The role of trehalose metabolism in plant growth and stress responses

(met een samenvating in het Nederlands)

Proefschrift

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Its appearance tells a story
A story about its journey of its life,
unbearable pain might be experienced

Its appearance tells its features
strugglefull, strong and never giving up
with a beautiful philosophy
pain it got sweet fresh it gives

Thank you Orange
you give me a deep lesson
if only I could take it

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CHAPTER 1

INTRODUCTION IN PLANT STRESS BIOLOGY AND TREHALOSE METABOLISM

1 Plant stress responses: societal significance and definition

Environmental conditions for crop production have always affected yields. When environmental conditions can be predicted, however, crops can be chosen and agricultural strategies can be employed that will produce predictable yield. The advantages of various combinations of crop and agricultural strategies are illustrated in the description of the Austronesian migrations in the Indonesian archipelago during the Neolithic period, for example (Munoz, 2006). Climate change is defined as a change in average daily weather pattern over a period of typically 10 years or more (Bernstein et al. 2007 Climate Change 2007: Synthesis report). Combined, climate change and the growing urbanization that pushes agricultural production to less favorable land and environments render yields much less predictable, they threaten the very livelihood of populations. The effect of environmental conditions on plant production impacts food security and the amount of commodities available for accommodation, clothing, transport, as well as industry and leisure. This impact is increasing as fossil fuels become depleted because fossil fuels momentarily have been substituting for raw materials and energy supply (Beddington, 2010). However, a return to a mostly bio-based economy is inevitable. Food security, the most essential for living beings, therefore is becoming harder to achieve as food demand parallels with world growing population, but is in anti-parallel with availability of agricultural land and fresh water for food production.

With a rapidly changing climate water availability and temperature maxima have been less predictable hence drought, flooding and extreme temperatures are reported more often globally. Where fresh water is lacking for agriculture because it is otherwise used or absent, salinity of the soils is often a problem accompanying drought. If an integrated agricultural or natural park management does not pre-empt the environmental changes then plants have to face and bear the impacts of exposure to extremes of environmental conditions. Yet, plants have not had time to adapt to these extremes or to be selected for enhanced resilience in the case of crop plants. Often, agricultural practices that permit resilience to climate change have not been implemented, at times for lack of knowhow or for social and economic reasons (Binternagel, et al., 2010). As a result, drought in Indonesia caused increasing losses of rice production from 100 thousand tons yearly between the year of 1981-1990 to 300 thousand tons yearly between the year of 1990-2000 (Boer and Las, 2003).

Crop choice and breeding are important components of agricultural production. In developing countries overall, breeding of modern and locally adapted varieties of crops contributed 50% to the increase of crop yield in the period 1981-2000 whilst it contributed only 21% in the period 1961-1980 (Evenson and Gollin, 2003). The breeding component is even higher in Sub-Saharan African countries with arid agro-ecosystems. Yet breeding for climate resilient varieties has only just begun with as an example, Scuba Rice, flooding tolerant rice varieties containing the *SUB1* locus (Singh, et al., 2010). Breeding crops resilient to extremes of environmental conditions to mitigate crop losses with climate change, however, implies a more complete understanding of the stresses that plants experience and of the individual stress responses activated in everyone crop variety.

What constitutes plant stress? Lichtenthaler (1998) defined plant stress as 'any unfavourable condition or substance that affects or blocks a plant's metabolism, growth or development'. Light which alters and is necessary for metabolism, growth and development is not defined as stress, therefore a plant stress is associated with the idea that metabolism, growth and development are inhibited or changed from a statistical norm (Lichtenthaler, 1998). Plants react to stress stimuli in a complex manner, yet breeders distinguish three differing strategies: stress escape, avoidance or tolerance (Levitt, 1972; Chaves, et al., 2003). Stress escape is achieved for example when the life cycle of the crop is shortened such that the crop plant may be grown to harvest within the period of reliable precipitation. Stress avoidance includes such adaptations as longer deeper roots maintaining access to water during drought (Price et al., 2002); stress avoidance further includes responses such as stomatal closure that reduce water loss. In contrast, stress tolerance goes beyond and implies plants

metabolically and physiologically adapted to the drought by osmotic adjustment and plants that sustain the re-watering stress after dehydration. A classic example of stress tolerance is the desert resurrection plant *Selaginella lepidophylla* that in the dormant dry state contains large amounts of the non-reducing glucose disacchharide trehalose which is thought to protect dried tissues (Zentella, et al., 1999). Plants with drought tolerance traits often undergo a tradeoff for survival in unfavorable environment slowing down growth, and compromising yield. This is the case for Barley and Wheat varieties bred at the International Center for Agricultural Research in the Dry Areas for example (Araus et al., 2002; Araus et al., 2008).

A stress may have differing effects depending on the developmental stage of the plant. When drought is encountered in the early stage of corn life, it affects growth mainly of the leaf area causing a yield reduction of 20% (Çakir, 2004). Drought during ear formation affects the size of the tassel causing a 30% yield reduction. The most severe effect is by drought during the silking stage; this shortens the duration of pollination from 6-8 days to 2-3 days because the silk dries before pollen tubes reach the tassel, resulting in up to 100% yield reduction. Drought at grain filling may cause about 10-30% yield reduction (Çakir, 2004). The interaction between stress susceptibility and developmental stages implies that physiological processes in development are inherently more or less susceptible to drought and/or that stress signals are differentially transduced depending upon the developmental stage or organ of the plant.

The complexity of the responses to stress, particularly to drought has been a hurdle to advance breeding towards drought resilient crops. An understanding of molecular mechanisms involved in drought responses and in controlling carbon allocation will undoubtedly assist modern molecular breeding approaches aiming at breeding the illusive “sustained yield under stress”.

Molecular mechanisms involved in stress responses include stress sensing, signal transduction and integration leading to differentiated responses. Stress sensing and signal transduction appear to take a great diversity of routes upstream in the signal transduction processes (Hey, et al., 2010). Some signal transduction mechanisms are specific for plants as for example that involving the hormone abscisic acid (ABA). ABA levels may rise 40 fold in plants exposed to drought and salt stress, this occurs both by *de novo* synthesis and by release of ABA from its glucosylated storage form (Lee, et al., 2011). ABA accumulation in leaves is further increased by ABA transport from roots. ABA signal perception and transduction until stomata closure is already well investigated : ABA receptors include the PYR1 proteins which when bound by ABA

inhibit the protein phosphatases PP2C that dephosphorylate SnRK2 kinase OST1; therefore when ABA accumulates phosphorylated OST1 kinase is active and phosphorylates the potassium channel KTA1 thus permitting K^+ influx, and the anion channel SLAC1 thus permitting Cl^- efflux and consequent stomata closure (Qin, et al., 2011b).

In contrast to many upstream sensing and signaling pathways, there are commonalities in downstream stress signaling pathways involved in the signal transduction and differential integration of a variety of stresses using second messengers such H_2O_2 and Ca^{2+} (Rentel and Knight, 2004) and Mitogen Activated Kinases or Snf Related Kinase 1 (SnRK1) (Baena-Gonzalez, et al., 2007; Baena-Gonzalez and Sheen, 2008). These downstream pathways are generally conserved between plants, animals and fungi and are therefore derived from evolutionary old pathways. The downstream pathway relating stress and energy signaling involves the AMP-dependent Protein Kinase, also Snf1 in yeast and SnRK1 in plants (Baena-Gonzalez and Sheen, 2008). Yet in plants, SnRK1 is also regulated by a trehalose metabolite that signals carbon utilization (Zhang, et al., 2009; Schluepmann, et al., 2003). Trehalose metabolism is further known as an ancient metabolic pathway associated with abiotic stress tolerance and energy storage (Gancedo and Flores, 2004; Chandra, et al., 2011; Paul, 2008). More knowledge of the molecular processes relating carbon utilization and energy stress is critically important as a basis to breeding sustained yield under stress. The role of trehalose metabolism in plant stress responses was thus chosen as the subject of study of this thesis.

2 Stress-induced changes and damage in plants resolved at the molecular level

Membranes are particularly sensitive to stress

The cell membrane maintains cell integrity and thus cellular function and homeostasis. Vital cellular processes depend upon membrane integrity: chemiosmosis, selective transport of molecules, membrane polarization, and cell compartmentalization. Despite it's crucial roles, the membrane is very susceptible to stress. Drought stress, for instance, affects primarily membranes (Crowe and Crowe, 1982; Crowe, et al., 1996). The membrane bilayer contains different lipids and proteins; these are not homogenously mixed but form rafts with in addition the inner face of the membrane bilayer often having a differing composition from the outer face. Each compound of the membrane has its own transition phase at a given temperature and a given water status. When dehydration or rehydration occurs, each part of the membrane behaves differently depending on its composition. Consequently, lateral separation, membrane fusion, removal of integral membrane proteins occur and in

more extreme cases the membrane undergoes phase transitions from fluid to gel or to non-bilayer (Lenne, et al., 2007). Water removal reduces the membrane surface causing transition to the gel phase with increased melting temperature (Bryant et al., 2001). Protecting membranes from drought is, therefore, likely an important mechanism to achieve desiccation tolerance (Crowe and Crowe, 1982). The exquisite sensitivity of membrane systems render them also a candidate sensor for changes in water availability, salt content and temperature. Evidence for this comes from the involvement of phosphatidic acid derived from membrane lipids in the responses to drought, salt and freezing stresses (Testerink and Munnik, 2011).

Impacts of different stresses and molecular responses in plants, a short description

A summary of the direct effects of different stresses on plants is presented in **Fig.1**. Drought, frost and salt stresses alter the availability of water, yet ninety percent of the cell cocktail is water. Water keeps macro molecules such as proteins, carbohydrates and nucleic acids in their hydrated conformation which is important for function in biochemical reactions. Water is further required in transport systems and

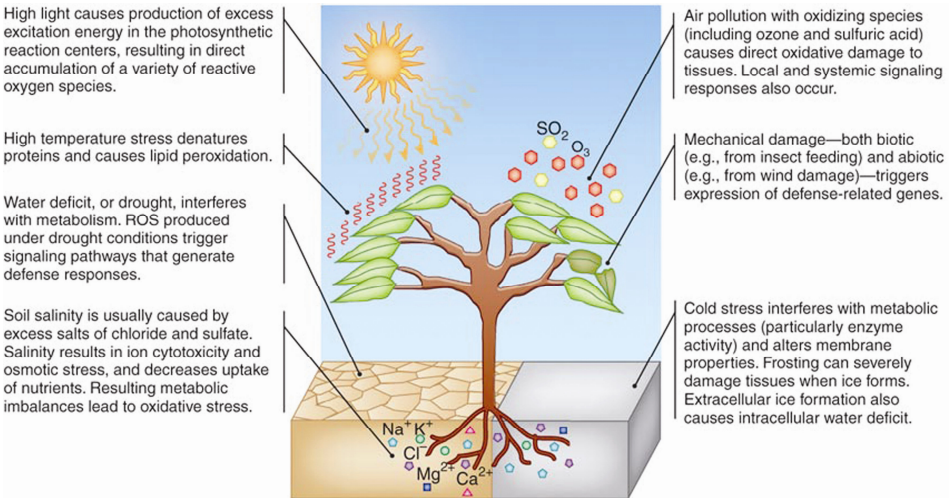

Figure 1. Effects and damages caused by a variety of stresses to plants, from Vickers et al. (2009). Excess light and heat, as well as exposure to oxidizing air pollutants, cause direct accumulation of ROS. High temperatures are often coincident with high light stress. Drought results in osmotic stress and intracellular water deficit; soil salinity and cold stress (particularly frosting) also result in water deficit, and the molecular responses to these three stresses are similar (though not identical). When stresses are combined, responses are often amplified; for example, high light/low temperature stress and high light/low water stress can result in very high production of ROS. ROS are particularly important for initiating signal cascades that trigger defense gene transcription and adaptive responses. Phytohormones are also important in these responses and are involved in signaling pathways. ABA is particularly important in water deficit responses, and ethylene, salicylic acid and jasmonic acid are often involved in wound responses.

as electron donor in photosystem II (PSII). Therefore stresses that affect availability of water may have a direct effect on the photosynthetic capacity of plants.

Reactive Oxygen Species (ROS) include free radical molecules such as $O_2^{\bullet -}$, superoxide radicals, $OH^{\bullet}$, hydroxyl radical, $HO_2^{\bullet}$, perhydroxy radical and $RO^{\bullet}$, alkoxy radicals. ROS further include non-radical molecules such as H_2O_2, hydrogen peroxide, and $1O_2$, singlet oxygen (Gill and Tuteja, 2010). In addition to the typically dehydration related stresses mentioned above, heat, photorespiration, mitochondrial respiration, pathogen attack, wounding and environmental stresses have been shown to disturb the balance of ROS production versus ROS scavenging and lead to elevated ROS levels (Gill and Tuteja, 2010; Hammond-Kosack and Jones, 1996). High ROS concentrations imply that ROS react uncontrolled and thence cause damage to proteins and lipid and carbohydrates. Yet ROS are not only a result of plant stress, they are known to mediate short distance (stress) signaling as well (Lee, et al., 2011; Osakabe, et al., 2010).

In addition to the general disturbances related to altered water availability, the abiotic stresses drought, salt, heat or cold stress also cause stress-specific disturbances and signaling as revealed by gene-expression profiling for example. Stress-inducible genes function not only in protecting cells by altering metabolism but also by regulating signal transduction in stress responses (Zhu, 2002; Bartels and Sunkar, 2005; Matsukura, et al., 2010; Tanaka, et al., 2012; Qin, et al., 2011a). Drought imposes osmotic stress that activates the transcription factor DREB2A (dehydration responsive element binding protein 2A) a central protein regulating transcription of a large number of stress related genes. However, over-expression of a constitutively active DREB2A protein improved significantly the plant's tolerance to dehydration but only slightly to freezing temperatures (Sakuma, et al., 2006a; Sakuma, et al., 2006b). Consistently, the metabolite profile of plants over-expressing constitutively active DREB2A was more similar to that of dehydrated than that of cold-treated plants (Munn, 2002; Maruyama, et al., 2009).

Salinity imposes both ionic toxicity and osmotic stress, leading to secondary stresses such as nutritional disorders and oxidative stress. The ionic toxicity is exemplified by high Na^+ concentrations in the cytosol directly inhibiting enzymes (Munn, 2002; Hasegawa, et al., 2000). Salt further disturbs nutrient uptake by roots, especially K^+ and Na^+ as they compete each other (Rodriguez-Navarro and Rubio, 2006). Osmotic stress caused by Na^+ accumulation in guard cells causes stomata opening and thence water loss, at very high Na^+ osmotic stress causes irreversible damage to stomata; not surprisingly, the effect of Na^+ accumulation on stomata differs in halophyte (plants growing in high salinity) compared to non-halophyte plants (Tester and Davenport, 2003). High salt uptake in plants not adapted to salt causes premature

leaf senescence and reduces photosynthesis and hence impairs growth (Perez-Alfocea, et al., 2011).

Heat stress typically activates the Heat shock factors (Hsf) in all eukaryotes studied, yet the family has radiated extensively in plants with the Arabidopsis genome containing some 21 members (von Koskull-Doring, et al., 2007). Group A Hsf function as transcription factors changing expression of target genes with heat stress elements (HSE: 5'-AGAAnnTTCT-3') in the promoters of heat shock-inducible genes of all eukaryotes. Hsf activate small heat shock proteins some of which were shown to act as molecular chaperones preventing denaturation and helping the folding of proteins synthesized *de novo*. Recent research, however, shows that Hsf activation may not only involve heat responses but also pathogen responses as in the case of the Arabidsopis TBF1 (Pajerowska-Mukhtar, et al., 2012). Moderate heat stress inactivates photosynthesis without affecting PSII integrity, possibly because of cyclic electron flow or membrane leakiness; with moderate heat rubisco deactivates in parallel with photosynthesis (Sharkey, 2005). Root thermotolerance in a perennial grass was associated with alternative respiration pathways and decreased turnover of proteins in energy metabolism (Huang, et al., 2012).

Damage due to ice crystal formation causes irreversible damage of cellular micro architecture (Wolfe and Bryant, 2001). So called "antifreeze proteins'' expressed by plants tolerant to freezing do not prevent freezing; instead, they control the size, shape, and aggregation of ice crystals and in the case of the plant proteins are very effective inhibitors of ice re-crystallization (Venketesh and Dayananda, 2008). Freezing tolerance of Arabidopsis expressing DREB1A was shown to be associated with altered expression of genes encoding starch-degrading enzymes, sucrose metabolism enzymes, and sugar alcohol synthases (Maruyama, et al., 2009). DREB1A over-expression further results in accumulation of monosaccharides, disaccharides and trisaccharides, and sugar alcohols. In contrast, DREB2A over-expression did not induce accumulation of sugars and sugar alcohols and resulted in significant tolerance to dehydration but not cold.

3 The functions of compatible solutes to prevent damage upon stress

Osmolytes are compounds that affect osmosis maintaining fluid balance and cell volume; natural osmolytes function as osmoprotectants. At times they may also be called compatible solutes which refers to the fact that they may also stabilize protein structures or play a role as ROS scavenger. An example of this is the amino acid proline (Krasensky and Jonak, 2012). Ion channel and membrane proteins are involved in sensing osmotic changes and signal for osmotic adjustments. Osmolytes can then be synthesized or be taken from the medium. They interact with molecules by hydrogen

bonding or electrostatic interaction (Wolfe and Bryant, 2001). Osmolytes can be organic or inorganic compounds; they can be ionic, non-ionic, nitrogenous solutes and small sugars. Organic osmolytes include amino acids and derivatives, amines and carbohydrates (Krasensky and Jonak, 2012). Non charged solutes include carbohydrates such as glycerol, myo-inositol, trehalose or sucrose and uncharged amino acids and peptides such as carboxamine and acetylated glutamine dipeptides. Anionic organic solutes include β-glutamate, β-hydroxybutyrate and derivatives and anionic polyols. Neutral osmolytes usually accumulate in bacteria, while negatively charged osmolytes are found in archea. The main effect of small solutes within cell compartments is on osmotic pressure. In contrast, when sugars are added to a solution, they may distort water structure, either enhance or reduce the tetrahedral coordinated hydrogen bond structure (THB) (Branca, et al., 2005). A solute enhancing THB is defined as water structure maker and a solute reducing THB as water structure breaker. Cryopreservation of biological membranes is achieved with a solute acting as water structure breaker which reduces the freezable water (Branca, et al., 2005). Solutes with water structure breaker properties are ClO_4^-, MnO_4^-, Br^-, Cl^-, K^+, Cs^+, sugars and I^-. Solutes with water structure maker increase nucleation of crystals; they are Li^+, Cu^+, Al^+, Mg^+, and OH^-. While Na^+, Ag^+ and Ba^+ are in borderline (Wiggins, 2001). Using both acoustic and viscosity measurements, the number of water molecules in the hydration shell, the hydration number, was determined to be higher for trehalose than for maltose or sucrose (Branca, et al., 2001). The high solvation energy of trehalose renders it more effective in reducing freezable water than maltose and sucrose.

A large body of work with over and stopped expression of genes from pathways of organic osmolyte metabolism supports the protective role of osmolytes/compatible solutes to a variety of stresses, a role predicted from physical chemistry and biochemical studies (Krasensky and Jonak, 2012). Metabolic profiling of Arabidopsis accessions with different freezing tolerances point to a crucial role of compatible solutes, including proline and raffinose, in freezing tolerance whilst trehalose levels remain often undetected in this plant species (Hannah, et al., 2006; Korn, et al., 2010). Comparison of the metabolite profiles from different crop cultivars or species also associates compatible solute accumulation with adaptation to salinity (Gong, et al., 2005; Janz, et al., 2010; Lugan, et al., 2010). Often combinations of compatible solutes were found to accumulate; these combinations differ from species to species and the combination was more effective than the simple additive effect of each.

4 Properties and protective effects of the disaccharide trehalose

Trehalose (α-D-glucopyranosyl [1-1]-α-D glucopyranose) is a disaccharide of glucose where the aldehydes at C_1 are linked by way of the alpha glycosidic bond (**Fig.2**

A); consequently, trehalose has no free aldehyde groups, is thus non-reducing and does not react with amino groups in a non-enzymic glycation reaction (Elbein, 1974). Trehalose is thence also very stable at a wide range of pH or temperature.

