I.Introduction

1. Abiotic stress

During their life history, plants are inadvertently exposed to unfavorable environmental (abiotic) conditions, such as high light intensity, water deficit, high salinity, temperature extremes and chemical pollution. An inability of plants to rapidly adapt to such conditions will consequently lead to damage of plant membranes and the subcellular milieu, potentially leading to the death of cells and whole organisms.

As different as the outlined stresses may seem, they bare commonalities (see Fig. I.1), which include osmotic and oxidative stresses that cause direct or indirect damage of proteins and membranes. Stress stimuli are sensed by the cells and via signal transduction cascades that trigger coordinated expression of transcription factors (e.g. CBF/DREB; Cook et al., 2004), transcriptional up-regulation of many genes are orchestrated (e.g. COR genes; Thomashow, 1999, metabolic enzyme genes; Guy et al., 2008; Wienkoop et al., 2008).

To cope with such unfavorable conditions on a short- and/or long-term basis, plants use different strategies. I will focus here on cold stress only, the main topic of my thesis.

1.1. Cold stress in plants

Cold stress includes low non-freezing temperatures (defined as temperatures below optimal for growth; i.e. temperatures of 1 to 14°C) and freezing temperatures (below 0°C). These phenomena are not mutually exclusive and often occur naturally in temperate zones where seasonal transitions are well defined.

The processes are complex, including both low and freezing temperatures and additionally water deficit (due to low air humidity and ice formation) and reduced light intensities (due to short day length and possible snow coverage). Water deficit (one of the mentioned stresses occurring during low and freezing temperatures) includes the risk of cell dehydration, resulting in protein denaturation and membrane fusion. In addition, intracellular ice crystal formation leads to subsequent membrane disruption. Low temperatures lead also to altered enzyme activities, which can lead to accumulation of toxic compounds and to respiration (due to altered enzyme activities of the photosynthetic pathway), leading to cell death.

Plants use different strategies to ensure survival under low and freezing temperatures, the simplest being surviving in the form of dormant seeds (annual plants, e.g. sunflower). Alternatively, survival occurs as deciduous woody plants with dormant nodes (many trees, bushes and shrubs). Some herbs and vegetables survive low temperatures in the form of tubers or roots, which are at least partly insulated by the soil and snow (e.g. *Verbascum spec.*, carrots). But many plants

survive low and freezing temperatures as whole living organisms with aboveground parts including leaves (e.g. *Ajuga reptans*, *Pinus spec.*); to be successful, they need to undergo a myriad of different cellular changes, outlined in this work.

