and outside of the “Bergische Universität Wuppertal” (Germany; 51.26°N, 7.18°E) or grown in the field outside of the “Bergische Universität Wuppertal”. Greenhouse temperature was 20 °C constantly with a light intensity of about 100 µmol/m^{-2} sec^{-1} at light period. Sample harvest was always performed after 6 h of illumination (except the darkness probes), the plant material was immediately shock frozen with liquid nitrogen and crushed to a very fine powder. Depending on their further usage different amounts were weighed in: 200 mg (sugar extraction), 150 mg (RNA-Isolation) and 5 g (per gradient of NAF) and either directly processed or stored at -80°C.

a) Non-aqueous fractionation

Ajuga reptans were grown outside of the “Bergische Universität Wuppertal” (Germany; 51.26°N, 7.18°E) at two separate locations. Warm treated plants were grown in summer months at 15 – 30 °C. Cold treated plants were grown at temperatures of -5° – +10 °C in winter months. Leaf samples were harvested at the end of August (about 14 h sunlight; about 20 - 25°C) and at the end of February (about 11 h sunlight; about 0 - 5°C) each at the end of the daylight period and the end of the dark period.

b) Tissue-specific analysis

Samples were taken from field grown *A. reptans* plants. Flowers, petals, stem, sink- and source-leaves were harvested during anthesis in spring (about 12.5 h sunlight; about 15 - 20 °C during the day and 5 - 10 °C during the night).

c) Temperature analysis (warm/cold)

Source leaf samples of *A. reptans* were harvested from pot grown plants in March (about 10 h sunlight) Temperature conditions for warm grown plants were 20 °C (day/night) and for cold grown plants 5 - 10 °C (day) and 0 °C (night).

d) Light-dependence analysis

Pot grown plants of *A. reptans* were transferred from natural d/n cycle (10h/14h) either from the green house (20°C) to a separated laboratory with 21 °C for warm acclimated plants or from outside with 0-5 °C at night and 10 °C during the day to a refrigeration room (5-8°C) for cold acclimated plants. Leaf samples were harvested before the transfer for light condition. After 24h and 48h in darkness leaf samples were harvested again.

2.2 Bacteria

Cloning of DNA fragments was performed using the *Escherichia coli* strain DH5α. Liquid cultures of *E. coli* cells were grown overnight at 37 °C in a roller or shaker in LB-medium supplemented with ampicillin and X-Gal (5-bromate-4-chlor-3-indoxyl-β-D-galactopyranoside). For growth on agar plates, 1.5% agar was added to the liquid medium before autoclaving. Antibiotics and other additives were pipetted into the melted agar medium under a clean bench after cooling the medium down to about 70 °C.

	Relevant Characteristics	Reference
E .coli DH5α	F-, φ80dlacZ ΔM15, endA1, recA1, hsdR17 (rk-, mk+), supE44, thi-1, gyrA96, relA1, Δ(lacZYA-argF) U169, λ-	Woodcock et al. (1989)

LB (Luria-Bertani) medium (1 l):

Select Pepton 140	10 g
yeast extract	5 g
NaCl	10 g

Additives	**stock solution**	**concentration in medium**
Antibiotics: ampicillin	100 mg/ml in dd H_2O	100 µg/ml (1:1000)
X-Gal	2% (w/v) in DMF	0.004% (1:500)

2.3 Plasmids

Plasmid	Used for	Used in Organism/ Strain	Relevant Characteristics	Selection Marker	Source
pGEM®-T Easy	cloning of PCR products	*E. coli* DH5α	AT-cloning, blue/white selection of transformants	Amp^R	Promega Madison WI. USA

2.4 Oligosaccharides

For the different PCR-reactions primer were designed and ordered at Eurofins Genomics (Germany) (Table 2.1).

Table 2.1 Primers used for RT reactions and/or PCR. Ambiguity code: N=A/C/G/T; R=G/A; Y=C/T.

Primer	Sequence (5´ → 3´)	Used for
degenerate primers		
ST1f	GCNGCNGGNRTNCARTTYGGNTGGGC	RT-PCR to search for cDNA fragments of SUT in *A. reptans* (Knop et al.2001)
ST1r	GCNACRTCNARDATCCARAANCC	
Sta-5´	GGNTGGTGYACNTGGGAYGC	RT-PCR to search for cDNA fragments of STS and RafS *A. reptans* (Voitsekhovskaja et al. 2009)
Sta-3´	TGRAACATRTCCCARTCNGG	
gene-specific primers		
ARST1-RACE-SP1rev	CGCGATCGCCTAATAACCAC	5´-RACE gene-specific primer for RT
ARST1-RACE-SP2rev	AGAAGCCGATGATGAGTACG	1st 5´-RACE gene-specific primer for PCR
ARST1-RACE-SP3rev	TAGTGCTGCGGTCGCTGAAGT	2nd 5´-RACE gene-specific primer for PCR
ARST1-RTq-1fwd	CTATTAGTCCAGCCGCTTG	3´-RACE gene-specific primer for the 1st PCR
ARST1-PFU1f	TACTCATCATCGGCTTCTCC	PCR of the full length cDNA of *ArSUT*
ARST1-PFU2r	CAAGTCAGGGCAGTAACAAG	
ARST1-PFU3f	AAACGCTTCAAAGTCCTCTC	
ARST1-PFU4r	CCTTGAAGCGGGTTATGGC	
oligo d(T)-Anchor Primer	GACCACGCGTATCGATGTCGACTTTTT TTTTTTTTTTV	5´-RACE anchor primer
PCR Anchor Primer	GACCACGCGTATCGATGTCGAC	3´-RACE adapter primer
dT_{20}	TTTTTTTTTTTTTTTTTTTT	RT reactions
HKG-actinF	ACCGAAGCCCCTCTTAACCC	control for traces of DNA in RNA samples or control of quality of cDNA and housekeeping Gene for qPCR (van den Berg et al. 2004)
HKG-actinR	GTATGGCTGACACCATCACC	
ARST1-RTq-7fwd	GCTAATAATATGACTCAGGGAC	qPCR of *ArSUT*
ARST1-RTq-8rev	CAACCGCCATAAATAAGGAG	
AR-RACE-SS16fwd	AAGACGATTCTACCCTCCC	qPCR of STS of *A. reptans*
AR-Q-STS1f	AAAGACGATTCTACCCTCCCT	

2.5 Non-aqueous fractionation

Non-aqueous-fractionation allows the separation of plant cell compartments and the subcellular distribution of metabolites using a density gradient. In the shock-frozen and lyophilized leaf material, metabolites bind to the membranes of chloroplast and vacuole and the plasma membrane in the water-free atmosphere. Because of the density of the membrane, a separation along the density gradient is possible (Gerhard and Held 1984, Riens et al. 1991, Nadwodnik and Lohaus 2008).

2.5.1 Making a density-gradient

The density-gradient consists of the two solutions n-heptane, with a density of 0.684 g/cm^3, and tetrachlorethylene (TCE), with a density of 1.632 g/cm^3. First of all the densities for the plant of interest needed to be identified. For that purpose, gradient densities of *Alonsoa meridionalis*, *Arabidopsis thaliana* and *Asarina barclaiana* were compared and a test gradient with ρ = 1.5 – 1.3 worked very well and was further used together with a gradient with ρ = 1.5 – 1.35. The winter harvested leaves needed a density gradient of 1.48 – 1.38.

To make sure that the solutions were completely free of water, a molecular sieve (0,4 nm) was used.

For making the gradient two solutions, solution A (high density) and solution B (low density), were needed. Five grams of the lyophilized leaf material (Christ alpha 2-4, Martin Christ; lyophilized for 5 days) was resuspended in 20 ml solution B, vortexed (2500 rpm, VV3, Biometra) and sonicated for three minutes at 30% cycle and 65% power (Sonopuls HD 60, Bandelin). During sonication the sample was kept on a water-ice bath for cooling. That following a filtration step using a nylon net with a pore size of 20 µm. The net was then rinsed with 30 ml n-heptane, the sample mixed by vortexing and centrifuged for 10 minutes at 3200 x g and 4°C (Centrifuge 5804R, Eppendorf). The supernatant was discarded and the pellet resuspended in 3 ml n-heptane. Thereof six aliquots of 50µl each were sampled as total fraction (F0) in 2 ml reaction tubes and dried in a vacuum concentrator (concentrator 5301, Eppendorf) for one hour. The remaining 2.7 ml sample was kept to put on the gradient.

The density gradient was made by combining 12 ml solution A and 13 ml solution B in 50 ml Falcontube using a double chamber gradient mixer. On top of the gradient the 2.7 ml of sample-heptane-mix was carefully transferred, letting it run down at the tube wall. After this the gradient was centrifuged for 70 minutes at 5000 rpm and 4°C (Centrifuge 5804R, Eppendorf) in a swing-out rotor (A-4-44), with lowest increase and decrease of the speed to prevent jerky movements that would swirl the gradient.
When the gradient was finished different fractions were observable, in summer-gradients about six, in winter gradients about five. These fractions were marked (F1 = high density to F5/6 = low density) and transferred into labeled 50 ml tubes, using a pasteur pipette. The fractions were filled up to 20 ml with n-heptane, vortexed, and centrifuged for 10 minutes at 3200 x g and 4°C (Centrifuge 5804R, Eppendorf). The supernatant was discarded and the pellet resolved in 3 ml n-heptane. Each fraction was aliquoted in six 2 ml reaction tubes, and dried in a vacuum concentrator (concentrator 5301, Eppendorf) for one hour.

2.5.2 Extraction of Metabolites

2.5.2.1 Protein extraction

The dried fraction pellets were resuspended in 500µl extraction buffer (STITT-buffer) containing

Hepes pH 7.4	50mM
$MgCl_2$	5mM
EDTA	1mM
Triton	0.1% (v/v)
Glycerol	10% (v/v)

and vortexed for one minute. After centrifugation (10 min, 13000 rpm, 4°C) the supernatant was kept on ice and used for enzyme activity determination (2.5.3) and protein contents (2.5.3.1).

2.5.2.2 Aqueous Chloroform/Methanol extraction

For determination of sugar contents and chlorophyll in each fraction the samples were dissolved in 5 ml Chloroform/Methanol 1.5/3.5 (v/v), mixed well and incubated

for 30 min on ice. Then 3 ml ddH_2O were added, mixed and centrifuged for 5 min at 5000 rpm. The upper watery phase was transferred into a round bottom flask and kept on ice. To the lower phase 2 ml ddH_2O were added, mixed and centrifuged (see above). Again the upper phase was transferred into the same flask. The sample was then evaporated until no liquid was remaining. The sugar was then dissolved in 1 ml ddH_2O and filtered, using a syringe filter (PA 0.2 µm). The extracts were then analyzed using HPLC.

For chlorophyll determination the remaining lower phase was used. The chloroform mixture was filled up to 10 ml with ethanol 96%, mixed well and centrifuged (5 min, 5000 rpm). The supernatant was used for photometric measurements at an absorption rate at 652 nm.

2.5.2.3 Extraction of anthocyanins

The anthocyanins were measured to confirm the results of the α-mannosidase activity (2.5.3.4). Each dried fraction was dissolved in 10 ml extraction medium (n-propanol - 32 weight % HCL - H_2O (18:1:81 v/v), incubated for 5 min at 100°C and kept for 3 h at RT in the dark. Afterwards the extracts were centrifuged (10 min, 10000 x g). The clear supernatant was transferred into a 1 cm cuvette and extinction was measured at 535 nm and 650 nm. Reference was pure extraction medium (Schopfer, Experimentelle Pflanzenphysiologie 2).

2.5.3 Determination of enzyme activities

The activities of the enzymes listed below (chap. 2.5.3.2 – 2.5.3.4) were measured from the protein extracts (chap. 2.5.2.1) of the density gradient fractions.

2.5.3.1 Protein determination

Proteins concentrations were measured in gradient fractions and untreated leaves of *A. reptans* according to Lowry (1951). Before an analysis, a solution containing A:B: C = 99:0.5:0.5 was freshly prepared from the stock solutions:

Solution A:	2% (w/v)	Na2CO3 in 0.1 M NaOH
Solution B:	1% (w/v)	$CuSO_4$ x $5H_2O$
Solution C:	2% (w/v)	Na-K-tartrate

130 µl water, 20 µl of the sample and 700 µl ABC mixture were pipetted together, vortexed and incubated for 15 min. Afterwards, 100 µl of Folin-Ciocalteu's phenol reagent (diluted 1:2) were added, and the samples were incubated for 10 min. From each sample, two aliquots were analyzed. A third aliquot was used to determine the background absorbance at 578 nm. For this purpose, the 20 µl aliquot was added to the reaction mixture only after both incubations were completed. The samples were centrifuged for 2 min at 13 000 rpm in an Eppendorf centrifuge and the extinction was measured at 578 nm. For each analysis, a calibration curve was made using BSA solutions in the same buffer as used for the sample extraction. The calibration curve points corresponded usually to 0.1, 0.2, 0.3 and 0.4 mg protein in the sample.

2.5.3.2 NADP-dependent Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

GAPDH is localized in the chloroplast as is part of glycolysis. Therefore it is a marker enzyme for that compartment. In that cycle two coupled reactions were taken advantage from: 3-Phosphoglyceratekinase catalyzes the formation of 1,3-bis-phosphoglycerate by transferring a phosphate-group from ATP to 3-Phosphoglycerate. In the next step 1,3-bis-phosphoglycerate is reduced to Glyeraldehyde-3-phosphate. The hydrogen required for that reaction results from the oxidation of NADPH. The method used is based on the decrease of NADPH, which reflects in the absorbance at 340 nm (Wirtz et al. 1980).

The enzyme activity was measured using 20µl of density gradient fraction protein extracts (chap. 2.5.2.1) in a total volume of 600µl.

The reaction mixture contained

HEPES pH 8.0	100 mM
$MgCl_2$	30 mM
KCl	20 mM
Na-EDTA	2 mM
DTT	6.7 mM
ATP	6.7 mM
NADPH	0.3 mM
Phosphoglyceratekinase	6 U

The reaction got started with the addition of 20 µl 3-Phosphoglycerate (200 mM) after 5 min of setting the graph and ran 15 min in all.

2.5.3.3 Phosphoenolpyrovate carboxylase (PEPCx)

PEPCx is an important enzyme in plant metabolism. Through carboxylating of phosphoenolpyruvate oxaloacetate is built. Oxaloacetate is a main intermediate in the citric acid cycle which provides reduction equivalents and precursors for amino acids and carbohydrates. In a following reaction oxaloacetate is turned into malate by the enzyme malate dehydrogenase and is attended by the oxidation of NADH. The decrease of NADH is measured at 340 nm (see chap. 2.5.3.2). The carboxylating reaction takes place in the cytoplasm of mesophyll cells and is therefore a marker enzyme for the cytosol.

The reaction mixture contained

Glycylglycin	50 mM
$MgCl_2$	10 mM
$KHCO_3$	4 mM
pH 7.9	

600 µl of reaction mixture, 10 µl NADH (20 mM), 20µl malate-dh (5 mg/ml) and 50 µl of protein extract were placed in a 1 ml cuvette. The reaction got started with the

addition of 10 µl Phosphoenolpyruvate (50 mM) after 5 min of setting the graph and ran 15 min in all.

2.5.3.4 α-Mannosidase

α-Mannosidase is a hydrolytic enzyme of the vacuole. It splits ρ-nitrophenyl-α-D-manno-pyranoside (pNP-mannosid) into mannose and ρ-nitrophenol, which has a yellow color in its dissociated state and can be detected photometrical at 405 nm.
As reaction mixture, 500 µl sodium citrate (50 mM, pH 4.5) was placed in 2 ml reaction tube with 50 ml of protein extract and 500 µl pNP-mannosid (10 mM= as starter. The reaction ran for 120 min and was stopped by adding 500 µl borate-buffer (800 mM, pH 9.8). The photometrical detection of pNP was performed immediately after stopping.

2.5.4 Calculation of the subcellular distribution of metabolites

To evaluate the subcellular distribution of metabolites (sugars, amino acids, anions) in chloroplast, cytosol and vacuole the measured data of enzyme activity and HPLC analysis were used for a calculation procedure following Riens et al. (1991). This procedure is based on the assumption that the metabolites are confined to the three compartments as described above, indicated by the corresponding marker enzymes. The evaluation is done by a computer program testing all possible cases for the distribution of a set metabolite between the three compartments at intervals of 1%. That means, there are 5151 possible distribution patterns available and the program calculates which of these yields the best agreement (best fit) with the experimental results. As a measure for this best fit one parameter was defined: Q = $\sqrt{\sum(\Delta_i)}$^2/ $n-1$, where Δ_i means the difference between the measured and the calculated distribution in each fraction, and n resembles the number of fractions. Only results with the lowest Q values were picked. From at least three gradients the “BestFit” values were averaged to avoid falsification by analytical errors.

2.5.5 Electron microscopy and determination of partial volumina of subcellular compartments

Source leaves of summer and winter plants (3 of each) were used for micrographs of minor veins and parenchyma cells (palisade and sponge). For Transmission Electron Microscopy (TEM), leaves of *A. reptans* were cut in pieces and fixed over night in Karnovsky`s solution (Karnovsky 1965), buffered with 0.1 mol/l sodium cacodylate (pH 7.4) at 4 °C. After postfixation for 120 min in 2% osmium tetroxide in the same buffer, the specimens were dehydrated in a graded series of acetone, and embedded in Spurr's medium (Spurr 1969). Ultrathin sections then were stained with uranyl acetate and lead citrate according to Reynolds (1963), and examined with a Hitachi TEM H600 at 70 kV. The whole process was conducted of Dr. Klaus Zanger and his associates (electron microscopy department in the institute for anatomy, University of Düsseldorf).

The partial volumes of the chloroplast, vacuoles and cytosol per mesophyll cell were determined using the obtained micrographs. The relative squares of the subcellular compartments on the sections were determined by an image-analysis technique (IMAGE J; public domain software, developed at US National Institutes of Health, available at http://rsbweb.nih.gov/ij/). The calculations were carried out using 15-20 sections of the mesophyll tissue (palisade parenchyma).

2.6 HPLC analysis

2.6.1 Sugars

Sugar of leaf extracts, phloem sap, density gradient fractions and stachyose synthase assay of were quantified using HPLC. An anion exchange column (CarboPAC10; Dionex Corp) was used for polyol, mono-, di- and polysaccharide detection. The column was eluted isocratically with NaOH 100 mM and a flow rate of 0.8 ml min^{-1}. The eluent was prepared with helium washed milipore water and NaOH (50 %) of purest quality (Baker, England). The sugars were detected by a thin layer amperometric cell (ESA, Model 5200) while a pulse amperometric detector (Coulochem II) set a pulse according to the scheme:

Table 2.2

pulse mode	voltage	duration	measure
measurement	50 mV	500 ms	400 ms
clearance	700 mV	540 ms	
regeneration	-800 mV	540 ms	

The calibration standards were composed of myo-Inositol, galactinol, sorbitol, mannitol, glucose, fructose, sucrose, raffinose, stachyose, maltose and verbascose and were used in the concentrations 50 mM, 100 mM, 250 mM and 500 mM. Leaf extraction samples were diluted 1:10 before measurement to ensure the values are in that range. Density gradient fractions were measured undiluted. The evaluation of chromatograms was performed with the integration program PeakNet 5.1 (Dionex, Germany).

2.6.2 Amino acids

The chloroform/methanol extract was also used for amino acid analysis. For detection via HPLC the amino acids were derivatized with ortho-phtaldialdehyde (OPA) and β-mercaptoethanol to fluophore apolar indole derivates. This derivatization step decreases the detection limit around the 20-fold of the derivatization with ninhydrine.

Reagents:

OPA-stock solution: 5-8 mg o-phtaldildehyde
1 ml methanol
125 µl potassium-borate (1 M; pH 10,4)
12,5 µl β-mercaptoethanol

working solution: 1 ml potassium-borate (1 M; pH 10,4)
0,5 ml OPA-stock solution

20 µl of working solution and 20 µl of extract were mixed and derivatized for 1 minute. 20 µl of that mixture were applied on a reverse phase column (Merck LiChro Cart 125-4; Supersphere 100 RP-18 endcapped) with a pre-column (LiChroCART 4-4; Lichrosphere 100 RP18-e) fixed first. Amino acid standards contained phosposerine, aspartic acid, glutamic acid, asparagine, serine, histidine, glutamine, glycine, threonine, arginine, alanine, tyrosine, valine, methionine, tryptophane, isoleucine, phenylalanine, leucine and lysine in the concentrations 4 µM, 10 µM and 20 µM.

HPLC-buffer:	H_2O	2.5 ml
	Phosphoric acid	3 ml
	NaOH	25 %
	EDTA	1,25 g
	pH 7,06 – 7,1 (with NaOH 25 %)	
Eluent A:	HPLC buffer	95 %
	Acetonitrile	5 %
Eluent B:	HPLC buffer	50 %
	Acetonitrile	50 %
Eluent C:	Acetonitrile	70 %
	H_2O	30 %

For eluting the derivates from the apolar stationary phase a gradient of acetonitrile and phosphate buffer was run.

Gradient run:

minute	% A	% B	% C	flow rate (ml/min)
0	100	0	0	0,9
12	81	19	0	0,9
20	81	19	0	0,9
38	52	48	0	0,9
41	52	48	0	0,9
48	32	68	0	0,9
51	0	0	100	1,2
66	0	0	100	1,2
68	100	0	0	0,9
78	100	0	0	0,9

The data analysis was again carried out with PeakNet 5.1 (Dionex).

2.6.3 Anions

For the anion analysis, an IonPac anion exchange column (AS11, Dionex, Idstein, Germany), connected with a conductivity detector module (CD20, Dionex, Idstein, Germany) was used. This column can detect organic and inorganic anions (Cl^-, NO^{-3}, oxalate, sulfate, malate, phosphate and citrate), but needed to run with a gradient. Additionally a suppressor was switched in to reduce the conductivity of the eluent.

Buffer A: H_2O

Buffer B: NaOH (40%)

Gradient run:

Buffer A 96 %

Buffer B 4 %

The suppressor current was 100 mA and the flow rate was 0.7 ml/min.

2.7 Isolation of nucleic acids

2.7.1 Isolation of total RNA from plant tissues

Plant tissue was harvested and frozen in liquid nitrogen immediately after cutting. For each method about 150-200 mg of frozen tissue was powdered transferred in a 2 ml reaction tube and kept frozen until extraction buffer was added. All consumable supplies were autoclaved and working surface was treated with RNase AWAY® (Roth) before starting an RNA-isolation.

2.7.1.1 RNA isolation using CTAB

The RNA was extracted following the procedure of Chang et al. (1993) using a CTAB extraction buffer containing

CTAB (hexadecyltrimethylammonium bromide)	2 %
PVP (polyvinylpyrrolidinone K 30)	2 %
Tris-HCL (pH8.0)	100 mM
EDTA	25 mM
NaCl	2 M
Mercaptoethanol (added just before use)	2 %

The buffer was heated to 65 °C, then mercaptoethanol was added and 800 µl was pipette to each sample and thoroughly vortexed. After incubation for 15 minutes at 65 °C and 1000 rpm (Thermo shaker TS1, Biometra), 400 µl Roti-Phenol (RNA) and 400µl chloroform were added. Again 15 minutes incubation (RT, 1400 rpm) following 5 minutes centrifugation (RT, 13000 rpm; 5254, Eppendorf). The upper phase was then transferred into a new 2 ml reaction tube. 800 µl chloroform:isoamylalcohol (24:1 (v/v)) were added, thoroughly vortexed (VV3, VWR) and centrifuged (see above) and upper phase transferred into a new reaction tube. This step was repeated until no longer any interphase (what means proteins in the sample) was visible. When the sample was “clean” the volume was determined and a quarter of the volume 10 M lithium chloride (-20 °C) was added and mixed. The sample was then stored on ice over night (maximum 18 hours to avoid precipitation of contaminations) to precipitate the nucleic acids. The next day the samples were centrifuged for 20 minutes (4 °C,

13000 rpm; Mikro 200R, Hettich) and the supernatant was discarded. For cleaning up, SSTE-buffer (1 M NaCl, 0.5 % SDS, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA (pH 8.0)) was warmed to 65 °C, 400µl added to each sample, and shook it for 10 minutes (42 °C, 850 rpm). Thereafter 400 µl of chloroform:isoamylalcohol (24:1 (v/v)) were added, mixed and centrifuged for 5 minutes (RT, 13000 rpm). The upper phase was transferred into a new 1.5 ml reaction tube and this washing step was repeated at least two times to remove any proteins. When the sample was clean for precipitating RNA two volumes of ethanol (96 %, -20 °C) were added, mixed and kept at -80 °C for one hour. That followed a centrifugation step (20 minutes, 4 °C, 13 000 rpm). The supernatant was discarded and the pellet was washed twice with ethanol (70 %, -20 °C), first 500 µl and second 80 µl ethanol. The pellet was then dried at 42 °C in the thermo shaker and when dry it was resolved in 33 µl of RNase and DNase free water. For further processing the samples were stored at -80 °C.

2.7.1.2 RNA isolation using Trizol

For samples that did not show good bands of rRNA on the gel with CTAB isolation using trizol appeared to be more efficient, but to remove the phenol from the sample was a bit more delicate because one can first check when the cDNA-synthesis didn`t work. Following Chomczynski and Sacchi (1987) the trizol extraction buffer was made of

38 %	Roti-Phenol (Roth, Karlsruhe, Germany)
800 mM	guanidinium thiocyanate
400 mM	ammonium thiocyanate
1M	sodium acetate (pH 5,0)
5 %	glycerol

To extract the RNA 1 ml of trizol was added to the frozen sample, thoroughly vortexed and incubated for 5 minutes at room temperature. The samples were then centrifuged for 10 minutes (4 °C, 20000 x g) and the supernatant was transferred into a new 2 ml reaction tube. 200 µl of chloroform were added, the samples were vortexed and incubated for 3 minutes at RT. After centrifugation for 15 minutes (4 °C, 15000 x g) the aqueous supernatant was transferred into a new reaction tube. The

chloroform extraction was repeated until no smell of phenol was detectable anymore (at least six times). When sure that all phenol was gone, the sample was mixed with half the volume of isopropanol and half the volume of high-salt-precipitation buffer (800 mM sodium citrate, 1.2 M sodium chloride) by inverting the tubes. After incubation for 10 minutes at RT the samples were centrifuged for 20 minutes (4 °C, 15000 x g). The supernatant was discarded and the pellet was washed twice with 900 µl ethanol (75 % (v/v)). The pellet was then dried at 42 °C in the thermo shaker and when dry it was resolved in 33 µl of RNase and DNase free water. For further processing the samples were stored at -80 °C.

2.7.2 DNA isolation from *E. coli*

2.7.2.1 Plasmid DNA miniprep isolation using STEL-buffer

Plasmid DNA was isolated from *E. coli* strains that were grown in overnight cultures. For checking the correct integration of the PCR-product in the pGEM®-T Easy cloning vector, a plasmid mini preparation was done. This method, using a STEL-buffer, is based on the lysis of the bacterial cell membrane by lysozyme and Triton-X. This method is not suited for sensitive reactions like sequencing due to a low purity degree of the sample, but sufficient enough for integration control.

150 µl of STEL-buffer for each sample preparation, containing

sucrose (w/v)	8 %
Triton X-100 (v/v)	5 %
Tris/HCL	50 mM
EDTA	50 mM

was mixed with 0.5 mg/ml lysozyme. 1 ml of the overnight culture was centrifuged for 1 minute (RT, 13 000 rpm) and the supernatant was discarded. The pellet was resolved with the buffer-lysozyme-mix and vortexed until complete resolving. After a short heat shock (30 sec, 100 °C) the samples were centrifuged again (20 min, RT, 13000 rpm). The formed pellet, made of cell walls i.a., was removed by using a pipette tip. The remaining liquid was mixed with 180 µl isopropyl for 10 min on the

thermo shaker to precipitate the DNA and centrifuged for 5 min and 13000 rpm afterwards. The supernatant was discarded and the pellet washed with 500 µl ethanol 70 %, centrifuged, supernatant discarded and pellet dried. The pellet was resolved in 50 µl DNase-free water at 37 °C.

2.7.2.1.1 Restriction hydrolysis and gel electrophoresis

The plasmid vector was digested with ECO RI (Thermo), which cut the plasmid at known sites in the multiple cloning site, therefore the resulted fragments had a proven size which could be detected using gel electrophoresis.

3 µl of DNA (STEL-Miniprep)
0.25 µl ECO R1 (10 u/µl)
2 µl ReAct 3 (10x)
14.75 µl H_2O

were incubated with for 90 min at 37 °C. The enzyme was deactivated by heating up to 65 °C for 20 min. A 1% agarose gel (chap. 2.9) was performed and analyzed. The expected product sizes were 3000 bp for the plasmid and 350 bp for the insert (PCR-product of ST1).

2.7.2.2 Plasmid DNA isolation using E.Z.N.A. Plasmid Miniprep Kit

When STEL-miniprep analysis was positive, thus the insert integrated, DNA was isolated from the overnight cultures of those samples using a plasmid preparation kit.
The remaining 2 ml of the sample of the overnight cultures were centrifuged for 2 minutes (8000 rpm, RT) and the supernatant completely removed.
The pellet was resolved in 250 µl resuspension solution and vortexed until the bacteria were dissolved. Then 250 µl of lysis solution were added and gently mixed but not vortexed. After addition of 350 µl of neutralization solution the samples were centrifuged for 5 minutes (13000 rpm, RT) and the supernatant was transferred into a HiBind® DNA Mini Column that was placed in a provided collection tube. This was centrifuged for 1 minute (13000 rpm, RT), supernatant was discarded and the column placed in the tube again. The next steps were done twice for absolute purity: 500 µl of wash solution were added, centrifuged for 30 – 60 seconds, supernatant

discarded, and column back into tube. To dry the column membrane another centrifugation step was done. In order to dissolve the bacterial DNA from the membrane, the column was placed into a new reaction tube and 50 µl of elution buffer were added to the sample. After 2 minutes of incubation at room temperature the samples were centrifuged for 2 minutes. The plasmid DNA was stored at -20 °C.

2.8 Determination of RNA and DNA by spectrophotometry

The concentrations of DNA or RNA in aqueous solution could be determined using a spectrophotometer (GENESYS 10S UV-VIS, Thermo Scientific). A special microlitre cuvette was used to determine the concentration without a dilution step. Two caps with different dilution factors (DF_{cap}) are able to cover various concentration ranges. The extinction of 1 µl of the nucleic acid solution was measured at 260 nm and 280 nm, respectively. The E_{260}/E_{280} ratio represents a measure for the protein contamination of the nucleic acid sample and should be between 1.8 and 2.1. The concentration of DNA or RNA was then calculated as follows:

$$\text{RNA [µg/µl]} = E_{260} \times 40 \times DF_{cap}$$

$$\text{DNA [µg/µl]} = E_{260} \times 50 \times DF_{cap}$$

The extinction coefficients are valid for double-stranded DNA and single-stranded RNA (Sambrook et al. 1989).

2.9 Agarose gel electrophoresis of RNA and DNA

2.9.1 Separation of DNA

Usually, 1% agarose gels in 1 x TEA buffer were used for RNA and 2% agarose gels for DNA fragments. 1 µl of loading dye was added to 5 µl DNA samples before loading them on a gel. Electrophoresis proceeded at 100 mA with 1 x TAE as running buffer. O'GeneRuler™ 1kb Plus DNA Ladder (Thermo) was used as size marker (1 µg per slot). After the successful separation, the agarose gel was incubated in an ethidium bromide solution (1 µl/ml) for 20 min. After that the DNA was photographed

under UV light using a transilluminator (INTAS UV-Systems) and the accompanying computer program.