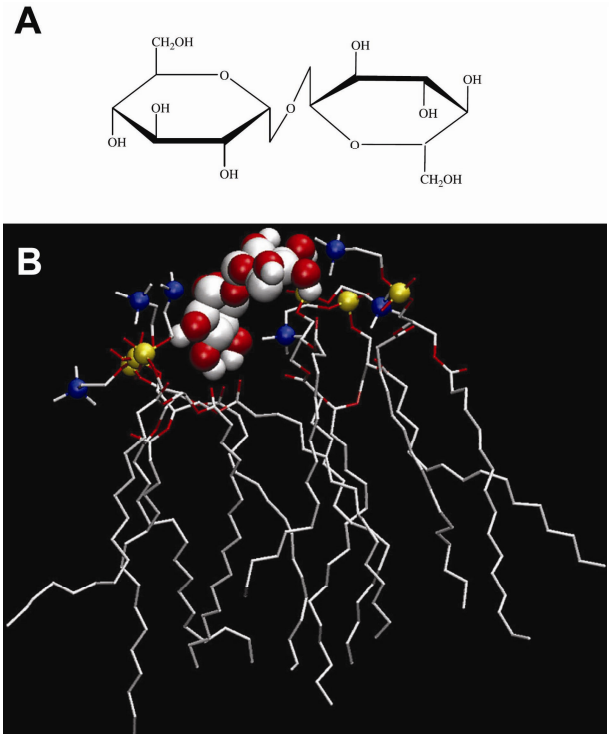

Figure 2. A Structure of trehalose from Pereira and Hunenberger (2008).

B Trehalose molecule simultaneously H-bonded to the head groups of six distinct lipid molecules, from Pereira and Hunenberger (2008). Image obtained from simulation of a dipalmitoylphosphatidylcholine bilayer in the presence of trehalose at 325º K. Trehalose is represented using a space fill model and the lipid molecules using a stick model. Nitrogen and phosphorous atoms are represented using a space fill model to better distinguish the lipid head groups.

Solutions of trehalose remain amorphous in complete dehydration. Its hydroxyl groups are thought to interact with biomolecules substituting water when water is scarce yet maintaining flex of proteins and maintaining fluid lipid bilayers (Crowe and Crowe, 1982; Crowe, et al., 1996; Lefort, et al., 2007). Additionaly, more water can be bound by trehalose than other sugars (Branca, et al., 2001; Lerbret, et al., 2005). Three hypotheses were proposed to explain the protection of biological structure and function by trehalose when water is scarce or frozen. Firstly, trehalose may function as water replacement when water is very limited upon dehydration and freezing. Trehalose undergoes hydrogen bonding around the polar and charged groups of lipid head groups (**Fig.2 B)** and proteins; hence their native structures are stabilized (Crowe, et al., 1996; Hackel, et al., 2012). Secondly, trehalose may entrap water, suggesting that trehalose concentrates water surrounding the biological molecules, thereby maintaining their native structures. Thirdly, trehalose as vitrifying agent may stabilize native structures by forming an amorphous matrix (Sun, et al., 1996; Sundaramurthi, et al., 2010). As protective agent, trehalose appears to be the most effective

disaccharide. Applications of the protective properties of trehalose are found in cryopreservation, for example inclusion of trehalose during the freezing and recovery improved semen motility, acrosomal and plasma membrane integrity, and improved maintenance of key enzyme activities (Hu, et al., 2010) or in the formulation of robust pharmacological preparations such as vaccines (Jin, et al., 2011).

To achieve protective functions, trehalose concentrations required in the suspension or in the cell are typically high, above 100 mM; consequently trehalose biosynthesis is generally induced in stressed microbes and eukaryotes alike, such as for example upon dehydration in the bacterium *E.coli*, the bread yeast *S.cerevisiae*, the desert plant *M. flabellifolia* or the Antarctic midge *B. antarctica* (Purvis, et al., 2005; Calahan, et al., 2011; Moore, et al., 2007; Benoit, et al., 2009). Trehalose accumulation in a variety of organisms may underlie tolerance to multiple stresses such as water stress (Kandror, et al., 2002), heat (Singer and Lindquist, 1998; Reinders, et al., 1999), salinity (Alarico, et al., 2005), oxidative stress (Alvarez-Peral, et al., 2002; Cao, et al., 2008), the combination of osmotic and heat stress (Gunasekera, et al., 2008), osmotic and oxidative stress (Giaever, et al., 1988; Fillinger, et al., 2001).

Trehalose however does not only function as a compatible solute or osmolyte, it also serves as a readily available energy storage molecule which when cleaved feeds directly in all pathways utilizing glucose such as glycolysis, the pentose phosphate pathway and glycogen metabolism. Trehalose is hence also a major metabolite of primary energy metabolism. It further serves as sugar transport form in the blood stream of most invertebrates where its metabolism is further associated with anaerobiosis (Elbein, et al., 2003). As a result, trehalose metabolism is found ubiquitously and thought to be evolutionary ancient (Avonce, et al., 2006). Only vertebrates that maintain physiological and biochemical homeostasis have seemingly lost the capability to synthesize trehalose yet they have retained the degradation pathway.

5 Trehalose biosynthesis pathways

There are at least 5 known pathways of trehalose synthesis (Avonce, et al., 2006) (**Fig. 3**). The first OtsA-OtsB pathway involves two steps of enzymatic reactions, converting UDP- or ADP-glucose and glucose-6-phosphate into the intermediate trehalose-6-phosphate (T6P) by Trehalose Phosphate Synthase (TPS), then in a second step, cleaving phosphate from T6P with T6P Phosphatase (TPP). This pathway is found in bacteria, archae, plant, metazoan and fungal kingdoms (Avonce, et al., 2006); its wide distribution is further confirmed by the numerous gene sequences in the phylogenetic tree obtained when TPS sequences are searched in the NCBI protein data base.

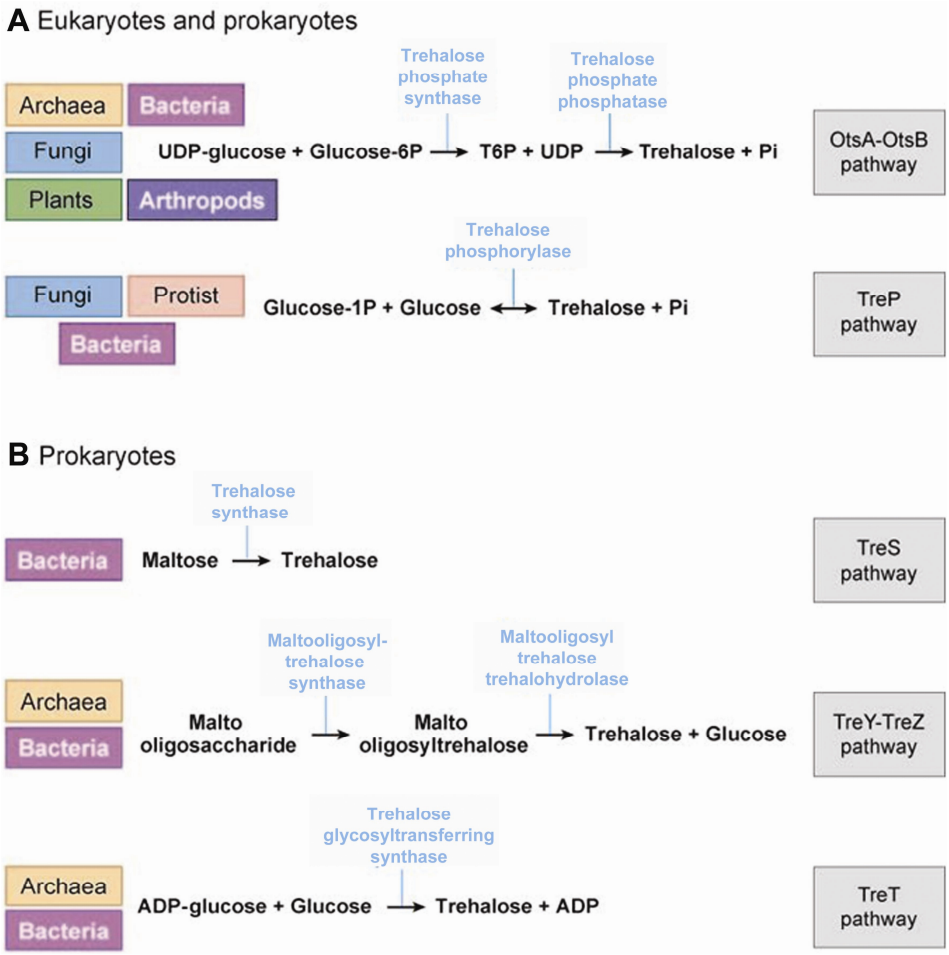

Figure 3. Known pathways of trehalose synthesis in eukaryotes and prokaryotes (A) and prokaryotes (B), figure from Paul et al. (2008). UDP, uridine diphosphate; Glucose-6P, glucose-6-phosphate; T6P, trehalose-6-phosphate; Glucose-1P, glucose-1-phosphate; ADP, adenosine diphosphate; Pi, inorganic phosphate.

The trehalose phosphorylase pathway, the TreP pathway is found in eukaryotes like the TPS/TPP pathway, but in eukaryotes it is restricted to protists and fungi. The TreP pathway is further present in bacteria and archea.

The remainder three trehalose biosynthesis pathways, the TreY-TreZ, TreS and TreT pathways are exclusively found in bacteria and archae (Paul, 2008). The TreY-TreZ pathway involves two steps: linkage of the terminal glucose of maltose oligomers or glycogen is converted from α-1-4 into α-1-1 linkage by TreY enzyme, then in a second reaction the terminal disaccharide trehalose is released by TreZ enzyme. This pathway was reported in *Brevibacterium helvolvum* (Kim, et al., 2000), *Arthrobacter* (Nakada, et

al., 1995), *Rhizobium* (McIntyre, et al., 2007) and *Archeon Sulfolobus acidocaldarius* (Maruta, et al., 1996).The TreS pathway uses maltose as substrate in a single step and reversible conversion to trehalose by TreS enzyme (Zhang, et al., 2011). At equilibrium 60% trehalose was found over 40% maltose (Wei, et al., 2004; Pan, et al., 2004). This pathway was reported in *Thermus aquaticus* (Tsusaki, et al., 1997), *Agaricus bisphorus* (Wannet, et al., 1998), *Pimelobacter* (Nishimoto, et al., 1996), *Thermobifida fusca* (Wei, et al., 2004). In the trehalose phosphorylase pathway, glucose-1-phosphate and glucose are converted to trehalose by TreP enzyme; example are found in *Thermus acidophyllus* and *Grifola frondosa* (Saito, et al., 1998). In contrast, in the pathway employing trehalose glycosyl transferring enzyme (TreT), ADP-glucose and glucose are converted to trehalose. The TreP reaction was shown to be reversible whilst the TreT reaction appears mostly irreversible unless the product is in 100 fold excess over the substrate (Ren, et al., 2005; Kouril, et al., 2008; Nobre, et al., 2008). Eubacteria generally use TreP whilst in archea TreT predominates (Avonce, et al., 2006).

Several prokaryotes utilize more than one pathway to synthesize trehalose. For example, *Mycobacterium tuberculosis*, *M. smegmatis*, *M. bovis* and *M. leprae* have the OtsA/OtsB, the TreY/TreZ and the TreS pathways for trehalose biosynthesis (De Smet, et al., 2000); trehalose in these organisms is a precursor for outer membrane glycolipids important for survival inside hosts. Another example is found with *Rhizobium* species. Combined induction of the TreY/TreZ and OtsA/OtsB pathways during drought stress causes accumulation of trehalose in the *Rhizobium leguminosarum* bv. Trifolii (McIntyre, et al., 2007).

6 The role of trehalose metabolism in plants

Unlike microbes when exposed to stress, plants generally do not accumulate trehalose in sufficient amounts to function as osmolyte. Moreover trehalose is only found at barely detectable levels in most plants. A few exceptions are found in desert plants such as *Selaginella lepidophylla* and *Myrothamnus flabellifolia* (Zentella, et al., 1999; Moore, et al., 2007).

Genes encoding enzymes from the OtsA/OtsB trehalose biosynthesis pathway, however, are found in all plants analyzed thus far (Fig.3). Surprisingly, a large number of genes containing the TPS and TPP domains are found in everyone plant genome. For example in the genome of the first plant ever sequenced *Arabidopsis thaliana*, 21 genes were identified that contained either the TPP domain only (TPPA-J) or both the TPS and TPP domains fused one after the other (TPS1-11) (Leyman, et al., 2001). This contrasts with the seemingly single gene encoding a trehalase enzyme TRE1 that

cleaves trehalose into glucose (Muller, et al., 2001). The situation is much the same in the monocotyledonous crop plant rice (Pramanik and Imai, 2005; Shima et al., 2007). Arabidopsis TPS1 enzyme is a functional TPS, but seemingly has no function as TPP (Blázquez et al., 1998). For Arabidopsis TPS11, both the TPS and TPP activities were demonstrated (Singh, et al., 2011). The TPS double domain structure found in plants is similar to the TPS double domain structure from the TPS in *S. cerevisiae* as well as other fungi (Avonce, et al., 2006; Wilson, et al., 2010; Wilson, et al., 2007). In contrast, the small single domain plant TPPs are active enzymes and resemble prokaryotic TPP (Vandesteene PhD thesis 2009). Together the large radiation of TPS/TPP protein families and the embryo lethality of Arabidopsis lacking TPS1 establish that trehalose metabolism is essential for plant development (Eastmond, et al., 2002; van Dijken, et al., 2004). The much altered branching pattern in maize plants mutated in the TPP RA3 is further consistent with a role in development (Satoh-Nagasawa, et al., 2006).

In contrast, Arabidopsis lacking TPS5 are sensitive to heat, suggesting a role in heat stress protection (Suzuki, et al., 2008). Arabidopsis TPS11 is further shown to mediate resistance to the green peach aphid *Myzus persicae* (Singh, et al., 2011). Rice over-expressing a fusion of *E.coli* TPS and TPP protein, OsTPS1 or OsTPP1 exhibit stress tolerance (Garg, et al., 2002; Ge, et al., 2008; Li, et al., 2011). Together, these results point to a role of trehalose metabolism in plant stress resistance. Analysis of the natural variation by scoring single nucleotide polymorphisms in 80 sequenced accessions of Arabidopsis reveals an astonishing conservation in the amino acid sequences of TPS5-7 and TPPJ and G compared to the somewhat less conserved yet essential TPS1 suggesting selective environmental pressure on these proteins of the trehalose pathway (Schluepmann, et al., 2011).

The demonstrated importance of trehalose metabolism in plants is in stark contrast with the low levels of trehalose metabolites, whether this be for the phosphorylated precursor T6P at less than 0.5 nmol.g^{-1} FW or for trehalose at less than 5 nmol.g^{-1} FW of plant tissue (Garg, et al., 2002; Lunn, et al., 2006; Delatte, et al., 2009). How then, does trehalose metabolism mediate its roles in either developmental or stress processes?

A possibility would be that trehalose metabolites together with proteins involved in trehalose metabolism affect key pathways in developmental or stress processes through allosteric or protein-protein interaction in conversion or signaling pathways. The pathways leading to activation of trehalose metabolism upon stress may have been retained in evolution, only the interactions between trehalose metabolites and downstream signals may have evolved in a plant specific manner (Hey, et al., 2010). To

regulate these interactions, levels of either metabolites may be tightly controlled inside cells and thus kept low. There is strong evidence in yeast and in rice that TPS proteins interact with each other in a specific manner in a complex (Bell, et al., 1998; Zang, et al., 2011). In the bread yeast, T6P was shown to inhibit Hexokinase 2 and affect downstream cAMP signaling and finally the respiratory chain (Blazquez, et al., 1993; Noubhani, et al., 2009). In the plant Arabidopsis, a role for T6P in the SnRK1 signaling pathway has been established: T6P inhibits SnRK1 activity *in vitro* and its accumulation *in vivo* contrasts the abundance of SnRK1 marker gene expression (Zhang, et al., 2009; Martinez-Barajas, et al., 2011). T6P inhibition of SnRK1 was further shown to inhibit seedling growth (in work presented in Chapter 2 of this thesis and in Delatte, et al., 2011 and likely underlies embryo development arrest of Arabidopsis lacking TPS1. T6P steady state further affects potato growth but the mechanism involved here is not clear (Debast, et al., 2011).

T6P by an unknown mechanism mediates sucrose induced AGPase redox activation and thence starch accumulation; interestingly, AGPase redox activation does not occur when SnRK1 is reduced in SnRK1 antisense potato (Geigenberger, 2011; Kolbe, et al., 2005; Tiessen, et al., 2003). Geigenberger (2011) therefore proposed that SnRK1 may in turn modulate T6P accumulation after sucrose feeding (Geigenberger, 2011). T6P was shown to be required for carbon utilization (Schluepmann, et al., 2003) and inhibition of SnRK1 is similarly known to mediate anabolic responses to nutrient supply (Baena-Gonzalez, et al., 2007; Baena-Gonzalez and Sheen, 2008; Paul, et al., 2010). It is possible therefore that the effect of trehalose metabolism on development and growth be mediated by T6P signaling and the interaction with SnRK1.

Recent results further demonstrate links between SnRK1 and ABA / GA hormone accumulation that is mediated by FUS3 (Tsai and Gazzarrini, 2012): SnRK1 phosphorylates FUS3, a transcription factor known to be critical during seed dormancy as it controls expression of the ABA biosynthesis gene ABI2. Extreme dormancy as well as late flowering and late senescence were caused by increased expression of the catalytic subunit of SnRK1 (KIN10) and FUS3 (Tsai and Gazzarrini, 2012). The developmental effects above were suppressed in the *fus3-3* mutant suggesting that the SnRK1 effect upon development be caused by FUS3. Over-expression of AKIN10 or FUS3 yields ABA-hypersensitive phenotypes that are dependent on the ABA biosynthetic gene ABI2 (Jossier, et al., 2009; Gazzarrini, et al., 2004). FUS3 is known to decrease GA and increase ABA; it could well be that the forms of stress resistance observed in AKIN10 or FUS3 over-expressors are due to these hormonal changes (Baena-Gonzalez, et al., 2007; Radchuk, et al., 2010; Radchuk, et al., 2006).

There is also a documented link between weak *tps1* mutations and ABA: germination of the weak *tps1-11, 12* and *13* mutants was hypersensitive to ABA (Gomez, et al., 2010; Gomez, et al., 2006). Taking into account the above results, putatively low T6P in the weak *tps1* mutants permits high SnRK1 activity and thus more stable and active FUS3 resulting in increased ABA and thus much delayed germination. In contrast, the potato tuber sprouting mechanism differs from seed maturation and dormancy: low T6P in potato was associated with low ABA accumulation and early sprouting (Debast, et al., 2011); possibly FUS3 is not expressed or stable in sprouting potato tubers.

Over-expression of TPS1 in Arabidopsis increased T6P steady state and conveyed sugar as well as ABA insensitive germination (Avonce, et al., 2004). Glucose-induction of HXK1 and ABI4 gene-expression and ABA accumulation no longer occur in the TPS1 over-expressors suggesting that low ABI4 in these plants may underlie sugar and ABA insensitivity; this may be explained by T6P inhibition of SnRK1 in these seedlings because SnRK1 was shown previously to be required to mediate responses to high sugar status (Tiessen, et al., 2003; Halford and Hey, 2009). Surprisingly however, TPS1 over-expressors exhibit drought tolerance. Further studies are required comparing the drought response mounted in TPS1 expressors and WT; particularly interesting will be the measurements of ABA, the behavior of ABI4, FUS3 and SnRK1 as well as the phosphorylation of ABA response element binding proteins of the bZIP transcription factor class. A possible link between SnRK1 activity and stress responses by way of phosphorylation of bZIP transcription factors is discussed in Hey et al. (2010). Whilst the T6P regulation in stress response processes is a possibility, the participation of trehalose in these processes cannot be excluded.