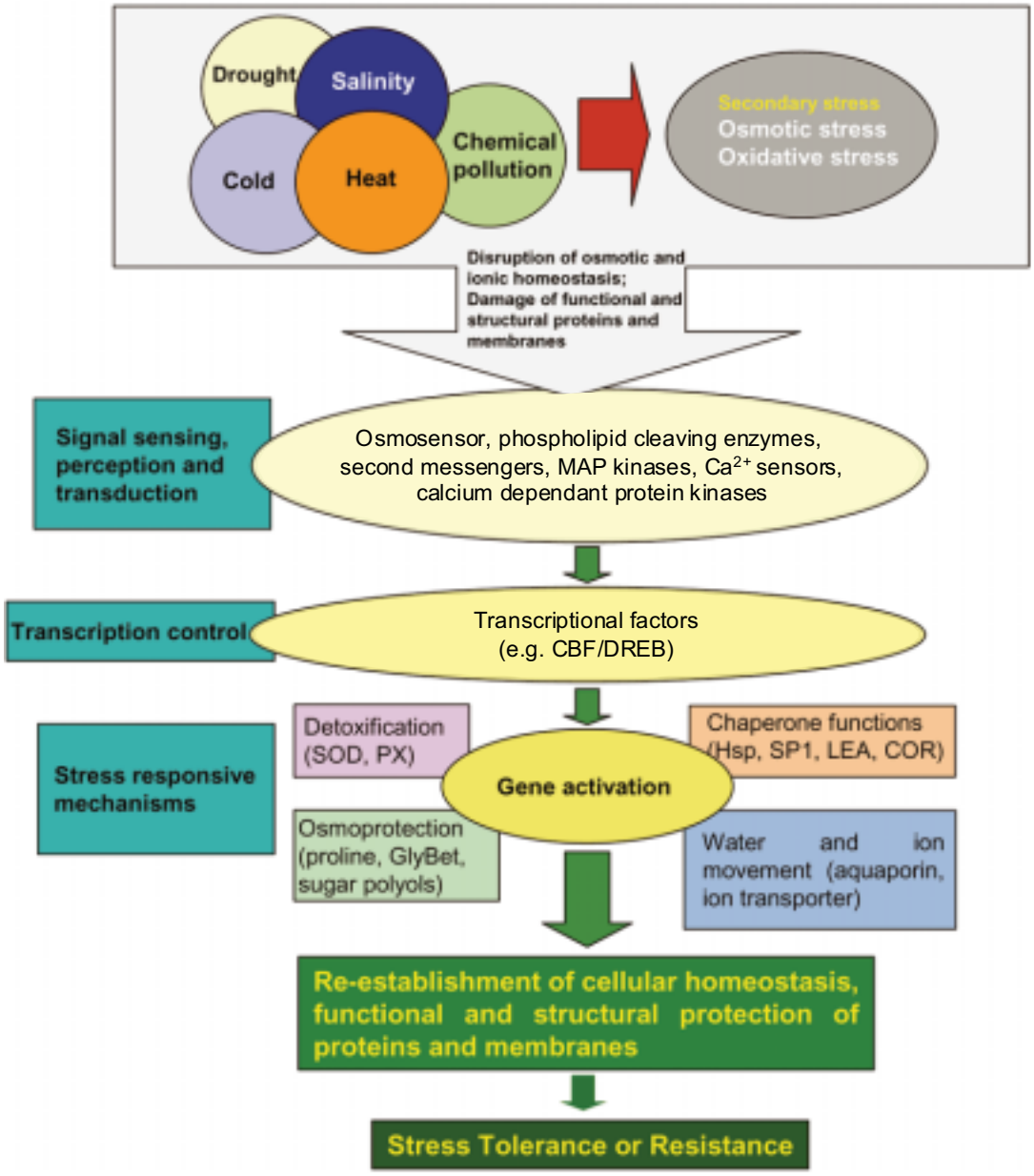

Fig. I.1 The complexity of the plant response to abiotic stresses. Primary stresses, such as drought, salinity, cold, heat and chemical pollution are often interconnected, and cause cellular damage and secondary stresses, such as osmotic and oxidative stress. The initial stress signals (e.g. osmotic and ionic effects, or temperature, membrane fluidity changes) trigger the downstream signalling process and transcription controls which activate stress-responsive mechanisms to re-establish homeostasis and protect and repair damaged proteins and membranes. Inadequate response at one or several steps in the signalling and gene activation may ultimately result in irreversible changes of cellular homeostasis and in the destruction of functional and structural proteins and membranes, leading to cell death. MAP, mitogen-activated protein; CBF/DREB, C-repeat binding/dehydration-responsive element binding; SOD, superoxide dismutase; PX, peroxidase; Hsp, heat-shock protein; SP1, stable protein 1; LEA, late embryogenesis abundant; COR, cold responsive gene/protein; GlyBet, glycinebetaine. Adapted from Wang et al. (2003).

1.1.1. Cold acclimation

Plants acquiring tolerance against low and/or freezing temperatures go through a phase of cold acclimation, which takes place during low non-freezing conditions. During this period, a lot of molecular changes occur, including expression of isoforms of proteins with a lower temperature optimum and antifreeze proteins (to maintain metabolism and stabilize membranes, Alberdi and Corcuera, 1991; Moffatt et al., 2006), insertion of unsaturated fatty acids into membranes (to maintain membrane fluidity and function, Palta et al., 1993; Nishida and Murata, 1996; Uemura and Steponkus, 1999; Gomès et al., 2000) and accumulation of compatible solutes (amino acids, quaternary ammonium compounds, cyclitols, disaccharides, sugar alcohols, oligosaccharides etc. to prevent dehydration and its consequences, Yancey et al., 1982; Tarczynski et al., 1993; Hoshino et al., 1999; Kaplan et al., 2004; see below). To this end, a lot of changes have to take place on the gene expression level (Thomashow, 1999; Kaplan et al., 2007). It has been shown that mutations in single components do not necessarily show an effect on the frost tolerance of the plant, but that a combination of different factors are needed (Zuther et al., 2004).