50 x TEA buffer:

Tris-acetate pH 8.3 2 M

EDTA 100 mM

2.10 DNA digestion

Isolation of RNA was always accompanied with contaminations of small amounts genomic DNA. In case of RACE and qPCR, the RNA needed to be treated with DNase I (RNase-free, Thermo) to avoid any DNA contamination in later analysis. Therefore 2 µg RNA, 2 µl Reaction buffer and 2 µl DNase I were filled up to 20µl with H_2O and were incubated for 30 min at 37°C. To inactivate the DNase 2 µl EDTA were added and incubated for 10 min at 65°C. The samples were then ready for further analysis.

2.11 cDNA Synthesis

cDNA-synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo). 1 µg of RNA was incubated with 1 µl oligo$(dT)_{18}$-primer and 12 µl H_2O for 5 min at 65 °C in order to break secondary structures. After a short chilling on ice the following components were added

5x Reaction Buffer	4 µl
RiboLock RNase Inhibitor	1 µl
dNTP-Mix (10 mM)	2 µl
reverse Transcriptase (200 U/µl; RevertAid M-MuLV)	1 µl

and incubated for 60 min at 42 °C. To inactivate the transcriptase a heating step (5 min, 70 °C) was conducted.

2.12 Amplification of DNA by polymerase chain reaction

2.12.1 Design of specific and degenerate primers

To obtain sequences of a SUT-transporter and a stachyose-synthase in A. *reptans* degenerated primers were used. Degenerated primers contain *wobble* bases that are able to bind to several nucleotides and are therefore not specific to species but to the template. It is then possible to isolate homologues sequences of a gene in a species that has not been analyzed yet. The first primer pair used was a degenerated primer pair designed from Knop et al. (2001). They were used to check, if at all a sucrose transporter is found in the mRNA of *A. reptans.* The second degenerated primer pair was designed from Voitsekhovskaja et al. (2009) and should result in 1100 bp sequence of the stachyose-synthase.

Primer design for both specific and degenerated should meet the following features: (1) a length of at least 18 nucleotides; (2) the primers should not form any strong secondary structure or (when using a primer pair) hybridize with each other; (3) the 3'-end of the primer should have one or two guanine or cytosine bases for stronger binding to the DNA-sequence; (4) the melting temperature (T_m) of the primer should not exceed the amplification temperature. T_m is the melting temperature at which 50% of the primer are bound to template and determines the annealing temperature ($T_{ann} = T_m - 3$) of the PCR. Primers used in the same reaction should have similar annealing temperatures. The T_m-values were adopted from MWG. For the design of specific primers used for RACE or RT-qPCR the software program PerlPrimer (version 1.1.21) was used.

2.12.2 PCR-reaction

The polymerase chain reaction was used for amplification of fragments of DNA (genomic) and cDNA using either specific or degenerate primers. PCRs were performed using a DNA thermal Cycler with heatable lid (Mastercycler® Gradient, Eppendorf). Usually, *Taq*-polymerase (Bio&Sell) or *Pfu*-polymerase (Thermo) was used. The reaction mixture contained if not otherwise indicated

DNA template	1 µl
10 x buffer B (Bio&Sell)	2.5 µl
$MgCl_2$ (25 mM)	2 µl
dNTP mixture (10 mM of each nucleotide)	0.5 µl
5´-primer (10 pmol/µl)	0.5 µl
3´-primer (10 pmol/µl)	0.5 µl
Polymerase (2 U/µl)	0.3 µl
ad dd H_2O 25 µl.	

Usually, a three step cycle program was used except for the amplification of full length ArSUT cDNA (2.12.4):

Table 2.3 Standard PCR-program

Temperature	Time	Step
94 °C	5 min	initial denaturation
94 °C	10 sec	denaturation
50 °C *	30 sec	annealing
72 °C	30 sec	elongation
		repeat of cycle 38-45x
72 °C	8 min	final elongation
4 °C		store

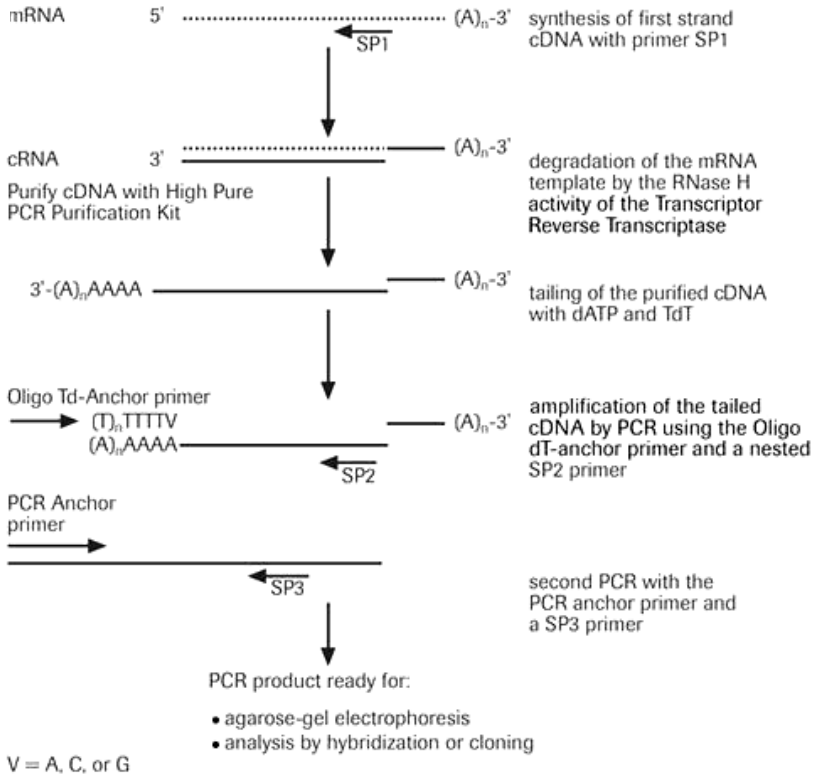
* *annealing temperature depends on the primers used for PCR*

.

2.12.3 Rapid amplification of cDNA ends (RACE)

RACE enables the generation of a full-length clone of a gene out of a short known sequence (Fig. 2.1). For the RACE-experiments is this study the 5'/3' RACE-Kit (2^{nd} Generation, Roche) was applied.

5‘ RACE



3‘ RACE

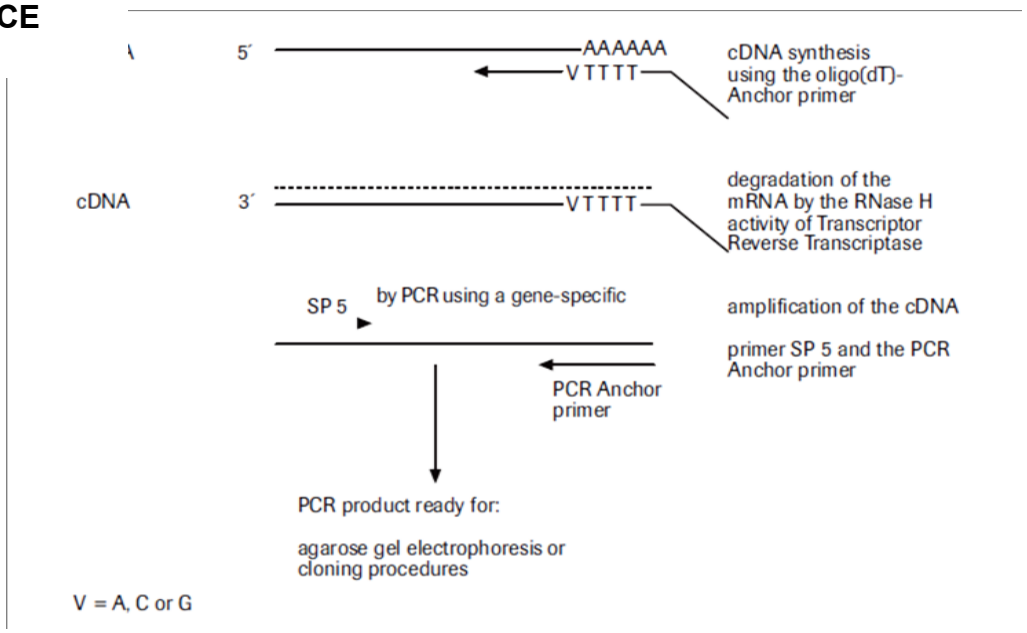


Figure 2.1 Schematic procedure of RACE (lifescience.roche.com)

2.12.3.1 3` RACE

For the amplification of the 3'-end of the cDNA an oligo(dT)-primer with an attached anchor sequence was used for cDNA-synthesis. That followed a PCR with one gene-specific primer (SP5) and one primer that binds specifically to the anchor-primer.

2.12.3.1.1 cDNA-synthesis

For reverse transcription

cDNA Synthesis buffer 5x	4 µl
dNTPs (10 mM each)	2 µl
Oligo dT-Anchor Primer	1 µl
Thermoscript RT	1 µl
total RNA	1 µg

were mixed and the total volume was filled up to 20 µl with dd H_2O. The mixture was incubated at 55 °C for 60 min and 5 min at 85 °C.

2.12.3.1.2 PCR-reaction

1 µl cDNA was used for a PCR reaction (50 µl total volume) with the PCR anchor primer and the gene specific primer ARST1-RTq-1fwd for 3´-RACE.

The following components were added to a reaction mix:

10x buffer B (Bio&Sell)	5 µl
$MgCl_2$ 25 mM	2.5 µl
dNTPs 10 mM	1 µl
gene-specific primer (SP5)	1 µl
PCR Anchor Primer 12.5 mM	1 µl
Taq-polymerase 5 U/µl (Bio&Sell)	0.5 µl

The temporal cycle differs from that of a standard PCR by a two period schedule line. The first time period follows a standard PCR time schedule and the second time period was extended about an additional elongation step with a time increment of 20 seconds in every new cycle because of the unknown length of the transcript.

Table 2.4 Schedule of 3‘RACE

Temperature	Time	Step
94 °C	2 min	initial denaturation
94 °C	15 sec	denaturation
60 °C	30 sec	annealing
72 °C	40 sec	elongation
		repeat of cycle 10x
94 °C	15 sec	denaturation
60 °C	30 sec	annealing
72 °C	40 sec	elongation
		repeat of cycle 10x
	+ 20 sec	time increment after every cycle
72 °C	7 min	final elongation
4 °C		store

If the PCR-product was too weak, a second PCR was conducted using the same primers and the PCR-product as template. For ArSUT a sequence length between 1.4 kb and 1.7 kb was expected according to its position in the gene of a sucrose-transporter (Knop 2001). Bands having the right size were cut out and extracted from an agarose gel, cloned into the pGEM®-T Easy vector (chap. 2.14.5) and sequenced from both ends.

2.12.3.2 5` RACE

The amplification of the 5’-ends of the cDNA requires additional steps. The 5’-end does not have a polyA-tail for primer binding and therefore needs an attachment of a poly-A-binding site to the 3’-end of the cDNA by means of a terminal transferase (TdT).

2.12.3.2.1 cDNA-synthesis

For reverse transcription the following components were added to a master mix:

5 x cDNA Synthesis Buffer	4 µl
dNTPs 10 mM	2 µl

gene-specific primer (SP1) 12.5 mM	1 µl
Thermoscrip RT	1 µl
total RNA	1 µg
dd H_2O up to	20 µl

The reaction proceeded at 55°C for 1 h and an inactivation step at 85 °C and 5 min. Before the polyA-tailing could happen purification needed to be done using the High Pure Purification Kit (Roche).

2.12.3.2.2 Terminale Transferase reaction

Terminal desoxynucleotidyl transferase (TdT; Promega, Germany) and dATP were added to the purified cDNA for the synthesis of a polyA-tail to the 3´ end of the cDNA. First, 11 µl of purified cDNA was incubated with 5 µl 10 x TdT buffer and 1.5 µl 2 mM dATP for 3 minutes at 94 °C. For the TdT reaction, 1 µl TdT (80 U/µl), were added to the chilled cDNA mixture and incubated at 37°C for 20 min. 5 µl of the TdT reaction mixture were then used for a PCR with the sequence-specific primer ARST1-RACE-SP2rev and the Oligo dT-Anchor primer (Table 2.1). The Oligo dT-Anchor primer contains an oligoT-sequence and therefore can bind to the polyA-tail at the 3´end of the first strand cDNA. This allowed the amplification of the cDNA fragment between the priming site of ARST1-RACE-SP2rev and the 5´end of the mRNA. The fragment was then cloned into pGEM®-T Easy (chap. 2.14.5) and both ends were sequenced. For safety, the 5´RACE was repeated with another gene-specific primer (ARST1-RACE-SP3rev), yielding the same 5´end sequence of the cDNA. This sequence was then used to design the primers ArST1-PFU1-2 which binds to the 5´ end of ArSUT gene, to amplify the full length cDNA with proof-reading control (chap. 2.12.4).

2.12.3.2.3 PCR-reaction

The attachment of the polyAs beforehand created a binding site for the Oligo(dT)-Anchor Primer in the first PCR-reaction that was composed in the following way:

10x buffer B (Bio&Sell)	5 µl
$MgCl_2$ 25 mM	2.5 µl

dNTPs 10 mM	1 µl
gene-specific primer (SP2) 12.5 mM	1 µl
Oligo(dT)-Anchor Primer 12.5 mM	1 µl
Taq-polymerase 5 U/µl (Bio&Sell)	0.5 µl
cDNA	5 µl
H_2O	34 µl

The PCR-program was identical to that in 3'RACE (Table 2.2).

A second PCR was conducted with a gene-specific primer (SP3) that was more internal positioned and the PCR-Anchor Primer. PCR-conditions were the same as before (chap. 2.12.3.1.2) except for the PCR-product as template (1:10 diluted and undiluted). The expected sequence length was around 500 – 600 bp. A band of that size was extracted from an agarose gel, cloned into the pGEM®-T Easy vector (chap. 2.14.5) and sequenced from both ends (chap. 2.12.4).

2.12.4 Amplification and cloning of ArSUT full length cDNA

Four gene-specific primers, ARST1-PFU1f, ARST1-PFU2r, ARST1-PFU3f and ARST1-PFU4r (Table 2.1**Fehler! Verweisquelle konnte nicht gefunden werden.**), were designed from the 5´ and 3´sequences of ArSUT obtained by 3´ and 5´RACE. PCR was performed with these primers and ArSUT cDNA obtained by RT reaction using oligo dT_{20} (2.11). For that PCR, a Phusion High-Fidelity DNA Polymerase (Thermo) with 3´-5´ exonuclease („proof-reading") activity was used to achieve high accuracy of the amplification.
The PCR program and the reaction conditions were based on the requirements of this special polymerase:

The PCR product was purified via agarose gel electrophoresis and ligated into pGEM®-T Easy. Cloning and selection of transformants were performed as described in chapter 2.14.5. Both strands of the full length cDNA were sequenced by Eurofins Genomics (Germany). The pGEM®-T Easy vector contains the promoter sequences of SP6 and T7. These flank the insert and make sequencing possible. For that

purpose 750-1500 ng of overnight cultures with the vector-insert construct (in 15 µl Tris/HCl, pH 8.5) were sent to Eurofins Genomics.

Table 2.5 PCR-program of *Pfu*-polymerase

Temperature	Time	Step
94 °C	3 min	initial denaturation
94 °C	20 sec	denaturation
56 °C	30 sec	annealing
72 °C	1 min	elongation
		repeat of cycle 44x
72 °C	5 min	final elongation
4 °C		store

.

2.13 Quantitative Real Time-PCR (*q*RT-PCR)

The *q*RT-PCR allows the determination of the relative transcription level of a gene of interest (GOI) compared to a housekeeping gene (HKG) that is constantly regulated (Higuchi et al. 1993, Nathan et al. 1995, Gibson et al. 1996, van Guilder et al. 2008). The signal of the fluorescent dye SYBR Green I is measured after every amplification cycle over the period of a standard PCR. The outcome of this is cycle threshold (Ct) that describes the point when the fluorescent signal exceeds the background signal and therefore the relative concentration of the target (van Guilder et al. 2008, www.lifetechnologies.com). To determine the relative expression level the ct-value of the GOI was related to Ct-value of the HKG, always with the assumption that the housekeeping gene is not influenced by the different experimental conditions (Wong and Medrano 2005).

2.13.1 cDNA Synthesis for quantitative RT-PCR

After RNA-isolation with subsequent DNA-digestion (2.10) a cDNA-synthesis using the standard oligo-dT_{20} primer, an RT from Fermentas/Thermo Scientific (RevertAid First Strand cDNA Synthesis Kit) with T_{opt} = 37°C was conducted. Reverse transcriptions were performed in a volume of 20 µl. 1 µg (= 11 µl from DNase-

Digestion) were applied. The reaction was performed as described in chapter 2.11, but without the 65° C step.

2.13.2 qRT-PCR- assay and analysis

To determine the accuracy of the gene expression level an efficiency analysis of the PCR-reaction was conducted. 100 % efficiency means a precise duplication of the amplicon. This was determined for every primer pair by pooling all cDNA of one experiment and the generation of a dilution series of 1:5. The subsequent standard curve and its regression line indicated the efficiency of the reaction. Values between 95 % and 105 % were used for the determination of the relative expression level.

Before starting the *q*RT-PCR assay the primer pair concentration needed to be optimized in order to achieve the best signal and to prevent the creation of primer dimers. Therefore different primer concentrations were tested.

For *q*RT-PCR reaction a master mix containing

2x qPCR Master Mix (Maxima SYBR Green,Thermo)	10 µl
f-primer (10 µM)	1.2 µl
r-primer (10 µM)	1.2 µl
ROX (5 µM)	0.08 µl
H_2O	3.52 µl

4 µl cDNA and 16 µl of the master mix were pipetted in each well of a 96-well plate. The reactions were operated with Mx3005P (Stratagene) and analyzed with the corresponding software program MxPro (version 4.10, Stratagene).

Table 2.6 Schedule for *q*RT-PCR

Temperature	Time	Step
95 °C	15 min	initial denaturation
95 °C	10 sec	denaturation
55 °C*, 60°C**	30 sec	annealing
		repeat of cycle 45x
55-95 °C		dissociation curve
95 °C		final denaturation

* STS, ** SUT

2.14 Gene cloning

2.14.1 Purification of PCR-products

Successful cloning of DNA-fragments requires purified DNA that was cleaned up by the E.Z.N.A.® Gel-Extraction Kit (Omega). Therefor an agarose gel was prepared with larger slots that can even collect several PCR-batches (for a better yield of DNA-amount identical PCR-batches can be pooled). The electrophoresis was run as described in chapter 2.9. After the ethidium-bromide bath, the band of interest was cut out on the UV-table, trying to expose the DNA as less degrading UV-light as possible and to keep the gel pieces small. The cut gel slices were transferred into a 2 ml reaction tube (prior weighed and labeled). The gel slice was weighed and the appropriate amount of binding buffer (XP2) was added. After an incubation of 10 minutes at 60 °C and 1400 rpm in a thermo shaker the gel slice was melted until no smears were visible, the pH of the solution was inspected. Yellowish color means the correct pH-range, reddish color needs an adjustment with 3 M sodium-acetate (pH 5.0) to reach the optimal pH-value of 7.5 that is necessary to bind the DNA on the silica membrane. The solution was now transmitted onto the HiBind DNA Mini Column (with a silica membrane) and centrifuged for 1 min at 10.000x *g*. If the volume the capacity of the column of 700 µl exceeded, the process was repeated. Thereby the flow-through was discarded. The membrane was washed with 300 µl binding buffer (XP2) (1 min, 13.000x *g*). The washing step was repeated at least twice to remove all agarose from the sample, because agarose is able to inhibit

enzymatic reactions like ligations. To remove any salt from the sample another washing step with SPW wash buffer was conducted. After discarding the flow-through the membrane was dried by centrifuging for 1 min at 13.000x *g.* The column was then placed on a new reaction tube and 30 µl elution buffer (10 mM Tris-HCl, pH 8.5) was added. After an incubation of 2 min at RT, the DNA was eluted by centrifugation for 1 min at 13.000x *g* and quantified as described in chapter 2.8.

2.14.2 A-Tailing

PCR-reactions using the proof-reading *Pfu*-polymerase produce an amplicon with blunt ends. These blunt ends impede the ligation into a pGEM®-T Easy vector that has overlapping Ts which are in turn complementary to the overlapping As from PCR reactions with *Taq*-polymerase. To perform such a T/A-cloning anyway, adenosine residues were attached to the 3'-ends of the amplicon. The reaction batch (10 µl) contained:

6.2 µl	purified PCR-product
1 µl	PCR-buffer B (10x)
0.8 µl	$MgCl_2$ (25 mM)
1 µl	ATP (2 mM)
1 µl	*Taq*-polymerase (5 U/µl)

The mix was incubated for 30 min at 72 °C. 2 µl of this batch were used for ligation.

2.14.3 Ligation

A ligation is necessary, when an insert (PCR-product) should be incorporated into a plasmid. Therefor either purified *Taq*-PCR-products or *Pfu*-PCR-products with attached adenosines were used for the pGEM®-T Easy vector.

The reaction volume (10 µl) contained 1 µl (1 U/µl) T4-DNA-ligase (Thermo), 2 µl 5 x ligase buffer (10 mM dATP, 50 mM $MgCl_2$, 10 mM DTT and 660 mM Tris-HCl, pH 7.6), 5 – 10 ng vector and the insert in an amount suited to provide a molar vector:insert ratio in the range of 1:3. The reaction took place overnight at 4°C.

2.14.4 Preparation of competent cells of *E. coli* strains DH5α with rubidium chloride

Competent cells of DH5α were used for transformation with the ligation products and should therefore possess a high competence for transformation.

A preparatory culture with *E. coli* cells (DH5α) was activated overnight in 5 ml SOB-Medium at 37 °C and 250 rpm. 50 ml SOC medium were inoculated with the DH5α preparatory culture to an OD_{578} of 0.05 and grown for 3 – 4 hours (37°C, shaking) until the OD_{578} reached a value of 0.5 – 0.7. The grown cells were poured into four 50 ml conical centrifuge tubes and sedimented for five minutes (3500 rpm, 4 °C). The following steps were performed on ice under a laminar flow hood. The pelleted cells were resuspended in 25 ml TMF 1 - medium per tube and incubated on ice for 30 min. The suspension was centrifuged again for 5 min (3500 rpm, 4 °C). The pellets were resuspended in 5 ml of TMF 1 – medium + 20% (v/v) glycerin. 200 µl aliquots of the competent cells were pipetted into pre-cooled 1.5 ml Eppendorf tubes and shock-frozen in liquid nitrogen. The cells were stored at -80°C.

SOB medium:

Trypton	2% (w/v)
yeast extract	0.5% (w/v)
NaCl	10 mM
KCl	2.5 mM

The pH-value was adjusted to pH 7.0 and the mixture was autoclaved for 10 min.

2 M $MgCl_2$ (filter-sterilized)	5 µl/ml
1 M Glucose (filter-sterilized)	20 µl/ml

TMF 1-medium:

$CaCl_2$	100 mM
RbCl	50 mM
$MnCl_2 * 4\ H_2O$	0 mM

The solution was filter-sterilized.

2.14.5 Transformation of *E. coli*

The competent cells were thawed on ice, 50 µl per reaction. 5-20 µl ligation reaction was added to the competent cell suspension and mixed by pipetting. After incubation on ice for 30 min, the cells were incubated at 42 °C for exactly 30 sec and transferred back to ice for 2 minutes. 1 ml SOC medium (1ml SOB-Medium + 5µl 2m $MgCl_2$ + 20µl 1M Gluc) were added to the cells under a laminar flow hood and the cells were incubated at 37 °C for 60 – 70 minutes in a thermo shaker at 250 rpm to allow the expression of antibiotic resistance genes. The cells were pipetted under a clean bench onto LB-X-Gal-Amp agar plates in two ways – diluted and concentrated. For the "diluted" application the cells were pipetted as they were and plated on the agar plate using a sterilized spreader rod to get single colonies. For the "concentrated" application the remaining solution was centrifuged for 2 min at 7000 rpm and 850 µl of the supernatant was removed and the pellet was dissolved in the left medium. This was pipetted onto a second plate and evenly spread using the sterilized spreader rod.

The plates were dried and incubated at 37 °C overnight. To proof the success of transformation a blue/white screening was used. The blue/white screening follows the principle given below:

The presence of lactose in the surrounding environment triggers the lacZ operon in *E. coli*. The operon activity results in the production of β-galactoisdase enzyme that metabolizes the lactose. When the plasmid vector is taken up by such cells, due to α-complementation process, a functional β-galatosidase enzyme is produced.

In the pGEM®-T Easy vector a multiple cloning site (MCS) is present within the lacZ sequence. This sequence can be nicked by restriction enzymes to insert the foreign DNA. When a plasmid vector containing foreign DNA is taken up by the host *E. coli*, the α-complementation does not occur, therefore, a functional β-galactosidase enzyme is not produced. If the foreign DNA is not inserted into the vector or if it is inserted at a location other than MCS, the lacZ gene in the plasmid vector complements the lacZ deletion mutation in the host *E. coli* producing a functional enzyme.

For screening the clones containing recombinant DNA, a chromogenic substrate known as X-gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside) is added to the agar plate. If β-galactosidase is produced, X-gal is hydrolyzed to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerizes to produce an insoluble blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo. The colonies formed by non-recombinant cells, therefore appear blue in color while the recombinant ones appear white. The desired recombinant colonies can be easily picked and cultured.
Single recombinant colonies were picked for growth overnight in LB-medium with ampicillin, following a Mini preparation of plasmid DNA (chap. 2.7.2.1) and characterization by restriction enzyme digestion with the following separation of DNA fragments by a gel electrophoresis (chap. 2.9).

2.15 Stachyose-synthase activity assay

2.15.1 Establishment of Sephadex-G25 columns

To desalt protein extracts that were later analyzed with HPLC Sephadex G25 columns needed to be established. Therefore per gram Sephadex G-25 (medium size, Sigma-Aldrich) 7 ml sodium phosphate buffer (50 mM, pH 7.0) was added and equilibrated overnight. The well soaked Sephadex granulate was then filled into prepared 1 ml disposable syringes (bottom opening plated with glass wool to prevent a flow-through of the granulate) up to a volume of 0.9 ml. The syringe itself was placed in a 15 ml tube. For storing the column was covered with sodium phosphate buffer (50 mM, pH 7.0) and sealed with parafilm. Before the application of the protein extracts, the columns were emptied by centrifugation for 5 min at 2000 rpm (modified after Voitsekhovskaja et al. 2009).

2.15.2 Stachyose-synthase activity

The activity of stachyose-synthase (STS) in *A. reptans* leaves and other plant tissues was measured by the time dependent formation of stachyose following the reaction galactinol + raffinose = myo-inositol + stachyose.

The procedure was modified after Voitsekhovskaja et al. (2009). 250 - 300 mg plant material was powdered and extracted in 1 ml extraction buffer (50 mM $NaPO_4$-buffer pH 7.0, 5 mM DTT, 50 mM sodium ascorbate) by vortexing and centrifugation (5 min, 4 °C, and 13.000 x *g*). The supernatant was transferred into a new reaction tube and stored on ice. 150 µl of that extract was then desalted on a 1ml sephadex G25 column (chap. 2.15.1) in order to remove all salts and sugars and concentrate proteins. Then the assay mix containing

$NaPO_4$-buffer (250 mM; pH 7.0)	3.5 µl
galactinol (10 mM)	10 µl
raffinose (225 mM)	3.5 µl
DTT (15 mM)	3.5 µl

was prepared in a 1.5 ml reaction tube and the reaction got started with the addition of 29.5 µ desalted extract. The reaction proceeded at 30 °C and was stopped after 0 min and 120 min by incubation at 100°C for 5 minutes. For the first timepoint, the tubes were placed at 100 °C immediately after the addition of the extract. Negative control reactions were performed for 120 min under the same conditions but galactinol was omitted from the assay mixture and substituted by $NaPO_4$-buffer (250 mM; pH 7.0).
Sugars were analyzed by HPLC according to Nadwodnik and Lohaus (2008)

3 Results

This study started with the analysis of sugar content in whole leave extracts and phloem exudates in Lamiaceae (*Ajuga reptans, Lamium album, Origanum majorana, Mentha piperata, Lavendula officinalis, Salvia officinalis, Caryopteris, Calamintha nepeta*), Oleaceae (*Ligustrum vulgare, Syringa vulgaris*), Cleastraceae (*Celastrus orbiculatus, Euonymus europaeus*), Bignoniaceae (*Catalpa bignioides*), Scrophulariaceae (*Buddleja davidii*) and Apiacaea (*petrosilenum crispum*). These plants are supposed to have an open minor vein configuration according to Gamalei (1989) and were analyzed on their RFO content in the whole leaf and the sugar composition in phloem exudates. In addition to sugar analysis a RNA-isolation with subsequent PCR with the degenerated primer pair for SUT1 (Knop et al. 2001) was done. Most of the species could be excluded for further analysis either because of their to low RFO content or because of no PCR result. For a further examination was *A. reptans* the plant of choice.

The results are separated into two manuscripts. The first manuscript was submitted for publication, the second is planned to publish, but needs more replications to verify the results.

Manuscript 3.1 addresses the issue of the subcellular compartmentation of metabolites (including RFOs) in summer and winter leaves of the perennial herb *A. reptans*. Furthermore it is about the different functions of raffinose-family oligosaccharides due to their subcellular localization and accumulation in summer and winter leaves of *A. reptans*.

Manuscript 3.2 addresses the issues of carbon partitioning, especially RFOs and sucrose, in different tissues (source leaves, sink leaves, stem, calyx and flower) of *A. reptans* and at different light and temperature conditions, respectively. Additionally, STS-expression and –activity and SUT4-expression was determined in the plant tissues and at the same varying conditions.

3.1 Manuscript 1: Subcellular distribution of raffinose-oligosaccharides and other metabolites in summer and winter leaves of *Ajuga reptans* (Lamiaceae)

Title: Subcellular distribution of raffinose-oligosaccharides and other metabolites in summer and winter leaves of *Ajuga reptans* (Lamiaceae)

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Main conclusion

In *Ajuga reptans* raffinose oligosaccharides accumulated during the cold season. Stachyose and verbascose were exclusively found in the vacuole whereas one fourth of raffinose was localized in the stroma.

Abstract

The evergreen labiate *Ajuga reptans* can grow at low temperature and even survive freezing. The central carbohydrate metabolism changes during the cold phase, e.g. raffinose family oligosaccharides (RFOs) accumulate. Additionally, *A. reptans* translocates high amounts of RFOs in the phloem. In the present study, subcellular concentrations of metabolites were studied in summer and winter leaves of *A. reptans* to gain further insight into regulatory instances involved in the cold acclimation process and into the function of RFOs.

Subcellular metabolite concentrations were determined by applying the non-aqueous fractionation technique and HPLC. Volumes of the subcellular compartments of summer and winter leaves were analyzed by morphometric measurements.

The metabolite content varied strongly between summer and winter leaves. Soluble metabolites (sugars, amino acids and malate) increased during cold season up to 10-fold whereas the starch content was decreased. In winter leaves the subcellular distribution showed a shift of carbohydrates from cytoplasm to vacuole and chloroplast. Despite this, the metabolite concentration was higher in all compartments in winter leaves compared to summer leaves because of the much higher total metabolite content in winter leaves. The different oligosaccharides did show different compartmentations. Stachyose and verbascose were almost exclusively found in the vacuole whereas one fourth of raffinose was localized in the stroma. Apparently, the subcellular distribution of the RFOs differs because they fulfill different functions in plant metabolism during cold season. Raffinose might function in protecting chloroplast membranes during freezing, whereas stachyose and verbascose might function primarily as carbon storage form.