What then is the role and mode of action of trehalose? Trehalose feeding at levels above 25 mM were shown to lead to T6P accumulation in Arabidospis seedlings which then causes SnRK1 inactivation and growth arrest (Schluepmann, et al., 2004; second chapter in this thesis and Delatte, et al., 2011). T6P accumulation when feeding trehalose could be due to inhibition of the rate of the TPP catalysis by the high trehalose levels compared to the minute amounts of T6P in tissues that synthesize T6P actively. It is therefore difficult to distinguish the effect of trehalose from that of T6P.

None the less trehalose accumulation appears important to mount abiotic stress responses with increased TPP activity (Shima, et al., 2007; Ge, et al., 2008). In addition, trehalose fed at low concentrations (5 $g.l^{-1}$) stimulates specific biotic stress responses that effectively protect wheat against powdery mildew, *Blumeria graminis*; papillae deposition in epidermis cells, phenylalanine ammonia-lyase and peroxidase activity were induced (Reignault, et al., 2001; Renard-Merlier, et al., 2007). Because trehalose

is present at high concentrations in phytopathogenic bacteria, fungi and in the larvae and adult herbivorous insects, trehalose may be perceived as an elicitor. The bi-functional TPS/TPP enzyme TPS11 from Arabidopsis was required for resistance against the green peach aphid (Singh, et al., 2011). Aphid induced trehalose synthesis mediated by AtTPS11 increased PAD4 expression which was necessary for resistance. Unlike the TPP domain in Arabidopsis TPS1-3 , the TPP domains of TPS4-11 contain all the elements required for function as phosphatase (Leyman, et al., 2001; Lunn, 2007): it is possible therefore that the TPS4-11 function in the synthesis of trehalose whilst TPS1-2 in the synthesis of T6P with TPS3 likely a pseudo gene. Given the cell-specific expression patterns of some of these genes, localized accumulation of trehalose may be of importance in mediating the responses. Feeding trehalose to Arabidopsis promotes expression of genes involved in detoxification and stress responses (Bae, et al., 2005a; Bae, et al., 2005b). Several genes of the trehalose metabolism are exquisitely sensitive to abiotic stresses; the TPP AtTPPD is induced by osmotic, salt, and cold stresses and in roots (Lordachescu and Imai, 2008). In roots as well, AtTPS4 is induced by osmotic, salt, drought, UV-B, wounding and heat stresses. In shoots, TPPD and TPS8 seem the most responsive to a variety of stresses.

7 Stress resistance of transgenic plants expressing genes of the trehalose metabolism

An increasingly large body of literature describes the stress resistance of transgenic plants expressing genes of the trehalose metabolism; this literature is summarized in Table 1. The first reports were from tobacco expressing yeast trehalose 6-phosphate synthase 1 (ScTPS1) with improved drought tolerance; yet the plants generated were small and had dark and lanceolate leaves compared to wild types; water retention by the leaves correlated with drought tolerance (Holmström, et al., 1990; Romero, et al., 1997; Karim, et al., 2007). A similar phenotype was observed in tobacco expressing *E.coli* TPS*, OtsA* (Goddijn, et al., 1997). Combined expression of TPS and TPP from either yeast or *E.coli* rectified most of the phenotypic aberrations and maintained a good degree of stress protection. Expression of TPS/TPP protein fusions of the *E.coli* OtsA and OtsB proteins allowed engineering of rice resistant to drought, cold and salt (Garg, et al., 2002; Jang, et al., 2003); the transgenic plants did not exhibit the phenotypic changes observed before and retained higher photosynthetic capacity under stress. T6P accumulation has previously been shown to mediate increased photosynthetic capacity (Pellny, et al., 2004). Expression of yeast TPS1 by transgenic chloroplasts allowed substantial trehalose accumulation and circumvented the effects seen when the TPS1 was expressed from a nuclear locus, the plants also displayed significant drought resistance (Lee, et al., 2003).

Table 1. Genes from the trehalose metabolism used to engineer stress tolerant plants in differing species, updated from Lordachescu and Imai, 2008.

Species	Promoter	*Genes*	Tolerance	Changes in phenotype	Trehalose	Reference
Tobacco	Rubisco	*ScTPS1*	Drought	No changes	800–3 200 $\mu g.g^{-1}$ DW (NS)	Holmström et al. 1996
Tobacco	CaMV35S	*otsA*	Drought	Aberrant root growth, lancet shaped leaves, stunted plants	20–110 $\mu g.g^{-1}$ FW (NS)	Goddijn et al. 1997
		otsA–otsB		Same as above, but not as pronounced, bleached interveinal tissue	30–60 $\mu g.g^{-1}$ FW (NS)	Pilon-Smits et al. 1998
Potato	Patatin	*otsA*	NA	No changes	3–20 $\mu g.g^{-1}$ FW (NS)	Goddijn et al. 1997
		otsA–otsB		NA	NA	
Tobacco	CaMV35S	*ScTPS1*	Drought	Loss of apical dominance, stunted growth, lancet shaped leaves, partial sterility	<170 $\mu g.g^{-1}$ FW (NS)	Romero et al. 1997
Tobacco	16SrRNA	*ScTPS1*	Drought	No changes	360–440 $\mu g.g^{-1}$ FW (NS)	Lee et al. 2003
Tobacco	Rubisco	*ScTPS1*	Drought	No changes	~8 $\mu g.g^{-1}$ FW (NS, DS)	Karim et al. 2007
		ScTPS1–TPS2	Drought	No changes	~16 $\mu g.g^{-1}$ FW (NS, DS)	
	AtRAB18 (drought inducible)	*ScTPS1*		No changes	1–2 $\mu g.g^{-1}$ FW (NS, DS)	
		ScTPS1–TPS2		No changes	~4 $\mu g.g^{-1}$ FW (NS, DS)	
Arabidopsis	Rubisco[a]	*ScTPS1*	Drought	No changes	NA	
Rice	ABRC1-rice actin1 (ABA-inducible)	*otsA::otsB*	Drought, salt, cold	No changes	~48 $\mu g.g^{-1}$ FW (NS)	Garg et al. 2002
					~508 $\mu g.g^{-1}$ FW (DS)	
	Rice rbcS[a]	*otsA::otsB*	Drought, salt, cold	No changes	~55 $\mu g.g^{-1}$ FW (DS)	
					~163 $\mu g.g^{-1}$ FW (NS)	
Rice	Ubiquitin	*otsA::otsB*	Drought, salt, cold	No changes	310–1 036 $\mu g.g^{-1}$ FW (NS)	Jang et al. 2003
Tomato	CaMV35S	*ScTPS1*	Drought, salt	Thick shoots with short internodes, rigid dark green leaves, aberrant root development	150 $\mu g.g^{-1}$ FW (NS)	Cortina and Culiáñez-Macià 2005
Arabidopsis	rd29A (stress inducible)	*ScTPS1–ScTPS2*	Drought, cold, salt, heat	No changes	8.2–16.7 $\mu g.g^{-1}$ FW (NS)	Miranda et al. 2007
	CaMV35S			Smaller dark green leaves, partial sterility, glucose insensitive	8.5–38.4 $\mu g.g^{-1}$ FW (NS)	
Rice	OsActin	ΔOsTPS1	Salt, cold, drought/PEG	No changes	80 $\mu g.g^{-1}$ FW (cold stress)	Li et al. 2012

Arabidopsis	CaMV35S	AtTPS11	Aphid resistance	No changes	12 $\mu g.g^{-1}$ FW	Singh et al. 2011
		OtsB	Aphid resistance		12 $\mu g.g^{-1}$ FW	
Tobacco	CaMV35S	*PsTreP*	Drought	No changes	6.3 $\mu mol.g^{-1}$ FW (NS)	Han et al. 2005
Sugarcane	2xCaMV35S	*GfTreP*	Drought	No changes	8.8–12.9 $mg.g^{-1}$ FW (NS)	Zhang et al. 2006

TPS/TPP pathway; treP pathway ¤With added transit peptide for chloroplast targeting; ABA, abscisic acid; DS, drought stressed; NS, not stressed; FW, fresh weight; NA, not available; *OtsA*, *Escherichia coli* trehalose-6-phosphate synthase (*TPS*); *OtsB*, *Escherichia coli* trehalose-6-phosphate phosphatase (*TPP*); *PsTreP*, *Pleurotus sajor-caju* trehalose phosphorylase; Sc*TPS1*, *Saccharomyces cerevisiae TPS*; *ScTPS2*, *Saccharomyces cerevisiae TPP*.

Expressing a truncated form of rice TPS1 (OsTPS1) that presumably lacks the N-terminal 131 amino acids yielded rice plants with high trehalose and a higher resistance to drought, cold and salt than the WT without any morphological defect (Li, et al., 2011). The N-terminus of the OsTPS1 therefore not only permits higher trehalose accumulation but also may mediate the pleiotropic phenotype observed when TPS are expressed.

Since biosynthetic enzymes from very differing species mediate protection, it is unlikely that the proteins in themselves mediate protection. It rather is likely that either trehalose or T6P or metabolites derived from them underlie the resistance phenotype. In every case, however the increase in concentrations achieved could not justify a role of trehalose as osmolyte (Table 1).

In addition to genes from the TPS/TPP pathway, genes from other trehalose biosynthesis pathways are now being used to engineer plants with increased abiotic stress resistances. Tobacco and sugarcane plants expressing trehalose phosphorylase TreP from the fungi *Grifola frondosa* or *Pleurotus sojar-caju* were found resistant against drought and salt (Zhang, et al., 2006; Zhang, et al., 2005; Han, et al., 2005).

Genes from the trehalose metabolism, however, are not the only group of genes capable of mediating stress protection, numerous genes have been reported to successfully mediate stress protection. This is consistent with genetic studies in crops and wild relatives showing that stress tolerance traits are generally polygenic (Price, et al., 2002; Katori, et al., 2010; Ribaut and Ragot, 2007).

8 Characterization of Trehalose Synthases

When commencing the present study we realized that in order to differentiate the role of trehalose from that of T6P and to engineer plants with improved stress resistance, trehalose synthases from bacterial or archae could be used that catalyse synthesis of trehalose whithout using T6P as an intermediate.

Information was therefore gathered regarding enzyme activity, pH, temperature, and K_m of trehalose synthases from the few organisms where trehalose synthase activities had been characterized. Enzymes with requirements matching the conditions prevailing in plant cytosol or plastid matrix (pH 6.5, low Km for substrate) and active at ambient temperatures were of particular interest as candidates for expression in plants.

A good candidate seemed TreP from *Grifola frondosa* (120 kDa); this protein was found active as a di-mer of identical 60 kDa subunits, it uses glucose and glucose-1-phosphate as substrate and is therefore a trehalose phosphorylase (Saito, et al., 1998). Optimum temperature and pH of this enzyme were 32.5-35^{o}C and 6.5-6.8 respectively, the enzyme appeared stable over a broad range of pH (6-9). The enzyme was very specific for its substrates glucose-1-phosphate and D-glucose for synthesis of trehalose and did not accept L-glucose, D-galactose, D-mannose, D-xylose, D-fructose, D-sorbitol, D-mannitol, nor D-fucose and β-glucose-1-phosphate, α-D-galactose-1-phosphate, α-D-mannose-1-phosphate, or α-D-xylose-1-phosphate. Also when the enzyme was assayed in the opposite direction for the phosphorylation reaction, only trehalose served as substrate whilst neotrehalose, palatinose, cellobiose, lactose, sucrose, maltose, or isomaltose did not. Equilibrium of the reaction was 90% towards biosynthesis of trehalose.

Another candidate seemed the TreS enzyme from *Pimelobacter:* it catalyses the conversion of maltose into trehalose with at equilibrium roughly 80% trehalose (Nishimoto, et al., 1996). Its optimum temperature was 25^{o}C and pH was relatively broad ranging from 6 to 8. Conversion was specific for maltose and trehalose: neotrehalose, kojibiose, nigerose, isomaltose and maltotriose were not substrates. The *TreS* gene of *Pimelobacter* is 1719-bp long and encodes a 573 amino acid sequence that in its 220 N-terminal residues is homologous with maltases from *Saccharomyces carlsbergenesis* and *Aedes aegypt*. TreS from *Thermobifida fusca* has a molecular weight of 66 kDa with optimum activity at pH 6.5, 25^{o}C and could therefore also be a candidate for expression in plants (Wei, et al., 2004). This enzyme was reported to convert matose into trehalose reversibly with at equilibrium about 60% trehalose concentration.

In contrast, TreS from *Thermus acidophyllus* is optimally active at the high temperature of 40-45^{o}C with a relatively high K_m of up to 4.2 mM trehalose. TreS from *Mycobacterium smegmatis* has an even higher K_m at 90 mM trehalose (Pan, et al., 2004).

9 Conclusion and structure of this thesis

Water deficit is common to drought, freezing and salt stresses, yet each stress also specifically affects plant growth, development and ultimately crop yield. In lower organisms trehalose is synthesized in large amounts and acts as a compatible osmolyte stabilizing surrounding compounds and structures by substituting for water upon water deficit. In plants, trehalose levels are tightly regulated and trehalose accumulates only in trace amounts. Its biosynthetic precursor T6P is essential for growth during all stages of development, acting as signaling molecule, but little is known about the role of trehalose in plants. Researchers have successfully manipulated trehalose accumulation in plants for crop improvement purposes. However, along with tolerances to a diversity of stresses, researchers observed pleiotropic effects on plant architecture, growth and metabolism. Manipulation was done by way of the intermediate T6P and therefore it has been difficult to distinguish the relative contribution of trehalose or T6P to the effects observed.

This thesis studies the role of trehalose in plants using two approaches: firstly by studying the effect of trehalose feeding to plants and secondly by manipulating levels of trehalose using pathways that do not also synthesize T6P. The thesis is divided into five chapters. Chapter 1 presents a general introduction serving as background information on stress biology and trehalose metabolism. Chapter 2 focuses on the study of molecular mechanisms involved when exogenous trehalose inhibits growth in Arabidopsis seedlings. Chapter 3 describes the surprising effects of trehalose feeding on Arabidopsis development in the dark. In Chapter 4, a strategy is taken to enhance endogenous levels of trehalose without affecting levels of the biosynthetic precursor T6P: genes encoding the bacterial trehalose synthases TreS and TreT are cloned and then expressed in Arabidopsis. The response of plants expressing these genes to abiotic stresses is then tested. Chapter 5 constitutes a general discussion and summary of the study.

The thematic choice for this thesis on plant stress combined with stress tolerance by engineering of the trehalose pathway was taken given the particular importance of drought in the agriculture of the Nusa Tenggara region in Indonesia, including in the South and East parts of Lombok also called the *lahan kritis* where water shortage is chronic both due to lack of precipitation and over-population (Klock and Sjah, 2007).

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CHAPTER 2

TREHALOSE FEEDING ARRESTS GROWTH OF ARABIDOPSIS SEEDLINGS:

over-expression of KIN10 or bZIP11 overcomes the growth arrest.

Based on:

Growth arrest by trehalose-6-phosphate: an astonishing case of primary metabolite control over growth by way of the SnRK1 signaling pathway

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ABSTRACT

The strong regulation of plant carbon allocation and growth by the trehalose metabolism is important for our understanding of mechanisms that determine growth and yield with obvious applications in crop improvement. To gain further insight on the growth arrest by trehalose feeding, we first established that starch deficient seedlings of the *pgm1* mutant were similarly affected as WT on trehalose. Starch accumulation in the source cotyledons therefore did not cause starvation and consequent growth arrest in the growing zones. We then screened the FOX collection of Arabidopsis expressing full-length cDNAs for seedling resistance to 100 mM trehalose. Three independent transgenic lines were identified with dominant segregation of the trehalose resistance trait that over-express the bZIP11 transcription factor. The resistance of these lines to trehalose could not be explained simply through enhanced trehalase activity or through inhibition of bZIP11 translation. Instead, trehalose-6-phosphate (T6P) accumulation was much increased in bZIP11 over-expressing lines suggesting that these lines may be insensitive to the effects of T6P. T6P is known to inhibit the central stress-integrating kinase SnRK1 (KIN10) activity. We confirmed that this holds true in extracts from seedlings grown on trehalose, then showed that two independent transgenic lines over-expressing KIN10 were insensitive to trehalose. Moreover, expression of marker genes known to be jointly controlled by SnRK1 activity and bZIP11 was consistent with low SnRK1 or bZIP11 activity in seedlings on trehalose. Results reveal an astonishing case of primary metabolite control over growth by way of the SnRK1 signaling pathway involving T6P, SnRK1 and bZIP11.

INTRODUCTION

Trehalose (α, α-1, 1 linked D-glucopyranosyl D-glucopyranoside) inhibits growth and is toxic when fed to seedlings of the dodder vine *Cuscuta reflexa* (Veluthambi et al., 1982a; Veluthambi et al., 1982b) or *Arabidopsis thaliana* (Fritzius et al., 2001; Ramon et al., 2007; Schluepmann et al., 2004; Schluepmann and Paul, 2009; Wingler et al., 2000). This effect is somewhat surprising as trehalose is a common sugar and synthesized in many organisms at high concentrations functioning as a carbon source and stress protection compound. In plants the trehalose pathway is ubiquitous and indispensible, but typically trehalose does not accumulate to high levels. A major function of the pathway in sugar signaling and regulation of growth and development has been elucidated in plants in recent years (Eastmond et al., 2002; Paul et al., 2010; Satoh-Nagasawa et al., 2006; Schluepmann et al., 2003; Zhang et al., 2009). The impact of trehalose feeding on growth is most likely related to this signaling function (Schluepmann et al., 2004). However, the precise mode of action of growth inhibition by trehalose is not known.

A striking feature of Arabidopsis seedlings grown on trehalose is starch accumulation in the source tissues whilst no starch accumulates in the tip of the roots in the columella, representing a reversal of carbon allocation. Starch accumulation in the cotyledons can be explained through upregulation of ADPglucose pyrophosphorylase (AGPase) transcriptionally (Wingler et al., 2000) and through AGPase redox activation (Kolbe et al., 2005) and also by inhibition of starch degradation (Ramon et al., 2007). However, interestingly, this effect is found only in source tissues such as cotyledons and not in sink tissues such as the columella of root tips, which no longer accumulate starch and appear to be starving due to lack of sufficient carbohydrate (Wingler et al., 2000). Feeding metabolisable sugar in combination with trehalose rescues growth and it would appear that the primary effect of trehalose on growth is related to utilization of sugar. This is also a feature of trehalose toxicity in cut dodder shoots where trehalose and sucrose uptake and accumulation were studied (Veluthambi et al., 1982a; Veluthambi et al., 1982b). Radio-labeled trehalose accumulated evenly throughout the shoot. Trehalose accumulation is associated with a decrease in the radio-labeled sucrose accumulation and starch content of shoot tips as well as growth arrest in the growing zone of the shoot tips (Veluthambi et al., 1982b).

Growth arrest of Arabidopsis seedlings on medium with 100 mM trehalose was previously attributed to trehalose 6-phosphate (T6P) accumulation under these particular conditions (Schluepmann et al., 2004). T6P has emerged as a powerful signal molecule in plants, a target of which has been identified as SnRK1 of the SNF1/ AMPK group of protein kinases (Martinez-Barajas et al., 2011; Paul et al., 2010; Zhang et al.,

2009). SNF1-related protein kinases perform a fundamental role in the physiological response of cells to energy limitation and starvation of carbon source through regulation of pathways and processes involved in metabolism, growth and development (Halford and Hey, 2009; Hardie, 2007; Polge and Thomas, 2007). SnRK1 integrates stress, sugar and specific developmental signals (Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008; Baena-Gonzalez, 2010). T6P inhibits SnRK1 from all plant tissues so far tested except for mature leaves. This can be explained through the requirement of an intermediary factor present in growing tissues but not in mature leaves and is consistent with the view that T6P promotes anabolic processes associated with growing tissues (Zhang et al., 2009). Whilst T6P promotes growth on sucrose (Paul et al., 2010; Zhang et al., 2009), T6P inhibits growth on trehalose (Schluepmann et al., 2004). SnRK1 activity was proposed to be inactivated by sugars (Baena-Gonzalez et al., 2007), consistent with the observation that sucrose feeding causes a rise in T6P concentrations (Lunn et al., 2006; Schluepmann et al., 2003) which subsequently inhibit SnRK1 activity, promoting growth. The link between SnRK1 and T6P is significant as it establishes T6P as a signaling metabolite integrating carbon metabolism with the activity of enzymes and gene expression reprogramming controlled by this central protein kinase (Paul et al., 2010; Zhang et al., 2009). It is quite possible that growth arrest on trehalose is mediated through SnRK1.