1.1.2. Compatible solutes

Many plants accumulate compatible solutes in response to desiccation, independent if it is induced by drought stress, salt stress or low and freezing temperature stress. Compatible solutes are defined as solutes which can accumulate to high concentrations without interfering with cellular structure or function. As mentioned above, they include soluble amino acids (e.g. proline; Hare and Cress, 1997), quaternary ammonium compounds [e.g. glycinebetaine (GlyBet); Chen and Murata, 2008], cyclitols [e.g. *myo*-inositol (Ino), pinitol; Vernon and Bohnert, 1992; Sheveleva et al., 1997] disaccharides [e.g. sucrose (Suc), trehalose; Fry et al., 1993; Garg et al., 2002], sugar alcohols (e.g. mannitol, sorbitol; Tarczynski et al., 1993; Sheveleva et al., 1998) and oligosaccharides [e.g. fructans, raffinose family oligosaccharides (RFOs); Hincha et al., 2006; Valluru and van den Ende, 2008] (for review see Hare et al., 1998).

One effect of compatible solutes is the decreasing of the osmotic potential of the cell, by accumulation to high concentrations. But often the absolute concentrations in cells are insufficient to increase water-holding capacities of the cells (Hare et al., 1998). Therefore, a more direct effect was proposed (Fig. I.2; Hoekstra et al., 2001). During moderate water loss, the compatible solutes can keep the surface of membranes and proteins hydrated and during more severe water loss, they can even replace the water shell and, therefore, prevent membrane fusion and denaturation of proteins (Hoekstra et al., 2001).

As abiotic stress resistance is of agricultural interest, different transgenic and breeding efforts have been made to either increase compatible solute concentrations

in plants or insert enzymes for the production of novel compatible solutes. The resulting plants showed not always the expected positive correlation between compatible solute concentration and stress resistance due to mistargeted intracellular localization of the accumulation of compatible solutes (e.g. GlyBet accumulation in chloroplasts of tomato plants was more effective than in the cytosol, Park et al., 2007). This indicates a relevance for a concerted combination of cellular compartmentation and compatible solute accumulation.

In addition, prolonged expression of high concentrations of compatible solutes can have undesired side effects, as has been observed in transgenic tobacco plants accumulating mannitol showing, besides salt and drought resistance, also reduced growth (Karakas et al., 1997).

Water soluble carbohydrates, a prominent class of compatible solutes, have the ability to form a glassy state by hydrogen bonding interactions. With increasing molecular weight of the carbohydrate it was observed that the higher the glass-to-liquid transition temperature is, the higher is the ability to protect cellular structures (Hoekstra et al., 2001). Intracellular glasses are mainly present in dry pollen and seeds (Koster, 1991). In seeds, they can play the dual roles of carbon storage (see also 2.6) and protective agent.

The disaccharide maltose (Malt, see also I. 4) has been proposed to be a compatible solute, being capable of protecting partly different enzymes and the electron transport chain activity *in vitro* (Kaplan and Guy, 2004), although it is a reducing carbohydrate (which can react with amino acids or proteins as part of a Maillard reaction; Maillard, 1916).

Further details will be given for the prominent compatible solutes studied in this thesis, the RFOs.