Keywords *Ajuga reptans*, cold acclimation, non-aqueous fractionation, subcellular metabolite concentration, raffinose, stachyose

Abbreviations

FW	Fresh weight
HPLC	High performance liquid chromatography
RFO	Raffinose oligosaccharides

3.1.1 Introduction

Raffinose oligosaccharides (RFOs) are α-1,6-galactosyl$_n$ extensions of sucrose that occur frequently in higher plants and the most common RFOs are raffinose, stachyose and verbascose. They are synthesized by sequential actions of α-galactosyltransferases which either transfers a galactosyl moiety from galactinol to sucrose, raffinose or stachyose (Peterbauer and Richter 2001). Together with sucrose, RFOs have several functions in plants, e.g. as carbon transport form in the phloem as well as carbon storage form in seeds or vegetative plant parts commonly related to desiccation or cold tolerance (Turgeon et al. 1993; Bachmann et al. 1994; Knaupp et al. 2011).

Many temperate and perennial plant species can grow at low temperature and even survive freezing. Exposure to low but non-freezing temperatures induces a multifaceted and complex process termed cold acclimation by which plants are able to increase their cold tolerance. During cold acclimation, numerous genetic, physiological and biochemical changes occur enabling plants to tolerate temperatures several degrees lower than before cold exposure (Strand et al. 1997; Stitt and Hurry 2002; Espinoza et al. 2010). Reprogramming of the central carbohydrate metabolism and concentrations of soluble sugars was shown to play a crucial role during cold acclimation (Scarth and Levitt 1937; Koster and Lynch 1992; Strand et al. 1997). Compatible solutes may act either nonspecifically as osmolytes, or as stabilizers for proteins and membranes during freezing (Knaupp et al. 2011). An increase of RFOs, especially raffinose concentration and also sucrose concentration were observed in plants like cabbage, *Ajuga reptans*, saltgrass or *Arabidopsis thaliana* exposed to low temperature (Santarius and Milde 1977, Bachmann et al. 1994, Shahba et al. 2003, Klotke et al. 2004). It was demonstrated that exogenous sucrose at high concentrations has a cryoprotective effect on cellular membranes (Uemura and Steponkus 2003) and also raffinose might function in protecting membranes at low temperatures (Schneider and Keller 2009). However, the accumulation of soluble sugars during cold exposure is insufficient to fully explain the process of cold acclimation (Hincha et al. 1996; Zuther et al. 2004). In plants, there is a large overlap between cold and circadian and/or diurnal regulated genes and metabolite contents (Espinoza et al. 2010). Expinoza et al. (2010) have shown that

about 80% of metabolites in *Arabidopsis* leaves that showed diurnal cycles maintained these during cold treatment including galactinol and raffinose.

RFOs are also common as transport form for carbon in the phloem of different plant families, e.g. Lamiaceae or Oleaceae (Knop et al. 2001; Öner-Sieben and Lohaus 2014). The transport of RFOs in the phloem is correlated to special forms of companion cells in the minor veins, so called intermediary cells (Turgeon et al. 1993). Intermediary cells are defined by abundant plasmodesmal connections towards the adjacent mesophyll cells or bundle-sheath-cells (Turgeon et al. 1993). To explain symplastic uphill transport of oligosaccharides into the sieve elements, a polymer trap mechanism has been proposed (Turgeon et al. 1993). According to this model sucrose diffuses through the numerous plasmodesmata into the intermediary cells. There raffinose and stachyose are synthesized from sucrose and galactinol by the activity of raffinose synthase and stachyose synthase (Holthaus and Schmitz 1991; Voitsekhovskaja et al. 2009). Furthermore, it was postulated that the size exclusion limit of plasmodesmata connecting intermediary cells to the bundle sheath cells enables the passage of disaccharides such as sucrose from mesophyll into the phloem whereas the tri- and tetrasaccharides raffinose and stachyose remain trapped in the phloem. Symplastic phloem loaders were proposed to be more cold-sensitive than apoplastic loading species (Gamalei 1991) although several freezing tolerant and evergreen species exist in the group of symplastic phloem loaders (Hoffmann-Thoma et al. 2001).

The perennial herb *Ajuga reptans* (Lamiaceae) is known to be freezing tolerant. Plants grown at low temperatures store large amounts of RFOs during cold seasons (Bachmann and Keller 1994; Peters and Keller 2009). *A. reptans* also translocates large amounts of RFOs in the phloem during all seasons and was classified as symplastic phloem loader (Bachmann and Keller 1994; Hoffmann-Thoma et al. 2001).

The accumulation of water-soluble carbohydrates is one of the most commonly observed responses of plants to cold or freezing conditions. However, such a general observation does not pay tribute to the fact that plant cells are highly compartmentalized and that local concentrations of potential stress protectants in particular locations are important (Lunn 2007; Schneider and Keller 2009; Nägele

and Heyer 2013). Changes in the subcellular concentration and distribution of sugars might provide a mechanism to protect specific compartments. Subcellular metabolite partitioning is already done for a number of plants, e.g. spinach (Riens et al. 1991), barley (Winter et al. 1993), tobacco (Heineke et al. 1994), *Plantago* (Nadwodnik and Lohaus 2008), or *A. thaliana* (Krueger et al. 2009; Knaupp et al. 2011; Krueger et al. 2011; Nägele and Heyer 2013) but subcellular localization of raffinose was mainly done for *Arabidopsis* (Iftime et al. 2011; Knaupp et al. 2011; Nägele and Heyer 2013) and only very few reports described the subcellular localization of stachyose (Voitsekhovskaja et al. 2006; Iftime et al. 2011) and probably none the localization of verbascose. *A. reptans* has been object to such studies as well, but only on isolated chloroplasts (Schneider and Keller 2009), vacuoles (Bachmann and Keller 1995) or protoplasts (Schneider and Keller 2009). The isolation of cell organelles or protoplast needs several hours and therefore these methods have the disadvantage that water-soluble, low-molecular weight substances, such as mono-, di- or oligosaccharides might putatively leak out of the organelles and protoplasts or may be redistributed or metabolized during this time (Schneider and Keller 2009). In contrast, non aqueous fractionation is a technique to separate subcellular compartments, and their molecular compositions, under conditions where biological activities are completely arrested due to rapid freezing and dehydration of the sample material.

In the present study the subcellular distribution of mono-, di-, and oligosaccharides, sugar alcohols, amino acids, and malate in summer and winter leaves of *A. reptans* have been evaluated using the non aqueous fractionation technique to gain further insight into temperature effects on subcellular localization of RFOs and other metabolites. For the calculation of the subcellular metabolite concentrations the subcellular volumes of mesophyll cells from summer and winter leaves were determined. In addition, primary metabolites were analyzed at the end of the light and dark period to better understand the response to cold during light-dark cycles.

3.1.2 Materials and methods

3.1.2.1 Plant materials

Ajuga reptans were grown outside of the "Bergische Universität Wuppertal" (Germany; 51.26°N, 7.18°E) at two separate locations. Warm treated plants were grown in summer months at 15 – 30 °C and in the following referred as summer leaves. Cold treated plants were grown at temperatures of -5° – 10 °C in winter months and below referred as winter leaves. Leaf samples were harvested at the end of August (about 14 h sunlight; about 20-25°C) and at the end of February (about 11 h sunlight; about 0-5°C) each at the end of the daylight period and the end of the dark period.

3.1.2.2 Non-aqueous fractionation of leaves

Leaf samples were ground with mortar and pestle to a fine powder on liquid nitrogen and lyophilized (Christ alpha 2-4; Martin Christ, Osterode am Harz, Germany). The procedure was conducted according to Riens et al. (1991), Nadwodnik and Lohaus (2008) and Krueger et al. (2014). For *A. reptans* leaves harvested in summer, a density gradient between 1.35 and 1.50 g ml^{-1} and for leaves harvested in winter between 1.38 and 1.48 g ml^{-1} were used. Six fractions were collected, aliquots of which were taken for the determination of the marker enzymes NADP-glycerine aldehyde phosphate (GAP)-dehydorgenase, PEP-carboxylase and α-mannosidase as markers for chloroplast, cytosol and vacuole, respectively (Riens et al. 1991; Klie et al. 2011; Krueger et al. 2014), and also aliquots for determination of metabolites. The cytosolic compartment was found to be enriched in the middle region of the gradient; the chloroplast material appeared in the region of lower density whereas the vacuolar material was mainly found in the fraction of highest density. For determination of metabolite concentrations in the gradient fractions, chloroform methanol extracts were prepared (see chapter extraction of soluble metabolites). For the evaluation of the subcellular distribution of metabolites between the stromal, cytosolic, and vacuolar compartment a calculation procedure according to Riens *et al*. (1991) was used.

3.1.2.3 Extraction of water soluble metabolites

The dried fractions of the gradients were used for the extraction of metabolites. 5 ml chloroform:methanol (1,5:3,5, v/v) was added to the pellet, and the sample was homogenized and kept on ice for 30 minutes. The homogenate was then extracted twice with 3 ml water. The aqueous phases were combined and evaporated in a rotatory evaporator (RV 10 Digital; IKA, Staufen, Germany). The dried residue was dissolved in 1 ml ultrapure H_2O (Millipore; Billerica, MA, USA), syringe-filtrated (0.20 µm nylon; Carl Roth, Germany) and stored at -80°C until analysis.

3.1.2.4 Metabolite analysis

Sugars and sugar alcohols in the different fractions were analyzed by HPLC with an amperometrical detector according to Nadwodnik and Lohaus (2008). Amino acids were analyzed on a reverse phase column (Merck LiChro Cart 125-4; Supersphere 100 RP-18 endcapped) with a precolumn derivatization with ortho-phtaldialdehyde (OPA) and β-mercaptoethanol derivatization according to Riens et al. (1991). Concentrations of anions and malate were analysed by ion chromatography (DX500, Dionex, Idstein, Germany) using an IonPac anion exchange column (AS4, 4 x 200 mm, Dionex, Idstein, Germany) connected with a conductivity detector module (CD 20, Dionex, Idstein, Germany). The ions were eluted with 1.8 mol m^{-3} Na_2CO_3 and 1.7 mol m^{-3} $NaHCO_3$ for 20 min (according to Lohaus et al. 2000). Starch was measured according to Riens et al. (1994) and protein was measured according to Lowry et al. (1951).

3.1.2.5 Electron microscopy

For Transmission Electron Microscopy (TEM), leaves of *A. reptans* (winter/summer) were cut in pieces and fixed over night in Karnovsky`s solution (Karnovsky 1965), buffered with 0.1 mol/l sodium cacodylate (pH 7.4) at 4 °C. After postfixation for 120 min in 2% osmium tetroxide in the same buffer, the specimens were dehydrated in a graded series of acetone, and embedded in Spurr's medium (Spurr 1969). Ultrathin sections then were stained with uranyl acetate and lead citrate according to Reynolds

(1963), and examined with a Hitachi TEM H600 at 70 kV. The cross-sectional areas of the subcellular compartments were quantified with analysis software (IMAGE J; public domain software, developed at US National Institutes of Health, available at http://rsbweb.nih.gov/ij/).

3.1.3 Results

3.1.3.1 Accumulation of assimilates in summer and winter leaves of *A. reptans* during light period

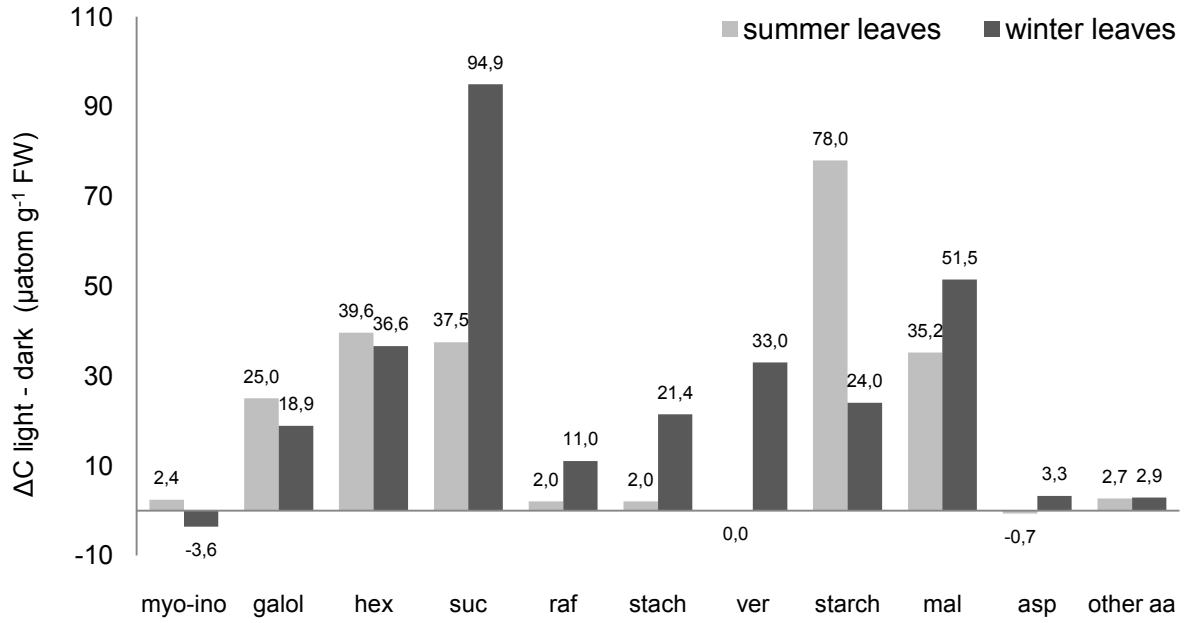
Leave samples of summer and winter grown plants of *A. reptans* were harvested and the contents of major photoassimilates (hexoses, sucrose, RFOs, starch, malate, and amino acids) were analyzed. In summer leaves of *A. reptans* the whole sugar content amounted about 35 µmol g^{-1} FW and the main sugars were glucose, fructose and sucrose (Table 3.1.1). The starch content (expressed in µmol glucose equivalent g^{-1} FW) was about 5-fold higher than the soluble sugar content. Summer and winter leaves differed vigorously in their sugar content because the sugar content in winter leaves was 4-5-fold higher than in summer leaves whereas the starch content was 4-5-fold lower (Table 3.1.1). In addition to sugars, leaves of *A. reptans* contained also high amounts of malate and amino acids. The content of aspartate was always higher than the sum of all other amino acids together.

Beside starch, sucrose and hexoses, which were the main products accumulated in summer leaves during the light period, considerable amounts of carbon were also accumulated in form of galactinol and malate (Fig. 3.1.1). In winter leaves also considerable amounts of carbon were accumulated during the light period in form of sucrose, hexoses, galactinol and malate. The accumulation of carbon in form of starch was less distinct than in summer leaves. In winter leaves the raffinose, stachyose and verbascose contents were also higher at the end of the light period in comparison to the end of the dark period. Although the contents of these oligosaccharides were only slightly altered in the course of the light-dark regime, an accumulation of carbon was observed during the light period because the carbon content per molecule was higher in these oligosaccharides than in the other sugars.

Table 3.1.1 Contents of sugars, amino acids and organic and inorganic ions in summer (*n* = 6) and winter (*n* = 6) leaves from *Ajuga reptans* at the end of the light and dark period. Mean values ± SD; hexoses: glucose and fructose; other amino acids: ala, arg, asn, gaba, gln, glu, gly, his, ile, leu, ly, met, phe, pser, ser, thr, trp, tyr, val. n.d. = not detectable

	summer leaves		winter leaves	
	end of light	end of dark	end of light	end of dark
Myo-inositol (µmol (g^{-1} FW)	6.6 ± 0.5	6.2 ± 0.9	6.7 ± 1.3	7.3 ± 1.2
Galactinol (µmol g^{-1} FW)	2.7 ± 0.8	0.6 ± 0.5	11.8 ± 2.9	10.2 ± 3.7
Hexoses (µmol g^{-1} FW)	14.8 ± 4.4	8.2 ± 2.9	70.0 ± 13.0	63.9 ± 8.5
Sucrose (µmol g^{-1} FW)	9.5 ± 2.2	6.4 ± 1.3	49.6 ± 7.0	41.7 ± 9.9
Raffinose (µmol g^{-1} FW)	0.3 ± 0.1	0.2 ± 0.0	5.5 ± 1.4	4.9 ± 1.1
Stachyose (µmol g^{-1} FW)	0.4 ± 0.2	0.3 ± 0.1	12.3 ± 1.3	11.4 ± 1.7
Verbascose (µmol g^{-1} FW)	< 0.1	< 0.1	4.5 ± 0.4	3.4 ± 0.6
Σ C in sugars (µatom C g^{-1} FW)	295	183	1731	1518
Starch (µmol glc equi g^{-1} FW)	185 ± 63	172 ± 46	43 ± 9	39 ± 6
Σ C in starch (µatom C g^{-1} FW)	1110	1032	258	234
Malate (µmol g^{-1} FW)	34.3 ± 5.7	25.5 ± 5.8	66.6 ± 6.9	53.8 ± 17.3
Aspartate (µmol g^{-1} FW)	10.3 ± 1.3	10.5 ± 2.2	9.4 ± 0.9	8.6 ± 0.7
Other amino acids (µmol g^{-1} FW)	8.6 ± 1.6	7.6 ± 2.9	7.5 ± 2.2	6.8 ± 1.2
Nitrate (µmol g^{-1} FW)	0.6 ± 0.2		0.1 ± 0.1	
Phosphate (µmol g^{-1} FW)	4.4 ± 1.2	4.4 ± 2.5	13.5 ± 1.9	11.1 ± 1.5
Sulfate (µmol g^{-1} FW)	8.3 ± 4.1	10.1 ± 4.1	7.2 ± 1.8	6.2 ± 1.4
Chloride (µmol g^{-1} FW)	15.1 ± 2.7	16.6 ± 4.1	12.9 ± 6.7	12.7 ± 3.9
Protein (mg g^{-1} FW)	7.7 ± 2.2	7.2 ± 2.9	11.6 ± 3.3	10.5 ± 2.7
Chlorophyll (mg g^{-1} FW)	1.7 ± 0.3	1.7 ± 0.3	0.9 ± 0.2	1.0 ± 0.3

Fig. 3.1.1 Difference of carbon assimilation in different metabolites at the end of the light and dark period in leaves of *Ajuga reptans*. The data are expressed as the carbon content of the various metabolites at the end of the light period minus the end of the dark period. Metabolite contents for calculation were taken from Table 1. Myo-ino: myo-inositol, galol: galactinol, hex: hexoses (glucose and fructose), suc: sucrose, raf: raffinose, stach: stachyose, ver: verbascose, starch: starch, mal: malate, asp: aspartate, other aa: other amino acids (ala, arg, asn, gaba, gln, glu, gly, his, ile, leu, ly, met, phe, pser, ser, thr, trp, tyr, val)



3.1.3.2 Subcellular distribution of sugars of summer and winter leaves

The subcellular distribution of sugars in summer and winter leaves is shown in Table 3.1.2. The data shown are mean values of each five independent experiments of summer and winter leaves. Metabolites that were attributed to only one compartment demonstrate are high reproducibility but metabolites that are distributed among three compartments or occurring in low contents a higher variation was found. The reproducibility of data is reflected in the standard deviations.

In summer leaves of *A. reptans* the different sugar alcohols, mono-, di-, and oligosaccharides showed different subcellular compartmentation (Table 3.1.2).

Table 3.1.2 Content and percentage distribution of sugars among the vacuolar, stromal and cytosolic compartments of summer and winter leaves from *Ajuga reptans* at the end of the light period. Mean values of *n* = 5 independent measurements are shown. Mean ± SD; hexoses: glucose and fructose; other amino acids: ala, arg, asn, gaba, gln, glu, gly, his, ile, leu, ly, met, phe, pser, ser, thr, trp, tyr, val. n.d. = not detectable

	Whole leaf content	Vacuole	Stroma	Cytosol
	µmol g^{-1} FW	%	%	%
Summer				
Myo-inositol	6.6 ± 0.5	59.7 ± 5.7	13.0 ± 1.7	27.3 ± 4.0
Galactinol	2.7 ± 0.8	44.7 ± 5.6	5.3 ± 6.8	50.3 ± 6.8
Hexoses	14.8 ± 4.4	98.4 ± 1.1	1.0 ± 1.0	0.6 ± 1.3
Sucrose	9.5 ± 2.2	38.6 ± 13.7	20.6 ± 19.0	40.8 ± 28.6
Raffinose	0.3 ± 0.1	60.0 ± 6.1	5.3 ± 4.7	34.7 ± 4.9
Stachyose	0.4 ± 0.2	97.7 ± 4.0	1.3 ± 2.3	1.0 ± 1.7
Verbascose	n.d.			
Malate	34.3 ± 5.7	98.8 ± 2.2	1.3 ± 1,5	0 ± 0
Aspartate	10.3 ± 1.3	97.0 ± 5.4	3.0 ± 5.4	0 ± 0
Other amino acids	8.6 ± 1.6	69.5 ± 18.2	7.8 ± 7.1	23.0 ± 16.0
Winter				
Myo-inositol	6.7 ± 1.3	92.0 ± 7.8	8.0 ± 7.8	0 ± 0
Galactinol	11.8 ± 2.9	78.6 ± 20.9	18.2 ± 15.5	3.2 ± 6.6
Hexoses	70.0 ± 13.0	93.6 ± 8.9	1.8 ± 4.0	4.6 ± 6.5
Sucrose	49.6 ± 7.0	75.8 ± 14.4	6.8 ± 7.3	17.4 ± 15.1
Raffinose	5.5 ± 1.4	75.3 ± 17.0	24.8 ± 17.0	0 ± 0
Stachyose	12.3 ± 1.3	100 ± 0	0 ± 0	0 ± 0
Verbascose	4.5 ± 0.4	99.0 ± 2.2	0 ± 0	1.0 ± 2.2
Malate	66.6 ± 6.9	98.8 ± 1.9	0 ± 0	1.0 ± 1.4
Aspartate	9.4 ± 0.9	91.3 ± 7.9	4.3 ± 6.7	4.5 ± 6.5
Other amino acids	7.5 ± 2.8	80.6 ± 11.1	8.8 ± 6.1	10.6 ± 7.4

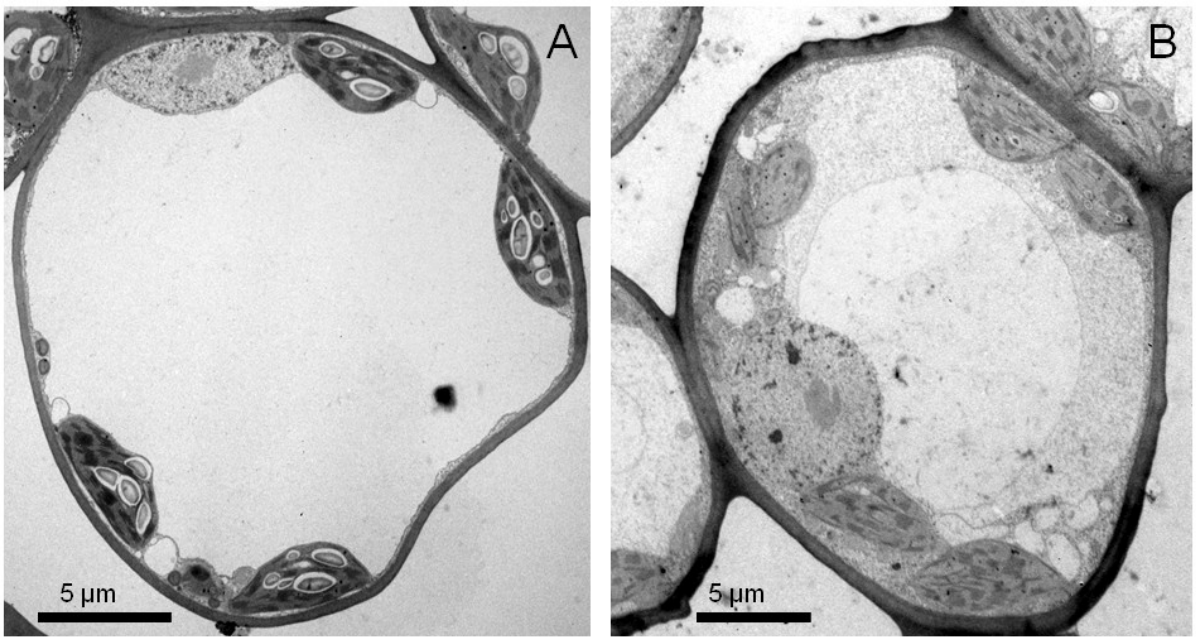
Stachyose and hexoses (glucose and fructose) were almost exclusively found in the vacuole. Sucrose and raffinose were distributed in all three compartments but mostly in the vacuole. Notably, in summer leaves no verbascose was found. The sugar alcohols were also distributed in all three compartments but myo-inositol mostly in the vacuole and galactinol in the vacuole and in the cytoplasm. *A. reptans* leaves contained high amounts of malate which was almost exclusively found in the vacuole (Table 3.1.2). Aspartate was the dominant amino acid. Aspartate was almost exclusively found in the vacuole whereas the other amino acids were distributed in all three compartments (Table 3.1.2).

The subcellular distribution of carbohydrates was similar in summer and winter leaves with the exception of mostly higher proportions in the vacuole during cold season. The raffinose and stachyose contents were 20-30-fold higher in winter leaves than in summer leaves (Table 3.1.2). In winter leaves also high amounts of verbascose were detectable. It should be noted that the subcellular distributions of raffinose, stachyose and verbascose were different (Table 3.1.2). Stachyose and verbascose were almost exclusively found in the vacuole whereas only three-quarter of raffinose was found in the vacuole and one-quarter in the chloroplast.

3.1.3.3 Subcellular volumes

The further conversion of subcellular metabolite proportions based on gram fresh weight (Table 3.1.2) into concentrations required the knowledge of the relative volumes of the subcellular compartments. A determination was done by morphometric analysis of light and electron micrographs. Table 3.1.3 shows the relative volumes of the vacuolar, chloroplastic and cytoplasmic (sum of cytosol, peroxisomes, mitochondria) and nuclear compartments of the mesophyll cells. The summer leaves of *A. reptans* contained mesophyll cells that exhibited a typical, large central vacuole surrounded by a thin layer of cytoplasm (Fig. 3.1.2). The large vacuole occupied 75.8 % of the total volume, followed by the chloroplasts with 11.9 %, the cytoplasm with 8.9 %, and nucleus with 3.4 % (Table 3.1.3). In winter leaves the vacuole was also the largest compartment with 55.4 % but the proportion of the

Fig. 3.1.2 Electron micrographs of *Ajuga reptans* mesophyll cells. A summer leaves, B winter leaves



cytoplasma (19.5 %) was about 2-fold increased in comparison to summer leaves (Fig. 3.1.2; Table 3.1.3). Similar results were shown for mesophyll cells of warm and cold grown *A. thaliana* plants (Strand et al. 1999) and a larger relative volume of the cytoplasm seems to be a more typical response to cold acclimation in plants. Also the proportion of chloroplasts at the cell volume in winter leaves (20.3 %) was about 60 % increased in comparison to summer leaves (Table 3.1.3). The relative portion of mitochondria and peroxisomes of the cytoplasm was about 13 to 15 % and the portion of the stroma of the chloroplast ranged from 48 to 58 % (Winter et al. 1993). In dicots the main part of the aqueous volume of the leaf is occupied by the mesophyll cells (Winter et al. 1993). Therefore, in summer leaves of *A. reptans* with an average water content of 841 µl g^{-1} FW the volumes of the vacuolar, stroma and cytoplasmic compartment can be estimated as 637, 50 and 75 µl g^{-1} FW. In winter leaves with an average water content of 694 µl g^{-1} FW the corresponding volumes are 384, 70 and 135 µl g^{-1} FW (Table 3.1.3).

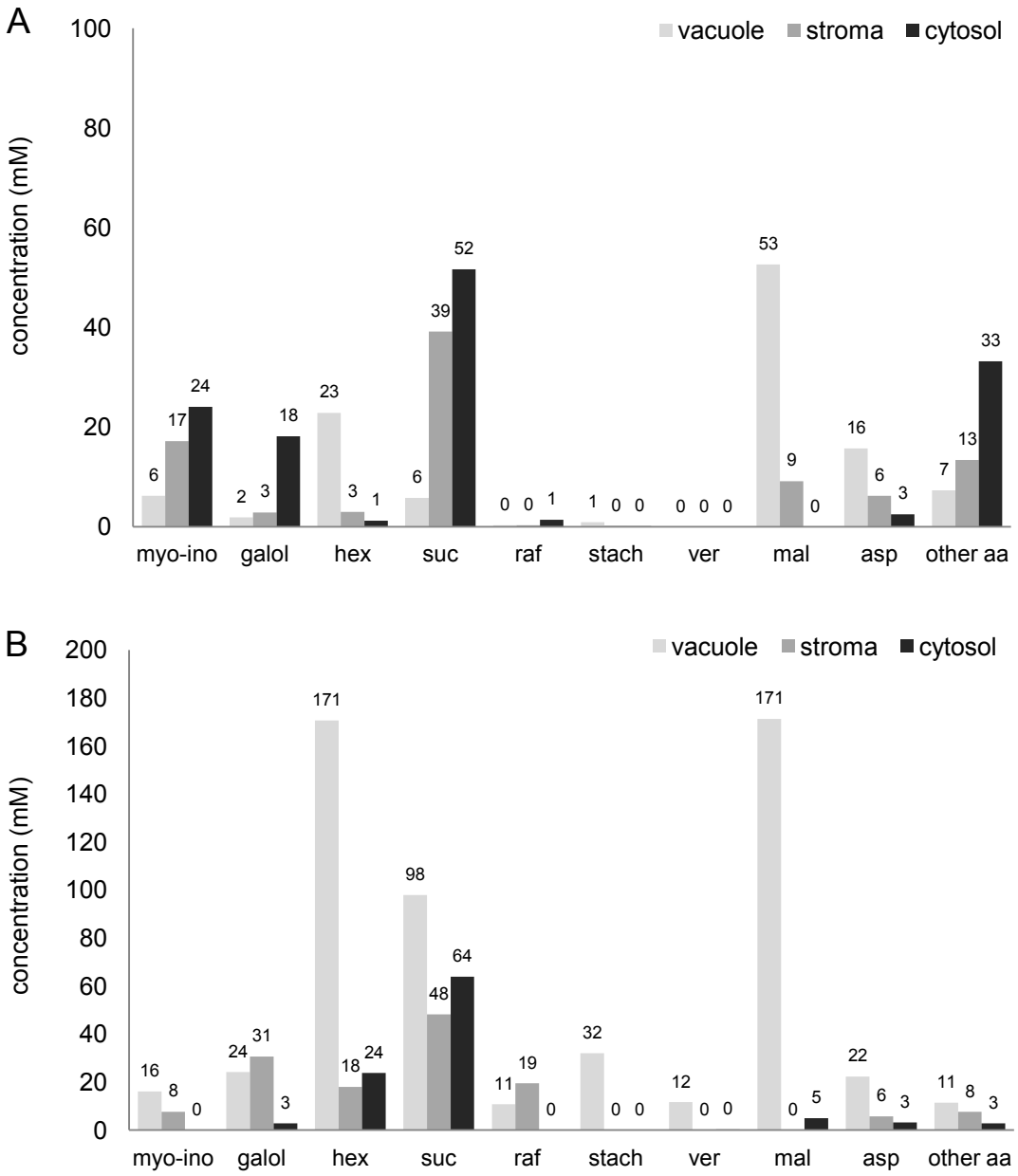
Table 3.1.3 Relative volumes (%) of the subcellular compartments at the total volume of mesophyll cells from summer and winter leaves of *Ajuga reptans*. Data were obtained from morphometric analysis (n = 20-22). The total volume of a mesophyll cell is defined as 100 %. The portion of the stroma at the chloroplast was about 50% (Winter *et al*. 1993). Cytoplasm is defined as cytosol, peroxisomes and mitochondria. Subcellular volumes were calculated from relative volumes of the subcellular compartments and the water space (summer leaves 841 ± 72 µl g^{-1} FW and winter leaves 694 ± 103 µl g^{-1} FW). Mean values ± SD are shown

	Summer leaves	Winter leaves
Vacuole (%)	75.8 ± 4.4	55.4 ± 6.0
Chloroplast (%)	11.9 ± 2.6	20.3 ± 5.2
Nucleus (%)	3.4 ± 0.8	4.8 ± 1.9
Cytoplasm (%)	8.9 ± 3.2	19.5 ± 3.7
Vacuole (µl g^{-1} FW)	637	384
Stroma (µl g^{-1} FW)	50	70
Cytoplasm (µl g^{-1} FW)	75	135

3.1.3.4 Subcellular metabolite concentrations

Subcellular concentrations were calculated for each sugar based on the subcellular volumes (Table 3.1.3), the subcellular proportions and the metabolite contents in the leaves (Table 3.1.2) measured as described above. In winter leaves sucrose was mainly concentrated in the vacuole, followed by the cytoplasm and the stroma (Fig. 3.1.3). In contrast, in summer leaves higher concentrations of sucrose were found in the stroma (Fig. 3.1.3) but the distribution of sucrose in this compartment was ambiguous (see Table 3.1.2). In addition, the small volume of the stroma (Table 3.1.3) led to high concentrations of metabolites which were localized in this compartment. The highest concentrations of hexoses and malate were found in the vacuole especially in winter leaves. In summer leaves the concentrations of raffinose, stachyose and verbascose in the different compartments were below 1 mM whereas in winter leaves concentrations of 10-35 mM of each oligosaccharide were found in the vacuole (Fig. 3.1.3). Only the raffinose concentration was also substantial in the stroma.