Expression of Asparagine Synthase 1 (ASN1) has previously been used as a reporter of SnRK1 activity; the expression of ASN1 increases when the catalytic subunit of SnRK1, KIN10, is over expressed (Baena-Gonzalez et al., 2007). The promoter of ASN1 was shown to contain a G-box sequence known to be bound by bZIP (basic region/leucine zipper motif) called GBF (G-box Binding Factor) transcription factors. Co-expression of several bZIP transcription factors with KIN10 was shown to potentiate the expression of ASN1 (Baena-Gonzalez et al., 2007). bZIP transcription factors regulate a range of processes in growth and development in relation to the environment including stress responses (Jakoby et al., 2002). A model was proposed by which several stress signals including hypoxia and darkness converge through SnRK1 signaling and are coordinated in part by the bZIP transcription factors 54, 18, 63, 1, 38 and 11 (Baena-Gonzalez et al., 2007). It is not known whether these bZIP proteins are phosphorylation targets of SnRK1 and/or if bZIP gene expression is controlled by SnRK1 directly. bZIP54, 18, 63, 1 and 38 gene-expression is induced whilst bZIP11 is repressed when KIN10 is increased (Supplemental Fig. S1A; Baena-Gonzalez et al., 2007) or when seedlings are starving; the opposite is true when seedlings have increased carbon access through sugar or CO_2 feeding (Supplemental Fig. S1B). Potentiation of KIN10 induced ASN1 expression when KIN10 and bZIP11 are co-expressed suggests that the SnRK1 and bZIP11 controls of ASN1 expression interact.

The changes in gene expression after KIN10 over-expression are comparable to those obtained during carbon starvation regimes and opposite those under high supply of glucose, sucrose or CO_2 (Supplemental Fig. S1B). The S1-class of bZIP transcription factors consists of bZIP 1, 2, 11, 44 and 53 and appears to be expressed specifically in sinks such as young leaves, anthers and seeds (Rook et al., 1998; Weltmeier et al., 2009); their expression is also affected by abiotic stresses in a tissue-specific manner (Kilian et al., 2007). S1-class bZIP are generally regulated at the post transcriptional level by Sucrose-Induced Repression of Translation (SIRT) at a conserved uORF located in the 5 prime of the mRNA (Hanson et al., 2008; Weltmeier et al., 2009; Wiese et al., 2004). bZIP11 has been proposed to alter nitrogen metabolism by controlling the expression of ASN1 and Proline Dehydrogenase (PDH; Hanson et al., 2008) and changes in bZIP1 alter the transcriptional response to the carbon/nitrogen ratio (Kang et al., 2010; Obertello et al., 2010). Whilst T6P and SnRK1, and bZIP11 and SnRK1 have been linked, a connection between all three components in the regulation of growth has not previously been established.

The strong regulation of carbon allocation and growth by the trehalose pathway is important for our understanding of mechanisms that determine plant growth and yield with obvious application in crop improvement. To gain further insight on the growth arrest by trehalose feeding, we first established that starch deficient seedlings of the *pgm1* mutant are similarly affected as WT on trehalose. Starch accumulation in the cotyledons, the source tissue therefore, does not cause starvation and consequent growth arrest in the growing zones. We then screened the FOX collection of Arabidopsis expressing full-length cDNAs behind the CaMV35S promoter (Ichikawa et al., 2006) for seedling resistance to 100 mM trehalose. Three independent transgenic lines from differing pools of the collection were identified with dominant segregation of the trehalose resistance trait that over-expressed the bZIP11 transcription factor. The resistance of these lines to trehalose could not be explained through enhanced trehalase activity or through inhibition of bZIP11 translation. Instead, T6P accumulation was much increased in bZIP11 over-expressing lines suggesting that these lines may be insensitive to the effects of T6P. T6P is known to inhibit SnRK1 activity and we confirmed that this holds true in extracts from seedlings grown on trehalose, then showed that two independent transgenic lines over-expressing KIN10 were insensitive to trehalose. Moreover, expression of a set of marker genes known to be jointly controlled by SnRK1 activity and bZIP11 was found to be consistent with low SnRK1 or bZIP11 activity in seedlings on trehalose. Results were consistent with the existence of a growth regulating pathway involving T6P, SnRK1 activity and bZIP11, regulating growth in the growing zones of Arabidopsis seedlings.

RESULTS

Primary and secondary screening of the FOX-collection on trehalose

Trehalose at 100 mM in half-strength MS inhibited the growth of Arabidopsis seedlings of all accessions thus far tested. Seedlings germinated on this medium developed short roots less than 3 mm long after 14 d and the leaf primordia did not extend into leaves (Fig. 1A WT tre; Fig. 1B WT) compared to normal development on 100 mM sorbitol osmoticum control (Fig. 1A WT sorb; Fig. 1B WT). Starch accumulated in large amounts in one or both cotyledons in seedlings grown on trehalose (Fig. 1A WT tre); such massive accumulation of carbon as starch in the source tissues of seedlings could possibly cause starvation in the growing zones of seedlings. The starch-less *pgm*1 mutant (Caspar et al., 1985), however, was inhibited on trehalose in a similar manner to WT (Fig. 1A *pgm1* tre). Starch accumulation in the source tissues of seedlings, the cotyledons, thus does not cause growth arrest on trehalose.

To further understand the mechanism of growth arrest on trehalose using a non hypothesis-driven approach, we used a genetic screen: seedlings from the Arabidopsis FOX collection (Ichikawa et al., 2006) were screened for growth on 100 mM trehalose. The entire collection was partitioned in 141 pools each containing 20-30 T1 seed from 100 independent transgenic lines; primary screening was carried out twice with 1000 seed per plate for each 141 pools. The germination frequency varied between 100% and as low as 30% entailing that not all transgenic lines were assayed for their resistance to trehalose and that thus the screen did not test all the cDNA of the FOX collection. T1 seedlings during the primary screening were chosen that displayed significantly longer roots than wild type and leaf primordia (Fig. 1A 93-1 tre). Secondary screening of T2 retained only lines where seedling roots after 14 d of growth on trehalose were at least 3 fold longer than WT and of the same length as WT on osmoticum control (Fig. 1B). In the case of lines 93-1 and 93-32 from pool 93, seedling root lengths were nearly as long on trehalose as they were on sorbitol (Fig. 1B). Seedlings from line 70 were assayed at a later stage because trehalose resistant seedlings obtained from pool 70 were difficult to grow and after re-screening only one plant grew to set seed. The T2 seedlings from lines 93-3, 33-1 and 70 were 100% resistant to trehalose, whilst those from 93-1 were not and thus still segregating.

An obvious cause of resistance to trehalose would be increased trehalase activity. Expression of the *E.coli* cytosolic trehalase in Arabidopsis seedlings resulted in seedlings with high trehalase activity that thrive on trehalose (Fig. 1B treF and Fig. 1C treF). Therefore activity of the Arabidopsis trehalase (TRE1) was assayed in lines identified during the secondary screening. We tested several membrane and cell wall preparations from WT seedlings but trehalase activities in these fractions were below

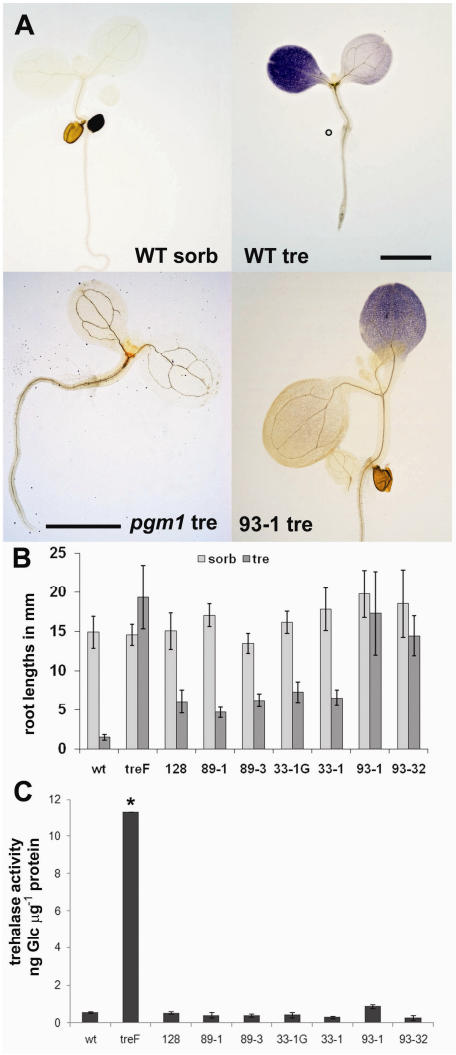

Figure 1. Carbon allocation, growth and trehalase activity of seedlings on 100 mM trehalose. Seedlings were grown under long-day conditions for 14 d on medium with 100 mM sorbitol osmoticum control (sorb) or trehalose (tre).

A, Starch staining. Seedlings were harvested at midday, stained with lugol and mounted in chloral hydrate. WT, wild type Col.0 seedlings; *pgm1*, seedlings lacking plastidic phosphoglucomutase 1 (Caspar et al., 1985); 93-1, seedlings from the FOX line 93-1. The bar represents 3 mm.

B, Root lengths. Average root-lengths from n> 20 seedlings of the differing genotypes with standard deviation. WT, wild type; treF, seedlings over-expressing *E.coli* trehalase treF; lines from the FOX collection of FOX pools 128, 89, 33 and 93 were line 128, 89-1, 89-3, 33-1G, 33-1, 93-1 and 93-32.

C, Trehalase activity in extracts of seedlings grown on trehalose for 14 d; the data are averages with standard deviations of 3 independent extracts. *ANOVA P< 0.050.

detection levels. Instead activity was readily detected in the soluble fraction and was not significantly different in FOX lines compared to WT, except for a small increase from pool 93 (ANOVA P=0.052). These activities were 10-20 fold lower compared to seedlings expressing *E.coli* trehalase treF (Fig. 1C).

Over-expression of the full-length cDNA of bZIP11 is linked with resistance to trehalose

Seedlings from the FOX lines were back crossed into WT and resistance to trehalose of F1 generation seedlings evaluated for each line. F1 seedlings of lines 33-1, 93-1 and 70

were resistant to trehalose, indicating that the trehalose resistance in these lines segregates as a dominant trait (Fig. 2A 33-1, 93-1). The cDNA present in the T-DNAs in the heterozygous F1 therefore caused trehalose resistance. The cDNA was then amplified using primers on the flanking sequences of the FOX vector for two different plants from each line (Fig. 2B). Fragments obtained from plants of the lines 33-1, 33-3, 70, 93-1 and 93-3 were of the same length. Subsequent cloning and sequencing revealed that these fragments all contained the full-length cDNA of bZIP11. The lines were from 3 differing pools, pools 33, 70 and 93. The lines therefore represented 3 independent transgenic events selected through screening which linked presence of the bZIP11 cDNA with trehalose resistance.

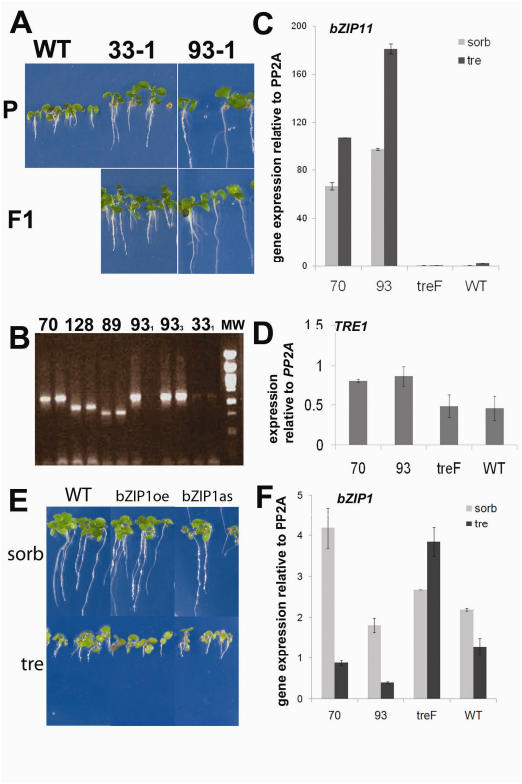

Figure 2. Characterization of the independent FOX lines expressing bZIP11 cDNA from the pools 33, 93 and 70. A, The trehalose resistance is a dominant trait as shown for lines 33-1 and 93-1. P, seedlings from the parental lines: WT, wild type; 33-1, FOX line 33-1; 93-1, FOX line 93-1. F1, the first generation from FOX line crosses with WT. B, PCR amplification of the FOX cDNA using DNA template from plants of the lines 70, 128, 89, 93-1, 33-1; MW, molecular weight marker lambda Pst1. C, Expression of *bZIP11* in 14-d-old seedlings from FOX lines 70, 93, the line expressing the *E.coli* trehalase treF (treF) and WT Col.0. D, Expression of the *TRE1* trehalase in the genotypes from C. E, Seedlings with altered expression of bZIP1 do not grow on medium with 100 M trehalose (tre) compared to sorbitol (sorb): WT, wild type; bZIP1oe and bZIP1as are the bZIP1 over-expressing and antisense lines described in Kang et al. (2010). F, Expression of bZIP1 in seedlings of the FOX lines 70 and 93, the line expressing treF and WT grown on medium with sorbitol (sorb) or trehalose (tre). Expression was determined by QPCR and is given relative to *PP2A* (At1g13320). Error bars represent

Expression of bZIP11 cDNA was tremendously increased in the FOX lines containing the bZIP11 cDNA compared to WT (Fig. 2C). In seedlings on trehalose, *AtTRE1* expression was less than two-fold increased compared to WT (Fig. 2D, ANOVA P=0.014), very much less increased than bZIP11 expression. Over- or antisense-expression of *bZIP1* in lines previously characterized (Kang et al., 2010) did not yield resistance to trehalose

suggesting that the function of bZIP11 could not be replaced by the other S1-class bZIP bZIP1 (Fig.2E). bZIP1 expression was high in treF (*E.coli* trehalase) -expressors on trehalose compared to WT or bZIP11 expressors (Fig.2F). Even though trehalase activity determinations suggested that the mechanism of trehalose resistance in bZIP11 over-expressors differed from that in treF expressors, we still wondered if the *in vitro* assays of trehalase (Fig. 1C) were conclusive.

AtTRE1 is not required for bZIP11 trehalose resistance

In further confirmation that TRE1 did not underlie resistance to trehalose in the FOX expressing bZIP11, a *tre1* knock out was characterized from the SALK collection (Salk 147073c, Alonso et al., 2003). PCR with primers on the left border of the T-DNA and in the start of the gene confirmed that the T-DNA was located 5 prime to the ATG of *TRE1*. Sequencing of the insertion site revealed an additional 25-bp insertion in the 5 prime and confirmed the T-DNA insertion site.

Gene expression quantification with Q-PCR further verified that the insertion causes a dramatic decrease in *TRE1* mRNA (Fig. 3A). Trehalase activity in seedlings and flowers of the *tre1-1* line was below detection (Fig. 3B). *tre1-1* was more susceptible to 25 and 50 mM trehalose than WT from the Col.0 accession (Fig. 3C) showing that TRE1 does contribute to the relative tolerance of WT seedlings to low levels of trehalose. The *tre1-1* seedlings were similarly sensitive to trehalose as the previously characterized *tre1-2* in the Ler accession (Fig. 3C; Vandesteene, 2009). The F1 seedlings from the cross *tre1-1* with the selected FOX lines were resistant to 100 mM trehalose (shown for the cross with FOX 93 in Supplemental Fig. S2). When analyzing the genotype of F2 seedlings with roots at least three-fold longer than WT, F2 seedlings could be found that were homozygous for the *tre1-1* T-DNA insertion (Fig. 3D). The trehalose resistance phenotype of seedlings homozygous for *tre1-1* and over-expressing *bZIP11* is shown in Supplemental Fig. S2. Together the data showed that AtTRE1 may contribute to but is not required for resistance to 100 mM trehalose and cannot explain rescue of seedlings on trehalose by bZIP11.

Translational repression at uORF2 does not occur on trehalose unlike on sucrose

bZIP11 cDNA is known to contain several upstream Open Reading Frames (uORFs) in the 5 prime region (Weltmeier et al., 2009; Wiese et al., 2004). uORF2 is conserved in the plant kingdom and found in all the S1-class bZIP mRNA. uORF2 mediates sucrose-induced repression of translation (SIRT) of the bZIP11 protein. The mechanism may also inhibit bZIP11 protein synthesis in seedlings grown on trehalose. To test this possibility seedlings of the UBQ10:5'UTR-GUS/GFP-I line (Wiese et al., 2004) that express the 5 prime UTR containing uORF2 in front of GUS/GFP were grown for 7 d,

then transferred to medium supplemented with either sucrose or trehalose for 48 h before GUS staining. GUS staining confirmed SIRT on sucrose medium supplemented with 100 mM sucrose throughout all tissues of the seedling (Fig. 3E 100 SUC). On trehalose medium, however, repression of translation was not observed (Fig. 3E 100 TRE). We therefore concluded that seedlings from the FOX lines translate bZIP11 unrestricted by SIRT when grown on trehalose.

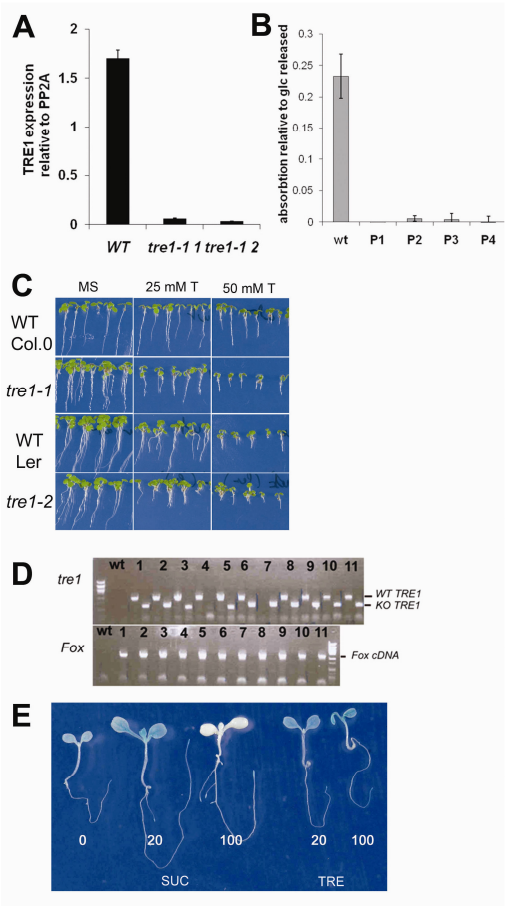

Figure 3. The roles of trehalase (TRE1) and of uORF2 in the FOX lines growing on trehalose. Error bars represent the standard deviation of 3 replicates. A, Expression of *TRE1* in wild type Col.0 (WT) and seedlings from two different plants of the *tre1-1* line (Salk 147073c). B, trehalase activity in flowers from wild type (WT) and several plants from the *tre1-1* line (P1-4). C, tre1-1 and tre1-2 seedling growth compared to their respective WT. Growth was on MS medium without (MS) or with 25 and 50 mM trehalose (25 mM T, 50 mM T). D, Genotype analysis of long root seedlings in the F2 generation of the cross 93-1 with wild type. DNA from wild type (WT) and 11 differing seedlings (1-10) was used as template. PCR was carried out to amplify the wild type sequence of *TRE1* (*WT TRE1*) or the T-DNA insertion at the *TRE1* locus (*KO TRE1*) in the above agarose gel (*tre1*). PCR was also carried out to amplify the FOX cDNA in the gel below (*FOX cDNA*). E, Unlike on sucrose, translational repression of *bZIP11* does not occur on trehalose. Seedlings expressing the 5 prime mRNA uORFs of the *bZIP11* mRNA fused to the *GUS* gene were grown for 7 d on MS medium, transferred for 48 h to medium with sucrose (SUC) or trehalose (TRE) at 0, 20 or 100 mM (0, 20, 100), then stained for GUS activity.

bZIP11 over-expressing seedlings accumulate T6P

WT seedlings growing on 100 mM trehalose accumulate T6P (Schluepmann et al., 2004) which has been linked to growth arrest under these particular conditions. We wondered whether the FOX line seedlings were insensitive to T6P or had just a low

accumulation of T6P. T6P in seedlings grown for 14 d on osmoticum control was 0.6-1 nmol g^{-1} FW in bZIP11 over-expressors, six- to tenfold higher than in WT (Fig. 4A). On trehalose medium, T6P accumulated in WT to 1.8 nmol g^{-1} FW as expected. T6P accumulation in bZIP11 over-expressors was, however, much higher reaching over 60 nmol g^{-1} FW (Fig. 4B). Data therefore showed that bZIP11 over-expression causes T6P accumulation.