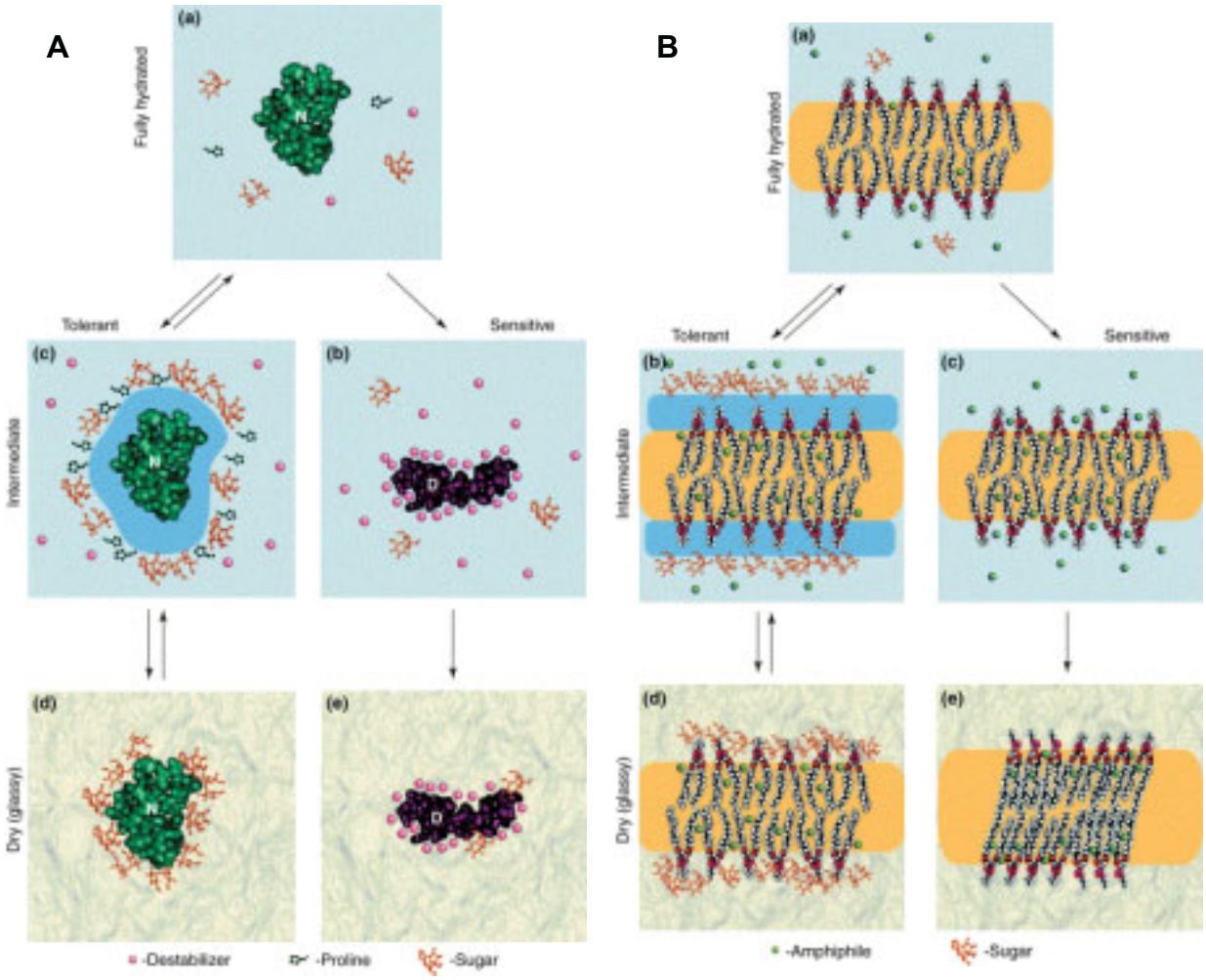

Fig. I.2 Mechanism of protein structure (A) and membrane (B) stabilization at different stages of water loss. (A) In fully hydrated cells (a), the native (folded) form of a protein (N) is thermodynamically favorable. Molecular crowding during water loss increases the probability of the cytoplasmic solutes interacting with the protein surface. In sensitive cells (b), the lack of compatible solutes causes preferential binding to dominate over preferential exclusion, which leads to protein unfolding and denaturation (D). In tolerant cells (c), preferential exclusion from the protein surface dominates over preferential binding, which maintains proteins in their native conformation at the intermediate water contents. Preferential exclusion of compatible solutes causes a preferential hydration of the protein surface (indicated as the blue ring around the protein). With the disappearance of the water shell from the proteins below 0.3 g H_2O g^{-1}dry weight, sugar molecules that were previously excluded from the protein surface replace water via hydrogen bonding, thus stabilizing the native protein structure in the dried (glassy) cytoplasm in tolerant cells (d). Compatible solutes other than sugars fail to stabilize proteins in the dried state. In dried, sensitive cells (e), the previously formed unfolded conformation (D) is fixed in a cytoplasmic glass. The reversibility of the processes occurring during dehydration and rehydration is indicated by arrows.
(B) In fully hydrated cells (a), membrane lipids are in an undisturbed liquid-crystalline state. Upon water loss (intermediate water contents), cytoplasmic amphiphilic compounds increase in concentration and partition into membranes, which can be considered as a preferential interaction. This causes membrane disturbance in both tolerant (b) and sensitive (c) cells. Amphiphile partitioning into membranes is complete with the dissipation of bulk water. In the intermediate water range, the presence of preferentially excluded solutes (sugars) in tolerant cells (b) keeps the membrane surface preferentially hydrated (indicated by the blue band) and prevents membrane fusion. The absence of these solutes in the sensitive cells (c) might result in membrane fusion, as in model membrane systems. On further drying below 0.3 g H_2O g^{-1}dry weight, the sugar molecules in tolerant cells replace water in the hydration shell of the membranes, thereby maintaining the spacing between phospholipid molecules. The bilayer remains in the liquid-crystalline phase. In sensitive cells, the removal of water from the hydration shell in the absence of sugars results in packing of the phospholipid molecules, which leads to a phase transition into the gel phase. This might lead to lateral