Fig. 3.1.3 Subcellular metabolite concentrations in summer (A) and winter (B) leaves of *Ajuga reptans*. Please note the different scale in figure A and B. Myo-ino: myo-inositol, galol: galactinol, hex: hexoses (glucose and fructose), suc: sucrose, raf: raffinose, stach: stachyose, ver: verbascose, starch: starch, mal: malate, asp: aspartate, other aa: other amino acids (ala, arg, asn, gaba, gln, glu, gly, his, ile, leu, ly, met, phe, pser, ser, thr, trp, tyr, val)



3.1.4 Discussion

3.1.4.1 Diurnal metabolite contents also during cold season

The perennial herb *A. reptans* can grow at low temperature and even survive freezing. Exposure to low but non-freezing temperatures induces a multifaceted and complex process termed cold acclimation, by which plants are able to increase their cold tolerance. The acclimation process includes the accumulation of solutes. In winter leaves of *A. reptans* a shift in the partitioning of carbon away from starch and towards soluble sugars including hexoses, sucrose and RFOs can be observed (Table 3.1.1). In cold acclimated *A. thaliana* leaves re-direction of newly fixed carbon towards sucrose synthesis rather than starch accumulation was also observed (Strand et al. 1997; 1999). The accumulation of soluble sugars can be mediated by the so-called CBF family of transcription factors, which trigger a large set of low temperature responses considered to be important for cold acclimation (Gilmour et al. 2000). The accumulation of free amino acids in winter leaves of *A. reptans* was not observed (Table 3.1.1) whereas the protein content was increased. Increased protein content was responsible for the increased activities of Rubisco and other Calvin-cycle enzymes as well as cFBPase and SPS in cold grown *A. thaliana* plants (Strand et al. 1999). This might be also the case for *A. reptans*.

In addition to temperature several other regulation processes influence primary metabolism. Diurnal regulation of starch, sugar or amino acid metabolism has been often demonstrated in warm grown plants (Riens et al. 1994). Also in *A. reptans* the carbon and nitrogen metabolism was influenced by these environmental factors, temperature and light-dark regime. During the dark period the sugar contents declined in summer leaves (Fig. 3.1.1). This decrease was less distinct than in other herbaceous plants (Riens et al. 1994). In crop plants like barley and spinach, carbon and nitrate assimilates showed diurnal rhythms with highest contents of sugars and amino acids at the end of the light period and the lowest at the end of dark, e.g. sucrose content at the end of the dark period (10-15 h) amounted to 10% of the content at the end of the light period (Riens et al. 1994). In plants, a large proportion of the carbon assimilated in leaves during the light period was deposited in the leaves for export during the night. Considerable phloem transport occurs in leaves at night, although at reduced rates (about 40% of the light rate; Riens et al. 1994). In *A.*

reptans, even after the dark period (10 h summer leaves, 13 h winter leaves) 64 % and 89 % of the sugar content measured at the end of the light period were still present (Table 3.1.1) and in the case of starch 93 and 91%, respectively. Therefore, in *A. reptans* probably much lower amounts of carbon assimilated at daylight are slated for export during dark periods than in crop plants like barley (Riens et al. 1994).

Higher contents of sugars and other metabolites at the end of the light period compared to contents at the end of the dark period could also be observed in winter leaves (Table 3.1.1, Fig. 3.1.1). These data support the assumption of the maintenance of sugar export in cold-acclimated winter leaves in *A. reptans* (Bachmann et al. 1994; Hoffmann-Thoma et al. 2001) although low temperatures might reduce phloem transport.

3.1.4.2 Nonaqueous fractionation technique and the subcellular distribution of RFOs

The non-aqueous fractionation technique and the three-compartment calculation program allowed us to determine the metabolite concentrations in the vacuolar, stromal and cytoplasmic compartments restrained in the state of active photosynthesis. For the analysis it was necessary to simplify the whole leaf to a consistent metabolic compartment, a mesophyll cell, subdivided into the three compartments listed above. It should be pointed out that the non-aqueous fractionation technique was developed for determination of subcellular concentrations of metabolites that are exclusively located in the mesophyll (Gerhardt and Heldt 1984). This is obviously a simplification of a more complex pattern, but until now it is one of the best ways to obtain information about subcellular metabolite levels *in vivo*. This method provides highly reproducible results for metabolites that were exclusively confined to a single compartment. A higher variation was found for metabolites located in more than one compartment and the variability was greatest when the proportion found in a particular compartment was low. However, the method yields reasonable results for the subcellular distribution of mono- and disaccharides, sugar alcohols, organic acids or amino acids. Voitsekhovskaja et al. (2006) have shown

that concentrations of hexoses and sucrose in mesophyll cells measured either by single-cell technique or by non-aqueous fractionation were similar.

In contrast, the non aqueous fractionation is less suitable for the determination of subcellular RFO concentrations. As one part of RFOs was synthesized in intermediary cells and the concentration in the phloem was at least one order of magnitude higher than in the mesophyll cells, it was to be expected that a considerable amount of RFOs associated to the mesophyll cells arose from the high content in the phloem which leads to somewhat overestimate concentrations in the mesophyll compartments. For *A. reptans* this portion can be estimated considering three assumptions: First, the sugar concentration in the phloem sap of *A. reptans* was similar to *Alonsoa meridionalis* with about 170 mM sucrose, 250 mM raffinose and 400 mM stachyose (Voitsekhovskaja et al. 2006) because both plant species are symplastic or mixed phloem loaders, second, the maximum volume of the sieve elements was about 2 µl g^{-1} FW (Winter et al. 1993), and third, no difference in minor vein structure of summer and winter leaves of *A. reptans* were detectable (Hoffmann-Thoma et al. 2001). Hence, the proportion of the leaf sugar contents that derives from the sieve elements was about 0.34 µmol g^{-1} FW for sucrose, 0.5 µmol g^{-1} FW for raffinose and 0.8 µmol g^{-1} FW for stachyose. Because the sucrose content in summer leaves of *A. reptans* was 9.5 µmol g^{-1} FW (Table 3.1.1), the “contamination” of the mesophyll by sucrose from the phloem can be neglected. In the case of the raffinose and stachyose contents (0.3 and 0.4 µmol g^{-1} FW) in summer leaves the proportion derived from the phloem represented the total leaf content of both oligosaccharides. In winter leaves the potential “contamination” of the mesophyll by the phloem was low, about 9 and 7%, respectively because the raffinose and stachyose contents in winter leaves were very high (see Table 3.1.1).

3.1.4.3 Subcellular distributions of metabolites in *A. reptans* in comparison with other plant species

The comparison of all measured carbohydrates in both, summer and winter leaves of *A. reptans* showed that most of the carbohydrates accumulate in the vacuolar compartment, about 71% in summer leaves and about 87% in winter leaves (Table

3.1.2). This is consistent with studies of other herbaceous plant species (Moore et al. 1997; Nadwodnik and Lohaus 2008; Farre et al. 2001; Voitsekhovskaja et al. 2006).

In summer and winter leaves hexoses (glucose and fructose) were almost exclusively found in the vacuole (Table 3.1.2). This corresponds to the results of other plant species which were also produced by the non-aqueous fractionation technique (Moore et al. 1997; Nadwodnik and Lohaus 2008; Farre et al. 2001; Voitsekhovskaja et al. 2006; Knaupp et al. 2011; Nägele and Heyer 2013). In contrast, Bachmann and Keller (1995) showed a different distribution for hexoses in cold treated *A. repta*ns leaves with the highest percentage in the cytoplasm (88 %). This divergence can derive from the different method used because Bachmann and Keller (1995) applied isolated protoplasts and vacuoles. The isolation procedure needed several hours. In this time metabolite transport at the membranes cannot be excluded, e.g. hexoses could be released from vacuole.

In several studies sucrose was localized mainly in the vacuole and in the cytoplasm (Winter et al. 1993; Moore et al. 1997; Voitsekhovskaja et al. 2006; Nadwodnik and Lohaus 2008). In cold acclimated *A. thaliana* the subcellular distribution of sucrose was different in that way that also the chloroplast contained a higher proportion of sucrose (Knaupp et al. 2011; Iftime et al. 2011; Nägele and Heyer 2013). This distribution was not found in winter leaves of *A. reptans* (Table 3.1.2) because the proportion of sucrose in the stroma decreased from summer to winter leaves. The proportion of sucrose in the cytoplasm of winter leaves was also lower than in summer leaves (Table 3.1.2). But the sucrose concentration in the cytoplasma was slightly higher in winter leaves than in summer leaves due to a higher total sucrose content in winter leaves (Fig. 3.1.3, Table 3.1.2). Nägele and Heyer (2013) have speculated that cytosolic sucrose accumulates rapidly after cold exposure, serving as a transient cryoprotectant for cellular membranes at early stages of cold exposure, while later it becomes replaced by a metabolic less critical compound, e.g. raffinose. This does not seem to be the case for *A. reptans* because the sucrose content was increased during the whole cold season.

In summer leaves of *A. reptans* myo-inositol was located in all three compartments whereas in winter leaves the main proportion was in the vacuole (Table 3.1.2). The chloroplastic pool of myo-inositol probably originates from its

synthesis by the stromal isoform of the myo-inositol synthesizing enzyme myo-inositol phosphate synthase (Adhikari et al. 1987).

Galactinol had been found previously in the mesophyll vacuoles of the apoplastic phloem loader *Antirrhinum majus* (Moore et al. 1997) and in the symplastic phloem loader *Alonsoa meridionalis* (Voitsekhovskaja et al. 2006). Although the galactinol-synthesizing enzyme is thought to be cytosolic in *A. reptans* (Bachmann and Keller 1995), in winter leaves galactinol occurred predominantly in the vacuoles of mesophyll cells, similar to the situation in *Antirrhinum* and *Alonsoa*, and in summer leaves in the vacuole and in the cytosol. This allowed suggesting that the pool of galactinol in the mesophyll of *A. reptans* is not directly related to RFO synthesis in the phloem. It is possible that galactinol is produced not only in the mesophyll but also within intermediary cells where it is used for the synthesis of raffinose and stachyose. This corresponds to the finding that *A. reptans* contained two isoforms of galactinol synthase in leaves, one mesophyll-specific and one intermediary cell-specific (Sprenger and Keller 2000). For the synthesis of galactinol, sucrose could be used after its hydrolysis by sucrose synthase producing UDP-glucose which can be further converted by UDP-glucose-4-epimerase into UDP-galactose, which is, together with *myo*-inositol, a substrate for galactinol synthase.

It exists only few data about the subcellular localization of stachyose (Voitsekhovskaja et al. 2006; Bachmann and Keller 1995) and these results indicate that stachyose is almost exclusively found in the vacuole. This corresponds to the findings in this study (Table 3.1.2). Stachyose and also verbascose in winter leaves of *A. reptans* were almost exclusively found in the vacuole. Iftime et al. (2011) have expressed a stachyose synthase from adzuki bean (*Vigna angularis*) in *A. thaliana*. In cold acclimated plants of this transgenic line stachyose accumulated in the cytosol but not in chloroplasts and vacuoles. Iftime et al. (2011) discussed that the *Arabidopsis* chloroplast envelope does not efficiently transport stachyose. This should also be true for the tonoplast in *Arabidopsis*.

In contrast to stachyose and verbascose a larger proportion of raffinose in winter leaves of *A. reptans* was located in subcellular compartments outside the vacuole (Table 3.1.2), about 25 % in the chloroplasts. It could not be excluded that carbohydrates located in chloroplasts by the non-aqueous fractionation technique or

by the isolation of chloroplasts might represent the fraction associated with the chloroplastic outer membrane (Voitsekhovskaja et al. 2006). So far, no evidence for a higher affinity of the chloroplastic outer membrane to raffinose than to stachyose exists. Apparently, the subcellular distribution of raffinose and stachyose is different because of their different functions in plant metabolism during cold season.

Malate is confined to the vacuole (Winter et al. 1994) and this finding is also true for *A. reptans*. Active transport of malate into leaf vacuoles has been shown (Martinoia et al. 1985). However, in C3 plants malate accumulates during the day with a maximum at the end of the light period, only being transported into the vacuole after reaching a threshold concentration (Martinoia and Rentsch 1994).

In several studies, most of the amino acids were highly abundant in the cytoplasma and chloroplasts (Riens et al. 1991; Krueger et al. 2011). In *A. reptans* a high proportion of amino acids was localized in the vacuole although the amino acid concentration was more similar in all three compartments because of the higher vacuolar volume in relation to the stromal or cytoplasmic volume (Table 3.1.3, Fig. 3).

3.1.4.4 Function of the different RFOs

RFOs, especially raffinose, are supposed to work as cryoprotectants (Bachmann et al. 1994; Bachmann and Keller 1995; Peters and Keller 2009) that are accumulated during the cold season. For raffinose a correlation of the tissue content with freezing tolerance has been demonstrated for some plant species, e.g. *A. thaliana* (Klotke et al. 2004). On the other hand, it was demonstrated that raffinose appears not to be essential for the freezing tolerance of *A. thaliana*, since a raffinose synthase mutant with a lack of raffinose showed the same freezing tolerance as wild type plants measured as electrolyte leakage from leaf cells after freeze-thaw cycles (Zuther et al. 2004).

To act as cryoprotectant, raffinose needs to be located in the cytoplasm (Koster and Lynch 1992) or in the chloroplast (Schneider and Keller 2009; Nägele and Heyer 2013). In winter leaves of *A. reptans* the main portion of raffinose was found either in the vacuole or in the stroma (Table 3.1.2, Fig. 3.1.3) which is in agreement with the findings of Schneider and Keller (2009). Contrary to Bachmann

and Keller (1995) or Nägele and Heyer (2013), we could not detect higher amounts of raffinose in the cytoplasm of mesophyll cells of *A. reptans*. This confirms the findings of Nägele and Heyer (2013) that the cytosolic raffinose content did not necessarily correlate with freezing tolerance.

It was shown that raffinose was re-allocated to the plastids during cold-acclimation in *A. reptans* as well as in *A. thaliana* (Table 3.1.2, Schneider and Keller 2009; Knaupp et al. 2011). Because of the probably lower raffinose concentration in the cytosol in comparison with the chloroplast stroma raffinose must be transported over the chloroplast envelop against a concentration gradient (Schneider and Keller 2009). It was recently demonstrated that raffinose (not only) was involved in stabilizing photosystem II against damage during freezing (Knaupp et al. 2011). Our compartmentation analysis of raffinose in winter leaves of *A. reptans* supports the possible role for raffinose in protection of photosystems. Based on metabolic profiling, Espinoza et al. (2010) could demonstrate that in *A. thaliana* accumulation of raffinose occurred earlier in comparison with other compatible solutes, such as proline. Accumulation of raffinose was in agreement with the earlier increase in the transcript encoding for galactinol synthase GolS3 and with the closely regulated raffinose synthase SIP1 (Espinoza et al. 2010).

Beside the role of raffinose as cryoprotectant, it could also function as a carbon storage compound because the concentration in the vacuole increased about 40-fold in the vacuole of winter leaves of *A. reptans* (Fig. 3.1.3). There are different reasons for higher raffinose contents in winter leaves, e.g. higher rates of synthesis, lower metabolic activity, and lower translocation rates of carbon compounds in the phloem.

The contents of stachyose and verbascose increased also about 40-fold in winter leaves of *A. reptans* in comparison to summer leaves (Fig. 3.1.3). In contrast to raffinose, stachyose and verbascose were almost exclusively found in the vacuole. Iftime et al. (2011) have shown that stachyose production in transgenic *A. thaliana* lines did not alter the freezing tolerance of cold acclimated plants. This shows that the cryoprotecting function seems unlikely for stachyose or verbascose. Instead, increased concentrations of these oligosaccharides more likely reflect metabolic changes at low temperatures, e.g. reduced metabolic activity or reduced carbon translocation rates in the phloem.

In addition to oligosaccharides all analyzed soluble carbon compounds were increased in winter leaves of *A. reptans* in comparison to summer leaves (Table 3.1.1). For different *A. thaliana* genotypes it was also shown that glucose, fructose and sucrose accumulated during cold acclimation but there was no correlation between the concentration of these substances and leaf freezing tolerance (Rohde et al. 2004). The only correlation they found was between the raffinose content and the freezing tolerance (Rohde et al. 2004).

SF and GL conceived and designed research. SF and GL conducted experiments. KZ and SK contributed analytical tools. SF and GL analyzed data. SF and GL wrote the manuscript. All authors read and approved the manuscript.

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3.1.4.5 References

Adhikari J, Bhaduri T J, DasGupta S, Majumder A L (1987) Chloroplast as a locale of L-*myo*-inositol-1-phosphate synthase. Plant Physiol 85: 611-164

Bachmann M, Matile P, Keller F (1994) Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L - Cold acclimation, translocation, and sink to source transition: discovery of chain elongation enzyme. Plant Physiol 105: 1335-1345

Bachmann M, Keller F (1995) Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L - Inter-and intracellular compartmentation. Plant Physiol 109: 991-998

Espinoza C, Degenkolbe T, Caldana C, Zuther E, Leisse A, Willmitzer L, Hincha D K, Hannah MA (2010) Interaction with diurnal and circadian regulation results in dynamic metabolic and transcriptional changes during cold acclimation in *Arabidopsis*. PLoS ONE 5: e14101

Farre E M, Tiessen A, Roessner U, Geigenberger P, Trethewey RN, Willmitzer L (2001) Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars, organic acids, amino acids, and sugar alcohols in potato tubers using a nonaqueous fractionation method. Plant Physiol 127: 685–700

Gamalei YV (1991) Phloem loading and its development related to plant evolution from trees to herbs. Trees 5: 50-64

Gerhard R, Stitt, M, Held, HW (1987) Subcellular metabolite levels in spinach leaves - Regulation of sucrose synthesis during diurnal alterations in photosynthetic partitioning. Plant Physiol 83: 399-407

Gilmour SJ, Sebolt AM, Salazar MP, Everard J D, Thomashow MF (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol 124: 1854-1865

Heineke D, Wildenberger K, Sonnewald U, Willmitzer L, Heldt HW (1994) Accumulation of hexoses in leaf vacuoles: Studies with transgenic tobacco plants

expressing yeast-derived invertase in the cytosol, vacuole or apoplasm. Planta 194: 29-33

Hincha D, Sonnewald U, Willmitzer L, Schmitt J (1996) The role of sugar accumulation in leaf frost hardiness: investigations with transgenic tobacco expressing a bacterial pyrophosphatase or a yeast invertase gene. J Plant Physiol 147: 604–610

Hoffmann-Thoma G, van Bel A, Ehlers K (2001) Ultrastructure of minor-vein phloem and assimilate export in summer and winter leaves of the symplasmically loading evergreens *Ajuga reptans* L , *Aucuba japonica* Thunb , and *Hedera helix* L. Planta 169: 231–242

Holthaus U, Schmitz K (1991) Distribution and immunolocalization of stachyose synthase in *Cucumis melo* L. Planta 162: 283-288

Iftime D, Hannah MA, Peterbauer T, Heyer AG (2011) Stachyose in the cytosol does not influence freezing tolerance of transgenic Arabidopsis expressing stachyose synthase from adzuki bean. Plant Science 180: 24-30

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27: 137-138

Klie S, Krueger S, Krall L, Giavalisco P, Flügge UI, Willmitzer L, Steinhauser D (2011) Analysis of the compartmentalized metabolome - a validation of the non-aqueous fractionation technique. Front Plant Sci 2: Art 55

Knop C, Voitsekhovskaja O, Lohaus G (2001) Sucrose transporters in two members of the Scrophulariaceae with different types of transport sugar. Planta 213: 80-91

Klotke J, Kopka J, Gatzke N, Heyer AG (2004) Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of *Arabidopsis thaliana* with contrasting cold adaptation – evidence for a role of raffinose in cold acclimation. Plant Cell Environ 27: 1395–1404

Knaupp J, Mishra KB, Nedbal L, Heyer AG (2011) Evidence for a role of raffinose in stabilizing photosystem II during freeze-thaw cycles. Planta 234: 477-486

Koster K, Lynch D (1992) Solute accumulation and compartmentation during the cold acclimation of Puma rye. Plant Physiol 98: 108-113

Krueger S, Niehl A, Lopez Martin MC, Steinhauser D, Donath A, et al. (2009) Analysis of cytosolic and plastidic serine acetyltransferase mutants and subcellular metabolite distributions suggests interplay of the cellular compartments for cysteine biosynthesis in *Arabidopsis*. Plant Cell Environ 32: 349–367

Krueger S, Giavaisco P, Krall L, Steinhauser MC, Büssis D, Usadel B, Flügge UI, Fernie AR, Willmitzer L, Steinhauser D (2011) A topological map of the compartmentalized *Arabidopsis thaliana* leaf metabolome. PLoS ONE 6 (3): e17806

Krueger S, Steinhauser D, Lisec J, Giavaisco P (2014) Analysis of subcellular metabolite distributions within *Arabidopsis thaliana* leaf tissue: A primer for subcellular metabolomics. Arabidopsis Protocols, Meth Mol Biol 1062: 575-596

Lohaus G, Hussmann M, Schneider H, Zhu JJ, Sattelmacher B (2000) Solute balance of a maize (*Zea mays* L) source leaf as affected by salt treatment with special emphasis on phloem re-translocation and ion leaching. J Exp Bot 51: 1721-1732

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275

Lunn J E (2007) Compartmentation in plant metabolism. J Exp Bot 58: 35-47

Martinoia E, Flügge U I, Kaiser G, Heber U, Heldt H W (1985) Energy-dependent uptake of malate into vacuoles isolated from barley mesophyll protoplasts. Biochem Biophys Acta 806: 311-319

Martinoia E, Rentsch D (1994) Malate compartmentation: responses to a complex metabolism. Annu Rev Plant Physiol Plant Mol Biol 45: 447-467

Moore B , Palmquist D , Seemann P (1997) Influence of plant growth at high CO_2 concentrations on leaf content of rinolose-1,5-bisphosphate carboxylase/oxygenase and intracellular distribution of soluble carbohydrates in tobacco, snapdragon, and parsley. Plant Physiol 115, 241-248

Nadwodnik J, Lohaus G (2008) Subcellular concentrations of sugar alcohols and sugars in relation to phloem translocation in *Plantago major*, *Plantago maritima*, *Prunus persica*, and *Apium graveolens.* Planta 227: 1079–1089

Nägele T, Heyer A G (2013) Approximating subcellular organisation of carbohydrate metabolism during cold acclimation in different natural accessions of *Arabidopsis thaliana.* New Phytol 198: 777–787

Öner-Sieben S, Lohaus G (2014) Apoplastic and symplastic phloem loading in *Quercus robur* and *Fraxinus excelsior*. J Exp Bot 65 (7): 1905-1916

Peterbauer T, Richter A (2001) Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. Seed Sci Res 11: 185-197

Peters S, Keller F (2009) Frost tolerance in excised leaves of the common bugle (*Ajuga reptans* L) correlates positively with the concentrations of raffinose family oligosaccharides (RFOs). Plant Cell Environ 32: 1099–1107

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17: 208-212

Riens B, Lohaus G, Heineke D, Heldt H W (1991) Amino acid and sucrose content determined in the cytosolic, chloroplastic and vacuolar compartments and in the phloem sap of spinach leaves. Plant Physiol 97: 227-233

Riens B, Lohaus G, Winter H, Heldt H (1994) Production and diurnal utilization of assimilates in leaves of spinach (*Spinacia oleracea* L.) and barley (*Hordeum vulgare* L.). Planta 192:497-501

Rohde P, Hincha D K, Heyer A G (2004) Heterosis in the freezing tolerance of crosses between two *Arabidopsis thaliana* accessions (Columbia-0 and C24) that will differences in non-acclimated and acclimated freezing tolerance. Plant J 38: 790-799

Santarius K A, Milde H (1977) Sugar compartmentation in frost-hardy and partially dehardened cabbage leaf cells. Planta 136: 163–166

Scarth G W, Levitt J (1937) The frost hardening mechanism of plant cells. Plant Physiol 12: 51-78

Schneider T, Keller F (2009) Raffinose in chloroplasts is synthesized in the cytosol and transported across the chloroplast envelope. Plant Cell Physiol 50: 2174–2182

Shahba MA, Qian Y L, Hughes HG, Koski AJ, Christensen D (2003) Relationships of soluble carbohydrates and freeze tolerance in saltgrass. Crop Science 43: 2148–2153

Sprenger N, Keller F (2000) Allocation of raffinose family oligosaccharides to transport and storage in *Ajuga reptans*: the roles of two distinct galactinol synthases. Plant J 21: 249-258

Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruc Res 26: 32-43

Stitt M, Hurry V (2002) A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in Arabidopsis. Curr Opin Plant Biol 5: 199-206

Strand A, Hurry V, Gustafsson P, Gardeström P (1997) Development of *Arabidopsis thaliana* leaves at low temperatures releases the suppression of photosynthesis and photosynthetic gene expression despite the accumulation of soluble carbohydrates. Plant J 12: 605–614

Strand A, Hurry V, Henkes S, Huner N, Gustafsson P Gardeström P, Stitt M (1999) Acclimation of *Arabidopsis* leaves developing at low temperatures increasing cytoplasmic volume accompanies increased activities of enzymes in the calvin cycle and in the sucrose-biosynthesis pathway. Plant Physiol 119: 1387-1397

Turgeon R, Beebe D U, Gowan E (1993) The intermediary cell: minor-vein anatomy and raffinose oligosaccharide synthesis in the Scrophulariaceae. Planta 191: 446-456

Uemura M, Steponkus P L (2003) Modification of the intracellular sugar content alters the incidence of freeze-induced membrane lesions of protoplasts isolated from *Arabidopsis thaliana* leaves. Plant Cell Environ 26: 1083-1096

Voitsekhovskaja O V, Koroleva O A, Batashev D R, Knop C, Tomos D, Gamalei Y V, Heldt H W, Lohaus G (2006) Phloem loading in two Scrophulariaceae species what can drive symplastic flow via plasmodesmata? Plant Physiol 140: 383–395

Voitsekhovskaja OV, Rudashevskaya E L, Demchenko K N, Pakhomova M V, Batashev D R, Gamalei Y V, Lohaus G, Pawlowski K (2009) Evidence for

functional heterogeneity of sieve element-companion cell complexes in minor vein phloem of *Alonsoa meridionalis.* J Exp Bot 60: 1873–1883

Wanner L A, Junttila O (1999) Cold-induced freezing tolerance in *Arabidopsis.* Plant Physiol 120: 391–400

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

Winter H, Robinson D G, Heldt H W (1994) Subcellular volumes and metabolite concentrations in spinach leaves. Planta 193: 530-535

Zuther E, Büchel K, Hundertmark M, Stitt M, Hincha D K, Heyer A G (2004) The role of raffinose in the cold acclimation response of *Arabidopsis thaliana.* FEBS Letters 576: 169-173

3.2 Manuscript 2: Partitioning and synthesis of raffinose-family oligosaccharides and sucrose in *A. reptans* (Lamiacea)

Title: Partitioning and synthesis of raffinose-family oligosaccharides and sucrose in *Ajuga reptans* (Lamiaceae)

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Main conclusion

In *A. reptans* stachyose was mainly synthesized in source leaves. STS-activity and –expression were temperature dependent but not dependent on light. A vacuolar SUT4 transporter was isolated from leaves of *A. reptans* and it was shown that the expression of that SUT4 was not temperature dependent but light-dependent.

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Abstract

The frost hardy evergreen plant *A. reptans* is classified as symplastic phloem loader. It translocates mainly raffinose and stachyose in addition to sucrose (Bachmann et al. 1994) and accumulates also raffinose oligosaccharides in leaves. Abiotic stress conditions like cold temperatures and different light conditions have impacts on sugar concentration and distribution, as well as enzyme activity (stachyose synthase = STS) and expression levels.

The highest RFO content and STS-expression was found in the source leaves of *A. reptans*, followed by the sink leaves and the stem. Also STS-activity was found to be highest in these tissues. Therefore it seems that stachyose is mainly synthesized in the source leaves of *A. reptans*, but also partially in sink leaves and the stem. STS-expression and –activity and therefore stachyose content are influenced by the temperature. In cold acclimated leaves expression as well as activity of STS, and RFO content was considerably increased. STS-expression was not light-dependent, as the expression level was constant after 24 h and 48 h of darkness.

Furthermore, a group 4 sucrose transporter, presumably localized in the tonoplast, was isolated from leaves of *A. reptans* and likewise analyzed with regard to its organ specific distribution and the effects by different temperatures and the light-dark regime on the expression levels. Due to its probable vacuolar localization the highest ArSUT4-expression was found in source leaves, but also in sink leaves and stem (parenchyma cells). ArSUT4-expression and sucrose concentration were diurnal regulated, given that both strongly decreased at ‘dark’ conditions. The temperature had only little impact on ArSUT4-expression, although the sucrose concentration increased together with the whole sugar content. Sucrose probably has only a secondary role in cold acclimation of *A. reptans* and its main function is the supply of a RFO precursor and carbon storage.