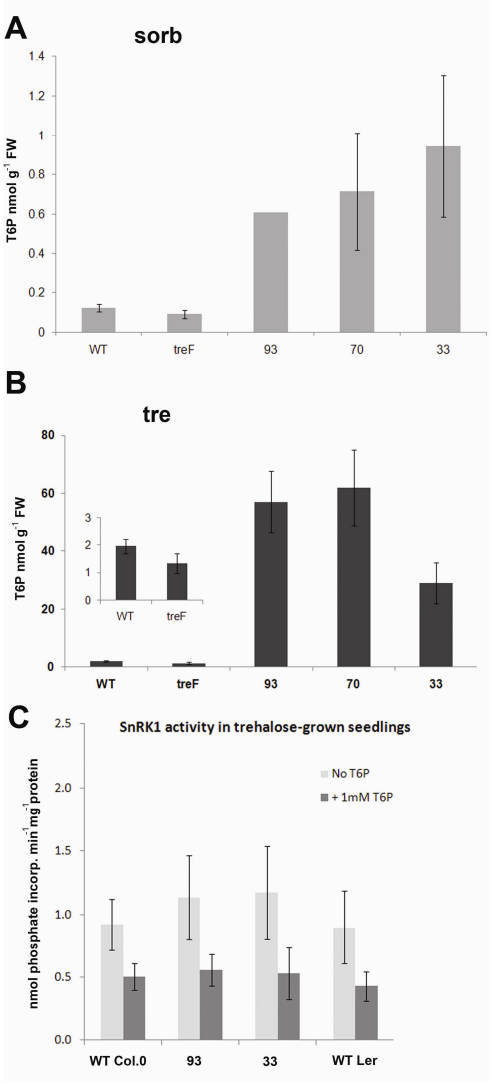

Figure 4. T6P accumulation and *in vitro* T6P inhibition of SnRK1 in seedlings grown on trehalose. WT, wild type Col.0; TreF, seedlings expressing *E.coli* trehalase treF; 93, 70 and 33 seedlings from the FOX lines 93, 70 and 33 respectively.

A, Seedlings grown on osmoticum control for 14 d (100 mM sorbitol).

B, Seedlings grown on 100 mM trehalose for 14 d.

C, SnRK1 activity assayed using the AMARA peptide in 14-d-old seedlings grown on 100 mM trehalose from wild type accession Col.0 (WT Col.0), Fox lines 93 and 33 and from wild type accession Ler (WT Ler). Error bars represent SEM of 3 biological replicates.

In contrast, T6P levels in seedlings expressing *E.coli* trehalase treF were similar to WT under control growth conditions. On trehalose, T6P levels in seedlings expressing treF accumulated to 1.5 nmol g^{-1} FW, a somewhat lesser extent than in WT, suggesting that

when trehalase is expressed unlikely all of the trehalose is cleaved. The differing accumulation of T6P in bZIP11 compared to treF expressing seedlings further supported the results which showed that trehalose resistance by bZIP11 is independent of trehalase. Instead the results point to the possibility that bZIP11 over-expression renders seedlings less susceptible to T6P accumulation.

T6P is known to inhibit seedling SnRK1 activity when seedlings are grown under normal conditions (Zhang et al., 2009). To test whether SnRK1 is inhibited by T6P in seedlings grown on trehalose, extracts from WT Arabidopsis seedlings grown in 100 mM trehalose were prepared from which small molecular weight compounds were removed using a desalting procedure. SnRK1 activity was then assayed in the absence or presence of 1 mM T6P. Results confirmed that SnRK1 from trehalose grown seedlings was significantly inhibited by T6P (Fig. 4C). In addition, SnRK1 activity assayed from extracts of bZIP11 over-expressing seedlings (93, 33) was similar to that of the WT (Fig. 4C). Over-expression of bZIP11 thus did not cause large changes in SnRK1 activity. We therefore hypothesized that the T6P accumulation and inhibition of SnRK1 in seedlings expressing constitutively high levels of bZIP11 may be overcome because bZIP11 likely controls a subset of genes responsive to SnRK1 activity. To test this possibility we first needed to establish whether SnRK1 activity limits the growth of seedlings on trehalose.

KIN10 over-expressing seedlings grew on 100 mM trehalose

To test whether reduced SnRK1 activity underlies the growth inhibition on trehalose, two lines O1 and O2 overexpressing KIN10 with high SnRK1 activity, previously characterized in detail were used (Baena-Gonzalez et al., 2007). Seedlings of both lines were resistant to 100 mM trehalose (Fig. 5A, only O2 shown). SnRK1 activity therefore likely is limiting when WT seedlings are grown on trehalose. The result further indicated that T6P inhibition of SnRK1 likely underlies growth arrest on trehalose and confirmed genetically previous results obtained by combining biochemical assay with gene-expression profiling (Zhang et al., 2009).

As with the bZIP11 over-expressors, KIN10 over-expression did not cause a significant induction of AtTRE1 activity (Fig. 5B) or of trehalase expression in seedling extracts (Fig. 5C). In addition, KIN10 over-expression did not affect the expression of bZIP11 (Fig. 5D); neither did bZIP11 over-expression affect SnRK1 activity (Fig. 4C) or KIN10 expression (Supplemental Fig. S3). Data therefore suggested that the SnRK1 and bZIP11 interaction described previously (Baena-Gonzalez et al., 2007) in seedlings on trehalose is likely post transcriptional with SnRK1 activity changes altering bZIP11 subcellular localization or activity.

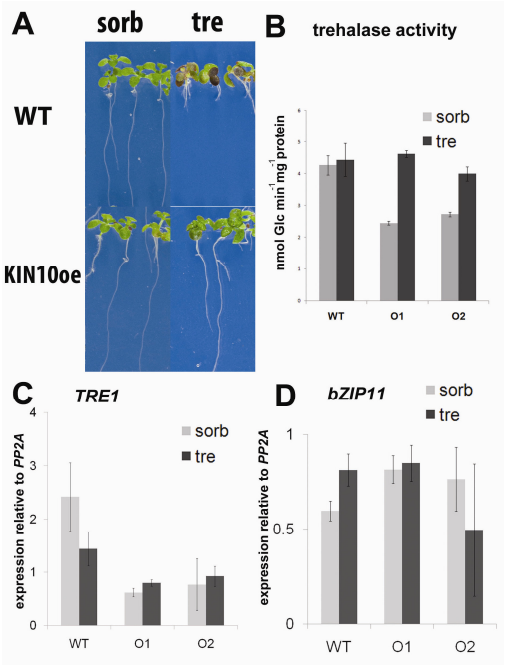

Figure 5. KIN10 over-expressing seedlings grow on trehalose without increased trehalase or bZIP11 expression. Seedlings were grown on trehalose (tre) compared to osmoticum control (sorb) for 14 d.

A, Phenotype of Ler wild type (WT) and KIN10 over-expression line O1.

B, trehalase activity in extracts from Ler WT and the lines over-expressing KIN10 O1 and O2.

C, *TRE1* expression determined relative to *PP2A* by Q-PCR in the genotypes from B.

D, *bZIP11* expression. The levels are averages of three biological replicates and error bars are standard deviation.

Regulation of targets common to bZIP11 and SnRK1

ASN1 is one of the known targets of SnRK1 and its expression was much reduced in WT grown on trehalose compared with sorbitol thus confirming that SnRK1 activity is likely inhibited by T6P on trehalose (Fig. 6A ASN1).

It is known that bZIP11 potentiates the induction of DIN6 (ASN1) gene-expression when SnRK1 activity is increased by KIN10 over-expression in protoplasts (Baena-Gonzalez et al., 2007). We compared genes known to be controlled by 6 h transient expression of KIN10 in protoplasts (511 up, 521 down; Baena-Gonzalez et al., 2007) with those known to be controlled in whole seedlings by the nuclear transfer of bZIP11 after 2 h (167 up, 96 down; Hanson et al., 2008). Table I lists the genes jointly controlled by KIN10 and bZIP11. In spite of the differing conditions used to identify regulation of gene expression, a large proportion of the bZIP11 induced genes, 32 out of 167, were also induced by KIN10. These include 4 genes of the 7 confirmed to be regulated by bZIP11 under its endogenous promoter: ASN1 (At3g47340), PRODH (At3g30775), BT2 (At3g48360) and PGPD14 (At5g22920). Nine genes from the 96 genes repressed by bZIP11 were also repressed by KIN10; only 3 genes from the total of 261 genes compared appeared differentially regulated by bZIP11 and KIN10. Results obtained from this comparison were thus consistent with the hypothesis that bZIP11 may be a target of SnRK1 regulation and may mediate a part of the output of SnRK1

Table I. Genes jointly controlled by SnRK1 and bZIP11 Two hundred and sixty one differentially expressed genes 2 h after nuclear transfer of bZIP11 (Hanson et al., 2008) were compared with the 1021 genes altered by 6 h transient KIN10 expression in protoplasts (Baena-Gonzalez et al., 2007), the commonly regulated genes are listed with TAIR annotation (May 2011).

Induced

At1g02660 alpha/beta-Hydrolases superfamily protein; putative triglyceride lipase activity
At1g10070 BCAT-2 chloroplast branched-chain amino acid aminotransferase
At1g18460 alpha/beta-Hydrolases superfamily putative lipase family
At1g32170 XTR4 xyloglucan endotransglycosylase-related protein
At1g62510 Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
At1g64660 MGL cytosolic methionine gamma lyase
At2g25200 Plant protein of unknown function (DUF868)
At2g30600 BTB/POZ domain-containing protein; involved in cell adhesion
At2g32150 Haloacid dehalogenase-like hydrolase (HAD) superfamily protein; nitrate responsive
At2g33380 CALEOSIN 3 calcium binding, induced by NaCl, ABA and dessication
At2g36220 expressed protein
At2g38400 AGT3 alanine:glyoxylate aminotransferase 2 homolog
At2g39570 ACT domain-containing protein; functions in amino acid binding
At2g47770 TSPO(outer membrane tryptophan-rich sensory protein)-related
At3g13450 DIN4 branched chain alpha-keto acid dehydrogenase E1 beta
At3g26510 Octicosapeptide/Phox/Bem1p family protein
At3g30775[a] ERD5, PRO1, PRODH, proline dehydrogenase
At3g47340[a] ASN1 glutamine-dependent asparagine synthetase
At3g48360[a] BT2 (AtBT-2) component of the TAC1-mediated telomerase activation pathway
At3g57520 RS2, SIP2 raffinose-specific alpha-galactosidase
At3g61060 PP2-A13, Phloem protein 2-A13
At3g61890 HB-12, homeobox-leucine zipper protein HB-12
At4g15530 PPDK pyruvate,orthophosphate dikinase
At4g28040 nodulin MtN21 /EamA-like transporter family protein (Drug/Metabolite Transporter)
At4g35770 SEN1 senescence-associated protein
At5g04040 SDP1, triacylglycerol lipase that is involved in storage lipid breakdown
At5g18670 BMY3, BAM9 glycosyl hydrolase family 14 (beta-amylase)
At5g22920[a] CHY-type/CTCHY-type/RING-type Zinc finger protein
At5g49360 BXL1, bifunctional βD-xylosidase/α-L-arabinofuranosidase
At5g53590 SAUR-like auxin-responsive protein family
At5g66170 STR18, Encodes a thiosulfate sulfurtransferase/rhodanase
At5g66650 Protein of unknown function DUF607

Repressed

At1g26770 EXP10 (Expansins(Alpha-Expansin Gene Family)) : expansin 10
At1g64060 RbohF(Respiratory burst oxidase family, Cytochrome b558 - H^+-channel)
At1g69530 EXP1 (Alpha-Expansin Gene Family)
At1g70230 TBL27, trichome birefringence-like, plant-specific DUF231
At1g76790 IGMT5, indole glucosinolate O-methyl transferase 5
At2g16660 Major facilitator superfamily protein, endomembrane system
At2g38170 CAX1, high affinity vacuolar calcium antiporter
At2g38940 PHT1;4 member of the Pht1 family of phosphate transporters
At3g09270 GSTU8 glutathione transferase belonging to the tau class of GSTs

Oppositely regulated

At2g14170 ALDH6B2, methylmalonate-semialdehyde dehydrogenase
At3g57040 ARR9 Response Regulator A- Type, cytokinin signalling
At5g17760 P-loop containing nucleoside triphosphate hydrolases superfamily protein

[a] also in the list of 7 genes which are induced by bZIP11 under the control of its own promoter.

signaling; they further showed that bZIP11 does in addition regulate transcription of a set of genes not included in regulation by SnRK1.

To test whether KIN10 and bZIP11-mediated rescue on trehalose may involve genes that are commonly controlled by both, we assayed the expression of a randomly chosen array of jointly induced (8) or repressed (5) genes in WT seedlings grown on trehalose compared to sorbitol (Fig. 6A). All but one of the 8 genes jointly induced by bZIP11 and KIN10 were significantly repressed on trehalose. Furthermore 3 of the 5 jointly repressed genes were significantly induced on trehalose compared to sorbitol. We conclude that a large proportion of genes jointly regulated by KIN10 and bZIP11 respond on trehalose as if KIN10 or bZIP11 were less active.

To compare the downstream events in KIN10 and bZIP11 over-expression, the levels of soluble sugars (Glc, Fru and Suc) were compared with those found in WT and in treF expressors (Fig. 6B). Similar results were found for hexoses and sucrose and data are presented as total soluble sugar. Soluble sugars accumulated in WT Col.0 or Ler seedlings grown on trehalose and in treF seedlings which were included for comparison. The data were consistent with carbon accumulation in source tissues whilst sugars are not used for sink growth. Strikingly, bZIP11 over-expression lines 93 or 33 had 10-20 times higher soluble sugar than WT (Fig. 6B 93, 33) irrespective of the medium. Sugars were also elevated (threefold compared to WT) in KIN10 over-expressors compared to WT and this increase was even more on trehalose (Fig. 6B O1, O2).

Arrest of hypocotyl elongation when wild type seedlings were grown in continuous darkness was another effect of feeding trehalose (Fig.6C, D WT tre). We thus concluded that trehalose feeding not only stopped use of carbon fixed by photosynthesis but also of carbon from seedling reserves needed for the for hypocotyl growth in the etiolation response. Over-expression of bZIP11 (Fig. 6C) or KIN10 (Fig. 6D) was able to suppress hypocotyl growth inhibition by trehalose in the dark. The observation suggested that KIN10 and bZIP11 rescue T6P inhibition of SnRK1 regardless of the source of carbon fueling the growth of sinks. Therefore convergence of the pathways involving KIN10 and bZIP11 in the control of carbon utilization for growth is likely.

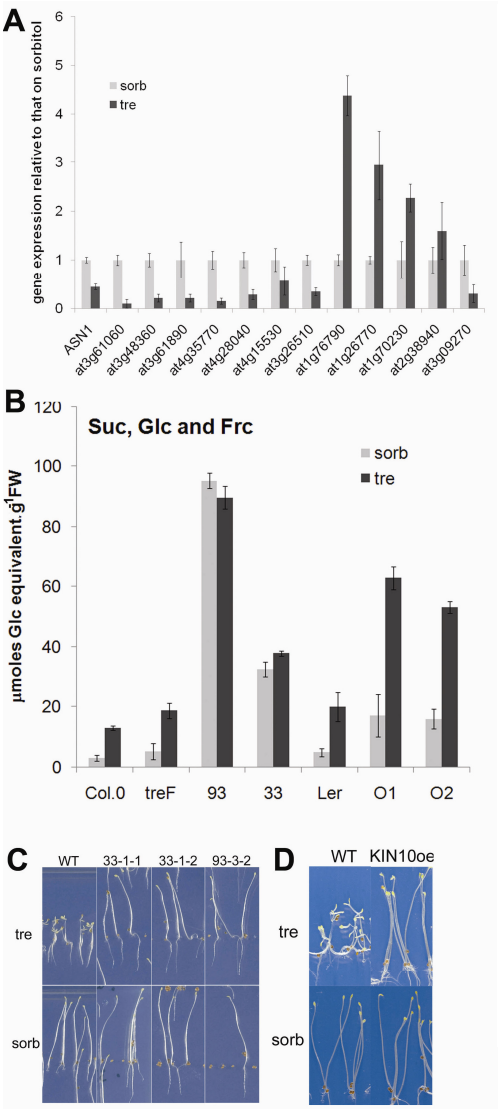

Figure 6. Do KIN10 and bZIP11 act in the same pathway?

A, Expression of targets common to KIN10 and bZIP11. Seedlings of WT were grown for 14 d on 100 mM of either sorbitol (sorb) or trehalose (tre), collected at midday. Expression was determined by Q-PCR relative to PP2A, then normalized to the level of expression on sorbitol.

B, Soluble sugars Suc, Glc and Fru in the seedlings with the genotype WT (Col.0), treF expressors (treF), bZIP11 expressors from line 93 (93) and 33 (33), wild type Ler (ler), KIN10 overexpressing lines O1 and O2. In A and B levels are averages of three biological replicates and error bars are standard deviation.

C, Phenotype of bZIP11-expressing seedlings on trehalose in continuous darkness. After 78 h at 4ºC, seed were exposed to light and 22ºC for 6 h then grown for 14 d in continuous darkness on medium with 100 mM of either sorbitol (sorb) or trehalose (tre). WT, wild type Col.0; 33-1-1 and 33-1-2 seed from two plants of the FOX-line 33-1; 93-3-2, seed from the FOX line 93. D, Phenotype of KIN10-expressing seedlings on trehalose in continuous darkness. Seed were treated as in C; WT, wild type Ler; KIN10oe, seed from the O2 line (Baena-Gonzalez et al., 2007).

DISCUSSION

Trehalose, a widespread disaccharide synthesized in all non vertebrate organisms functions widely as a carbon source and stress protection compound. When fed exogenously to plants, however, it can have a surprisingly strong inhibitory effect on growth and carbon allocation (Veluthambi et al., 1982a; Veluthambi et al., 1982b; Wingler et al., 2000). The trehalose pathway in plants has evolved a role that is distinct from other organisms in the regulation of metabolism in relation to growth and

development (Paul et al., 2010; Schluepmann and Paul, 2009; Zhang et al., 2009). The signal transduction mechanisms involved in trehalose signaling therefore are of great interest as potential targets for crop improvement. Here we provide evidence that growth arrest by trehalose-6-phosphate is an astonishing case of primary metabolite control over growth by way of the SnRK1 signaling pathway also involving bZIP11. Our work highlights the importance of bZIP11 and SnRK1 specifically in the growth response to trehalose and more generally in the regulation of growth by carbon availability to growing sinks.

Growth arrest is not due to excessive starch accumulation but to impaired utilization of sugar

The phenotype of Arabidopsis seedlings on trehalose is interesting because it involves a large change in carbon allocation within the seedling in addition to growth arrest. Starch accumulates in cotyledons, whilst growing sinks appear carbon limited. Starchless *pgm 1* mutants are similarly impaired as wild type on trehalose, which rules out the possibility that sinks are carbon limited because starch accumulation sequesters available carbon. Feeding metabolisable sugar in combination with trehalose alleviates growth arrest, therefore, growth arrest is likely due to insuficient carbon in growing sinks (Schluepmann et al. 2004). Feeding trehalose could affect sucrose transport with high levels of trehalose displacing sucrose at the sucrose transporters and so reducing phloem loading and unloading processes. Growth arrest by trehalose, however, already occurs at trehalose concentrations of 25 mM when trehalose is fed in combination with the trehalase inhibitor Validamycin A (Wingler et al. 2000). At these concentrations, trehalose is unlikely having an effect on sucrose transport just by displacing sucrose. In contrast, T6P has previously been shown to cause the growth inhibition on trehalose and to have an important signaling function (Schluepmann et al. 2004; Kolbe et al. 2005; Zhang et al. 2009). One of these signaling functions is in plastid redox processes (Kolbe et al. 2005; Geigenberger, 2011). Starch accumulation in trehalose fed leaves of Arabidospis is caused by T6P accumulation stimulating AGPase redox activation in plastids (Kolbe et al. 2005). This is consistent with the massive accumulation of starch seen in cotyledons of seedlings. The *pgm1* mutants show that the effect of T6P on AGPase redox activation in cotyledons is not involved in the mechanism leading to growth arrest.