phase separations and irreversible membrane damage. Below 0.1 g H_2O g^{-1}dry weight, cytoplasmic components are immobilized in a glassy matrix, which might differ in properties between tolerant (d) and sensitive (e) cells. Whether preferential exclusion has an influence on amphiphile partitioning into membranes is not known. The arrows indicate the reversibility of the processes during rehydration. The slow repartitioning of amphiphiles from membranes into the cytoplasm on rehydration might cause transient leakage of cytoplasmic solutes from tolerant cells. From Hoekstra et al.(2001).

2. Raffinose family oligosaccharides (RFOs)

RFOs are sucrosyl galactosides of which the smallest member the trisaccharide raffinose (Raf) is. It consists of a galactose (Gal) molecule linked by an α-1,6 bond to the C6 of the glucose (Glc) moiety of Suc (Fig. I.3). Longer-chain RFOs have one (stachyose; Sta) or more additional Gal residues linked by α-1,6 bonds to the C6 of Gal.

RFOs are widespread in the plant kingdom. They are both water soluble (with the exception of Raf which is only moderately soluble, with only 203 mg/ml water at 20°C) and non-reducing, which enable them to fulfill their roles as storage carbohydrates (Keller and Matile, 1985; Bachmann et al., 1994; Peterbauer and Richter, 2001), phloem sugars (mainly Sta; Bachmann et al., 1994; Sprenger and Keller, 2000) and stress protectants (Zuther et al., 2004; Hincha et al., 2006).

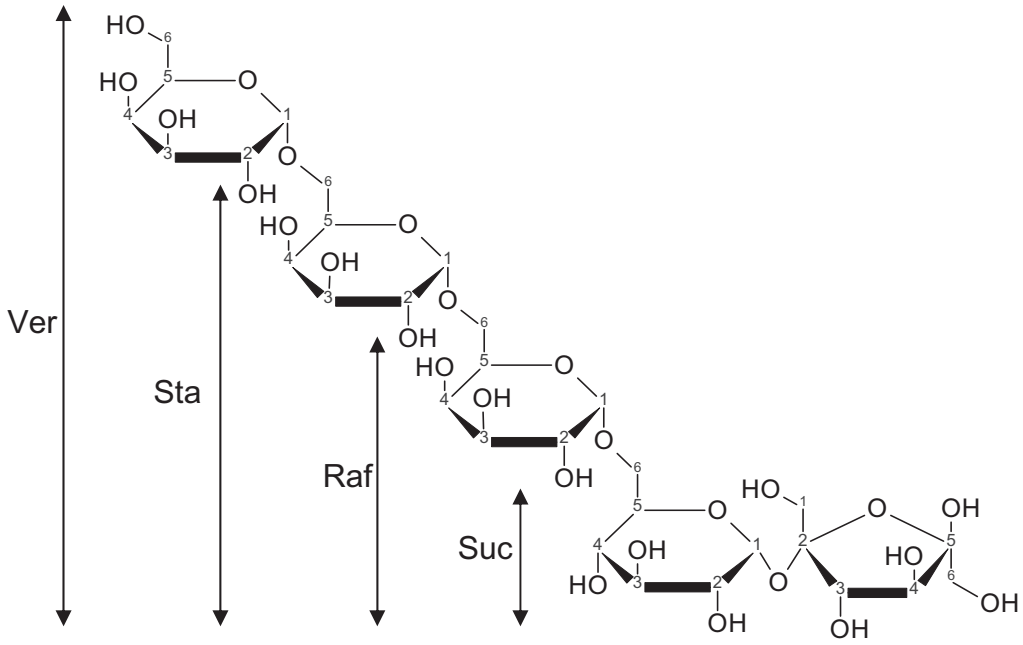

Fig. I.3 Chemical structure of raffinose family oligosaccharides (RFOs). Shown are the three smallest RFO members. Raf (raffinose) has one Gal (galactose) unit attached to the C6 of the sucrosyl Glc. Sta (stachyose) has a second Gal unit attached to the C6 of the Gal of Raf and Ver (verbascose) has a third Gal unit attached to the C6 of the terminal Gal unit of Sta.