Key words Ajuga *reptans*, raffinose-family-oligosaccharides, stachyose-synthase, SUT4, RFO

Abbreviations

BSC	Bundle-sheath cell
CC	Companion cell
cDNA	Complementary DNA
dp	Degree of polymerization
HPLC	High performance liquid chromatography
FW	Fresh weight
IC	Intermediary cell
MC	Mesophyll cell
RFO	Raffinose oligosaccharides
RT-PCR	Reverse transcription PCR
RT-*q*PCR	Real-Time quantitative PCR
SUT	Sucrose Transporter

3.2.1 Introduction

Raffinose family oligosaccharides (RFOs) are important carbohydrates in several plant species and the most common RFOs to be found in plants are raffinose, stachyose and verbascose. RFO synthesis starts with the formation of galactinol from UDP-gal and myo-inositol catalyzed by the galactinol synthase (GS; EC 2.4.1.123). Raffinose synthesis is catalyzed by the raffinose-synthase (RS; EC 2.4.1.82) which transfers a galactosyl moiety from galactinol to sucrose (Lehle and Tanner 1973). Further, stachyose-synthase (STS; EC 2.4.1.67) adds one more galactosyl residue to the raffinose molecule to synthesize stachyose (Tanner and Kandler 1968). Together with sucrose, RFOs have several functions in plants, e.g. as carbon transport form in the phloem, as carbon storage form in seeds or vegetative plant parts and as compatible solutes commonly related to desiccation or cold tolerance (Turgeon et al. 1993; Bachmann et al. 1994; Knaupp et al. 2011).

The transport of RFOs, in addition to the ubiquitous sucrose, in the phloem is correlated with the symplastic phloem loading mechanism. The minor veins of symplastic phloem loaders have special forms of companion cells (CCs), so called intermediary cells (ICs) with abundant plasmodesmal connections towards the adjacent mesophyll cells or bundle-sheath-cells (BSCs) (Turgeon et al. 1993). To explain symplastic uphill transport of oligosaccharides into the sieve elements, a polymer trap mechanism has been proposed (Turgeon et al. 1993). According to this model sucrose diffuses through the numerous plasmodesmata into the intermediary cells where RFO synthesis starts. Stachyose-synthase (STS) was localized in the intermediary cells (Holthaus and Schmitz 1991; Voitsekhovskaja et al. 2009). Furthermore, it was postulated that the size exclusion limit of plasmodesmata connecting intermediary cells to the bundle sheath cells enables the passage of disaccharides such as sucrose from mesophyll into the phloem whereas the tri- and tetrasaccharides raffinose and stachyose remain trapped in the phloem.

The accumulation of non-reducing and soluble carbohydrates, like sucrose or RFOs is one of the most commonly observed responses of plants to abiotic stresses (Bachmann et al. 1994, Peters and Keller 2009, Wanner and Junttila 1999). Although all RFOs increase during cold temperatures mostly raffinose was associated with cold acclimation as it may stabilize membranes or photosystems during freezing

temperatures (Gaffney et al. 1988, Bachmann and Keller 1995, Nishizawa et al. 2008, van den Ende and Valluru 2009) or during drought and heat stress conditions (Santarius 1973). The accumulation of RFOs in winter leads to the assumption that RFOs were stored firstly for their cryo-protecting function and secondly as a stock for energy remobilization towards growth during spring time (Peters and Keller 2009). Also the cold induced expression of the galactinol synthase gene *AtGolS3* in *Arabidopsis* plants support the participation of RFOs in cold acclimation and freezing tolerance (Taji et al. 2001). Gathering from that, one would expect that the other RFO-synthesizing enzymes RS and STS act similar. But although a higher RFO content during the cold season was observed, the activity of STS did not show any differences between warm and cold treated plants (Bachmann et al. 1994). This could imply a higher expression and thus an increased availability of the enzyme in the leaf mesophyll cells, as it is already shown for STS (Bachmann and Keller 1995) and for RS (Nishizawa et al. 2008). An increased sucrose content should also be involved in cold acclimation which was already observed in *Arabidopsis* (Nägele and Heyer 2013), spinach (Guy et al. 1992), ivy (Steponkus and Lanphear 1968) and citrus (Guy et al. 1980).

Furthermore, sucrose and RFOs are also part of the carbon storage pool in vegetative parts of the plant or in seeds (Peterbauer and Richter 2001). Several studies (Holthaus and Schmitz 1991, Beebe and Turgeon 1992, Sprenger and Keller 2000) showed that RFO-synthesis may occur in different cell types in leaves and it seems that there is a correlation between the place of RFO synthesis and its function in plant metabolism. In addition to the intermediary cells RFOs were also synthesized in mesophyll cells of some plant species (Bachmann and Keller 1995), stored in the vacuole during the day and catabolized at night (Beebe and Turgeon 1992). Moreover, stachyose that is transported into the vacuole can be synthesized to long-chain RFOs (verbascose, ajugose, etc.) by the galactinol independent enzyme galactan:galactan galactosyltransferase (GGT) that catalyzes the direct transfer of a Gal-residue from one RFO molecule to another, resulting in the next higher and lower RFO oligomers (Haab and Keller 2002)

Metabolites like sucrose or RFOs produced in excess in the light period are transported into the vacuole, which serves as a temporary storage pool, and released to the cytoplasm when required for metabolism. The transmembrane distribution of

sucrose is catalyzed by small families of transporter proteins, typically named SUC or SUT, which can classified into four distinct groups (Sauer 2007). Group 4 transporters were localized in the tonoplast of barley, *Arabidopsis*, melon, tomato or wheat (Endler et al. 2006, Schneider et al. 2012, Deol et al. 2013). Their physiological function is probably the transport of sucrose from the vacuole into the cytoplasma. Until now the corresponding raffinose - or stachyose transporter were not characterized.

The frost hardy and perennial labiate *A. reptans* synthesizes different amounts of RFOs during vegetation period (Bachmann et al. 1995) and is able to survive freezing temperatures. Further, it is classified as symplastic phloem loader because it has intermediary cells in the minor veins and translocates RFOs, predominantly stachyose in the phloem (Hoffmann-Thoma et al. 2001).

In the present study effects of different temperature- and light-conditions on sugar content and distribution as well as on the regulation of stachyose synthesis and sucrose transport into the vacuole in different plant tissues and leaves of *A. reptans* have been analyzed. Therefore sugar analysis, transcript expression of STS and SUT4 using quantitative RT-PCR and STS-activity were performed in order to better understand the accumulation of soluble sugars and their regulating processes in matters of environmental adaptations (temperature and light).

3.2.2 Materials and methods

3.2.2.1 Plant materials

Ajuga reptans plants were either grown in 3 l pots in compost soil in a green house and outside of the “Bergische Universität Wuppertal” (Germany; 51.26°N, 7.18°E) or grown in the field outside of the “Bergische Universität Wuppertal”. Greenhouse temperature was 20 °C constantly with a light intensity of about 100 µmol/m^{-2} sec^{-1} at light period. Sample harvest was always performed after 6 h of illumination (except the darkness probes), the plant material was immediately shock frozen with liquid nitrogen and either directly processed or stored at -80 °C.

Experimental conditions

a) tissue specific analysis: Samples were taken from field grown *A. reptans* plants. Flowers, petals, stem, sink- and source-leaves were harvested during anthesis in spring (about 13 h of daylight; about 15-20 °C during the day and 5-10 °C during the night).

b) warm/cold conditions: Source leaf samples were harvested from pot grown plants in March (about 13 h of daylight) Temperature conditions for warm grown plants were 20 °C (day/night) and for cold grown plants 5-10 °C (day) and 0 °C (night).

c) light/24h darkness/48h darkness: Pot grown plants were transferred either from the green house (20 °C) to a separated laboratory with 20 °C for warm acclimated plants or from outside (0-5 °C at night and 10 °C during the day) to a refrigeration room (5-8 °C) for cold acclimated plants. Leaf samples were harvested at the end of the light period, after 24h and 48h in darkness.

For every condition listed above sugar analysis, RTq-PCR for SUT- and STS-expression, and a STS-activity assay was performed as described in the following.

3.2.2.2 Extraction of sugars

Frozen leaf material was mortared and weighed in to 200 mg portions. 5 ml chloroform:methanol (1,5:3,5, v/v) was added, the sample was homogenized and kept on ice for 30 minutes. The homogenate was then extracted twice with 3 ml water. The aqueous phases were combined and evaporated in a rotatory evaporator (RV 10 Digital; IKA, Staufen, Germany). The dried residue was dissolved in 1 ml ultrapure H_2O (Millipore; Billerica, MA, USA), syringe-filtrated (0.20 µm nylon; Carl Roth, Germany) and stored at -80 °C until analysis.

3.2.2.3 Sugar analysis

Sugars were analyzed by HPLC according to Nadwodnik and Lohaus (2008).

3.2.2.4 Stachyose synthase assay

The activity of stachyose synthase (STS) in *A. reptans* leaves and organs was measured by the time dependant formation of stachyose and *myo*-inositol from galactinol and raffinose. 250 - 300 mg plant material was extracted with NaH_2PO_4/Na_2PO_4-buffer (50 mM; pH 7.0). 150 µl of the extract was desalted on a 1ml sephadex-G25 column before application in the assay. STS assay (final volume 50 µl) containing 2.5 mM galactinol, 15 mM raffinose, 1 mM DTT (all in homogenization buffer), and 30 µl of desalted crude extract. Reaction proceeded at 30 °C and was stopped after 120 minutes by incubation at 100 °C for 5 minutes and centrifugation at 13,000*g*. Sugars were analyzed by HPLC according to Nadwodnik and Lohaus (2008).

3.2.2.5 Preparation of total RNA

RNA from whole leaves was isolated using a modified protocol from Chang et al. (1993). 100 - 200 mg leaf material was used. Integrity was checked by agarose gel electrophoresis and concentration was measured at 260 nm wavelength.

3.2.2.6 Isolation of cDNA and polymerase chain reaction (PCR)

First-strand cDNA was synthesized from 1 ng of total RNA isolated from leaves (sink, source), stem, calyx and petals using the ReverdAid^{TM} First Strand cDNA Synthesis Kit (Fermentas; St. Leon-Rot, Germany) with oligo $(dT)_{18}$ primer.

The single-strand cDNA was used for PCR reaction with the following degenerated primers: (1) SUT: forward: 5'-GCI GCI GGI RTI CAR TTY GGI TGG GC-3', reverse: 5'-GCI ACR TCI ARD ATC CAR AAI CC-3' (Knop et al. 2001). The degenerated primers were designed from two conserved regions that are about 330 bp apart from each other, using sequence data from the published amino acid sequences of several sucrose uptake transporters (Knop et al. 2001). To validate the sequences of the fragments a BLASTX search was performed (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Applying these fragments to RACE (5'/3' RACE Kit; Roche, Mannheim) full-length clones of a putative sucrose transporter,

named ArSUT was obtained. (2) STS: Forward: 5'- GGNTGGTGYACNTGGGAYGC-3', reverse: 5'-TGRAACATRTCCCARTCNGG-3' (Voitsekhovskaja et al. 2009). To validate the sequence of the fragment a BLASTX search was performed.

The cDNA fragments of ArSUT and ArSTS were used for gene specific primer design to run a qPCR analysis (Kit: Maxima SYBR Green; Thermo Scientific). Primer Reactions were performed on MX3005P (Stratagene, Agilent Technologies, USA) and analyzed with the associated software MxPro 4.1 (Stratagene, USA). (1) ArSUT: forward 5'-GCT AAT AAT ATG ACT CAG GGA C-3', reverse 5'- CAA CCG CCA TAA ATA AGG AG-3', (2) ArSTS: forward 5'-AAG ACG ATT CTA CCC TCC C-3', reverse 5'- AAA GAC GAT TCT ACC CTC CC T-3'.

3.2.3 Results

3.2.3.1 Isolation of a cDNA of STS from source leaves of *A. reptans*

Using the degenerated primer, we obtained a DNA sequence of about 1100 bp. This partial sequence was translated into a protein sequence of 400 amino acids and aligned together with STS protein and protein-like sequences *Alonsoa meridionalis*, *Vitis vinifera*, *Solanum lycopersicum*, *Citrus sinensis*, *Genlisea aurea.* Phylogenetic analysis (maximum likelihood plus bootstrap analysis) showed that the sequence obtained from *A. reptans* ranks among other STS proteins (Fig. 3.2.1).

3.2.3.2 Isolation of a SUT4 sucrose transporter from source leaves of *A. reptans*

Using the degenerated primer, we obtained a DNA sequence of about 350 bp. Taking this as starting point for RACE, the received sequence of the sucrose transporter (2171bp) proved to have over 68 % homologies with SUT4 of *Solanum lycopersicum,* with SUT4 of *Solanum tuberosum,* the SUT4 of *Daucus carota,* and with SUT1 of *Vitis vinifera.* Phylogenetic analysis (maximum likelihood plus bootstrap analysis) revealed that ArSUT is clearly assembled within the SUT4 transporters (Fig. 3.2.2A). Sequence analysis showed the putative vacuolar targeting dileucine-motif in

the N-terminus of ArSUT (Fig. 3.2.2B) (Deol et al. 2013). The obtained sequence contained an open reading frame of 502 amino acids, corresponding to a calculated molecular weight of 54.2 kDa. Topology predictions indicated that the protein possesses 12 membrane-spanning regions with a longer central loop.

Figure 3.2.1 Phylogenetic analysis of stachyose synthase protein of different plants together with STS of *A. reptans*. Protein alignment was performed using Clustal W within the MEGA 6 software. Aligned were about 400 amino acids (partly conserved, partly non homologues) of the protein sequences. Maximum likelihood tree with 500 bootstrap repetitions was generated using MEGA 6 software. Numbers indicate percent of bootstrap analysis. Bar indicates evolutionary distance. ArSTS is marked with an asterisk. AmSTSp (*Alonsoa meridionalis*; CAD31704), VvSTSl (*Vitis vinifera*; XP 002273065), SlSTSl (*Solanum lycopersicum;* XP 004229378), CsSTSl (*Citrus sinensis*; XP 006488987), GaSTS (*Genlisea aurea*; EPS58292).

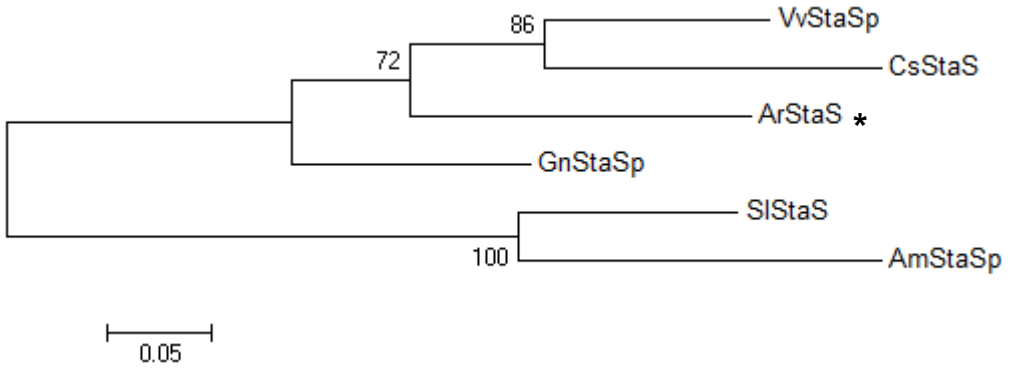
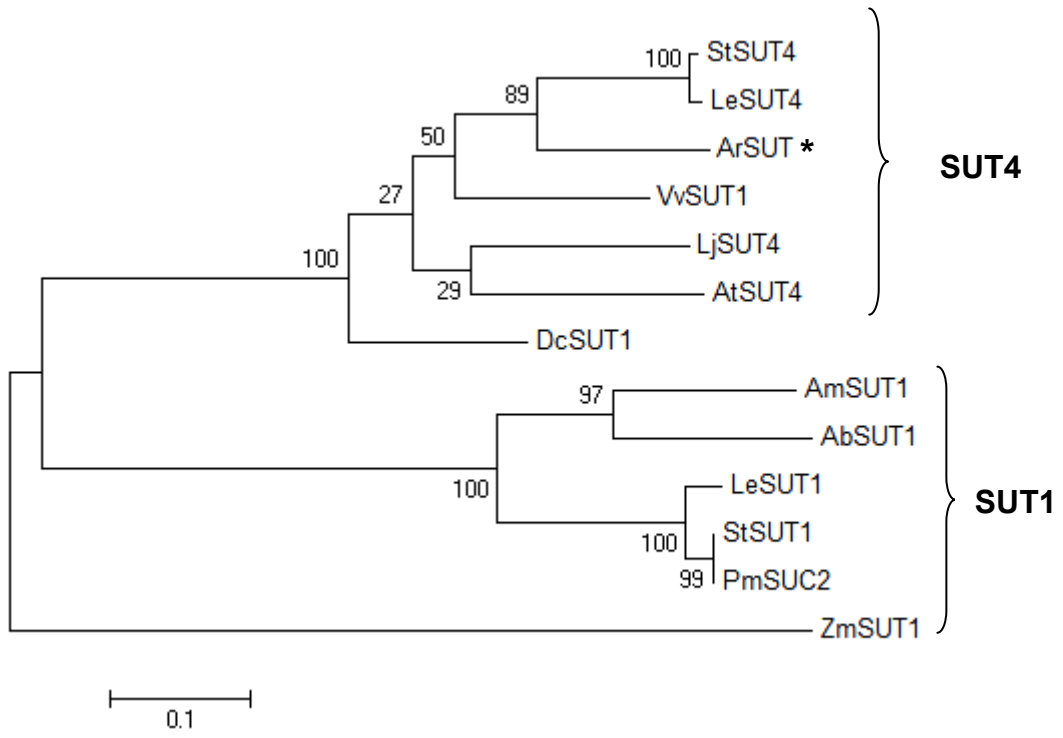


Figure 3.2.2 (A) Phylogenetic analysis of sucrose transporters of different plants together with ArSUT. Protein alignment was performed using Clustal W within the MEGA 6 software. The non homologues variable C- and N-termini of the protein sequences were shortened. Maximum likelihood tree with 500 bootstrap repetitions was generated using MEGA 6 software. Numbers indicate percent of bootstrap analysis. Bar indicates evolutionary distance. *ArSUT* is marked with an asterisk. *AmSUT1 (Alonsoa meridionalis;* AF191025), *AbSUT1* (*Asarina barclaiana*; AF191024), *AtSUT4* (*Arabidopsis thaliana*; AAG09191), *DcSUT1a* (*Daucus carota*; CAA76367), *LeSUT1, LeSUT4,* (*Lycopersicum esculentum*; CAA57726, AAG09270), *LjSUT4* (*Lotus japonicus*; CAD61275), *PmSUC2* (*Plantago major;* CAA48915), *StSUT1, StSUT4* (*Solanum tuberosum*, CAA48915, AAG25923), *ZmSUT1* (*Zea mays*, BAA83501), VvSUT1 (*Vitis vinifera*, AAD55269). **(B)** Partial Alignment of amino acid

sequences of the in (A) described species. In the box is the putative vacuolar targeting dileucine motif shown. All SUT4 sequences possess this motif except *AtSUT4* which instead has KRVLL.

A



B

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ZmSUT1    LILAGMVAGGVQYGWALQLSLLTPYVQTLGLSHALTSFMWLCGPIAGLVVQPLVGLYSDRCTARWGRRRPFILIGCML
AbSUT1    IIVVASIAAGVQFGWALQLSLLTPYVQLLGIPHKFASFIWLCGPISGMIVQPVVGYYSDNCSSRFGRRRPFIAAGAAL
AmSUT1    IILVAAIAAGVQFGWALQLSLLTPYVQLLGIPHTWTAFIWLCGPVSGMLVQPIVGYYSDNCTLRFGRRKPFIAGGAGL
StSUT1    IIVVAAIAAGVQFGWALQLSLLTPYVQLLGVPHVWAAFIWLCGPISGLLVQPIVGYYSDNCTSRFGRRRPFIAAGAGL
PmSUC2    IIVVASIAAGVQFGWALQLSLLTPYVQLLGIPHKFASFIWLCGPISGMIVQPVVGYYSDNCSSRFGRRRPFIAAGAAL
LeSUT1    IIVVASIAAGVQFGWALQLSLLTPYVQLLGIPHRFASFIWLCGPISGMIVQPVVGYYSDNCSSRFGRRRPFIAAGAAL