The transcription factor bZIP11 overcomes growth inhibition on trehalose

The FOX collection was utilized to screen for rescue of growth on trehalose. Strikingly we isolated three independent transgenic lines over-expressing bZIP11 a transcription factor shown to be important in the regulation of growth (Hanson et al., 2008). Translation of bZIP11 is repressed by sucrose (Wiese et al., 2004), however no such

effect was produced by trehalose. A simple way of explaining rescue on trehalose is through breakdown of trehalose by trehalase (Schluepmann et al., 2004). However, trehalase activity was not elevated in seedlings over-expressing bZIP11 and neither did backcrossing bZIP11 into trehalase knockout compromise the rescue of seedlings by bZIP11, showing that bZIP11 rescues growth on trehalose without increasing the breakdown of the exogenously supplied trehalose. This implies that bZIP11 is part of a growth regulatory process that can be invoked to overcome the growth inhibition normally imposed by trehalose. The results obtained differ from those in Ma et al. 2011 where bZIP11 protein was fused to a nuclear targeting domain and where targeting of the fusion protein to the nucleus induced the expression and activity of TRE1. TRE1 was not detected as a target of native bZIP11 (Hanson et al. 2008), however, and this is consistent with results obtained with the FOX lines here. bZIP11 is expressed along the vasculature (Rook et al. 1998) and is proposed to regulate nitrogen metabolism by controlling the expression of ASN1 and PDH (Hanson et al., 2008). Sugar supply induces expression of bZIP11, therefore it was proposed that bZIP11 is important to relate nitrogen and carbon metabolism. Plants over-expressing KIN10 with active SnRK1 and primed to respond to starvation conditions consistently have reduced expression of bZIP11 (Baena-Gonzalez et al., 2007).

bZIP11 over-expression results in unprecedented levels of T6P recorded in Arabidopsis

T6P accumulation in plants grown on trehalose previously shown to be causally related to growth inhibition (Schluepmann et al., 2004). This was shown through specific reduction in T6P content through expression of a trehalose 6-phosphate hydrolase which rescued growth. Therefore it was surprising that bZIP11 overexpressors on trehalose exhibited huge accumulation of T6P to the highest levels so far reported for Arabidopsis (Fig. 4B). Sugars were also elevated in bZIP11 (Fig. 5E). A strong relationship between sucrose (Lunn et al., 2006) and sucrose and hexoses (Martinez-Barajas et al., 2011) and T6P has previously been found. It is thus possible that elevated T6P can be accounted for as a response to elevated sugar found in bZIP11 over-expressors compared with WT. Given the recent evidence that SnRK1 is a target of T6P (Paul et al., 2010; Zhang et al., 2009), that SnRK1 would be inhibited by T6P accumulation on trehalose (Fig. 4C) and that bZIP11 over-expression increases the impact of endogenous SnRK1 activity (Baena-Gonzalez et al., 2007) we hypothesised that high SnRK1 activity could also be a mechanism of rescue of growth on trehalose.

KIN10 over-expression rescues growth on trehalose

SnRK1 is essential for carbon utilization in growth. KIN10 over-expression increases seedling growth compared to WT when carbon availability is limiting, whereas

seedlings with low SnRK1 activity through KIN10 antisense thrive when carbon availability is high (Baena-Gonzalez et al., 2007). We found that as with bZIP11, over-expression of SnRK1 also rescues seedling growth on trehalose (Fig. 5A). Like seedlings expressing bZIP11, KIN10 expressors also accumulate free sugars compared with either WT or treF when they are grown in osmoticum control conditions (Fig. 5E). This would imply that SnRK1 too is part of a mechanism that regulates growth in the presence of trehalose and that T6P through inhibition of SnRK1 prevents growth on trehalose. This conclusion is supported by previous work which shows the inhibition of SnRK1 by T6P in Arabidopsis seedlings, by direct *in vitro* assay and indirectly by profiling known targets of SnRK1 in seedlings with altered T6P steady state (Zhang et al., 2009).

Interaction of bZIP11 and SnRK1

A subset of S-class bZIP transcription factors are proposed to mediate some of the transcriptional re-programming in response to altered SnRK1 activity (Baena-Gonzalez et al., 2007). The mechanism linking kinase activity signaling and transcriptional control by S-Class bZIP proteins is not known, but possibly involves phosphorylation of the bZIP protein which would explain potentiation of the SnRK1 response when KIN10 and bZIP11 are over-expressed simultaneously in protoplasts (Baena-Gonzalez et al., 2007). Recombinant bZIP EEL/DPBF4 and ABI5 were substrates of SnRK1 after immuno-precipitation of KIN10GFP protein fusion (Bitrián et al. 2011). We confirm KIN10 and bZIP11 interaction when seedlings are on trehalose: 1) over-expression of bZIP11 has the effect of overcoming the effects of low SnRK1 activity on trehalose, 2) the majority of genes tested that are jointly controlled by KIN10 and bZIP11 are expressed in a manner consistent with low activity of either KIN10 or bZIP11 in WT seedlings on trehalose (Table I; Fig. 6A), 3) under differing conditions, KIN10 and bZIP11 permit growth of sinks in seedlings on trehalose and 4) T6P accumulates in bZIP11 over-expressing seedlings grown on sorbitol. Comparison of data from Baena-Gonzalez et al. (2007) and Hanson et al. (2008) shows that 44 genes may be jointly regulated by bZIP11 and KIN10 (Table I). Constitutive and high expression of bZIP11 may therefore counteract T6P inhibition of SnRK1 activity on trehalose and impact at least a subset of the targets that are commonly regulated by bZIP11 and KIN10. These targets of bZIP11 are likely important for the control of carbon utilization for growth.

S1-Class bZIP transcription factors are thought to act redundantly but have a differential expression responsiveness and pattern (Hanson et al., 2008; Weltmeier et al., 2006; Weltmeier et al., 2009). Seedlings over-expressing or with antisense to bZIP1 (Kang et al., 2010) were sensitive to trehalose however (Fig. 2E), suggesting that bZIP11 has a different function from bZIP1. Control of the bZIP11 gene-expression is opposite to that of the other S-class bZIP capable of potentiating the effect of KIN10

expression: bZIP11 is repressed under carbon starvation conditions or by high SnRK1 activity (Baena-Gonzalez et al., 2007 and Fig. S1). bZIP11 expression is furthermore induced by increased carbon availability and this is consistent with results presented here where bZIP11 is required for carbon utilization for growth.

The arrest of hypocotyl growth by trehalose in seedlings in the dark was not reported previously. Seedlings on the combination of trehalose and Validamycin in the dark were reported not to accumulate starch and therefore it was concluded that carbon accumulating as starch in light grown seedlings on trehalose was of photosynthetic origin (Wingler et al., 2000). Inhibition of hypocotyl growth in dark grown seedlings on trehalose, and suppression thereof by over-expression of either KIN10 or bZIP11 indicates that the pathway of growth inhibition by trehalose in the dark and in the light likely involves the same mechanism. In the dark, gluconeogenesis from lipids stored in both the endosperm and the cotyledons fuels hypocotyl growth (Penfield et al., 2004). In light or dark therefore, KIN10/bZIP11 activities are limiting growth of sink tissues in seedlings as T6P accumulates on trehalose. Inhibition of the growth of sink tissues when SnRK1 was reduced by antisense inhibition has previously been reported in barley pollen grains and in potatoes (Purcell et al., 1998; Zhang et al., 2001). Importantly the work with segregating pollen grains by Zhang et al. 2001 shows that the effect is in the cells where SnRK1 is low and is not due to low SnRK1 in surrounding tissue.

A possible model of interactions that control carbon availability in growing sinks

T6P inhibition of SnRK1 could be part of a regulatory loop that relates SnRK1 activity with the amount of sucrose (Fig. 7A). In this regulatory loop sucrose-induced T6P increase inhibits SnRK1 when sucrose is plenty. As sucrose decreases T6P decreases and active SnRK1 signals nutrient stress such that more carbon is then made available to the heterotrophic growing cells. This regulatory loop could explain why both too little and too much of (either SnRK1 or) T6P are growth inhibitory (Schluepmann et al. 2003; Schluepmann et al. 2004; Debast et al., 2011). It also would explain the reported correlation between levels of sucrose and T6P (Lunn et al., 2006; Martínez-Barajas et al., 2011).

Glucose (Glc) and sucrose (Suc) feeding cause AGPase redox activation and thus increased starch synthesis by two differing pathways that are likely also relevant for the growth responses to these sugars (Tiessen et al., 2003; Michalska et al., 2009; Geigenberger, 2011). When feeding Glc, T6P does not accumulate (Delatte et al., unpublished) and glucose-6-phosphate (G6P) is shunted through the plastidic oxidative part of pentose phosphate pathway (OPP) generating NADPH for NADPH Thioredoxin Reductase C (NTRC) dependent reduction of AGPase and thus activation (Michalska et

al., 2009). In contrast, feeding sucrose or trehalose leads to T6P increase (Schluepmann et al., 2004; Lunn et al., 2006). T6P increase may result from the increased amount of substrate uridine diphosphate glucose (UDPG) when sucrose is cleaved by sucrose synthase.

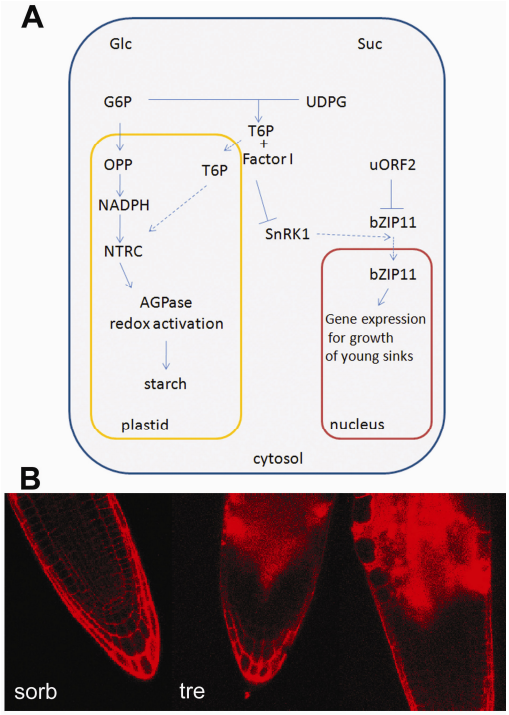

Figure 7. A, Model of interactions affecting growth and starch accumulation on trehalose when T6P accumulates. Glucose (Glc) and sucrose (Suc) feeding cause AGPase redox activation and thus starch synthesis by differing pathways that are likely also relevant for the growth responses to these sugars (Tiessen et al., 2003; Michalska et al., 2009; Geigenberger, 2011). When feeding Glc, T6P does not accumulate (Delatte et al., unpublished) and glucose-6-phosphate (G6P) in plastids is shunted through the oxidative part of the pentose phosphate pathway (OPP) generating NADPH for NADPH-Thioredoxin Reductase C (NTRC) dependent reduction of AGPase and thus activation. In contrast, feeding sucrose or trehalose leads to T6P increase which acts upon AGPase redox by an unknown mechanism (Schluepmann et al., 2004; Kolbe et al., 2005; Lunn et al., 2006; Michalska et al., 2009). Sucrose inhibits translation of bZIP11 by way of uORF2 (Wiese et al., 2004), but trehalose does not. When feeding trehalose, T6P accumulates. T6P accumulation inhibits SnRK1; this inhibition of SnRK1 depends on an intermediary Factor I present in young tissues (Zhang et al. 2009). Possibly, SnRK1 phosphorylation activates bZIP11 transfer to the nucleus or complexing of the transcription factor in such a way that bZIP11 controls a part of the SnRK1 output that is required for growth. Thus when T6P accumulates and inhibits SnRK1 in young tissues, over-expression of bZIP11 may act as a surrogate for SnRK1. **B,** Antisense SnRK1 restricted to individual pollen of barley in particular (Zhang et al., 2001) but also work in developing potato tubers (Purcell et al., 1998) show that SnRK1 is required in growing heterotrophic cells for growth and starch accumulation. It therefore is possible that SnRK1 is needed to respond to nutrient stress so as to make carbon available in growing sinks. SnRK1 inhibition (by artificially increasing T6P when feeding trehalose or by antisense SnRK1) would hence uncouple growth from carbon starvation responses leading to the swollen cells observed in the growing zones of roots of Arabidopsis seedlings on trehalose (tre) compared to sorbitol (sorb). Root tips were stained with propidium iodide in water immediately prior to visualization under the confocal microscope.

Alternatively T6P increase in response to sucrose feeding may result from a sensing/signaling system affecting either T6P synthesis or degradation. The sucrose pathway to AGPase redox activation was shown to depend upon SnRK1 activity (Thiessen et al., 2003) but we have yet to know at which step SnRK1 is required. SnRK1 may not be required when trehalose grown seedlings convert the carbon fixed by

chloroplasts into starch, alternatively Factor I may be absent in cotyledons. A number of Class II T6P synthases (TPS), including TPS5 are likely targets of SnRK1 but until now these enzymes were not shown to synthesize T6P (Harthill et al., 2006). When feeding sucrose or trehalose, T6P also causes NTRC dependent AGPase redox activation in the chloroplasts (Kolbe et al., 2005; Michalska et al., 2009). Although the interaction with redox-signaling may be an important aspect of T6P signaling, we have shown here that T6P accumulation on trehalose and consequent accumulation of starch in cotyledons does not cause growth arrest.

When feeding trehalose, T6P accumulation inhibits SnRK1. Inhibition of SnRK1 depends on and intermediary factor (Factor I) present in seedling extracts but not in leaf extracts (Zhang et al., 2009). Possibly, SnRK1 activates bZIP11 transfer to the nucleus or complexing of the transcription factor in such a way that bZIP11 controls a part of the SnRK1 output that is required for growth. Thus when T6P accumulates and inhibits SnRK1, over-expression of bZIP11 may act as a surrogate for SnRK1 in the growing zones. Antisense SnRK1 restricted to individual pollen of barley in particular (Zhang et al., 2001) but also work in developing potato tubers (Purcell et al., 1998) suggest that SnRK1 is required in individual cells of growing sinks for growth and starch accumulation.

In short-term trehalose feeding experiments of potato slices AGPase redox activation was also found and it was dependent on the presence of SnRK1 (Kolbe et al., 2005). SnRK1 may therefore coordinate substrate availability for starch synthesis and AGPase redox activation in sink tissues. In this respect, it is important to take into account that the subcellular localization of SnRK1's catalytic subunit KIN10 was shown to differ in the different tissues of the seedlings (Bitrián et al., 2011): this may also change SnRK1 susceptibility to T6P. In growing zones of root and shoot KIN10 was reported in the nucleus, whilst in the hypocotyls it was not. It is thus possible that in growing sinks SnRK1 is required to signal nutrient stress so as to activate processes that will make carbon available to growing cells. We conclude that SnRK1 inhibition (by artificially increasing T6P when feeding trehalose or by antisense SnRK1) may uncouple carbon starvation from growth responses leading to the swollen cells observed in the growing zones of roots of Arabidopsis seedlings on trehalose (tre) compared to sorbitol (sorb) (Fig. 7B). More research is needed to understand the precise role of bZIP11 in the carbon allocation responses and to understand where and how SnRK1 as well as bZIP11 over-expression lead to the accumulation of free sugars in seedlings.

METHODS

Plant materials and growth conditions

Lines in Col.0 accession are: Tref line 42 (Schluepmann et al., 2003), *tre1-1* is Salk 147073c (http://signal.salk.edu Alonso et al., 2003), FOX lines (Ichikawa et al., 2006), the bZIP1 over and antisense lines (Kang et al., 2010) and finally the line to test translational repression of bZIP11 that contains the 5 prime untranslated leader of bZIP11 mRNA fused to GUS/GFP under the control of the *UBIQUITIN10* promoter (*UBQ10*:*5'UTR-GUS*/*GFP*, (Wiese et al., 2004)). Lines in Ler accession: *tre1-2* (Vandesteene, 2009), KIN10 O1 and KIN10 O2 (Baena-Gonzalez et al., 2007). Seed were generally vapor-phase sterilized (Clough and Bent, 1998), plated on medium and cold treated at 4^0C for 72 h in darkness. Medium used for growth of seedlings was generally agar-solidified (0.8 % w/v) half-strength MS (Murashigue and Skoog, 1962) with 100 mM of filter-sterilized sorbitol or trehalose. The plates were then transferred for growth at 22^oC in a long-day light cycle (16 h light/8 h dark) regime with 100 µM m^{-2} s^{-1} light intensity and 80% humidity. When grown in the dark, seedlings were first exposed to 6 h light at 22^oC to promote germination before transfer to darkness at 22^oC.

Seedling screening for trehalose resistance

Seed from the Arabidopsis FOX collection (Ichikawa et al., 2006; T1 generation) was collected using a bamboo skewer with a small pinhole at one end serving as a measure for 20-30 seed; collection was at the Plant Science Center, RIKEN Yokohama (Japan). Twenty to thirty T1 seed from 100 differing transgenic lines were pooled in one single pool; 141 pools covered the entire collection. Seedlings from differing pools are therefore necessarily from a differing transgenic event. During the primary screen, 1000 seed per pool of 100 independent transgenic lines were screened twice on plates containing half-strength MS salts and vitamins supplemented with 100 mM trehalose. The germination frequency varied between 100% and 30%. Seedlings were chosen over a period of 1 to 3 weeks of growth that displayed longer roots and more growth of leaf primordia than wild type (Fig. 1a 93-1 tre). For seven of the pools, more than one seedling of the pool (1-6 seedlings) was identified to be resistant. The seedlings were transferred to soil and grown for T2 seed set; a total of 157 seedlings were grown resulting in seed of 121 lines for a secondary screen. Seedlings recovered from the pool 70 mostly died upon transfer to soil, after repeated screening only one plant survived to set seed and was dealt with later in a separate secondary screening. Secondary screening was carried out on the T2 generation seed for each line by testing seedling growth after 14 d on 100 mM trehalose and 100 mM sorbitol to test specificity of the resistance to trehalose, and on medium with 12 µg l^{-1} hygromycin B to

evaluate expression of the marker gene associated with the presence of the T-DNA insertion. Lines from 6 different pools were retained after the secondary screening with average root length unchanged on sorbitol control but at least 3-fold longer than WT on trehalose medium. Lines obtained from differing pools necessarily are of independent transgenic origin, whilst lines obtained from the same pool likely are not. FOX Lines from the T2 generation were subsequently grown to generate T3 and T4 seed stock with 100 % trehalose resistance and homozygous for the presence of bZIP11 cDNA.

Lugol and GUS staining

Seedlings were grown for 14 d in long-day light cycle. Seedlings were harvested at midday, then washed in 70% ethanol before starch staining with 43.4 mM KI/5.7 mM I_2 (Lugol solution from Sigma, Wingler et al., 2000) and mounted in a mixture of chloral hydrate/glycerol/water (vol 8:1:2). To test the inhibition of bZIP11 translation, seedlings were grown in long-day light cycle for 7 d on MS medium, then transferred for 48 h to MS supplemented with either Suc or trehalose at 0, 20, 100 mM concentration. GUS staining was in GUS buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 10 mM EDTA, 0.1% (v/v) Triton X-100, and 1 mg mL^{-1} of 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid) according to (Wiese et al., 2004). The experiment was repeated with seedlings grown for 14 d on 100 mM of either sucrose or trehalose with results similar to those obtained after only 48 h incubation.