2.1. RFO biosynthesis in *Ajuga reptans*

A. reptans accumulates a series of long-chain RFO oligomers up to a degree of polymerization (DP) of 15 (Ver is DP 5). Ver biosynthesis in this plant comprises a four step reaction including both a galactinol (Gol)-dependent and a Gol-independent part (Fig. I.4). The Gol-dependent biosynthesis occurs in the cytosol, with the short-chain RFOs Raf and Sta being the principal products. Gol formation is catalyzed by Gol synthase (GolS; EC 2.4.1.123; Pharr et al., 1981; Liu et al., 1998; Sprenger and Keller, 2000) which uses the substrates UDP-Gal and *myo*-inositol (Ino) to form Gol. Subsequently, the galactosyl moiety of Gol is transferred to Suc by Raf synthase (RafS; EC 2.4.1.82; Lehle and Tanner, 1973), leading to the production of Raf. The final cytosolic step is the synthesis of Sta by Sta synthase (StaS; EC 2.4.1.67; Lehle and Tanner, 1972; Peterbauer and Richter, 1998; Peterbauer et al., 1999), where a galactosyl moiety from Gol is transferred to Raf. Further elongation of the tetra-saccharide Sta takes place via Gol-independent steps in the vacuole, as shown for leaves of *A. reptans* (Bachmann et al., 1994; Inan Haab and Keller, 2002; Tapernoux-Lüthi et al., 2007). This suggests that a transport mechanism exists across the tonoplast of *A. reptans* vacuoles to import Sta into the vacuoles. The elongation enzyme is galactan: galactan galactosyl transferase (GGT; Tapernoux-Lüthi et al., 2004) which transfers a Gal subunit from one RFO to another, leading to two RFO species, one with a DP higher and one with a DP lower than before ($RFO_m + RFO_n \rightarrow RFO_{m+1} + RFO_{n-1}$). The only other reported evidence of GGT activity and long-chain RFO accumulation comes from *Coleus blumei* leaves, although in this study the compartmentation of RFO accumulation was not investigated (Gilbert et al., 1997).

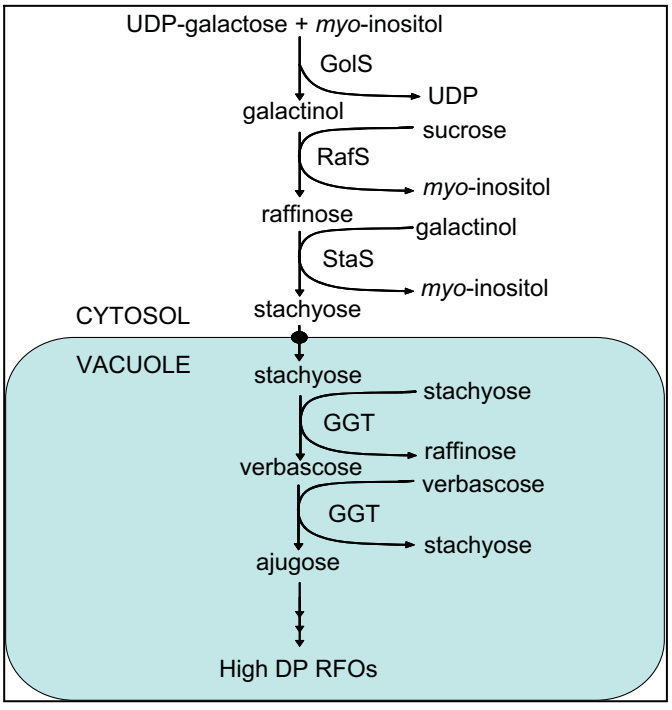

Fig. I.4 Simplified scheme of the RFO biosynthetic pathways in the mesophyll of *A. reptans*. GolS, galactinol synthase; RafS raffinose synthase; StaS, stachyose synthase; GGT, galactan:galactan galactosyltransferase [simplified from Bachmann and Keller (1995)].