LjSUT4    LLRVASVASGIQFGWALQLSLLTPYVQQLGIPHQWASIIWLCGPVSGLFVQPLVGHLSDKCTSRFGRRRPFILAGAAS
* ArSUT   LFRVSSVACGIQFGWALQLSLLTPYVQELGIPHAWASIIWLCGPLSGLLVQPLVGHFSDRSTSRFGRRRPFILAGATS
VvSUT1    LLRVASVACGIQFGWALQLSLLTPYVQELGIPHAWSSIIWLCGPLSGLLVQPLVGHLSDRCNSRFGRRRPFIVAGATS
LeSUT4    LLRVASVAGGIQFGWALQLSLLTPYVQELGIPHAWASIIWLCGPLSGLLVQPLVGHMSDKCTSRFGRRRPFIVAGAAS
DcSUT1    LLRVASVACGIQFGWALQLSLLTPYVQELGIPHAWSSIIWLCGPLSGLLVQPIVGHMSDQCTSKYGRRRPFIVAGGTA
StSUT4    LFRVASVAGGIQFGWALQLSLLTPYVQELGIPHAWASIIWLCGPLSGLLVQPLVGHMSDKCTSRFGRRRPFIVAGAVS
AtSUT4    LLRVASVACGIQFGWALQLSLLTPYVQELGIPHAWASVIWLCGPLSGLFVQPLVGHSSDRCTSKYGRRRPFIVAGAVA
```

SUT1

SUT4

3.2.3.3 Sugar content and distribution, STS-expression and -activity and SUT-expression level in different plant tissues

Different sugars (myo-inositol, galactinol, glucose, fructose, sucrose, and the RFOs raffinose, stachyose and verbascose) were detected by HPLC analysis in stem, sink- and source-leaves, petals and calyx of *A. reptans*. The total sugar content ranged from 23 µmol g^{-1} FW in the calyx to 338 µmol g^{-1} FW in the stem (Fig. 3.2.3A). The highest RFO contents were detected in stem and source leaves. The stachyose content in source leaves was 16.6 µmol g^{-1} FW. The stem, predominantly consisting of sieve elements and xylem vessels, showed a stachyose concentration of 14.7 µmol g^{-1} FW and almost the double amount of sucrose. The content of sucrose and RFOs in sink leaves was lower than in source leaves. In calyx and petals were nearly solely hexoses (not shown) and sucrose detected, RFOs were either very low or not existent.

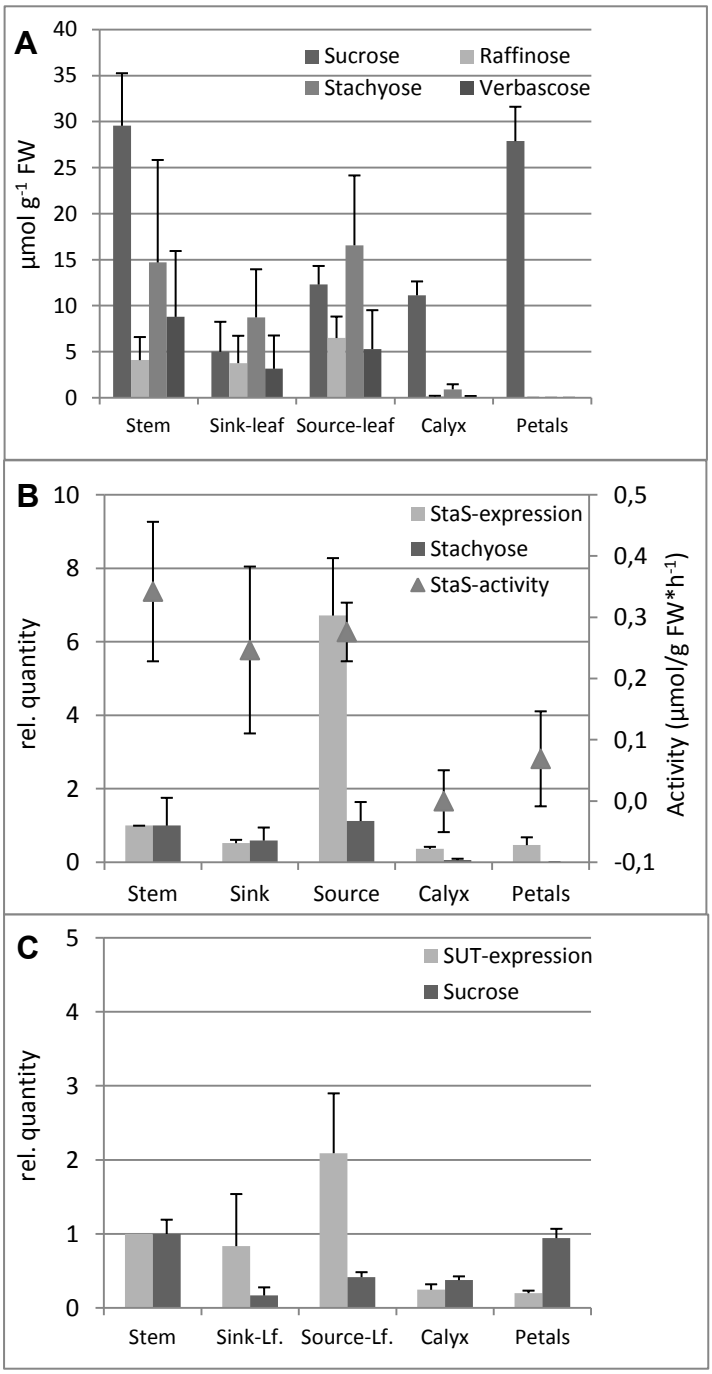
STS-expression was found in all plant tissues of *A. reptans*, but transcript levels in petals and calyx were very low and negligible. This corresponds to the finding that no stachyose was found in these two tissues (Fig. 3.2.3B). The highest level of STS-expression was found in the source leaves.

The activity of STS was determined by measuring the time dependant stachyose concentration. The highest STS-activity was found in the stem, closely followed by source and sink leaves (Fig. 3.2.3B). Similar to the STS-expression level, little or no activity could be detected in petals and calyx. Although the STS-expression in source leaves was about 6 to 7-fold higher than in the stem, the STS-activity was less compared to the stem (Fig 3.2.3B). In calyx and petals the values of STS-expression and -activity fit in all.

ArSUT expression levels were likewise determined in all analyzed tissues and had its highest expression, in relation to the stem, in the source leaves followed by stem and sink leaves and the lowest level was found in the petals (Fig. 3.2.3C). The expression in the flowers (petals and calyx) was could be neglected.

The sugar distribution and content increased during cold treatment of leaves of *A. reptans* (Fig. 3.2.4A). Sucrose concentration was 1.5-fold higher in cold treated leaves compared to warm treated ones. But the proportion of sucrose at the total

Figure 3.2.3 (A) Contents of sucrose and the RFOs raffinose, stachyose, verbascose in the different tissues of *Ajuga reptans*. **(B)** and **(C)** show the relative quantity of STS-expression/stachyose content and STS-activity and the relative quantity of SUT-expression/sucrose content, referring to values of the stem (=1). Mean values of three independent measurements ± SD are shown.



3.2.3.4 Sugar content and distribution, STS-expression and -activity and SUT-expression level in warm and cold treated plants

The sugar distribution and content increased during cold treatment of leaves of *A. reptans* (Fig. 3.2.4A). Sucrose concentration was 1.5-fold higher in cold treated leaves compared to warm treated ones. But the proportion of sucrose at the total sugar content stayed constant. In the case of RFO the content as well as the proportion at the total sugar content increased significantly. Raffinose rose 4-fold, stachyose almost 6-fold and verbascose from 0 µmol g^{-1} FW to 2.2 µmol g^{-1} FW.

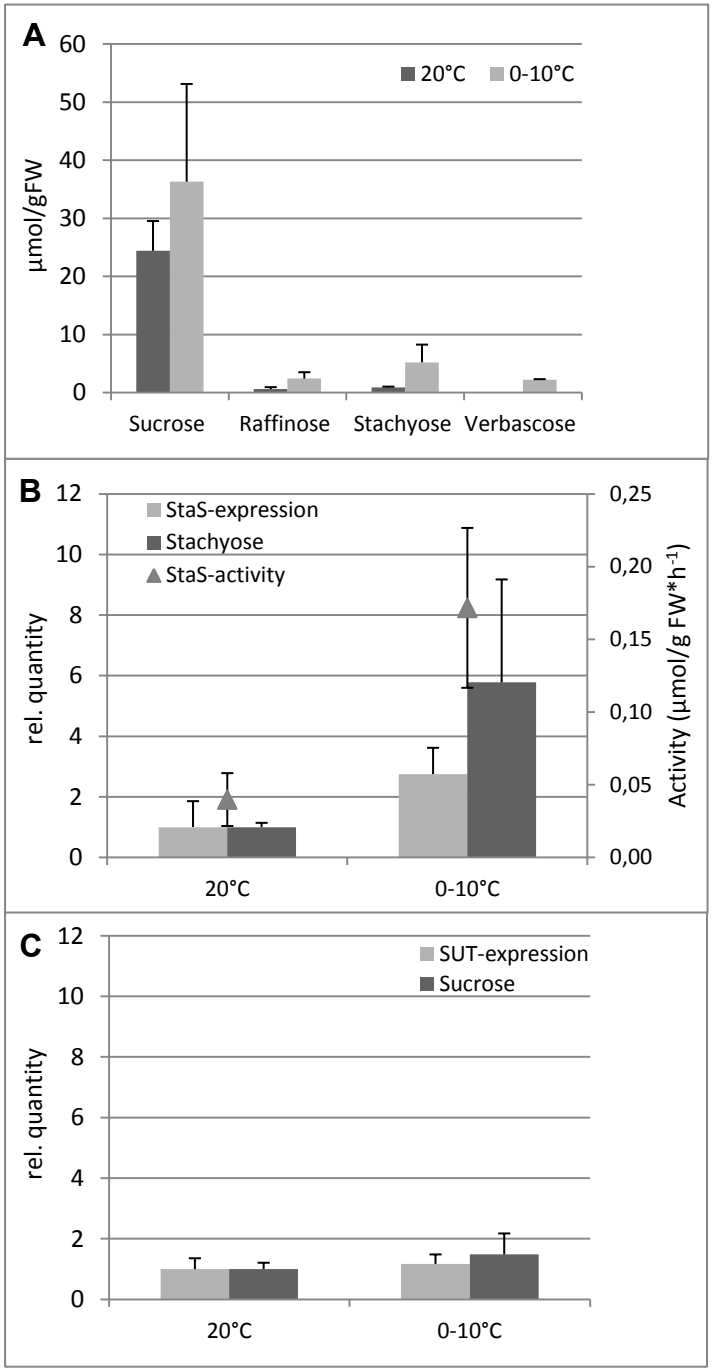
The increase of the RFO content was also reflected by the expression level of STS in source leaves of cold and warm treated plants (Fig. 3.2.4B). The STS-expression level increased significantly about 3-fold and the stachyose synthase activity about 4-fold in leaves of cold treated plants.

A slightly, but significantly elevated expression followed by an increase of the sucrose concentration was observed for SUT (Fig. 3.2.4C).

3.2.3.5 Sugar content and distribution, STS-expression and -activity and SUT-expression level in constant darkness

For this experiment plants grown with a 10 h light/14 h dark period were exposed to darkness for 24 h and 48 h, respectively. Additionally to the light stress, the plants were put to different temperatures (20°C and <10°C). Source leaves of cold treated plants at light condition had a sucrose concentration of 12.5 µmol/g FW and a raffinose concentration of 6.3 µmol g^{-1} FW, stachyose had 13.0 µmol g^{-1} FW and verbascose 4.9 µmol g^{-1} FW (Fig. 3.2.5A), a total RFO content of 24.2 µmol g^{-1} FW, which is almost twice the number of sucrose. All sugars, despite of verbascose in cold treated plants, decreased after 24 h and 48 h in darkness, especially sucrose and raffinose decreased significantly. After two days in complete darkness and temperatures between 5 – 10 °C the sucrose concentration constituted 2.6 µmol/g FW and that of RFOs 16 µmol/g FW. Compared to cold treated plants, the ones kept at 20 °C showed about one fifth on average of the total sugar content. The sucrose content decreased significantly from 4.2 µmol g^{-1} FW in light to 0.4 µmol g^{-1} FW after

Figure 3.2.4 (A) Content of sucrose and the RFOs raffinose, stachyose and verbascose in source leaves of warm (20°C) and cold (0 – 10°C) treated *Ajuga reptans.* **(B)** and **(C)** show the relative quantity of STS-expression/stachyose and STS-activity and the relative quantity of SUT-expression/sucrose and at the two temperature conditions, referring to values of the stem (=1). Mean values of three independent measurements ± SD are shown.

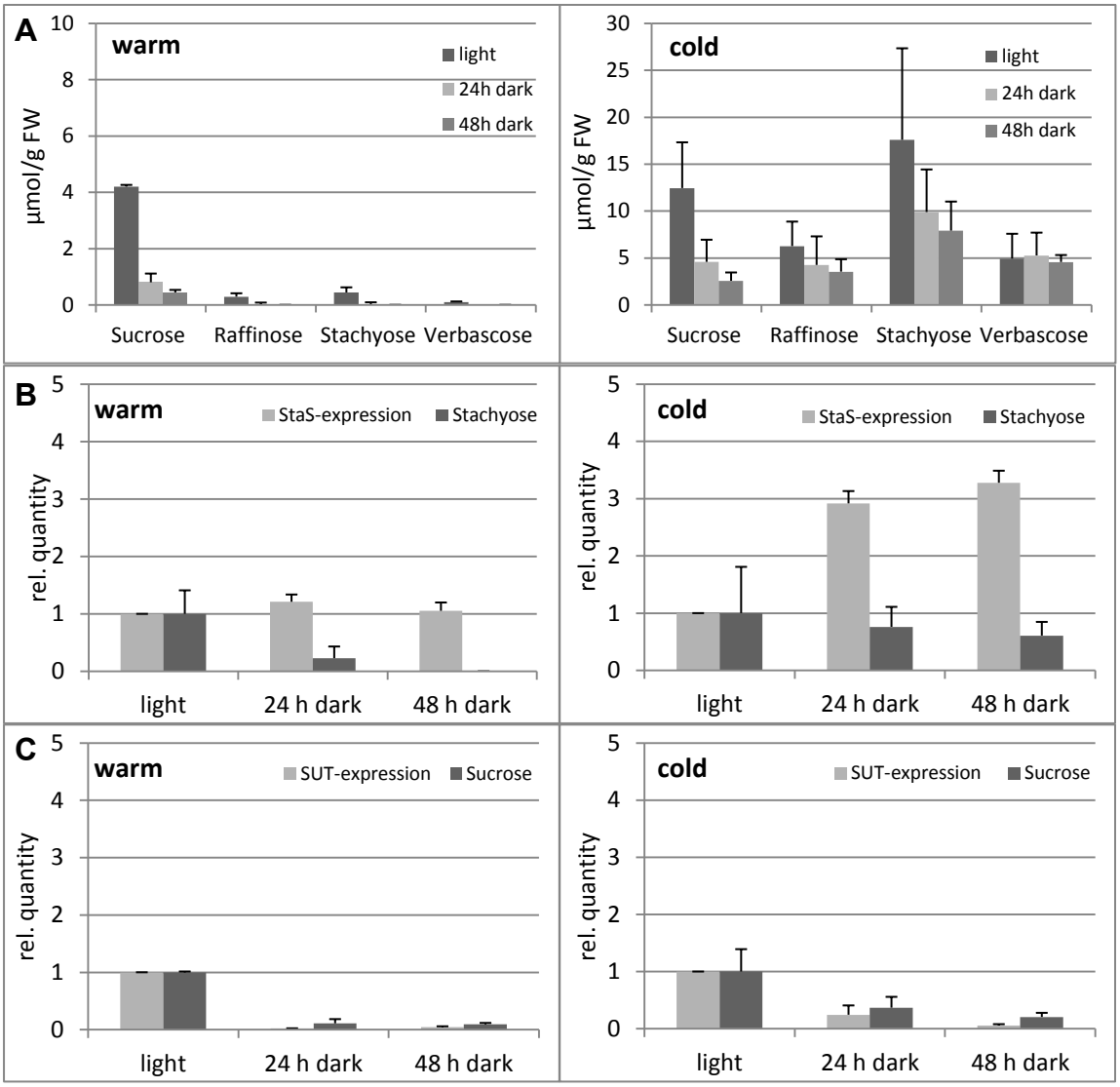


48 h in darkness. Similar was observed for the RFOs, because they decreased significantly from 0.82 µmol g^{-1} FW to 0 µmol g^{-1} FW after two days in darkness.

STS expression levels showed a different pattern; in warm treated plants the STS expression remained almost constant, but in cold treated plants it rose significantly two- and threefold after 24 h and 48 h, respectively (Fig. 3.2.5C).

In both cold and warm treated plants SUT expression levels decreased significantly towards zero after 24 and 48 hours of darkness related to the light conditions (Fig. 3.2.5B).

Figure 3.2.5 (A) Content of sucrose and the RFOs raffinose, stachyose and verbascose of light, 24 hours darkness, and 48 hours darkness treatment in source leaves of *Ajuga reptans.* Additionally to light stress, the plants were treated with temperature stress (20°C and 0-10°C). **(B)** and **(C)** show the relative quantity of SUT-expression/sucrose and STS-expression/stachyose at different light conditions (see above), referring to values of the stem (=1). Mean values of three independent measurements ± SD are shown.



3.2.4 Discussion

3.2.4.1 Organ specific distribution of stachyose and sucrose in *A. reptans*

The analysis of the different plant tissues stem, sink-leaves, source-leaves, calyx and petals of *A. reptans* showed that sucrose was present in every tissue whereas RFOs were only found in stem and leaves (sink and source) (Fig. 3.2.3A). The high amounts of RFOs, particularly verbascose, are due to harvest time in autumn with temperatures between 5 °C (night) and 20 °C (day), when RFO accumulation for cold acclimation had already started.

It is not to be eliminated that the sink leaves analyzed here are already in the transition to source leaves, but assuming that these sink leaves are in fact sink organs stachyose seems to be synthesized not only in source leaves and but also in sink leaves and the stem (Fig. 3.2.3C). Bachmann et al. (1994) also demonstrated for *A. reptans* that STS together with the other anabolic enzymes GolS and RS showed already some activity in young sink leaves, before the transition from sink to source leaf started. Contrary to that Holthaus and Schmitz (1991) described that STS-activity was supposed to occur only in RFO-exporting mature leaves of *Cucumis melo* and stachyose found in young leaves should only be imported. The findings in this study argue for the assumption that stachyose synthesis in leaves of *A. reptans* is not linked with the ability to export stachyose via the phloem. STS-activity found in the stem could imply that either some translocated sucrose and galactinol is synthesized to stachyose before unloading into the sink organs or that some stachyose is synthesized in the parenchyma cells of the stem. Similar to STS-expression in *A. reptans* was RS-expression in leaves of *Cucumis sativus* higher than in other plant tissues (stem, fruit, roots) (Sui et al. 2012). These results indicate that the RFO-synthesis pathway occurs predominantly in source leaves but also at some level in sink leaves and stems.

Although no stachyose and no STS-activity were found in the floral parts of *A. reptans*, very low STS-expression levels were shown in both tissues. Similar observations were made by Sui et al (2012) for RS in *C. sativus* fruits. Miao et al. (2007) and Sui et al. (2012) assumed that after long distance transport into flowers raffinose is converted into sucrose, which is quickly catabolized into hexoses. A similar process is possible for stachyose in *A. reptans*. This in turn could imply that

RFOs translocated in the phloem, were likely to be hydrolyzed before they reach the flower. Peterbauer and Richter (2001) could demonstrate high contents of stachyose and high activity of STS in pea seeds. Richter et al. (2000) also demonstrated the occurrence of STS-mRNA in developing seeds of adzuki bean, thus it is possible that little STS-mRNA is also expressed in flowers of *A. reptans* and show only activity at the time of seed development or the enzyme is transported into the seed where it is responsible for *de novo* stachyose synthesis.

The determined pH-optimum for STS in *A. reptans* was pH 7.0 (data not shown). This is conforming to pH-optima of STS-activities in *Phaseolus vulgaris* (Tanner and Kandler 1966), *Lens culinaris* (Hoch et al. 1999), *Vigna angularis* (Peterbauer and Richter 1998) and *Cucumis melo* (Holthaus and Schmitz 1991). The results are also in accordance with the pH values of STS of *A. reptans* measured by Bachmann et al. (1994).The neutral pH-value indicates a probable localization of the enzyme in the cytoplasm and not in the vacuole, although the highest portion of stachyose (98 % in warm grown plants and 100 % in cold grown plants) was found in the vacuole in source leaves of (different article). Some authors (Sprenger and Keller 2000; Holthaus and Schmitz 1991, Peterbauer and Richter 2001) already described the localization of STS in the cytosol. Keller (1992) and Bachmann and Keller (1995) demonstrated that all precursors (myo-inositol, galactinol, raffinose) and synthesis required enzymes (galactinol synthase, raffinose synthase) are extravacuolar and stachyose and higher RFOs almost exclusively vacuolar. They assumed stachyose synthesis has to happen in the cytoplasm followed by a transport into the vacuole by a Sta/H^+-antiporter (Keller 1992, Greutert and Keller 1993). Vacuolar stachyose is either stored or converted into higher RFOs using a galactan:galactan galactosyltransferase (GGT) that is galactinol independent and transfers a galactosyl residue from one RFO to another (Haab and Keller 2002).

Photosynthetically produced sucrose is either used for transport, RFO-synthesis or storage in the vacuole. The low ArSUT4-expression in sink leaves are indicative for its role in sucrose release from the vacuole since mainly phloem translocated sucrose is being used for primary metabolism and only small amounts of stored sucrose were intended to be used up for metabolic processes. The high sucrose contents in the floral parts (calyx and petals; Fig. 3.2.3A) were presumably

formed only by transport given that at least the petals are not able to perform photosynthesis and to produce their own sucrose. Flowers are, similar to very young leaves, sink tissues and can only use imported or stored sucrose for metabolic processes. This is also conforming to the almost not existing expression level of ArSUT4 in calyx and petals (Fig. 3.2.3B) that shows that no sucrose release into cytoplasm occurs via this sucrose transporter, but other transport options are still possible. The high sucrose concentration in the stem (Fig. 3.2.3A) is mainly allocated to the phloem transport, and partly to the phloem surrounding parenchyma cells, where sucrose is produced by photosynthetic activity. As already described above, the sucrose in the stem could partly be synthesized to stachyose before entering the sink. The observed ArSUT4-expression in the stem occurs also presumably in the phloem surrounding parenchyma cells and in the ordinary companion cells, given that sieve elements do not contain vacuoles (Behnke 1989). Meyer et al. (2000) described the identification of AtSUC3, a H^+/sucrose-symporter that could not been classified among the three existing types of SUTs so far, in large parenchymatic cells between the mesophyll and the phloem stems and suggests i.a. that these large cells could represent transient storage compartments. The sucrose exporter ArSUT4 could impact on the sucrose export from the vacuoles of parenchymatic cells adjacent to the phloem, by releasing sucrose for phloem transport or RFO synthesis.

3.2.4.2 Influence of cold temperatures on carbohydrates (RFOs and sucrose) and stachyose synthesis

One of the functions of raffinose family oligosaccharides is to transport carbohydrates from source tissues (mature leaves) to sink tissues (roots, flowers, young leaves). But they also function in carbon storage (Sprenger and Keller 2000) and as antioxidant in abiotic stress accomplishment like cold temperatures (Wanner and Junttila 1999, Nishizawa et al. 2008, ElSayed et al. 2014). Cold acclimation and freezing tolerance are processes at which numerous genetic, physiological and biochemical changes enable plants to tolerate temperatures several degrees lower than before cold exposure (Strand et al. 1997; Stitt and Hurry 2002; Espinoza et al. 2010). Significant differences can be seen even after one day at low temperatures and Arabidopsis and spinach reach their maximum tolerance after a few days

(Wanner and Junttila 1999, Kurkela et al. 1988, Gilmour et al. 1988). GolS increased after cold inducement in leaves of soy bean and kidney bean (Castillo et al. 1990). RS-expression and –activity increased notably in leaves of *C. sativus* under low temperature (Sui et al. 2012). This is also confirmed by Taji et al. (2002) who found an accumulation of raffinose due to the overexpression of GolS in leaves of a transgenic Arabidopsis. As a frost-hardy evergreen plant, *A. reptans* increases the RFO concentrations in leaves at temperatures below 10 °C about 4 – 5 fold after at least one week exposure to low temperature. At the same time the STS-expression level increases almost three-fold and STS-activity four-fold (Fig. 3.2.4B). Although Haab and Keller (2002) remarked that RFOs play only a secondary role in cold tolerance, this is an obvious adaption to cold temperatures, for altered gene expression, including changes in mRNA-expression and enzyme activity, that can be observed within a few hours in leaves of Arabidopsis (Wanner and Junttila 1999).

Exogenous sucrose at higher concentrations has a direct cryoprotective effect on cellular membranes (Uemura and Steponkus 2003). But neither sucrose concentration nor the ArSUT4-expression level seemed to be affected by low temperatures as both increase only marginally as reaction to cold temperature exposure. Contrary to that did different SUTs in *Juglans regia* show increased transcript levels at low temperatures (Decourteix et al. 2006). Stachyose content or STS-expression and -activity in turn did increase significantly, and therefore it is likely that the major respond to cold temperature in leaves of *A. reptans* is RFO-accumulation and sucrose plays only a secondary role in cold acclimation. Therefore the increased sucrose levels in the vacuoles of winter leaves (different article) were a result of long-term stored carbohydrates.

Due to the increased RFO concentration (Fig. 3.2.4A) and especially of stachyose, during winter, a temperature optimum of stachyose synthase activity at lower degrees would have been a logic consequence. But this was not the case; the temperature optimum was around 30 °C (data not shown). However, the STS-activity in *A. reptans* seems to be less sensitive to declining temperatures than the STS-activity in other plant species (Gaudreault and Webb 1981). This means that although the highest activity of STS was observed at 30 °C, the impairment at lower temperatures is not highly distinct and the enzyme still functions well enough at temperatures far below its original temperature optimum. Although Bachmann et al.

(1994) described that the STS-activity was not affected by cold treatment, this study showed an increased STS-expression as well as an increased STS-activity in cold treated leaves. Interestingly, STS-activity seems to react differently to temperature stress: only cold stress seems to affect enzyme-activity, but not heat stress (Gil et al. 2012). So temperature stress accomplishment works only in one direction. However, RFOs seem to have an important role in the matter of cold acclimation (Santarius and Milde 1977, Gaffney et al. 1988, Bachmann and Keller 1995, Klotke et al. 2004, Schneider and Keller 2009). Raffinose in the chloroplast functions in stabilizing photosystem II (Santarius 1973, Knaupp et al. 2011). The accumulation of raffinose in vacuoles and chloroplasts in leaves of *A. reptans* support this function. Contrary to that are the main functions of stachyose transport and storage and it is not supposed to function directly in cold acclimation or freezing tolerance. Although Zuther et al. (2004) do not preclude a participation of stachyose (together with raffinose) in freezing tolerance; this may not be the case for *A. reptans*, since almost the whole stachyose portion is accumulated in the vacuole. The increased stachyose concentration, STS-expression and –activity during low temperatures in this study rather point to an increased need to produce long-term storage carbohydrates, that can be supplied during cold winter months to maintain metabolism at impaired photosynthesis (Bachmann et al. 1994).

3.2.4.3 “Changes in RFO-and sucrose content in different light-dark regimes”

A major fraction of photosynthetically produced sucrose is stored in leaf vacuoles during the light period and released during the night to maintain carbon supply in the dark (Kaiser and Heber 1984, Martinoia et al. 1987). Diurnal changes for sucrose have been observed in *A. reptans* as well (data not published). The influence of a varying light-dark regime on carbohydrate contents, STS- and SUT- expression as well as STS-activity had to be examined. Although very low amounts of sugars were found in the warm treated plants at light conditions (low light condition (about 100 µmol m^{-2} sec^{-1}) and a constant temperature at day and night), a reasonable interpretation of the results is still possible due to the self-containment of the experimental set-ups. In warm and cold treated plants of *A. reptans* both, sucrose

and RFO contents decreased after 24 and 48 hours darkness. It is noticeable that the consumption of stored sucrose and RFOs is less in cold treated plants compared to the plants kept at 21 °C. Reasons for a delayed consumption could be on the one hand their utilization as “frost protection agent”; the plant needs at least a defined amount of RFOs for that special purpose and/or a reduced phloem transport that were caused by cold temperatures (Strand et al. 1999, Stitt and Hurry 2000). This is also reflected by the observation that the STS-expression level increased at cold and dark treatment and remained constant at warm and dark treatment. That leads to the assumption that STS-expression is only slightly influenced by light and stronger by temperature. RFO-synthesis can be maintained until sucrose and starch are depleted and no sucrose can be supplied because of the lacking synthesis from photosynthesis products.

Contrary to STS-expression the ArSUT4-expression level decreased almost towards zero after 24 h and 48 h darkness, independent of the temperature. Probably ArSUT4 translocates sucrose over the tonoplast into the cytoplasm and can fulfill this function until all sucrose (also catabolized starch and RFOs) are depleted. This seems to happen faster in warm treated plants due to the fact that the sugar content is almost 10-fold lower compared to cold treated plants (Fig 3.2.5A).

3.2.4.4 Conclusions

Stachyose is mainly synthesized in source leaves of *A. reptans*, but additional synthesis occur in sink leaves and stem. The insensitivity of STS to low temperatures plays an important role in cold acclimation. STS has a higher activity at low temperatures, even after 48 h of darkness. Apart from that, the light and dark regime has only a slight effect on STS-expression and STS-activity.

This was different for the isolated SUT4 transporter of *A. reptans*. ArSUT4-expression and sucrose content were diurnal regulated as both were strongly decreased at darkness. The temperature had only little effect on ArSUT4-expression, although the sucrose content increased slightly together with the whole sugar content.

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3.2.5 Literature

Ayre BG (2011) Membrane-Transport Systems for Sucrose in Relation to Whole-Plant Carbon Partitioning. Mol Plant: 1-18

Bachmann M, Matile P, Keller F (1994) Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L - Cold acclimation, translocation, and sink to source transition: discovery of chain elongation enzyme. Plant Physiol 105: 1335-1345

Bachmann M, Keller F (1995) Metabolism of the Raffinose Family Oligosaccharides in Leaves of *Ajuga reptans* L. – Inter- and Intracellular Compartmentation. Plant Physiol 109: 991-998

Beebe DU, Turgeon R (1992) Localization of galactinol, raffinose, and stachyose synthesis in *Cucurbita pepo* leaves. Planta 188: 354-361

Behnke HD (1989) Structure of the phloem. Transport of photoassimilates: 79-137

Castillo EM, de Lumen BO, Reyes PS, de Lumen HZ (1990) Raffinose Synthase and Galactinol Synthase in Developing Seeds and Leaves of Legumes. J Agric Food Chem 38: 351-355

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11(2): 113-116

Decourteix M, Alves G, Brunel N, Améglio T, Guilliot A, Lemoine R, Pétel G, Sakr S (2006) *JrSUT1*, a putative sucrose transporter could mediate sucrose influx into xylem parenchyma cells and be regulated by freeze-thaw cycles over the autumn-winter period in walnut tree (*Juglans regia* L.). Plant, Cell & Environment 29: 36-47

Deol KK, Mukherjee S, Gao F, Brûlé-Babel A, STSolla C, Ayele BT (2013) Identification and characterization of the three homeologues of a new *sucrose transporter* in hexaploid wheat (*Triticum aestivum* L.). BMC Plant Biol 13: 181-195

ElSayed AI, Rafudeen MS, Golldack D (2014) Physiological aspects of raffinose oligosaccharides in plants: protection against abiotic stress. Plant Biol 16: 1-8

Endler A, Meyer S, Schelbert S, Schneider T, Weschke W, Peters SW, Keller F, Baginsky S, Martinoia E, Schmidt UG (2006) Identification of a Vacuolar Sucrose

Transporter in Barley and Arabidopsis Mesophyll Cells by a Tonoplast Proteomic Approach. Plant Physiol 141: 196-207

Espinoza C, Degenkolbe T, Caldana C, Zuther E, Leisse A, Willmitzer L, Hincha DK, Hannah MA (2010) Interaction with diurnal and circadian regulation results in dynamic metabolic and transcriptional changes during cold acclimation in Arabidopsis. PLoS ONE 5: e14101

Gaffney SH, Haslam E, Lilley TH, Ward TR (1988) Homotactic and heterotactic interactions in aqueous solutions containing some saccharides. Experimental results and an empirical relationship between saccharide solvation and solute–solute interactions. J Chem Soc, Faraday Trans 84: 2545-2552

Gaudreault PR, Webb JA (1981) Stachyose synthesis in leaves of *Cucurbita pepo. Phytochem 20 (12): 2629-2633*

Gil L, Ben-Ari J, Turgeon R, Wolf S (2012) Effect of CMV infection and high temperatures on the enzymes involved in raffinose family oligosaccharide biosynthesis in melon plants. J Plant Physiol 169: 965-970

Gilmour SJ, Hajela RK, Thomashow MF (1988) Cold acclimation in Arabidopsis. Plant Physiol 87: 745-750

Greutert H, Keller F (1993) Further Evidence for Stachyose and Sucrose/H^+ Antiporter s on the Tonoplast of Japanese Artichoke (*Stachys sieboldii*) Tubers. Plant Physiol 101: 1317-1322

Guy CL, Yelenosky G, Sweet HC (1980) Light exposure and soluble sugars in citrus frost hardiness. Fla Sci 43: 268-273

Guy CL, Huber JLA, Huber SC (1992) Sucrose Phosphate Synthase and Sucrose Accumulation at Low Temperature. Plant Physiol 100: 502-508

Haab CT, Keller F (2002) Purification and characterization of the raffinose oligosaccharide chain elongation enzyme, galactan:galactan galactosyltransferase (GGT), from *Ajuga reptans* leaves. Plant Physiol 114: 361-371

Hoch G, Peterbauer T, Richter A (1998) Purification and Characterization of Stachyose Synthase from Lentil (*Lens culinaris*) Seeds: Galactopinitol and Stachyose Synthesis. Archives of Biochemistry and Biophysics 366 (1): 75-81

Hoffmann-Thoma G, van Bel A, Ehlers K (2001) Ultrastructure of minor-vein phloem and assimilate export in summer and winter leaves of the symplasmically loading evergreens *Ajuga reptans* L., *Aucuba japonica* Thunb., and *Hedera helix* L. Planta 212: 231–242

Holthaus U, Schmitz K (1991) Stachyose synthesis in mature leaves of *Cucumis melo,* Purification and characterization of stachyose synthase (EC. 2 .4. 1. 67). Planta 184: 525-531

Holthaus U, Schmitz K (1991) Distribution and immunolocalization of stachyose synthase in *Cucumis melo* L. Planta 185: 479-486

Kaiser G, Heber U (1984) Sucrose transport into vacuoles isolated from barley mesophyll protoplasts. Planta 161: 562-568

Keller F (1992) Transport of Stachyose and Sucrose by Vacuoles of Japanese Artichokes (*Stachys sieboldii*) Tubers. Plant Physiol 98: 442-445

Klotke J, Kopka J, Gatzke N, Heyer A G (2004) Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of *Arabidopsis thaliana* with contrasting cold adaptation – evidence for a role of raffinose in cold acclimation. Plant, Cell & Environment 27: 1395–1404

Knaupp J, Mishra KB, Nedbal L, Heyer AG (2011) Evidence for a role of raffinose in stabilizing photosystem II during freeze-thaw cycles. Planta 234: 477-486

Knop C, Voitsekhovskaja OV, Lohaus G (2001) Sucrose transporter in two members of the Scrophulariaceae with different types of transport sugar. Planta 213: 80-91.

Kurkela S, Francke M, Heino P, Lång V, Palva ET (1988) Cold induced gene expression in Arabidopsis L. Plant Cell Rep 7: 495-498

Lehle L, Tanner W (1973) The function of myo-inositol in the biosynthesis of raffinose. Purification and characterization of galactinol:sucrose 6-galactosyl-transferase from Vicia faba seeds. European J Biochem 38: 103-110

Martinoia E, Kaiser G, Schramm MJ, Heber U (1987) Sugar-transport across the plasmalemma and the tonoplast of barley mesophyll protoplasts: evidence for different transport systems. Plant Physiol 131: 467-487

Miao MM, Xu XF, Chen XX, Xue LB, Cao BS (2007) Cucumber carbohydrate metabolism and translocation under chilling night temperature. J Plant Physiol 164: 621-628

Nadwodnik J, Lohaus G (2008) Subcellular concentrations of sugar alcohols and sugars in relation to phloem translocation in *Plantago major*, *Plantago maritima*, *Prunus persica*, and *Apium graveolens*. Planta 227: 1079–1089.

Nägele T, Heyer AG (2013) Approximating subcellular organisation of carbohydrate metabolism during cold acclimation in different natural accessions of *Arabidopsis thaliana.* New Phytol 198: 777–787

Nishizawa A, Yabuta Y, Shigeoka S (2008) Galactinol and Raffinose Constitute a Novel Function to Protect Plants from Oxidative Damage. Plant Physiol 47: 1251–1263

Peterbauer T, Richter A (1998) Galactosylononitol and Stachyose Synthesis in Seeds of Adzuki Bean - Purification and Characterization of Stachyose Synthase. Plant Physiol 117: 165-172.

Peterbauer T, Richter A (2001) Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. Seed Sci Res 11: 185-197

Peters S, Keller F (2009) Frost tolerance in excised leaves of the common bugle (*Ajuga reptans* L.) correlates positively with the concentrations of raffinose family oligosaccharides (RFOs). Plant, Cell & Environment 32: 1099–1107

Richter A, Hoch G, Puschenreiter M, Mayer U, Peterbauer T (2000) The role of stachyose synthase in the oligosaccharide metabolism of legume seeds. Seed biology: advances and applications. Proceedings of the Sixth International Workshop on Seeds: 75-84

Santarius KA (1973) The protective effect of sugars on chloroplast membranes during temperature and water stress and its relationship to frost, desiccation and heat resistance. Planta 113 (2): 105-114

Santarius KA, Milde H (1977) Sugar compartmentation in frost-hardy and partially dehardened cabbage leaf cells. Planta 136: 163–166

Sauer N (2007) Molecular physiology of higher plant sucrose transporters. FEBS Letters
581: 2309–2317

Schneider S, Hulpke S, Schulz A, Yaron I, Höll J, Imlau A, Schmitt B, Batz S, Wolf S, Hedrich R, Sauer N (2012) Vacuole release sucrose via tonoplast-localised SUC4-type transporters. Plant Biol 14: 325-336

Sprenger N, Keller F (2000) Allocation of raffinose family oligosaccharides to transport and storage in *Ajuga reptans*: the roles of two distinct galactinol synthases. Plant J 21: 249-258

Steponkus PL, Lanphear FO (1968) The relationship of carbohydrates to cold acclimation in Hedera helix L. cv. Thorndale. Physiol Planta 22: 777-791

Stitt M, Hurry V (2002) A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in *Arabidopsis*. Curr Opin Plant Biol 5: 199-206

Strand A, Hurry V, Gustafsson P, Gardeström P (1997) Development of *Arabidopsis thaliana* leaves at low temperatures releases the suppression of photosynthesis and photosynthetic gene expression despite the accumulation of soluble carbohydrates. Plant Journal 12: 605–614

Strand A, Hurry V, Henkes S, Huner N, Gustafsson P Gardeström P, Stitt M (1999) Acclimation of *Arabidopsis* leaves developing at low temperatures increasing cytoplasmic volume accompanies increased activities of enzymes in the calvin cycle and in the sucrose-biosynthesis pathway. Plant Physiol 119: 1387-1397

Sui X, Meng F, Wang H, Wei Y, Li R, Wang Z, Hu L, Wang S, Zhang Z (2012) Molecular cloning, characteristics and low temperature response of raffinose synthase gene in *Cucumis sativus* L.. J Plant Physiol 169: 1883-1891

Sun AJ, Xu HL, Gong WK, Zhai HL, Meng K, Wang YQ, Wei XL, Xiao GF, Zhu Z (2008) Cloning and Expression Analysis of Rice Sucrose Transporter Genes *OsSUT2M* and *OsSUT5Z*

Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2002) Important roles of drought- and cold-inducible genes for

galactinol synthase in stress tolerance in *Arabidopsis thaliana*. Plant J 29 (4): 417-426

Tanner W, Kandler O (1966) Biosynthesis of Stachyose in *Phaseolus vulgaris.* Plant Physiol 41: 1540-1542