Propidium iodide staining of roots and confocal microscopy

Seedlings were grown for 5 d on MS supplemented with 100 mM of either sorbitol or trehalose. Seedlings were mounted with the root only between the cover slips and in 10 μM propidium iodide, mounting was immediately before visualization under the confocal microscope (63· NA 1.4 Plan apochromat water immersion objective, Leica SP2 inverted laser confocal microscope with an Ar 488-nm laser excitation, dichroic 488/543/633 and emission settings 562-588 nm).

DNA extractions, PCR and sequencing

To genotype the F2 generation seedlings obtained from the crosses of WT with FOX lines, seedlings were grown for 14 d on MS with 100 mM trehalose, then DNA was extracted from a single seedling with long root; the DNA extraction was as previously described (Cheung et al., 1993). Primers GS4 and GS6 (Supplemental Table SI) were used for PRC-amplification of the cDNA in the FOX lines as described in Ichikawa et al.,

2006. Genotyping of *tre1-1* was as recommended (http://signal.salk.edu) using primers LBb1, LP1tre1, RP1tre1, LP2tre1, RP2tre1 listed in Supplemental Table I.

Assays for trehalase activity

Trehalase activity was assayed as previously described with some modifications (Brodmann et al., 2002). Seedlings (T2) were grown on trehalose medium, short root WT seedlings or seedlings with long roots from the FOX lines were used. Seedlings were pooled as 70 mg FW and ground in 50 µl Buffer (0.1 M MES/KOH (pH 6.3), 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM DTT, 0.01% (vol/vol) Triton-X-100) at 4^{o}C. Subsequently, 100 ul Buffer was added, the crude extract mixed then centrifuged 5 min, 13000 rpm at 4^{o}C to remove the insoluble fraction. Sugars in the soluble supernatant were then removed by repeated dilution then concentration of the proteins in the extract using regenerated cellulose membrane (Amicon Ultra- 0.5 ml 10K Ultracel, Millipore), dilution was with 3 consecutive additions of 300 µl 20 mM MES/KOH (pH 6.3). The final volume of extract was adjusted to 50 µl. To assay trehalase, extract (10 µl) was incubated in triplicate with 15 mM trehalose for 1 h at 37^{o}C, then boiled for 10 min. Alternatively control assays were boiled immediately. Glc released from trehalose was quantified (Enzytec D-Glucose kit , scil Diagnostics, Viemheim, Germany) as the difference between boiled controls and samples which were assayed for 1 h. Values were averaged from 3 biological replicates with standard deviations.

T6P determinations

Seedlings were grown under long-day growth conditions for 14 d. Five replicate samples of 50 mg each were harvested at midday by snap-freezing. Lactose-6-phosphate (5 nmol) was added as an internal standard. Materials were then ground frozen, extracted and analysed as described in (Delatte et al., 2009). Briefly, seedling extracts obtained by the subsequent liquid–liquid and solid-phase extractions were reconstituted in water and analysed by anion-exchange chromatography combined with electrospray ionization mass spectrometry. The method provided baseline resolution of T6P and allowed its specific detection at m/z 421 with good linearity. T6P concentrations were inferred from a five-point calibration curve using the signal obtained for the internal standard to correct for potential recovery losses.

Assays for SnRK1 activity

Seedlings were grown for 14 d in 100 mM sorbitol or trehalose, snap frozen at midday as 100 mg FW replicates. Three biological replicates for each data point were each ground frozen then extracted and T6P as well as sugars removed by the desalting

procedure previously described (Zhang et al., 2009). SnRK1 activity determinations were using the AMARA peptide as substrate.

Sugar determinations

Soluble sugars were extracted from samples of 14 d old seedlings; samples were 3 biological replicates for each data point. The extraction procedure was as previously described (Schluepmann et al., 2003). Sucrose, glucose and fructose were assayed enzymatically (Enzytec D-Glucose/D-Fructose/Sucrose kit, scil Diagnostics, Viemheim, Germany).

RNA extraction and Q-PCR

RNA was extracted from flash frozen seedlings collected at midday (50 mg FW). Samples were in triplicate biological replicates for each data point and the extraction protocol was according to the instruction manual (Spectrum Plant Total RNA Kit, Sigma-Aldrich). DNase treatment, reverse transcription and Q-PCR were as described previously (Hanson et al., 2008). Primers used for the Q-PCR reactions are described in Supplemental Table SII. Primers from the genes jointly targeted by KIN10 and bZIP11 were taken from the CATMA site http://www.catma.org/.

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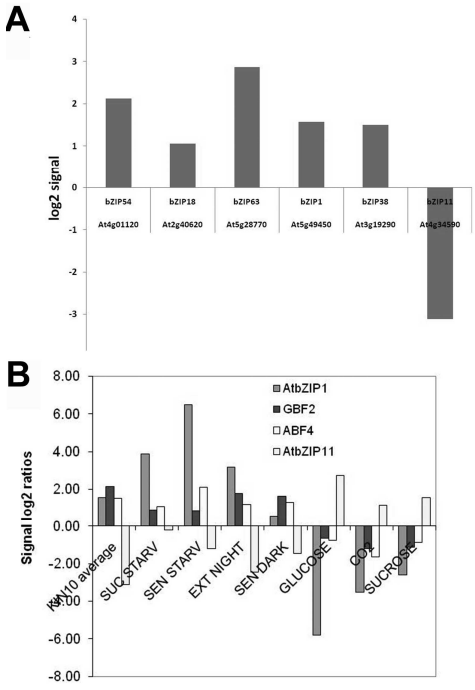
Supplemental Table SI. Primers for characterization of the FOX lines and the SALK T-DNA insertion line Salk 147073c

Primer Name	**Primer sequence**
FoxGS4 (Ichikawa et al., 2006)	ACATTCTACAACTACATCTAGAGG
FoxGS6 (Ichikawa et al., 2006)	CGGCCGCCCCGGGGATC
LBb1 (T-DNA Salk)	GCGTGGACCGCTTGCTGCAACT
LP1tre1	TGAATTGGATCTCCTTATGGC
RP1tre1	AGTGACGAGTTTGGTTGTTGC
LP2tre1	TGTGATTCCATCTCTTCATCC
RP2tre1	GTGTCTGTGTCCGAGTCCAAC

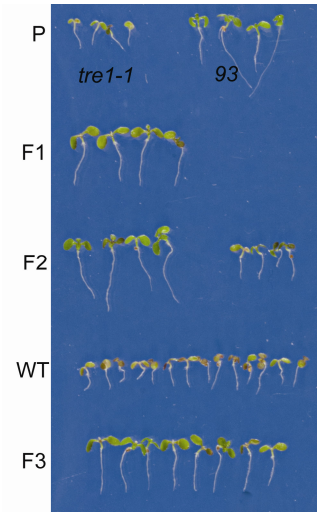
Supplemental Table SII. Primers for quantitative real time RT-PCR

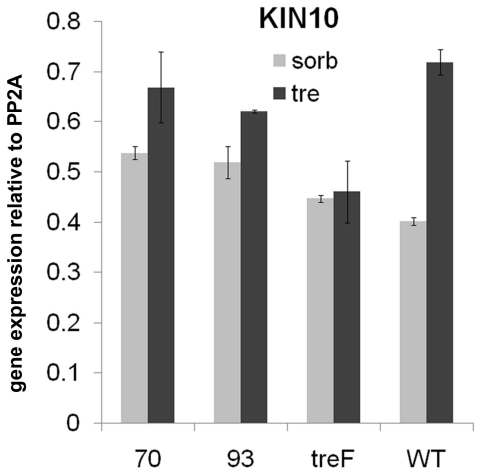
Annotation	AGI	sense	antisense
PP2A	At1g13320	TAACGTGGCCAAAATGATGC	GTTCTCCACAACCGCTTGGT
TRE1	At4g24040	AGCGAGAGAGAAAGCGTTTC	CCTTCCATGTCTCAGATTC
bZIP11	At4g34590	TCGTCAGGATCGGAGGAGAGT	GATCGTCTAGGAGCTTTTGTTTCTTC
bZIP1	At5g49450	AGAGGCTTGACTGGGTCGAA	GGTAACTCGAGAGCCAAATCTTC
KIN10	At3g01090	CCGCTCCAGAGGTAATTTCG	CACACCACAGCTCCAGACATC
ASN1	At3g47340	ATCGGAGTTCACCTTTCG	GCCTTAAGTGGTGGTATGG
PP2-A13	At3g61060	CGAATATCTCGGGAGGTTCAC	AATTCGCAGGCAACTTTGAT
BT2	At3g48360	AAGGGGTTTAATCAAAACCAGGAAG	CAGCCAATTTAAAAGGGTTTACTCC
HB-12	At3g61890	TGCAGAGACTAAACGAAGAG	ACCAAAACTCCCACCAGT
SEN1	At4g35770	GCTTCACGGCGATCACAGA	GATGATTGATACTTGCGTTGAGAAC
	At4g28040	AAATTGCTCAATGCCTTGCT	GATACAGAGCGCCGAAGAAA
PPDK	At4g15530	TTCTTGATCAGCAAGGTGTAG	TGAAAGGAGAACAAGAGACA
	At3g26510	AAGTCCTAGATCTCCGTCTCTATCA	AAGATTCTTCACGTTATTGCCAGTG
IGMT5	At1g76790	TCGTGATGACGTCACCGGAT	AATATCGACAAATCGGCTCTGCTT
EXP10	At1g26770	TAATGTATTACTGATGCAGAGGTGG	GTGATCAATCCCCCAATTTTC
TBL27	At1g70230	TTGCGGCGATGGAAGGTT	TTTTGTACTCCTCCATGGTTTGAAG
PHT1;4	At2g38940	CTGAATCTAAAGGTAAGTCACTCG	CAAAGAACAACTTGAGTTGCTAGAG
GSTU8	At3g09270	ATTTTCCAAGAAGCTTCAGGCGT	TAGATGTAACACTTCCAAACATGGC



Supplemental Figure S1. Over-expression of KIN10, the catalytically active subunit of SnRK1, in Arabidopsis seedlings results in decreased bZIP11 expression and in a physiological state much alike a carbon starvation response. (Data extracted from Supplemental Tables S3 and S4 (Baena-Gonzalez et al., 2007)). **A**, Log2 scale expression of bZIP genes in Arabidopsis seedlings over-expressing KIN10. **B**, Comparing the behavior of bZIP gene expression under carbon starvation and excess carbon conditions. KIN10 average, average values for protoplasts expressing KIN10 transiently for 6 h (Baena-Gonzalez et al., 2007); SUC STARV, cultured cells after 24 h sucrose starvation (Contento et al., 2004); SEN STARV, starvation-induced senescence (Buchanan-Wollaston et al., 2005); EXT NIGHT, extended night resulting in carbon deprivation (Thimm et al., 2004); SEN DARK, darkness-induced senescence (Lin and Wu, 2004), 1 day; GLUCOSE, 3% glucose addition (3h in the dark) to 24 h-starved seedlings (Price et al., 2004); CO2 fixation, 4-h treatment of plants with ambient [CO2] (350 ppm) vs. with low [CO2] (<50 ppm) (Blasing et al., 2005); SUCROSE, 1% sucrose addition (8 h 70 µmol m^{-2} s^{-1} light) to 48 h starved seedlings (Palenchar et al., 2004). Expression of the S-class bZIP transcription factors bZIP1 and bZIP11 is affected by KIN10 expression and the carbon status. GBF2 (At4g01120) and ABF4 (At3g19290) are bZIP but not S-class bZIP.

Supplemental Figure S2. Segregation of trehalose resistance in the F1-F3 generations after crossing *tre1-1* with FOX line 93. Seedlings were grown on 100 mM trehalose for 14 days. P1 are seedlings from the parental lines *tre1-1* and FOX line 93. F1 are first generation seedlings obtained from the cross of tre1-1 with FOX 93. F2 seedlings were obtained from seed of F1 plants and could be separated in typically long or short root phenotypes. WT are wild type Col.0 seedlings, F3 are trehalose resistant F3 seedlings obtained from an F2 plant with the bZIP11 cDNA and *tre1*|*tre1*.





Supplemental Figure S3. Expression of *KIN10* in lines over-expressing *bZIP11* from the FOX collection. Expression of *KIN10* was determined by Q-PCR in 14 d seedlings from FOX lines 70 and 93, the line expressing the *E.coli* trehalase treF (treF) and wild type Col.0 (WT). Expression is given relative to the expression of *PP2A* (At1g13320).

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CHAPTER 3

TREHALOSE FEEDING CAUSES DEETIOLATION OF ARABIDOPSIS SEEDLINGS IN THE DARK: altering PIF4/DELLA and SnRK1 pathways but not the ethylene pathway suppresses the effect

Based on:

Trehalose-6-phosphate dependent deetiolation of seedlings in the dark: linking carbon availability to developmental decisions

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To be submitted.

ABSTRACT

How developmental switches are coupled to available energy and carbon resources within the plant is not yet understood. We show here that depending on available metabolizable carbon, trehalose-6-phosphate (T6P) accumulation induces deetiolation in seedlings in the dark, effectively acting as carbon control over seedling development. T6P is an established signaling metabolite that is known to inhibit the activity of SnRK1 kinase, the plant homolog to the animal AMP dependent protein kinase and a central integrator of energy and nutrient status. T6P dependent deetiolation in the dark was suppressed when metabolizable carbon was available. T6P dependent deetiolation in the dark was furthermore partially suppressed in the absence of DELLA proteins and fully suppressed by over-expression of PIF4, a phytochrome-interacting DNA binding factor that is known to suppress deetiolation. DELLA proteins have been shown to mediate the integration of many environmental responses including light, cold, salt and heat, abiotic stresses that affect levels of bioactive gibberellins (GA). T6P dependent deetiolation in the dark was insensitive to GA, however, and was instead suppressed by increased KIN10, the catalytic subunit of SnRK1. T6P/SnRK1 and the PIF4/DELLA pathways commonly impact the expression of photosynthesis genes and data presented is consistent with T6P/SnRK1 gating growth and physiological responses otherwise mediated by PIF transcription factors.

INTRODUCTION

Trehalose is the alpha 1,-1-linked glucose disaccharide and because of the absence of reducing ends is an uncommonly stable sugar (Crowe, 2007). Trehalose biosynthesis occurs in most organisms including archaebacteria, bacteria and most eukaryotes with the exception of vertebrates and is therefore considered an ancient pathway (Avonce, et al., 2006; Avonce, et al., 2010). In plants trehalose is generally synthesized from the two central primary metabolites glucose-6-phosphate and uridine diphospho-glucose by trehalose-6-phosphate synthase (TPS). In *Arabidopsis thaliana* the biosynthetic intermediate trehalose-6-phosphate (T6P) is essential for development and required for carbon utilization for growth (Eastmond, et al., 2002; Schluepmann, et al., 2003). T6P is not sufficient for growth, however: three-fold accumulation of T6P in transgenic plants over-expressing TPS causes stunted growth, whilst its 10-fold accumulation when feeding high trehalose concentrations was shown to cause growth arrest (Schluepmann, et al., 2003; Schluepmann, et al., 2004). T6P was shown to induce indirectly the redox-activation of AGPase and so promote starch synthesis, it was also shown to inhibit SnRK1 activity, a kinase central to integration of energy and primary metabolisms (Kolbe, et al., 2005; Zhang, et al., 2009; Paul, et al., 2010). Trehalose metabolism has thus evolved into a regulatory role in plants and T6P that is present at below micro-molar concentrations has an essential signaling role relating carbon with energy status in Arabidopsis seedlings.

T6P metabolism was shown to affect development in Arabidopsis at several growth stages of Arabidopsis including, embryo development, seedling growth, floral induction and in Maize culm development but the underlying mechanisms remain unknown (Eastmond, et al., 2002; Satoh-Nagasawa, et al., 2006; van Dijken, et al., 2004). Data is presented here that sheds light on how T6P metabolism commands light development in Arabidopsis seedlings grown in the dark. When germinating under the soil surface in darkness, seedlings typically promote hypocotyl elongation and repress cotyledon development with the non-green cotyledons remaining small and folded and the apical hook piercing the soil, a development called etiolation. When reaching the surface, seedlings undergo deetiolation as they switch to light development (photomorphogenesis). Many of the molecular mechanisms triggered by light that ultimately result in the repression of hypocotyl elongation, promotion of the unfolding of the apical hook and greening and growth of the cotyledons are known (Alabadi, et al., 2008; Lau and Deng, 2010). A key pathway is that mediated by the phytochrome light receptor B (PHYB), which when activated by light leads to the degradation of the

Phytochrome Interacting Factors (PIF). PIF are bHLH transcription factors mediating repression and promotion of gene expression necessary for light or dark development. In darkness PIF4 and PIF3 accumulate, suppress deetiolation and promote hypocotyl growth. The Phytochrome/PIF pathway is further controlled by the GA/DELLA pathway known to regulate growth because DELLA proteins bind PIF at their G-box DNA binding domain thus interfering with PIF activity. In darkness of a day/night cycle, bioactive GA accumulate leading to DELLA destruction thus allowing PIF4 and PIF3 promotion of hypocotyl growth (de Lucas, et al., 2008; Feng, et al., 2008). GA by means of DELLA is not the only factor besides light regulating the PIF growth integrators: high temperature and brassinolide signals were recently shown to also be mediated by PIF (Koini, et al., 2009; Stavang, et al., 2009; Oh, et al., 2012; Leivar and Quail, 2011; Sun, et al., 2012; Hao, et al., 2012). Here we report that seedlings with increased T6P accumulation underwent deetiolation in the dark that was suppressed by removal of DELLA, over-expression of PIF4 or KIN10. KIN10 is the catalytic subunit of SnRK1. T6P dependent deetiolation was not influenced by GA or ethylene pathways but by additional supply of metabolizable carbon. This ads metabolizable sugar with the T6P/SnRK1 pathway to other factors such as light (quality), high temperature, GA and brassinolides that regulate growth through PIF factors.

RESULTS

Deetiolation and T6P accumulation in seedlings grown in constant darkness

Seedlings expressing *E.coli* TPS OtsA, accumulate three-fold the levels of T6P compared to wild type (WT) (Schluepmann, H. et al. 2003). When grown in the dark on osmoticum control, these seedlings exhibited shorter hypocotyls and larger cotyledons compared to WT (Fig. 1a OtsA/WT sorb). Moreover, when grown on trehalose medium WT and OtsA expressing seedlings accumulate high levels of T6P (Schluepmann, et al., 2004; Delatte, et al., 2011) and in the dark consistently developed even shorter hypocotyls, opened apical hook and enlarged cotyledons compared to OtsA expressors on sorbitol (Fig. 1 a,b WT tre, sorb). The symptoms associated with T6P accumulation in dark grown seedlings much resembled those during deetiolation. By 4 days after germination, hypocotyl elongation in WT on trehalose was arrested entirely (Fig. 1b).

Inhibition of hypocotyl elongation was overcome by expression of *E.coli* T6P hydrolase TreC (Fig.1 a and c TreC) that specifically cleaves T6P into Glucose-6P and glucose. Suppression by TreC suggested that T6P accumulation underlies the trehalose effect. The trehalose effect was further overcome by a simultaneous supply of sucrose (Fig.1

d) or of glucose by the expression of *E.coli* trehalase TreF (Fig. 1a) suggesting that absence of metabolizable carbon was critical when T6P accumulated.