Tanner W, Kandler O (1968) Myo-Inositol, a Cofactor in the Biosynthesis of Stachyose. European J Biochem 4: 233-239

Turgeon R, Beebe DU, Gowan E (1993) The intermediary cell: Minor-vein anatomy and raffinose oligosaccharide synthesis in the Scrophulariaceae. Planta 191: 446-456

Uemura M, Steponkus PL (2003) Modification of the intracellular sugar content alters the incidence of freeze-induced membrane lesions of protoplasts isolated from *Arabidopsis thaliana* leaves. Plant, Cell and Environment 26: 1083-1096

van den Emde W, Valluru R (2009) Sucrose, sucrosyl oligosaccharides, and oxidative stress: scavenging and salvaging? J Exp Bot 60: 9-18

Voitsekhovskaja OV, Rudashevskaya EL, Demchenko KN, Pakhomova MV, Batashev DR, Gamalei Y V, Lohaus G, Pawlowski K (2009) Evidence for functional heterogeneity of sieve element-companion cell complexes in minor vein phloem of *Alonsoa meridionalis.* Journal of Experimental Botany 60: 1873–1883

Wanner LA, Junttila O (1999) Cold-Induced Freezing Tolerance in Arabidopsis. Plant Physiol 120: 391-399

Weschke W, Panitz R, Sauer N, Wang Q, Neubohn B, Weber H, Wobus U (2000) Sucrose transport into barley seeds: molecular characterization of two transporters and implications for seed development and starch accumulation. Plant J 21(5): 455-467

Zuther E, Büchel K, Hundertmark M, Stitt M, Hincha DK, Heyer AG (2004) The role of raffinose in the cold acclimation response of *Arabidopsis thaliana.* FEBS Letters 576: 169-173

4 Discussion

4.1 RFOs and other metabolites accumulate in vacuoles and chloroplasts in winter leaves of *A. reptans*

The accumulation of non-reducing water soluble carbohydrates is one of the most commonly observed responses of plants to abiotic stress. In winter leaves of *A. reptans* the whole sugar concentration increased strongly and therefore a subcellular localization of the different sugars was analyzed. The comparison of all measured carbohydrates in both, summer and winter leaves of *A. reptans* showed that most of the carbohydrates accumulate in the vacuolar compartment, about 71% in summer leaves and about 87% in winter leaves (Table 3.1.2). This is consistent with studies of other herbaceous plant species (Moore et al. 1997; Nadwodnik and Lohaus 2008; Heineke et al. 1994; Voitsekhovskaja 2006) that are listed in Table 4.1.

Existing data so far localize stachyose almost exclusively in the vacuole (Voitsekhovskaja et al. 2006; Bachmann and Keller 1995). This corresponds to the findings in this study (Table 3.1.2) where almost the whole portion of stachyose and verbascose was found in the vacuole in winter leaves of *A. reptans*. In contrast to transgenic *Arabidopsis* (Table 4.1; Iftime et al. 2011) *A. reptans* has probably transporter systems for stachyose in the chloroplast and the vacuole considering that stachyose synthesis occurs in the cytosol of mesophyll cells (Holthaus and Schmitz 1991, Peterbauer and Richter 2001).

Contrary to stachyose and verbascose about 25 % of raffinose was located in the chloroplasts (Table 3.1.2) in winter leaves of *A. reptans*. It can be assumed that the different subcellular distribution of raffinose and stachyose is due to their different functions in plant metabolism during the cold season (see 4.2).

Also the sucrose content increased in winter leaves, but although a shift of the sucrose portion from cytoplasm and chloroplast towards the vacuole could be observed in winter leaves, the sucrose concentration in the cytoplasm increased as well, due to higher total sucrose content in winter leaves (Table 3.1.2). These findings are not conforming to studies on cold acclimated *Arabidopsis* where large portions of sucrose were also localized in the chloroplasts (Table 4.1; Knaupp et al. 2011, Iftime et al. 2011, Nägele and Heyer 2013).

Table 4.1 Percentage distribution of carbohydrates among the stromal (Chl), cytoplasmic (Cyt) and vacuolar (Vac) compartments of leaf cells in different plant species. WT = wildtype, CA = cold acclimated, STS = stachyose synthase, NAF = non-aqueous fractionation

	myo-Inositol			Galactinol			Glucose + Fructose			Sucrose			Raffinose			Stachyose			Verbascose		
	chl	cyt	vac	chl	cyt	vac	chl	cyt	vac	chl	cyt	vac	chl	cyt	vac	chl	cyt	vac	chl	cyt	vac
Ajuga reptans[*1]	13	27	60	5	50	45	1	1	98	21	41	38	5	35	60	1	1	98	n.d.		
Ajuga reptans (CA)[*1]	8	0	92	18	3	79	2	4	94	7	17	76	25	0	75	0	0	100	0	1	99
Ajuga reptans (CA)[*2]	36	58	6	12			6	88	6	31	59	10	19	21	60						
Alonsoa meridionalis[*3]	81	13	6	2	2	96	1	1	98	21	44	35				0	6	94			
Asarina barclaiana[*3]	53	24	23				1	1	98	14	35	51				n.d.					
Arabipopsis thaliana (WT; CA)[*4]							0	12	28	37	55	8	33	46	21						
A. thaliana (transgen azuki bean STS, CA)[*4]							2	2	96	52	47	1	36	59	5	13	64	23			
A. thaliana (Col-0, CA)[*5]				33	17	50	0	2	98	41	18	41	29	5	66						
A. thaliana (Col-0)[*5]				19	35	46	6	5	89	14	68	18	12	68	20						
A. thaliana (Col-24)[*6]							2	21	77	15	56	29	28	56	16						
A. thaliana (Col-24, CA)[*6]							5	36	59	18	78	4	9	66	25						
Anthirrinum majus[*7]	55	27	18	4	0	96	0	0	100	1	84	15									
Petroselinum hortense[*7]	43	23	34				3	0	97	5	57	38									
Apium graveolens[*8]	44	9	48				1	1	98	1	20	79									
Plantago major[*8]	60	35	6				3	1	96	1	44	55									
Plantago maritima[*8]	82	1	17				1	1	98	1	56	44									

*[1]this thesis (NAF); *[2] Schneider and Keller 2009 (protoplasts and chloroplasts), Bachmann and Keller 1995 (vacuoles); *[3] Voitsekhovskaja et al 2006 (NAF); *[4] Iftime et al. 2011 (NAF); *[5] Knaupp et al. 2011 (NAF); *[6] Nägele and Heyer 2013 (NAF), *[7]Moore et al. 1997 (NAF); *[8] Nadwodnik and Lohaus 2008 (NAF).

Other metabolites involved in RFO synthesis like myo-inositol and galactinol likewise accumulated in mesophyll vacuoles in winter leaves of *A. reptans* (Table 3.1.2). Similar was found for *Antirrhinum majus* (Table 4.1; Moore et al. 1997) and *Alonsoa meridionalis* (Table 4.1; Voitsekhovskaja et al. 2006). Vacuolar galactinol is therefore assumed to be related to the RFO storage pool in mesophyll cells rather than to the transport pool in ICs and can be used for prospective RFO synthesis or together with sucrose and raffinose as osmoprotectants (Nishizawa et al. 2008). Galactinol in chloroplasts of winter leaves could together with raffinose act as ROS scavengers (Nishizawa et al. 2008).

In summer and winter leaves hexoses (glucose and fructose) were almost exclusively found in the vacuole (Table 3.1.2). This corresponds to the results of other plant species which were also produced by the non-aqueous fractionation technique (Table 4.1; Moore et al. 1997, Nadwodnik and Lohaus 2008, Farre et al. 2001, Voitsekhovskaja 2006, Knaupp et al. 2011, Nägele and Heyer 2013), but is contrary to findings of Bachmann and Keller (1995) who showed the highest percentage of hexoses in the cytoplasm of cold treated *A. reptans* leaves. This divergence could be a result of the different method used, given that they applied protoplasts and vacuoles for this analysis.

4.2 RFOs have different functions in *A. reptans*: raffinose functions in cold temperature response, whereas stachyose and verbascose play a role in carbohydrate storage

Cold acclimated plants that are frost hardy are able to produce and accumulate soluble sugars without suppression of photosynthetic metabolism (Strand et al. 1999). In *A. reptans* these solubles are raffinose oligosaccharides. RFOs fulfill different functions in the plant: In symplastic phloem loaders it is the main transport carbon in leaves, but it also acts as carbon storage in seeds and in desiccation tolerance in the non-frost hardy cucurbits and legumes, or they function in osmotic regulation by altering turgor pressure and the vacuolar osmotic potential (Bachmann and Keller 1995, Nishizawa et al. 2008). But RFOs, and especially raffinose, are also supposed to work as cryoprotectants (Bachmann and Keller 1995; Bachmann et al.

1994, Wanner and Junttila 1999, Nishizawa et al. 2008, Peters and Keller 2009, ElSayed et al. 2014), that are accumulated during the cold season. Cold acclimation and freezing tolerance are processes at which numerous genetic, physiological and biochemical changes enable plants to tolerate temperatures several degrees lower than before cold exposure (Strand et al. 1997; Stitt and Hurry 2002; Espinoza et al. 2010). Different enzymes that are involved in RFO-synthesis showed increased expression and activity after cold inducement (Castillo et al. 1990, Taji et al. 2002, Sui et al. 2012).

A. reptans reveals such adaptions to cold temperatures also: It increases the RFO concentrations in leaves at temperatures below 10 °C about 4 – 5 fold after at least one week exposure to low temperature. The role of raffinose in freezing tolerance in Arabidopsis is still discussed (Klotke et al. 2004, Zuther et al. 2004), but for *A. reptans* it clearly seems to function as freeze protectant. To act as such cryo-protectant, raffinose needs to be located in the cytoplasm (Koster and Lynch 1992) or in the chloroplast (Schneider and Keller 2009; Nägele and Heyer 2013). In winter leaves of *A. reptans* the main portion of raffinose was found either in the vacuole or in the stroma (Table 3.1.2, Fig.3.1.3) which is in agreement with the findings of Schneider and Keller (2009). Contrary to Bachmann and Keller (1995) or Nägele and Heyer (2013), we could not detect higher amounts of raffinose in the cytoplasm of mesophyll cells of *A. reptans*. This confirms the findings of Nägele and Heyer (2013) that the cytosolic raffinose content did not necessarily correlate with freezing tolerance.

It was shown that raffinose was re-allocated to the plastids during cold-acclimation in *A. reptans* as well as in *A. thaliana* (Table 3.1.2, Schneider and Keller 2009; Knaupp et al. 2011). Because of the probably lower raffinose concentration in the cytosol in comparison with the chloroplast stroma, raffinose must be transported by an energized transport system over the chloroplast envelop against a concentration gradient (Schneider and Keller 2009). It was recently demonstrated that raffinose (not only) was involved in stabilizing photosystem II against damage during freezing (Knaupp et al. 2011). The compartmentation analysis of raffinose in winter leaves of *A. reptans* in this study supports the possible role for raffinose in the protection of photosystems. Based on metabolic profiling, Espinoza et al. (2010) could demonstrate that in *A. thaliana* accumulation of raffinose occurred earlier in

comparison with other compatible solutes, such as proline. Accumulation of raffinose was in agreement with the earlier increase in the transcript encoding for galactinol synthase GolS3 and with the closely regulated raffinose synthase SIP1 (Espinoza et al. 2010). Beside its role as cryoprotectant in *A. reptans*, it is also possible that raffinose functions as a carbon storage compound since the concentration in the vacuole increased about 40-fold in winter leaves (Fig. 3.1.3). These higher raffinose contents in winter leaves are due to e.g. higher rates of synthesis, lower metabolic activity, and lower translocation rates of carbon compounds in the phloem.

The contents of stachyose and verbascose increased also about 40-fold in winter leaves of *A. reptans* in comparison to summer leaves (Fig. 3.1.3). In contrast to raffinose, stachyose and verbascose were almost exclusively found in the vacuole. Iftime et al. (2011) have shown that stachyose production in transgenic *A. thaliana* lines did not alter the freezing tolerance of cold acclimated plants. This shows that the cryoprotecting function seems unlikely for stachyose or verbascose. Instead, increased concentrations of these oligosaccharides more likely reflect metabolic changes at low temperatures, e.g. reduced metabolic activity or reduced carbon translocation rates in the phloem. Therefore, the main functions of stachyose are transport and storage and it is not supposed to function directly in cold acclimation or freezing tolerance. Although Zuther et al. (2004) do not preclude a participation of stachyose (together with raffinose) in freezing tolerance; this may not be the case for *A. reptans*, since almost the whole stachyose portion is accumulated in the vacuole. The increased stachyose concentration, STS-expression and –activity during low temperatures in this study (Fig. 3.2.4) rather point to an increased need to produce long-term storage carbohydrates, that can be supplied during cold winter months to maintain metabolism at impaired photosynthesis (Bachmann et al. 1994).

In addition to the oligosaccharides all analyzed soluble carbon compounds were increased in winter leaves of *A. reptans* in comparison to summer leaves (Table 3.1.1). For different *Arabidopsis* genotypes it was also shown that glucose, fructose and sucrose accumulated during cold acclimation but there was no correlation between the concentration of these substances and leaf freezing tolerance (Rohde et al. 2004). The only correlation they found was between the raffinose content and the freezing tolerance. The increased sucrose concentration in winter leaves of *A. reptans* (Table 3.1.1), that was also found in winter grown spinach, winter rape and

wheat, and rye (Strand et al 1999) indicates an indirect role of sucrose in the cold acclimation. It possibly serves as metabolic substrate for the synthesis of cryo-protecting compounds like the raffinose oligosaccharides and does not have a cryo-protecting function itself, as a higher sucrose level alone could not improve freezing tolerance in transgenic *Arabidopsis* plants (Klotke et al. 2004). *A. reptans* stores large amounts of sucrose in the vacuole during the winter in addition to the high sucrose concentration in the cytosol (Fig. 3.1.3) to facilitate a constant carbon supply. In summer leaves the situation is different: although nearly the same percentage of sucrose is found in cytosol and vacuole the actual sucrose concentration in the vacuole is lower than in the cytosol (Fig. 3.1.3). The different sucrose amounts and distribution in summer and winter leaves result in diverging concentration gradients between the cytosol and the vacuole that are 0.65 in winter leaves and 8.7 in summer leaves. This let assume that the SUT4 sucrose transporter found in *A. reptans* has probably no function in summer leaves as there is obviously too little sucrose in the vacuole that could be translocated into the cytosol. ArSUT-expression seemed not to be affected by low temperatures as it did not decrease significantly in warm treated leaves compared to cold treated leaves (Fig. 3.2.4 C) and does therefore neither contradict nor support the hypothesis. This was different for SUTs in *Juglans regia* that showed increased transcript levels at low temperatures (Decourteix et al. 2006).

Contrary to the findings of Bachmann et al. (1994) STS-expression and -activity did increase significantly at cold temperatures, and therefore it is likely that the respond to cold temperature in leaves of *A. reptans* is the accumulation of RFOs and sucrose plays only a secondary role in cold acclimation (Fig. 3.2.4). Therefore the increased sucrose levels in the vacuoles of winter leaves are a result of long-term stored carbohydrates.

4.3 RFOs and sucrose are synthesized and distributed differently in *A. reptans*

The analysis of the different plant tissues stem, sink-leaves, source-leaves, calyx and petals of *A. reptans* showed that sucrose was present in every tissue whereas RFOs were only found in stem and leaves (sink and source) (Fig. 3.2.3 A).

Stachyose contents, STS-expression and –activity (Fig. 3.2.3) indicate that stachyose seems to be synthesized not only in source leaves, but also in sink leaves and the stem (Fig. 3.4.3 C). Therefore, it is probable that stachyose synthesis in leaves of *A. reptans* is not linked with the ability to export stachyose via the phloem, but to other functions RFOs do fulfill in *A. reptans* (see 4.2). STS-activity found in the stem could imply that either some translocated sucrose and galactinol is synthesized to stachyose before unloading into the sink organs or that some stachyose is synthesized in the parenchyma cells of the stem. Similar to STS-expression in *A. reptans* was RS-expression in leaves of *Cucumis sativus* higher than in other plant tissues (stem, fruit, roots) (Sui et al. 2012). These results indicate that the RFO-synthesis pathway occurs predominantly in source leaves but also at some level in sink leaves and stems.

Although no stachyose and no STS-activity were found in the floral parts of *A. reptans*, very low STS-expression levels were shown for both tissues. It is assumed that after long distance transport into flowers raffinose is converted into sucrose, which is quickly catabolized into hexoses (Miao et al. 2007, Sui et al. 2012). In *A. reptans* stachyose could be translocated in the phloem and might be hydrolyzed before reaching the flower. Furthermore, it is possible that little STS-mRNA is also expressed in flowers of *A. reptans* to show some activity only during seed development as already shown for pea seeds and adzuki beans (Richter et al. 2000, Peterbauer and Richter 2001).

Photosynthetically produced sucrose is either used for transport, RFO-synthesis or storage in the vacuole. Sucrose transporter of *Lotus japonicus* (LjSUT4) and potato (StSUT4) showed the highest expression level in sink leaves (Flemetakis et al. 2003, and Chincinska et al. 2008). The low ArSUT-expression in sink leaves of *A. reptans* could be indicative for its role in sucrose release from the vacuole since mainly phloem translocated sucrose is being used for primary metabolism in sink

leaves and only small amounts of stored sucrose were intended to be used up for metabolic processes. The high sucrose contents in the floral parts (calyx and petals; Fig. 3.2.3 A) were presumably formed only by transport given that at least the petals are not able to perform photosynthesis (they lack of photosynthesis pigments) and to produce their own sucrose. The low expression level of ArSUT in calyx and petals (Fig. 3.2.3 B) shows that only little sucrose is released into cytoplasm. If the sucrose concentration in the vacuole is lower than in the cytosol, sucrose transporters are not able to perform export. The high sucrose concentration and ArSUT-expression in the stem (Fig. 3.2.3 A) is mainly allocated to the phloem transport and partly to the companion cells and the phloem surrounding parenchyma cells, where sucrose is produced by photosynthetic activity and transiently stored (Meyer et al 2000). ArSUT could have also impact on the sucrose export from the vacuoles of parenchymatic cells adjacent to the phloem, by releasing sucrose for phloem transport or RFO synthesis.

4.4 STS-expression and ArSUT-expression are differently affected by light and temperature

In plants, a large proportion of the carbon, especially of sucrose, assimilated in leaves during the light period was deposited in leaf vacuoles for export during the night to maintain carbon supply in the dark (Kaiser and Heber 1984, Martinoia et al. 1987). Considerable phloem transport occurs in leaves at night, although at reduced rates (about 40% of the light rate; Riens et al. 1994). The consumption of assimilates should be restricted to maintain metabolism only (Riens et al. 1994). Diurnal changes for sucrose and other carbons have been observed in crop plants like barley and spinach (Riens et al. 1994), and in *A. reptans* in summer- as well as in winter leaves (see 3.1.4.1). The influence of a varying light-dark regime on carbohydrate contents, STS- and SUT- expression as well as STS-activity was one question to be answered in this study. In warm and cold treated plants of *A. reptans* both, sucrose and RFO contents decreased after 24 and 48 hours darkness. The consumption of stored sucrose and RFOs is less in cold treated plants compared to the plants kept at 21 °C. This effect can have different reasons: (1) RFOs are needed as “frost protection

agent"; (2) a reduced phloem transport caused by cold temperatures (Strand et al. 1999, Stitt and Hurry 2002), although a reduced phloem transport could also be observed for *A. reptans* in summer leaves at a normal light-dark regime (see 3.1.4.1). The results of this study lead to the assumption that STS-expression is only slightly influenced by light and stronger by temperature, what in turn favors the probable function of stachyose as long term storage carbohydrate. Furthermore, RFO-synthesis can only be maintained until sucrose and starch are depleted and no sucrose can be supplied because of the lacking synthesis from photosynthesis products in the dark.

Contrary to STS-expression the ArSUT-expression level decreased almost towards zero after 24 h and 48 h darkness, independent of the temperature (Fig. 3.2.5). The probable localization of ArSUT is the vacuolar membrane (see 3.2.3.2) therefore it can be assumed that the transporter translocates sucrose over the tonoplast into the cytoplasm and can fulfill this function only until all sucrose (also catabolized starch and RFOs) are depleted or the cytoplasmic sucrose concentration is higher than the vacuolar. This seems to happen faster in warm treated plants due to the fact that the sugar content is almost 10-fold lower compared to cold treated plants (Fig. 3.2.5 A) and a reduced phloem transport in the dark.

4.5 Does *A. reptans* use a combined phloem loading mechanism?

Although *A. reptans* is classified as an exclusive symplastic phloem loading plant (Bachmann et al. 1994), it is still under debate that *A. reptans* combines skills of both symplastic and apoplastic phloem loading mechanisms as it was already demonstrated for *Alonsoa meridionalis* (Voitsekhovskaja et al. 2009). Both possess ICs and ordinary CCs in their minor veins (Hoffmann-Thoma et al. 2001, Knop et al. 2004) and it is assumed that they have a similar phloem sap composition due to similar transport sugars and subcellular sugar concentrations (Voitsekhovskaja et al. 2006, this study). The combined phloem loader *A. meridionalis* has a phloem sap composition containing about 175 mM sucrose, 250 mM raffinose and 400 mM stachyose (Voitsekhovskaja et al. 2006). In apoplastic phloem loaders the translocated sucrose concentration is about 1 M (in phloem sap) (Lohaus et al.

1995). So far, only concentrations resulting from stem cut-offs of *Cucumis melo* leaves have been reported by Mitchell et al. (1992) and Haritatos et al. (1996), but with diverging sucrose concentrations around 50 mM on the one hand and 130 mM on the other hand. But those cut-offs are most probably contaminated from injured parenchyma cells at the cut surface and do contain apoplastic solutes as well and therefore do not show pure phloem sap. It was not possible to get phloem sap by the laser aphid stylet technique from leaves of *A. reptans.*

Usually, there is a large concentration gradient between the cytosol of mesophyll cells and the phloem in apoplastic loading plants ranging between 5 in barley and maize (Lohaus et al. 1995, Lohaus et al. 1998) and 22 in *Asarina scandens* (Voitsekhovskaja et al. 2006), due to a high concentration in the phloem and a low concentration in the cytosol. The high sucrose concentration in the phloem sap of apoplastic loaders can be achieved by the use of transporter systems that translocate sucrose actively against that concentration gradient. In compliance with the polymer-trap model of symplastic loading plants, sucrose is expected to be present in the cytosol of mesophyll cells of symplastic species in higher concentrations than in the phloem. This is already demonstrated for *Cucumis melo* (Haritatos et al. 1996) with a concentration gradient of 0.7, but not absolutely reliable (see above). The concentration gradient found for the combined phloem loader *A. meridionalis* was 2 (Voitsekhovskaja et al. 2006) and implies a slightly increased sucrose concentration in the phloem compared to the cytosol for that loading mechanism.

Subcellular compartmentation analysis from *A. reptans* revealed a cytosolic sucrose concentration of 52 mM (Table 3.1.2). Compared to *A. meridionalis* (with 71 mM) and *A. scandens* (with 43 mM) (Voitsekhovskaja et al. 2006) the concentration draws near to the apoplastic loader. If the sucrose concentration in the phloem is almost as high as in *A. meridionalis* with 175 mM, what phloem exudates (not shown) let suggest, a concentration gradient higher than 1 and only symplastic loading of sucrose by diffusion is not likely and an additional active transport would be necessary.

All this could indicate what Fisher (1986) already proposed for the prior classified symplastic loading *Coleus blumei* and Slewinski et al. (2013) assumed for all symplastic loading plants: the simultaneous use of polymer trapping and apoplastic loading also for *A. reptans*.

Considering that several SUT homologues genes were already found in the *Arabidopsis* or rice genome (Sauer et al. 2004, Aoki et al. 2003), it is possible that also *A. reptans* has more than one SUT-gene. Assuming that *A. reptans* uses symplastic and apoplastic phloem loading, it is most likely that there is also a sucrose transporter that is involved in phloem loading and unloading. Like vacuolar sucrose transporters, phloem loading SUTs (group 1) have 12 transmembrane helices with a cytoplasmic N- and C-terminus and mediate sucrose uptake from the apoplast in source leaves and sucrose release to the apoplast in sink tissues (Sauer 2007). They are localized to the plasma membrane of ordinary CCs and transfer cells in the phloem of apoplastic loading plants (Kühn et al. 1996, Kühn et al. 1997, Kühn 2003). Such a SUT1 sucrose transporter has already been identified for *A. meridionalis* (Knop et al. 2004) that has also been classified as strict symplastic loader before a heterogeneous loading mechanism was demonstrated (Voitsekhovskaja et al. 2009). They described that the two types of transport carbohydrates, sucrose and RFOs, enter the phloem on two different ways. Sucrose loading occurs from ordinary companion cells via the sucrose transporter *AmSUT1* that was identified by Knop et al. (2001) and RFOs were synthesized in the ICs directly in the phloem. If actually *A. reptans* performs the combined loading mechanism it might give several advantages under various environmental conditions and multiple phloem loading allows the plant to adapt faster to biotic and abiotic stresses (Slewinski et al. 2013).

Further analysis in terms of additional sucrose transporters needs to be done. Aoki et al. (2003) could demonstrate that the sequence identities range about 80% within the different SUT groups from different plant species. Combined with a sequence identity between 45-97 % of different SUTs in one species (Sauer et al. 2004, Aoki et al. 2003) it should be possible to create a new degenerate primer pair based on conserved regions of all SUT1s and the already identified SUT in *A. reptans* to obtain further SUT genes from *A. reptans*.

Concluding, the combined phloem loading for *A. reptans* is possible, but could not been verified.

5 Summary

This thesis aimed to investigate the compartmentation of phloem-translocated carbohydrates, amino acids and anions, and of the synthesis of one specific transport form, stachyose, in the process of phloem loading and stress response in leaves of symplastic phloem loaders. The model plant *A. reptans* is characterized by the presence of a special type of companion cells in the minor veins, the intermediary cells, and the translocation of raffinose family oligosaccharides (RFOs), mainly stachyose, in the phloem and last it`s ability to survive freezing temperatures.

To elucidate how the compartmentation of carbohydrates, especially RFOs can support this mechanism, and to understand which role the different RFOs have in the plant, the compartmentation between subcellular compartments of mesophyll cells was analyzed.

Furthermore, the partitioning of carbohydrates in different tissues (source leaves, sink leaves, stem, calyx and flowers) of *A. reptans* was analyzed by means of the expression level and activity of stachyose synthase (STS) and the isolation and identification of a probable vacuolar sucrose transporter (SUT), together with its expression level in these tissues.

To understand the role and effects of light and temperature on stachyose synthesis and sucrose transport, sequences of the STS-gene and SUT-gene were cloned, sequenced and used to examine the expression levels of stachyose synthase and the identified sucrose transporter ArSUT. Based on phylogenetic analysis and a dileucine-motif ArSUT is probably localized to the vacuolar membrane.

The metabolite content varied strongly between summer and winter leaves. Soluble metabolites (sugars, amino acids and malate) increased during cold season up to 10-fold whereas the starch content was decreased. In winter leaves the subcellular distribution showed a shift of carbohydrates from cytoplasm to vacuole and chloroplast. Despite this, the metabolite concentration was higher in all compartments in winter leaves compared to summer leaves because of the much higher total metabolite content in winter leaves. The different oligosaccharides did show a different compartmentation. Stachyose and verbascose were almost exclusively found in the vacuole whereas one fourth of raffinose was localized in the stroma of chloroplasts. Apparently, the subcellular distribution of the RFOs differs

because they fulfill different functions in plant metabolism during cold season. Raffinose might function in protecting chloroplast membranes during freezing, whereas stachyose and verbascose might function primarily as carbon storage form. STS-expression, –activity and stachyose content were influenced by the temperature. In cold acclimated leaves expression as well as activity and RFO content were considerably increased. Changes in the light-dark regime had no effects on STS-expression, although the RFO content decreased at darkness. The decreasing sugar content is probably the result of sugar consumption and reduced phloem transport.

That was different for ArSUT-expression and sucrose concentration. Both were strongly decreased at darkness. Assuming that ArSUT is a vacuolar transporter it can only release sucrose as long as it is still available. The temperature had only little effect on the ArSUT-expression, although the sucrose concentration increased together with the whole sugar concentration. This indicates that no or only little sucrose transport into the cytoplasm occurs and that vacuolar sucrose is stored there for longer periods instead for remobilization into the cytoplasm.

Furthermore, a combined phloem loading mechanism could not be excluded for *A. reptans*, but a more elaborate analysis concerning that subject needs to be done.

6 Abbreviations

Amp	ampicillin
Amp^R	ampicilin resistance
AP	adapter primer
ArSUT	*Ajuag reptans* sucrose transporter
ATP	adenosine 5´ triphosphate
bp	base pair
BSC	bundle sheath cell
BSA	bovine serum albumin
CC	companion cell
cDNA	complementary DNA
Ch	chloroplasts
Chl	chlorophyll
CMV	cucumber mosaic virus
CTAB	hexadecyltrimethylammonium bromide
Cyt	cytoplasm
dATP	deoxyadenosin 5´ triphosphate
dd H_2O	double distilled water
DDSA	Dodecenyl Succinic Anhydride hardener for epoxy resin
DIG	digoxigenine
DMF	dimethylformatide
DMP-30 2,4,6-tris	(dimethylaminomethyl) phenol, a tertiary amine epoxy accelerator
DMSO	dimethylsulfoxide
DNA d	esoxyribonucleic acid
dNTPs	deoxyribonucleotides
DP	degree of polymerization
dpm	decays per minute
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum

EtOH	ethanol
FW	fresh weight
G6PDH	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GS	galactinol synthase (UDP-galactose:myo-Inositol galactosyltransferase)
HEPES	hydroxyethyl-piperazinethane sulfonic acid
HPLC	high pressure liquid chromatography
IC	intermediary cells
IPTG	isopropyl-β-D-thiogalactopyranoside
IWF	intercellular washing fluid or apoplastic extract
K_m	Michaelis-Menten constant
kb	kilobase pair
kDa	kilodalton
MC	mesophyll cell
MCS	multiple cloning site
NAD	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
OC	ordinary cells
OD	optical density
ORF	open reading frame
PAGE	polyacrylamid-gel electrophoresis
PCMBS	para-chloro-mercuribenzene sulphonate
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PEPCx	phosphoenolpyruvate carboxylase
pf	plasmodesmal field
PM	plasma membrane
PMF	proton motive force
PP	phloem parenchyma

PVP	polyvinylpyrrolidon
RACE	rapid amplification of cDNA ends
RFOs	raffinose family oligosaccharides
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
RS	raffinose synthase (galactinol:sucrose 6-α-D-galactosyltransferase)
RT	reverse transcriptase
SD	standard deviation
SE	sieve element
SE-CCC	sieve element-companion cell complex
STEL	sucrose – Triton-X-100 – Tris– EDTA –lysozyme buffer
STS	stachyose synthase (galactinol:raffinose 6-α-D-galactosyltransferase)
SUT	sucrose transporter
T_{ann}	annealing temperature
Taq	*Thermus aquaticus*
TC	transfer cells
TdT	terminal desoxynucleotidyl transferase
T_m	melting temperature
T_{op}	temperature optimum
Tris	tris-(hydroxymethyl)-aminomethane
U	enzyme activity unit (1 unit corresponds to the conversion of 1 µmol substrate min^{-1})
UTP	uridine 5´ triphosphate
UV	ultraviolet light
Vac	vacuoles
v/v	volume/volume
w/v	weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XV	xylem vessel

7 List of Figures

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9 Literature

Adhikari J, Bhaduri TJ, DasGupta S, Majumder AL (1987) Chloroplast as a locale of L-*myo*-inositol-1-phosphate synthase. Plant Physiol 85: 611-164

Amiard V, Mueh KE, Demming-Adams B, Ebbert V, Turgeon R, Adams III WW (2005) Anatomical and photosynthetic acclimation to the light environment in species with differing mechanisms of phloem loading. PNAS 102 (36): 12968-12973

Aoki N, Hirose T, Scofield GN (2003) The Sucrose Transporter Gene Family in Rice. Plant Cell Physiol 44 (3): 223-232

Asada K (2006) Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol 144: 391-396

Ayre BG (2011) Membrane-Transport Systems for Sucrose in Relation to Whole-Plant Carbon Partitioning. Mol Plant: 1-18

Bachmann M, Keller F (1995) Metabolism of the Raffinose Family Oligosaccharides in Leaves of *Ajuga reptans* L. – Inter- and Intracellular Compartmentation. Plant Physiol 109: 991-998

Bachmann M, Matile P, Keller F (1994) Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L - Cold acclimation, translocation, and sink to source transition: discovery of chain elongation enzyme. Plant Physiol 105: 1335-1345

Beck E, Ziegler P (1989) Biosynthesis and Degradation of Starch in Higher Plants Annu Rev Plant Physiol Plant Mol Biol 40: 95-117

Beebe DU, Turgeon R (1992) Localization of galactinol, raffinose, and stachyose synthesis in *Cucurbita pepo* leaves. Planta 188: 354-361

Behnke HD (1989) Structure of the phloem. Transport of photoassimilates: 79-137

Carpaneto A, Geiger D, Bamberg E, Sauer N, Fromm J, Hedrich R (2005) Phloem-localized, Proton-coupled Sucrose Carrier ZmSUT1 Mediates Sucrose

Efflux under the Control of the Sucrose Gradient and the Proton Motive Force. J Biol Chem 280: 21437-21443

Castillo EM, de Lumen BO, Reyes PS, de Lumen HZ (1990) Raffinose Synthase and Galactinol Synthase in Developing Seeds and Leaves of Legumes. J Agric Food Chem 38: 351-355

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11(2): 113-116

Chincinska I, Liesche J, Krügel U, Michalska J, Geigenberger P, Grimm B, Kühn C (2008) Sucrose Transporter StSUT4 from Potato Affects Flowering, Tuberization, and Shade Avoidance Response. Plant Physiol 146: 515-528

Chomczynski P, Sachi N (1987) Single step method of RNA-isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162 (1): 156-159

Crowe JH, Hoekstra FA, Ngyen KHN, Crowe LM (1996) Is vitrification involved in depression of the phase transition temperature in dry phospholipids? Biochem Biophys Acta 1280: 187-196

Decourteix M, Alves G, Brunel N, Améglio T, Guilliot A, Lemoine R, Pétel G, Sakr S (2006) *JrSUT1*, a putative sucrose transporter could mediate sucrose influx into xylem parenchyma cells and be regulated by freeze-thaw cycles over the autumn-winter period in walnut tree (*Juglans regia* L.). Plant, Cell & Environment 29: 36-47

Deol KK, Mukherjee S, Gao F, Brûlé-Babel A, Stasolla C, Ayele BT (2013) Identification and characterization of the three homeologues of a new *sucrose transporter* in hexaploid wheat (*Triticum aestivum* L.). BMC Plant Biol 13: 181-195

ElSayed Al, Rafudeen MS, Golldack D (2014) Physiological aspects of raffinose oligosaccharides in plants: protection against abiotic stress. Plant Biol 16: 1-8

Endler A, Meyer S, Schelbert S, Schneider T, Weschke W, Peters SW, Keller F, Baginsky S, Martinoia E, Schmidt UG (2006) Identification of a Vacuolar Sucrose Transporter in Barley and Arabidopsis Mesophyll Cells by a Tonoplast Proteomic Approach. Plant Physiol 141: 196-207

Espinoza C, Degenkolbe T, Caldana C, Zuther E, Leisse A, Willmitzer L, Hincha DK, Hannah MA (2010) Interaction with diurnal and circadian regulation results in dynamic metabolic and transcriptional changes during cold acclimation in Arabidopsis. PLoS ONE 5: e14101

Farre EM, Tiessen A, Roessner U, Geigenberger P, Trethewey RN, Willmitzer L (2001) Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars, organic acids, amino acids, and sugar alcohols in potato tubers using a nonaqueous fractionation method. Plant Physiol 127: 685–700

Fisher DG (1986) Ultrastructure, plasmodesmatal frequency, and solute concentration in green areas of variegated *Coleus blumei* Benth. leaves. Planta 169: 141-152

Flemetakis E, Dimou M, Cotzur D, Efrose RC, Aivalakis G, Colebatch G, Udvardi M, Katinakis P (2003) A sucrose transporter, LjSUT4, is up-regulated during *Lotus japonicas* nodule development. Journal of Experimental Botany 54: 1789-1791

Gaffney SH, Haslam E, Lilley TH, Ward TR (1988) Homotactic and heterotactic interactions in aqueous solutions containing some saccharides. Experimental results and an empirical relationship between saccharide solvation and solute–solute interactions. J Chem Soc, Faraday Trans 84: 2545-2552

Gahrtz M, Stolz J, Sauer N (1994) A phloem-specific sucrose-H^+ symporter from *Plantago major* L. supports the model of apoplastic phloem loading. The Plant Journal 6 (5): 697-706

Gamalei YV (1989) Structure and function of leaf minor veins in trees and herbs - A taxonomic review. Trees 3: 96-110

Gamalei YV (1991) Phloem loading and its development related to plant evolution from trees to herbs. Trees 5: 50-64

Gamalei YV, van Bel AJE, Pakhomova MV, Sjutkina AV (1994) Effects of temperature on the conformation of the endoplasmic reticulum and on starch accumulation in leaves with the symplasmic minor-vein configuration. Planta 194: 443-453

Gaudreault PR, Webb JA (1981) Stachyose synthesis in leaves of *Cucurbita pepo.* Phytochem 20 *(*12): 2629-2633

Gerhard R, Held HW (1984) Subcellular Metabolite Levels in Leaves by Fractionation of Freeze-Stopped Material in Nonaqueous Media. Plant Physiol 75: 542-547

Gerhard R, Stitt M, Held HW (1987) Subcellular metabolite levels in spinach leaves - Regulation of sucrose synthesis during diurnal alterations in photosynthetic partitioning. Plant Physiol 83: 399-407

Gibson UEM, Heid CA, Williams PM (1996) A Novel Method for Real Time Quantitative RT-PCR. Genome Res 6: 995-1001

Gil L, Ben-Ari J, Turgeon R, Wolf S (2012) Effect of CMV infection and high temperatures on the enzymes involved in raffinose family oligosaccharide biosynthesis in melon plants. J Plant Physiol 169: 965-970

Gilmour SJ, Hajela RK, Thomashow MF (1988) Cold acclimation in Arabidopsis. Plant Physiol 87: 745-750

Gilmour SJ, Sebolt AM, Salazar MP, Everard J D, Thomashow MF (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol 124: 1854-1865

Górecki RJ, Brenac P, Clapham WM, Willcott JB, Obendorf RL (1996) Soluble Carbohydrates in White Lupin Seeds Matured at 13 and 28 °C. Crop Science 36 (5): 1277-1282

Gottwald JR, Krysan PJ, Young JC, Evert RF, Sussman MR (2000) Genetic evidence for the *in planta* role of phloem-specific plasma membrane sucrose transporters. PNAS 97 (25): 1379-1384

Guy CL, Yelenosky G, Sweet HC (1980) Light exposure and soluble sugars in citrus frost hardiness. Fla Sci 43: 268-273

Guy CL, Huber JLA, Huber SC (1992) Sucrose Phosphate Synthase and Sucrose Accumulation at Low Temperature. Plant Physiol 100: 502-508

Haab CT, Keller F (2002) Purification and characterization of the raffinose oligosaccharide chain elongation enzyme, galactan:galactan galactosyltransferase (GGT), from *Ajuga reptans* leaves. Plant Physiol 114: 361-371

Handley LW, Pharr DM, Mcfeeters RF (1983) Relationship between Galactinol Synthase Activity and Sugar Composition of Leaves and Seeds of Several Crop Species. J Amer Soc Hort Sci 108 (4): 600-605

Hannah, M A, Wiese D, Freund S, Fiehn O, Heyer A G, Hincha DK (2006) Natural genetic variation of freezing tolerance in *Arabidopsis.* Plant Physiol 142: 98-112

Haritatos E, Keller F, Turgeon R (1996) Raffinose oligosaccharide concentrations measured in individual cell and tissue types in *Cucumis melo* L. leaves: implications for phloem loading. Planta 198: 614-622

Hegi G (1964) Flora von Mitteleuropa. Band V, 4. Teil. Carl Hanser Verlag (München): S. 2542

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

Higuchi R, Fockler C, Dollinger G, Watson R (1993) Kinetic PCR Analysis: Real-time Monitoring of DNA Amplification Reactions. Bio/Technology 11: 1026-1030

Hincha D, Sonnewald U, Willmitzer L, Schmitt J (1996) The role of sugar accumulation in leaf frost hardiness: investigations with transgenic tobacco expressing a bacterial pyrophosphatase or a yeast invertase gene. J Plant Physiol 147: 604–610

Hoch G, Peterbauer T, Richter A (1998) Purification and Characterization of Stachyose Synthase from Lentil (*Lens culinaris*) Seeds: Galactopinitol and Stachyose Synthesis. Archives of Biochemistry and Biophysics 366 (1): 75-81

Hoffmann-Thoma G, van Bel A, Ehlers K (2001) Ultrastructure of minor-vein phloem and assimilate export in summer and winter leaves of the symplasmically loading evergreens *Ajuga reptans* L., *Aucuba japonica* Thunb., and *Hedera helix* L.. Planta 212: 231–242

Holthaus U, Schmitz K (1991) Stachyose synthesis in mature leaves of *Cucumis melo,* Purification and characterization of stachyose synthase (EC. 2 .4. 1. 67). Planta 184: 525-531

Holthaus U, Schmitz K (1991) Distribution and immunolocalization of stachyose synthase in *Cucumis melo* L.. Planta 185: 479-486

Huber JLA, Pharr DM, Huber SC (1990) Partial purification and characterization of stachyose synthase in leaves of *Cucumis sativus* and *Cucumis melo*: utilization of a rapid assay for *myo*-inositol. Plant Science 69: 179-188

Iftime D, Hannah MA, Peterbauer T, Heyer AG (2011) Stachyose in the cytosol does not influence freezing tolerance of transgenic Arabidopsis expressing stachyose synthase from adzuki bean. Plant Science 180: 24-30

Kaiser G, Heber U (1984) Sucrose transport into vacuoles isolated from barley mesophyll protoplasts. Planta 161: 562-568

Kandler O, Hopf H (1982) Oligosaccharides Based on Sucrose (Sucrosyl Oligosaccharides). Plant Carbohydrates I (Loewus et al. eds.) Springer Verlag: 348-383

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27: 137-138

Keller F (1992) Transport of Stachyose and Sucrose by Vacuoles of Japanese Artichokes (*Stachys sieboldii*) Tubers. Plant Physiol 98: 442-445

Keller F, Matile P (1985) The Role of the Vacuole in Storage and Mobilization of Stachyose in Tubers of *Stachys sieboldii*. J Plant Physiol 119 (4): 369-380

Keller F, Pharr DM (1996) Metabolism of carbohydrates in sinks and sources: galactosyl-sucrose oligosaccharides. Photoassimilate Distribution in Plants and Crops: Source-Sink Relationships (Zamski E and Schaffner AA, eds.) New York: Marcel Dekker, 157-183

Klie S, Krueger S, Krall L, Giavalisco P, Flügge UI, Willmitzer L, Steinhauser D (2011) Analysis of the compartmentalized metabolome - a validation of the non-aqueous fractionation technique. Front Plant Sci 2: Art 55

Klotke J, Kopka J, Gatzke N, Heyer AG (2004) Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of *Arabidopsis thaliana* with contrasting cold adaptation – evidence for a role of raffinose in cold acclimation. Plant Cell Environ 27: 1395–1404

Knaupp J, Mishra KB, Nedbal L, Heyer AG (2011) Evidence for a role of raffinose in stabilizing photosystem II during freeze-thaw cycles. Planta 234: 477-486

Knop C, Voisekhovskaja OV, Lohaus G (2001) Sucrose transporters in two members of the Scrophulariaceae with different types of transport sugar. Planta 213: 80-91

Knop C, Stadler R, Sauer N, Lohaus G (2004) AmSUT1, a Sucrose Transporter in Collection and Transport Phloem of the Putative Symplastic Phloem Loader *Alonsoa meridionalis.* Plant Physiol 134: 204–214

Koster K, Lynch D (1992) Solute accumulation and compartmentation during the cold acclimation of Puma rye. Plant Physiol 98: 108-113

Kratsch HA, Wise RR (2000) The ultrastructure of chilling stress. Plant Cell Environ 23: 337-350

Krueger S, Niehl A, Lopez Martin MC, Steinhauser D, Donath A (2009) Analysis of cytosolic and plastidic serine acetyltransferase mutants and subcellular metabolite distributions suggests interplay of the cellular compartments for cysteine biosynthesis in *Arabidopsis*. Plant Cell Environ 32: 349–367

Krueger S, Giavaisco P, Krall L, Steinhauser MC, Büssis D, Usadel B, Flügge UI, Fernie AR, Willmitzer L, Steinhauser D (2011) A topological map of the compartmentalized *Arabidopsis thaliana* leaf metabolome. PLoS ONE 6 (3): e17806

Krueger S, Steinhauser D, Lisec J, Giavaisco P (2014) Analysis of subcellular metabolite distributions within *Arabidopsis thaliana* leaf tissue: A primer for subcellular metabolomics. Arabidopsis Protocols, Meth Mol Biol 1062: 575-596

Kühn C, Quick WP, Schulz A, Riesmeier JW, Sonnewald U, Frommer WB (1996) Companion cell-specific inhibition of the potato sucrose transporter SUT1. Plant, Cell and Environment 19: 1115-1123

Kühn C, Franceschi VR, Schulz A, Lemoine R, Frommer WB (1997) Macromolecular Trafficking Indicated by Localization and Turnover of Sucrose Transporters in Enucleate Sieve Elements. Science 275: 1298–1300

Kühn C (2003) The Sucrose Transporter *StSUT1* Localizes to Sieve Elements in Potato Tuber Phloem and Influences Tuber Physiology and Development. Plant Physiol 131: 102–113

Kurkela S, Francke M, Heino P, Lång V, Palva ET (1988) Cold induced gene expression in Arabidopsis L. Plant Cell Rep 7: 495-498

Lalonde S, Tegeder M, Throne-Holst M, Frommer WB, Patrick JW (2003) Phloem loading and unloading of sugars and amino acids. Plant Cell Environ 26: 37-56

Lauterbach C, Niedermeier M, Besenbeck R, Stadler R, Sauer N (2007) Immunolocalization of the PmSUC1 Sucrose Transporter in *Plantago major* Flowers and Reporter-Gene Analyses of the *PmSUC1* Promoter Suggest a Role in Sucrose Release from the Inner Integument. Plant Biol 9 (3): 357-365

Lehle L, Tanner W (1973) The function of myo-inositol in the biosynthesis of raffinose. Purification and characterization of galactinol:sucrose 6-galactosyl-transferase from Vicia faba seeds. European J Biochem 38: 103-110

Lemoine R (2000) Sucrose transporters in plants: update in function and structure. Biochem Biophys Acta 1465: 246-262

Lohaus G, Winter H, Riens B, Heldt HW (1995) Further studies of the phloem loading process in leaves of barley and spinach. The comparison of metabolite concentrations in the apoplastic compartment with those in the cytosolic compartment and in the sieve tubes. Bot Acta 108: 270-275

Lohaus G, Büker M, Hußmann M, Soave C, Heldt HW (1998) Transport of amino acids with special emphasis on the synthesis and transport of asparagine in the Illinois Low Protein and Illinois High Protein strains of maize. Planta 205: 181-188

Lohaus G, Hussmann M, Schneider H, Zhu JJ, Sattelmacher B (2000) Solute balance of a maize (*Zea mays* L) source leaf as affected by salt treatment with special emphasis on phloem re-translocation and ion leaching. J Exp Bot 51: 1721-1732

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275

Lunn J E (2007) Compartmentation in plant metabolism. J Exp Bot 58: 35-47

Martinoia E, Flügge U I, Kaiser G, Heber U, Heldt H W (1985) Energy-dependent uptake of malate into vacuoles isolated from barley mesophyll protoplasts. Biochem Biophys Acta 806: 311-319

Martinoia E, Kaiser G, Schramm MJ, Heber U (1987) Sugar-transport across the plasmalemma and the tonoplast of barley mesophyll protoplasts: evidence for different transport systems. Plant Physiol 131: 467-487

Martinoia E, Rentsch D (1994) Malate compartmentation: responses to a complex metabolism. Annu Rev Plant Physiol Plant Mol Biol 45: 447-467

Meyer S, Melzer M, Truernit E, Hümmer C, Besenbeck R, Stadler R, Sauer N (2000) *AtSUC3*, a gene encoding a new *Arabidopsis* sucrose transporter is expressed in cells adjacent to the vacuolar tissue and in a carpel cell layer. Plant J 24 (6): 869-882

Miao MM, Xu XF, Chen XX, Xue LB, Cao BS (2007) Cucumber carbohydrate metabolism and translocation under chilling night temperature. J Plant Physiol 164: 621-628

Mitchell DE, Gadus MV, Madore MA (1992) Patterns of Assimilate Production and Translocation in Muskmelon *(Cucumis melo* L.). Plant Physiol 99: 959-965

Moore B , Palmquist D , Seemann P (1997) Influence of plant growth at high CO_2 concentrations on leaf content of rinolose-1,5-bisphosphate carboxylase/oxygenase and intracellular distribution of soluble carbohydrates in tobacco, snapdragon, and parsley. Plant Physiol 115, 241-248

Nadwodnik J, Lohaus G (2008) Subcellular concentrations of sugar alcohols and sugars in relation to phloem translocation in *Plantago major*, *Plantago maritima*, *Prunus persica*, and *Apium graveolens.* Planta 227: 1079–1089

Nägele T, Heyer AG (2013) Approximating subcellular organisation of carbohydrate metabolism during cold acclimation in different natural accessions of *Arabidopsis thaliana.* New Phytol 198: 777–787

Nathan M, Mertz LM, Fox DK (1995) Optimizing Long RT-PCR. Life Tech 17 (3): 78-80

Nishizawa A, Yabuta Y, Shigeoka S (2008) Galactinol and Raffinose Constitute a Novel Function to Protect Plants from Oxidative Damage. Plant Physiol 47: 1251–1263

Obendorf RL, Horbowicz M, Dickerman AM, Brenac P, Smith ME (1998) Soluble Oligosaccharides and Galactosyl Cyclitols in Maturing Soybean Seeds in Planta and in Vitro. Crop Science 38 (1): 78-84

Öner-Sieben S, Lohaus G (2014) Apoplastic and symplastic phloem loading in *Quercus robur* and *Fraxinus excelsior*. J Exp Bot 65 (7): 1905-1916

Oparka KJ, Turgeon R (1999) Sieve Elements and Companion Cells – Traffic Control Centers of the Phloem. Plant Cell 11: 739-750

Peterbauer T, Richter A (1998) Galactosylononitol and Stachyose Synthesis in Seeds of Adzuki Bean - Purification and Characterization of Stachyose Synthase. Plant Physiol 117: 165-172

Peterbauer T, Richter A (2001) Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. Seed Sci Res 11: 185-197

Peterbauer T, Mucha J, Mach L, Richter A (2002) Chain Elongation of Raffinose in Pea Seeds. J Biol Chem 277: 194-200

Peters S, Keller F (2009) Frost tolerance in excised leaves of the common bugle (*Ajuga reptans* L.) correlates positively with the concentrations of raffinose family oligosaccharides (RFOs). Plant, Cell & Environment 32: 1099–1107

Reinders A, Sivitz AB, Starker CG, Gantt JS, Ward JM (2008) Functional analysis of LjSUT4, a vacuolar sucrose transporter from *Lotus japonicus*. Plant Mol Biol 68: 289-299

Reinders A, Sivitz AB, Ward JM (2012) Evolution of plant sucrose uptake transporters. Frontiers Plant Sci 3 (22): 1-12

Rennie EA, Turgeon R (2009) A comprehensive picture of phloem loading strategies. PNAS 106 (52): 14162-14167

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17: 208-212

Richter A, Hoch G, Puschenreiter M, Mayer U, Peterbauer T (2000) The role of stachyose synthase in the oligosaccharide metabolism of legume seeds. Seed biology: advances and applications. Proceedings of the Sixth International Workshop on Seeds: 75-84

Riens B, Lohaus G, Heineke D, Heldt H W (1991) Amino acid and sucrose content determined in the cytosolic, chloroplastic and vacuolar compartments and in the phloem sap of spinach leaves. Plant Physiol 97: 227-233

Riens B, Lohaus G, Winter H, Heldt HW (1994) Production and diurnal utilization of assimilates in leaves of spinach (*Spinacia oleracea* L.) and barley (*Hordeum vulgare* L.). Planta 192:497-501

Riesmeier JW, Willmitzer L, Frommer WB (1994) Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. EMBO Journal 13 (1): 1-7

Rohde P, Hincha DK, Heyer AG (2004) Heterosis in the freezing tolerance of crosses between two *Arabidopsis thaliana* accessions (Columbia-0 and C24) that will differences in non-acclimated and acclimated freezing tolerance. Plant J 38: 790-799

Sambrook JEF, Fritsch EF, Maniatis T (1989) Molecular Cloning. J Biol Chem 264

Santarius KA (1973) The protective effect of sugars on chloroplast membranes during temperature and water stress and its relationship to frost, desiccation and heat resistance. Planta 113 (2): 105-114

Santarius KA, Milde H (1977) Sugar compartmentation in frost-hardy and partially dehardened cabbage leaf cells. Planta 136: 163–166

Sauer N, Ludwig A, Knoblauch A, Rothe P, Gahrtz M, Klebl F (2004) *AtSUC8* and *AtSUC9* encode functional sucrose transporters, but the closely related *AtSUC6* and *AtSUC7* genes encode aberrant proteins in different *Arabidopsis* ecotypes. Plant J 40: 120-130

Sauer N (2007) Molecular physiology of higher plant sucrose transporters. FEBS Letters 581: 2309–2317

Scarth GW, Levitt J (1937) The frost hardening mechanism of plant cells. Plant Physiol 12: 51-78

Schneider T, Keller F (2009) Raffinose in chloroplasts is synthesized in the cytosol and transported across the chloroplast envelope. Plant Cell Physiol 50: 2174–2182

Schneider S, Hulpke S, Schulz A, Yaron I, Höll J, Imlau A, Schmitt B, Batz S, Wolf S, Hedrich R, Sauer N (2012) Vacuole release sucrose via tonoplast-localized SUC4-type transporters. Plant Biol 14: 325-336

Schopfer P (1989) Experimentelle Pflanzenphysiologie 2: 336-337. Springer Verlag.

Schulz A, Beyhl D, Marten I, Wormit A, Neuhaus E, Poschet G, Büttner M, Schneider S, Sauer N, Hedrich R (2011) Proton-driven sucrose symport and antiport are provided by the vacuolar transporters SUC4 and TMT1/2. Plant J 68: 129-136

Shahba MA, Qian Y L, Hughes HG, Kosk AJ, Christensen D (2003) Relationships of soluble carbohydrates and freeze tolerance in saltgrass. Crop Science 43: 2148–2153

Shakya R, Sturm A (1998) Characterization of Source- and Sink-Specific Sucrose/H^+ Symporters from Carrot. Plant Physiol 118: 1473-1480

Slewinski TL, Meeley R, Braun DM (2009) *Sucrose transporter1* functions in phloem loafing in maize leaves. J Exp Bot 60 (3): 881-892

Slewinski TL, Braun DM (2010) Current perspectives on the regulation of whole-plant carbohydrate partitioning. Plant Science 178 (4): 341-349

Slewinski TL, Zhang C, Turgeon R (2013) Structural and functional heterogeneity in phloem loading and transport. Frontiers in Plant Science 4: Art 244

Sprenger N, Keller F (2000) Allocation of raffinose family oligosaccharides to transport and storage in *Ajuga reptans*: the roles of two distinct galactinol synthases. Plant J 21: 249-258

Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruc Res 26: 31-43

Stadler R, Brandner J, Schulz A, Gahrtz M, Sauer N (1995) Phloem Loading by the PmSUC2 Sucrose Carrier from Plantago major Occurs into Companion Cells. The Plant Cell 7: 1545-1554

Stadler R, Truernit E, Gahrtz M, Sauer N (1999) The ATSUC1 sucrose carrier may represent the osmotic driving force for anther dehiscence and pollen tube growth in Arabidopsis. Plant J 19 (3): 269-278

Steponkus PL, Lanphear FO (1968) The relationship of carbohydrates to cold acclimation in Hedera helix L. cv. Thorndale. Physiol Planta 22: 777-791

Stitt M, Hurry V (2002) A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in *Arabidopsis*. Curr Opin Plant Biol 5: 199-206

Stone JM, Palta JP, Bamberg JB, Weiss LS, Harbage JF (1993) Inheritance of freezing resistance in tuber-bearing Solanum species: Evidence for independent genetic control of nonacclimated freezing tolerance and cold acclimation capacity. PNAS 90: 7869-7873

Strand A, Hurry V, Gustafsson P, Gardeström P (1997) Development of *Arabidopsis thaliana* leaves at low temperatures releases the suppression of photosynthesis and photosynthetic gene expression despite the accumulation of soluble carbohydrates. Plant Journal 12: 605–614

Strand A, Hurry V, Henkes S, Huner N, Gustafsson P Gardeström P, Stitt M (1999) Acclimation of *Arabidopsis* leaves developing at low temperatures increasing cytoplasmic volume accompanies increased activities of enzymes in the calvin cycle and in the sucrose-biosynthesis pathway. Plant Physiol 119: 1387-1397

Sui X, Meng F, Wang H, Wei Y, Li R, Wang Z, Hu L, Wang S, Zhang Z (2012) Molecular cloning, characteristics and low temperature response of raffinose synthase gene in *Cucumis sativus* L.. J Plant Physiol 169: 1883-1891

Sun AJ, Xu HL, Gong WK, Zhai HL, Meng K, Wang YQ, Wei XL, Xiao GF, Zhu Z (2008) Cloning and Expression Analysis of Rice Sucrose Transporter Genes *OsSUT2M* and *OsSUT5Z*

Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. Plant J 29 (4): 417-426

Tanner W, Kandler O (1966) Biosynthesis of Stachyose in *Phaseolus vulgaris.* Plant Physiol 41: 1540-1542

Tanner W, Kandler O (1968) Myo-Inositol, a Cofactor in the Biosynthesis of Stachyose. European J Biochem 4: 233-239

Tapernoux-Lüthi EM, Böhm A, Keller F (2004) Cloning, Functional Expression, and Characterization of the Raffinose Oligosaccharide Chain Elongation Enzyme, Galactan:Galactan Galactosytransferase, from Common Bugle Leaves. Plant Physiol 134: 1377-1387

Taylor AO, Craig AS (1971) Plants under Climatic Stress. Plant Physiol 47: 719-725

Truernit E, Sauer N (1995) The promoter of the *Arabidopsis thaliana* SUC2 sucrose-H+ symporter gene directs expression of β-glucuronidase to the phloem: Evidence for phloem loading and unloading by SUC2. Planta 196: 564-570

Turgeon R (1989) The Sink-Source Transition in Leaves. Annu Rev Plant Physiol Plant Mol Biol 40: 119-138

Turgeon R, Gowan E (1990) Phloem loading in *Coleus blumei* in the absence of carrier-mediated uptake of export sugar from the apoplast. Plant Physiology 94**:** 1244–1249

Turgeon R, Gowan E (1992) Sugar synthesis and phloem loading in Coleus blumei leaves. Planta 187: 388-394

Turgeon R, Beebe DU, Gowan E (1993) The intermediary cell: minor-vein anatomy and raffinose oligosaccharide synthesis in the Scrophulariaceae. Planta 191: 446-456

Turgeon R, Medville R (1998) The absence of phloem loading in willow leaves. PNAS 95: 12055-12060

Turgeon R, Medville R (2004) Phloem Loading. A Reevaluation of the Relationship between Plasmodesmatal Frequencies and Loading Strategies. Plant Physiol 136: 3795–3803

Uemura M, Steponkus PL (2003) Modification of the intracellular sugar content alters the incidence of freeze-induced membrane lesions of protoplasts isolated from *Arabidopsis thaliana* leaves. Plant Cell Environ 26: 1083-1096

van Bel AJE (1993) Strategies of Phloem Loading. Annu Rev Plant Physiol Plant Mol Biol 44: 253-281

van Bel AJE (1996) Interaction between sieve element and companion cell and the consequences for photoassimilate distribution. Two structural hardware frames with associated physiological software packages in dicotyledons? J. Exp. Bot. 47: 1129-1140

van den Berg N, Crampton BG, Hein I, Birch PRJ, Berger DK (2004) High-throughput screening of suppression subtractive hybridization cDNA libraries using DNA microarray analysis. BioTechniques 37: 818-824

van den Emde W, Valluru R (2009) Sucrose, sucrosyl oligosaccharides, and oxidative stress: scavenging and salvaging? J Exp Bot 60: 9-18

van Guilder HD, Vrana KE, Freeman WM (2008) Twenty-five years of quantitative PCR for gene expression analysis. Bio Tech 44: 619-626

Voitsekhovskaja OV, Koroleva OA, Batashev DR, Knop C, Tomos D, Gamalei YV, Heldt HW, Lohaus G (2006) Phloem loading in two Scrophulariaceae species what can drive symplastic flow via plasmodesmata? Plant Physiol 140: 383–395

Voitsekhovskaja OV, Rudashevskaya EL, Demchenko KN, Pakhomova MV, Batashev DR, Gamalei YV, Lohaus G, Pawlowski K (2009) Evidence for functional heterogeneity of sieve element-companion cell complexes in minor vein phloem of *Alonsoa meridionalis.* Journal of Experimental Botany 60: 1873–1883

Wanner L A, Junttila O (1999) Cold-induced freezing tolerance in *Arabidopsis.* Plant Physiol 120: 391–400

Weschke W, Panitz R, Sauer N, Wang Q, Neubohn B, Weber H, Wobus U (2000) Sucrose transport into barley seeds: molecular characterization of two transporters

and implications for seed development and starch accumulation. Plant J 21(5): 455-467

Weise A, Barker L, Kühn C, Lalonde S, Buschmann H, Frommer WB, Ward JM (2000) A new subfamily of Sucrose Transporters, SUT4, with Low Affinity/High Capacity Localized in Enucleate Sieve Elements of Plants. The Plant Cell 12: 1345-1355

Widders IE, Kwantes M (1995) Ontogenic changes in seed weight and carbohydrate composition as related to growth of cucumber (*Cucumis sativus* L.) fruit. Sci Hort 63 (3-4): 155-165

Williams LE, Lemoine R, Sauer N (2000) Sugar transporters in higher plants – a diversity of roles and complex regulation. Trends in Plant Science 5: 283–290

Winter H, Lohaus G, Heldt HW (1992) Phloem Transport of Amino Acids in Relation to their cytosolic levels in Barley leaves. Plant Physiol 99: 996-1004

Winter H, Robinson DG, Heldt HW (1993) Subcellular volumes and metabolite concentrations in barley leaves. Planta 191: 180-190

Winter H, Robinson DG, Heldt HW (1994) Subcellular volumes and metabolite concentrations in spinach leaves. Planta 193: 530-535

Wirtz W, Stitt M, Heldt HW (1980) Enzymic Determination of Metabolites in the Subcellular Compartments of Spinach Protoplasts. Plant Physiol 66: 187-193

Wright KM (2003) Structural and Functional Vein Maturation in Developing Tobacco Leaves in Relation to *AtSUC2* Promoter Activity. Plant Physiol 131: 1555–1565

Wong ML, Medrano JF (2005) Real-time PCR for mRNA quantitation. Bio Techniques 39 (1): 1-11

Xin Z, Browse J (2000) Cold comfort farm: the acclimation of plants to freezing temperatures. Plant, Cell & Environment 23: 893–902

Ziegler H (1975) Nature of transported substances. Transport in plants 1: 59-100

Zimmermann MH, Ziegler H (1975) List of sugars and sugar alcohols in sieve-tube exudates. Encycl Plant Physiol New Ser:

Zuther E, Büchel K, Hundertmark M, Stitt M, Hincha DK, Heyer AG (2004) The role of raffinose in the cold acclimation response of *Arabidopsis thaliana.* FEBS Letters 576: 169-173

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Erklärung

Hiermit versichere ich, Sarah Findling, dass ich die vorliegende Arbeit in allen Teilen selbst angefertigt habe. Dabei habe ich keine anderen als die angegeben Quellen und Hilfsmittel verwendet, und die Stellen, die im Wortlaut anderen Werken entnommen wurden als solche gekennzeichnet. Desweiteren erkläre ich hiermit, dass diese Arbeit in keinem anderen Fachbereich oder wissenschaftlichen Hochschule in dieser oder einer ähnlichen Fassung vorgelegen hat.

Wuppertal, den 15. August 2014