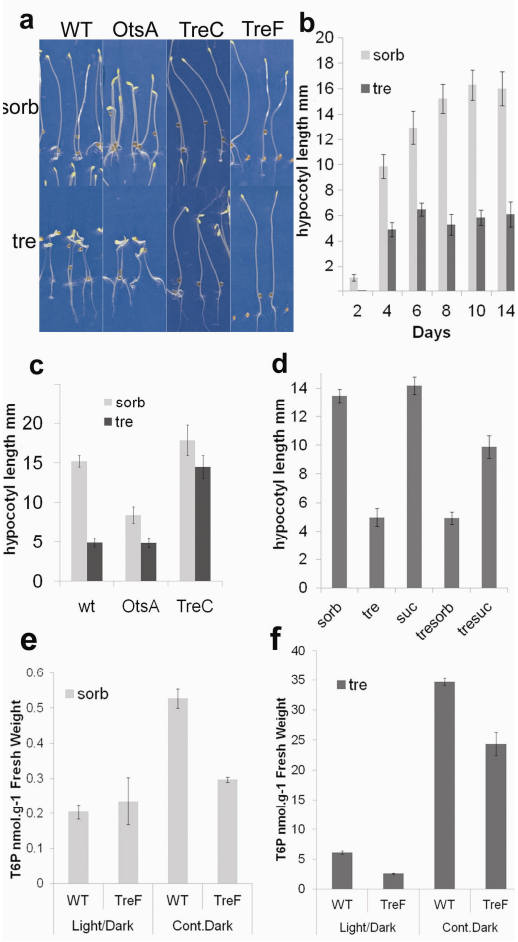

Figure 1. Deetiolation in the dark when seedlings accumulate T6P. **a** Phenotype of seedlings grown in continuous darkness on medium with 100 mM of either sorbitol osmoticum control (sorb) or trehalose (tre). WT Col.0 control (WT), the *E.coli* TPS expressing line 19.3 (OtsA), the *E.coli* T6P phosphorylase expressing line 25.6 (TreC); the *E.coli* trehalase expressing line 42.6 (TreF). Germination was induced by transfer to 22ºC and light for 6 h before growth in darkness at 20ºC for 14 days. **b** Hypocotyl lengths in mm of WT Col.0 determined from pictures using ImageJ, readings are averages of at least 20 measurements and the standard deviation thereof. **c** Hypocotyl lengths of seedlings from differing genotypes grown as in a). **d** Hypocotyl lengths of WT Col.0 seedlings grown on 100 mM sorbitol (sorb), trehalose (tre), or sucrose (suc) or the combination 100 mM trehalose with 50 mM sorbitol (tresorb) or sucrose (tresuc). **e** T6P accumulation in seedlings on 100 mM sorb grown 14 d in 16h light /8h dark cycles and harvested at midday (Light/Dark) or in continuous darkness (Cont. Dark); WT Col.0 (WT), the *E.coli* trehalase expressing line (TreF). **f** T6P accumulation in seedlings growing as in e but on 100 mM of tre. T6P measurements are averages from 5 biological replicates with standard deviation.

T6P measurements in the seedlings grown in continuous darkness revealed that trehalose feeding caused a very high accumulation of T6P, reaching 35 nmole.g^{-1} Fresh Weight (FW) in WT seedlings, more than 50-fold the levels in osmoticum control (Fig. 1 e,f). In continuous darkness, T6P levels were generally higher compared to the mid-day levels of seedlings grown in long-day conditions whether on sorbitol (2-fold) or trehalose (7-fold).

Together the data suggested that T6P accumulation is required for the observed symptoms of deetiolation in the dark. Seedlings expressing *E.coli* trehalase TreF,

however, were completely etiolated on trehalose medium (Fig1.a TreF) in spite of high T6P accumulation at 25 nmole.g^{-1} FW (Fig. 1f). Accumulation of T6P in the absence of metabolizable sugar therefore underlies the deetiolation symptoms.

T6P dependent deetiolation and the PIF/DELLA pathway

To test whether the deetiolation symptoms were those of known pathways, we tested the behavior of mutants from the Phytochrome B/PIF/DELLA pathway on trehalose medium (Fig. 2a). Seedlings over-expressing PIF4 suppressed the effect of trehalose, suggesting that the deetiolation on trehalose may be due to limited PIF activity. Consistent with this, seedlings of the quadruple mutant of PIF1, PIF3, PIF4 and PIF5, *pif1,3,4,5* had a much reduced hypocotyl elongation during etiolation (Leivar, et al., 2008) which was not further altered by trehalose medium (data not shown). The double mutant of PIF4 and PIF5, *pif4,5*, behaved like the WT consistent with repression of PIF activity on trehalose medium. The phytochrome B (PHYB) mutant also behaved like WT suggesting that PHYB is not required and therefore does not influence PIF stability on trehalose.

Since PIF protein stability is affected by the GA/DELLA pathway (de Lucas, M. et al. 2008, Feng, S. et al. 2008), we next tested if DELLA proteins are necessary for deetiolation on trehalose. The quadruple mutant (*qdella*) in the DELLA proteins RGA, GAI, RGL1 and RGL2 (*gai-t6, rga-t2, rgl1-1, rgl2-1*) had significantly longer hypocotyls compared to the WT Ler seedlings when grown on 100 mM trehalose in the dark (Fig. 2b,c). Single DELLA mutants, such as *rga24*, *gai2* (Fig. 2c) and the *rgl1*, *rgl2* double mutant (not shown) were much like the WT on trehalose medium confirming that DELLAs likely act redundantly. Results thus suggest that DELLA proteins likely repressed PIF4 activity when seedlings were grown in continuous darkness on trehalose medium.

Requirement of DELLA proteins for the repression of PIF activity in seedlings grown in constant darkness was surprising because seedlings in the dark of a day/night cycle are known to accumulate bioactive GA due to the dark-induced *GA20ox1* and repression of *GA2ox1* gene expression; the high bioactive GA concentrations in the dark of the day night cycle cause DELLA protein degradation. On the other hand several forms of stress, including salt stress were shown to reduce the accumulation of bioactive GA and thus stabilize DELLA proteins (Achard, et al., 2008a; Achard, et al., 2006; Achard, et al., 2008b). T6P accumulation in the absence of metabolizable sugars may cause a nutrient-stress response and thus affect GA levels. To test this, we studied seedlings on trehalose with increasing concentrations of GA_3. GA_3 did not affect hypocotyl length of seedlings on osmoticum control (Fig. 2d sorb GA), except at high concentrations (10 µM, supplemental Fig.1) when it delayed the time of germination and therefore reduced apparent hypocotyl length measured in 14d seedlings. Importantly, addition

of GA_3 did not affect deetiolation in seedlings grown on trehalose medium (Fig. 2d tre GA). Deetiolation of seedlings on trehalose in continuous darkness was thus insensitive to GA_3.

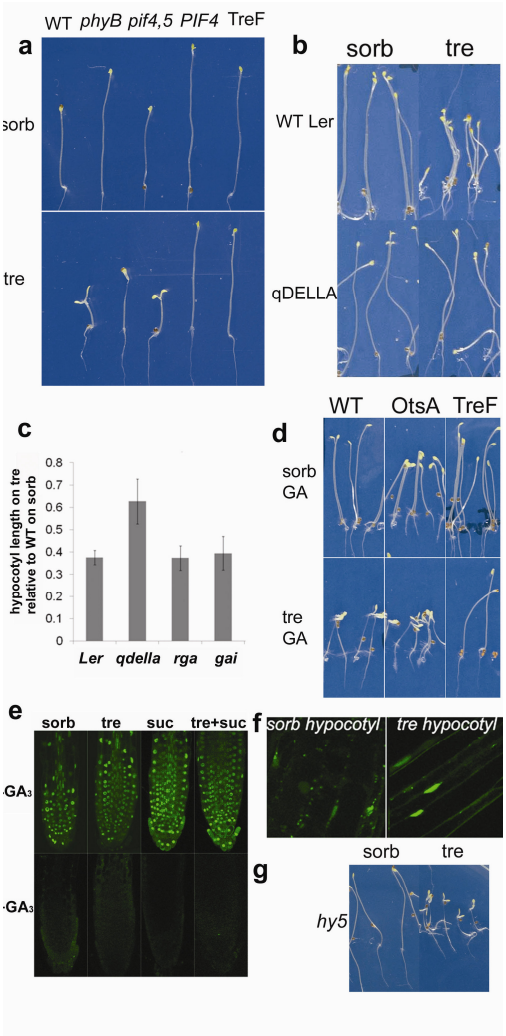

Figure 2. T6P dependent deetiolation and the PIF/DELLA pathway. a Phenotype of dark grown seedlings from WT, *phyB* mutant in Phytochrome B, *pif4,5* mutant lacking both PIF4 and PIF5, PIF4 line with 35S::PIF4 or TreF line expressing the *E.coli* trehalase. Seedlings were grown for 14 days on medium with 100 mM sorbitol (sorb) or trehalose (tre). **b** Seedling phenotypes from the quadruple mutant of the DELLA proteins RGA, GAI, RGL1 and RGL2 (qDELLA) compared to those of the WT (WT Ler) dark grown for 14 days on medium with 100 mM sorbitol (sorb) or trehalose (tre). **c** Hypocotyl lengths of seedlings grown for 14 day in darkness on trehalose medium (tre) from the WT (Ler), the quadruple DELLA knockout (qDELLA) and mutants in the DELLA proteins RGA (*rga*) and GAI (*gai*). **d** Seedlings from WT, OtsA and TreF were grown on medium containing 10 uM GA_3 with 100 mM of either sorbitol (sorb GA) or trehalose (tre GA). **e** Seedlings from the line expressing the RGA::GFP protein fusion behind the RGA promoter were grown for 2 to 7 days on media without (-GA_3) or with 10 uM GA (+GA_3) combined with 100mM of either sorbitol (sorb), trehalose (tre), sucrose (suc) or trehalose and sucrose at 50 mM each (tresuc). Under low light, seedlings were rapidly mounted in water and immediately viewed under the confocal microscope. The images represent typical root tips at 4 days. **f** RGA::GFP fluorescence in hypocotyls of seedlings grown on 100 mM of either sorbitol (sorb hypocotyl) or trehalose (tre hypocotyl). **g** Seedlings from the *hy5-1* mutant on sorbitol (sorb) or trehalose (tre).

GA and HY5 in seedlings grown continuously in the dark

If *qdella* mutants suppressed the inhibition of hypocotyl elongation in the dark on trehalose, then DELLA proteins must be present under these conditions. The line expressing the protein fusion RGA:GFP behind the RGA promoter (*pRGA-RGA::GFP*) has

previously been used as a valid reporter of DELLA accumulation in seedlings *in vivo* (Achard, et al., 2007; Silverstone, et al., 2001) and was therefore grown on medium supplemented with sugars in the dark. Confocal microscopy revealed accumulation of the fusion RGA::GFP in cells of roots and hypocotyls of seedlings from this line grown in continuous darkness irrespective of whether or not metabolizable carbon or/and trehalose was added to the medium (Fig. 2e). The RGA::GFP was found throughout the seedlings including roots and the upper third of hypocotyls where elongation occurs (Fig. 2f). Counterstaining of the nuclei with DAPI confirmed that RGA::GFP fusion protein accumulation was prominently in nuclei (not shown). We conclude that continuous darkness and not carbon nutrient stress commanded RGA accumulation. Addition of exogenous GA_3 lead to RGA removal from the nuclei (Fig. 2e). Data therefore suggested that GA_3 did not accumulate in continuous darkness unlike in the dark period of a day/night cycle; this was also observed by (Cheminant, et al., 2011). Importantly, trehalose feeding did not cause an enhanced accumulation of RGA (Fig. 2e), therefore trehalose feeding unlikely increased DELLA inactivation of PIF proteins. Another pathway than the DELLA pathway may thus be influenced by T6P.

LONG HYPOCOTYL5 (*HY5*) is a positive regulator of photomorphogenesis (Ma, et al., 2002; Holm, et al., 2002). In the dark period of a day/night cycle GA causes the COP1 proteasome mediated degradation of HY5 (Alabadi, et al., 2008). To test the possibility that HY5 was specifically stabilized on trehalose medium, we grew the *hy5* mutants *hy5-1* (Koornneef, et al., 1980) in the Landsberg erecta (ler) and *hy5-ks50* (Oyama, et al., 1997) in the Wassilewskija (Ws) genetic backgrounds on trehalose in the darkness. The mutants of both backgrounds displayed a deetiolated phenotype on trehalose (Fig. 2g, only *hy5-1* is shown). We concluded that deetiolation of seedlings grown in the dark on trehalose was independent of HY5. This conclusion was further consistent with the observation that GA did not influence T6P dependent deetiolation of seedlings grown in continuous darkness.

T6P dependent deetiolation in the dark and the SnRK1 pathway

We had previously shown that inhibition of growth by T6P accumulation when seedlings were grown in the day/night cycle could be suppressed by over-expression of KIN10, the catalytic subunit of SnRK1 (Delatte, et al., 2011). KIN10 over-expression also suppressed T6P dependent deetiolation in the dark (Fig. 3a) indicating that T6P inhibition of SnRK1 in the dark also caused T6P dependent deetiolation.

Analysis of the expression of marker genes for photosynthesis such as CAB2 and PLASTOCYANIN 1C (PC) after 10 days of growth in continuous darkness revealed that when T6P accumulated either by way of TPS expression or trehalose feeding these genes also accumulate (Fig. 3 b,c). T6P inhibition of SnRK1 in the absence of added

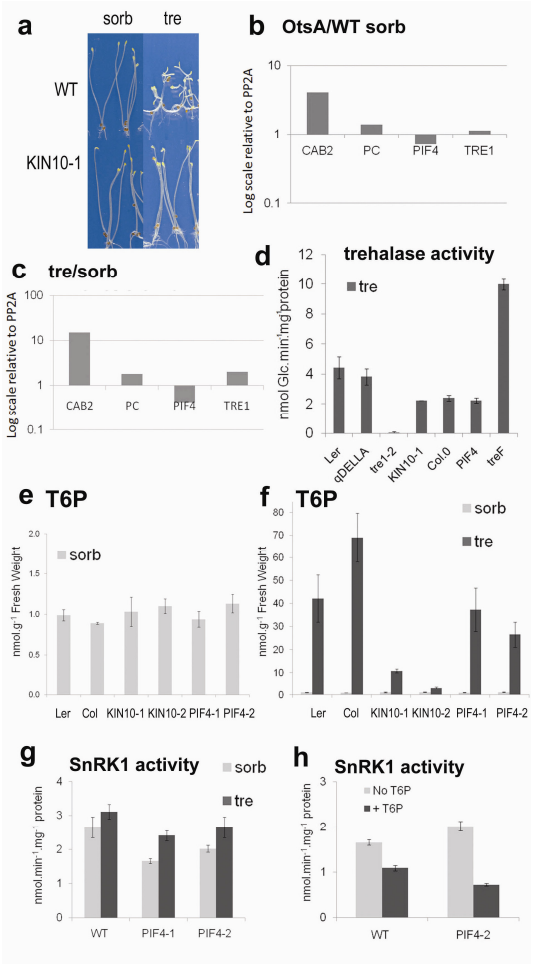

Figure 3. T6P dependent deetiolation and the SnRK1 pathway. a Phenotype of dark grown WT ler (WT) and 35S::KIN10 line 01 (KIN10-1) on sorbitol (sorb) or trehalose (tre). **b** Gene-expression in seedlings expressing *E.coli* TPS compared to Col.0 WT (OtsA/WTsorb). Seedlings were grown for 10 days in continuous darkness on sorbitol for RNA extraction and QPCR quantification of the expression of CAB2 (AT1G29920), PC (AT1G76100), PIF4 and TRE1. Readings are in Log2 scale and are averages of 3 determinations. **c** Gene-expression in WT seedlings after 10 days in the dark on trehalose compared to sorbitol (tre/sorb). **d** Trehalase acitivity in soluble extracts of seedlings grown in the dark on 100 mM trehalose: WT ler (ler), (qdella), trehalase knockout in ler background (*tre1-2*), KIN10 over-expressor line 01 (KIN10-1), WT col.0 (Col.0), 35S::PIF4 line2 (PIF4) and TreF (TreF). **e** T6P accumulation in dark-grown seedlings on sorbitol (sorb); WT ler (Ler), WTCol.0 (Col), (KIN10-1), 35S::KIN10 line 02 (KIN10-2), 35S::PIF4 line1 (PIF4-1), 35S::PIF4 line2 (PIF4-2). **f** T6P accumulation in dark-grown seedlings on trehalose (tre) compared to sorbitol (sorb). **g** SnRK1 activity of extracts from dark grown seedlings on trehalose (tre) or sorbitol (sorb). **h** SnRK1 activity assayed without (No T6P) or with 10uM T6P (+T6P).

metabolizable sugars therefore not only reduced hypocotyl elongation and induced growth of cotyledons but also induced expression of photosynthesis genes otherwise induced during photomorphogenesis. PIF4 or PIF5 are known to repress expression of photosynthesis genes and photomorphogenesis during growth in the dark (Table S1 in (Lorrain, et al., 2009)). Furthermore, a large proportion of genes switched on in the *pif1,3,4,5* mutant belongs to the ontology terms covering photosynthesis (Oh, et al., 2012; Leivar, et al., 2009). PIF and SnRK1 thus target common processes. Whilst the effects of PIF have been characterized in the dark, the SnRK1 pathway has mostly been characterized in seedlings grown in the light when PIF proteins are absent; sucrose feeding was shown to decrease SnRK1 activity and consequently repress photosynthesis of seedlings in light (Baena-Gonzalez, et al., 2007). We therefore set

out to study the interactions between the T6P/SnRK1 and DELLA/PIF pathways in seedlings grown in continuous darkness.

Neither the *qdella* mutant nor the PIF4 over-expressor had increased trehalase activity in their soluble extracts compared with WT (Fig. 3d) consistent with no measurable increase in the expression of the TRE1 gene encoding Arabidopsis trehalase (data not shown). The possibility that DELLA/PIF4 influence T6P dependent deetiolation in the dark by promoting endogenous trehalase and so provide metabolizable sugar is thus not likely.

DELLA/PIF4 may influence T6P accumulation and thereby may act upstream of T6P. Neither PIF4 nor KIN10 over-expression altered T6P accumulation in dark grown seedlings on osmoticum control (Fig. 3 e). On trehalose, T6P accumulated in WT and PIF4 over-expressors to astonishingly high levels (50-fold those in seedlings on sorbitol), but the levels are similar in these genotypes (Fig. 3 f). PIF4 therefore does not alter T6P synthesis or degradation rates. In the KIN10 over-expressors on trehalose, however, accumulation of T6P was significantly less, yet still 6-fold that in the osmoticum control (Fig.3 f). KIN10 therefore likely decreased T6P biosynthesis rates, yet the 6-fold accumulation observed was still higher than the 3-fold accumulation in the *E.coli* OtsA expressing line that exhibited the deetiolation phenotype in the dark on trehalose whilst the KIN10 over-expressors did not. We conclude that the DELLA/PIF4 pathway does not influence T6P accumulation. The DELLA/PIF4 pathway also does not have the effect KIN10 has on T6P accumulation in seedlings grown on trehalose.

DELLA/PIF4 may interfere with T6P's inhibition of SnRK1 (Zhang et al. 2009). Over-expression of PIF4 did not alter *in vitro* SnRK1 activity levels when compared to WT (Fig. 3g), and this was consistent with PIF4 over-expression not altering expression of KIN10 (data not shown). Over-expression of PIF4 also did not alter the susceptibility of SnRK1 inhibition by T6P *in vitro* (Fig. 3h). PIF4 thus did not alter T6P inhibition of KIN10 when assayed *in vitro*.

Alternatively, T6P/SnRK1 pathway is upstream of and alters the DELLA/PIF pathway. Expression of PIF4, PIF1, PIF3 and PIF5 was unaltered in the KIN10 over-expression lines (Supplemental Fig. 2), which confirmed previous observations in seedlings grown in the light (Baena-Gonzalez et al. 2007). Neither of the PIFs are therefore direct targets of SnRK1 regulation of gene-expression. Consistently, when T6P accumulated by way of TPS expression or trehalose feeding in continuous darkness, PIF4 gene expression was not repressed (less than 2-fold) to an extent which would be consistent with PIF4 absence and consequent deetiolation of the seedlings (Fig. 3 b,c). We conclude that the two pathways do not interact by altering the expression of the important players PIF4 or KIN10.

The two pathways may interact post-transcriptionally and this may be visible in shared transcriptional targets. Targets had previously been identified in seedlings grown in the dark or in red light in the case of PIF (Leivar et al., 2009) and in protoplasts in the light in the case of SnRK1 (KIN10; Baena-Gonzalez et al. 2007). Comparing the 1252 genes altered in *pif1,3,4,5* compared to WT with the 1021 genes altered after 6h transient expression of KIN10 compared to WT reveals 120 common targets, roughly 10 % of either gene set. Ninety one of the common targets were induced by KIN10 and were controlled by promoters enriched with the i-box motif GATAA (Lopez-Ochoa, et al., 2007; Athena analysis of the list obtained in R; http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/analysis_select.pl). Seven of these 91 genes belonged to photosynthesis (GO:0015979 with 196 members in Arabidopsis) yielding a GO enrichment with significant adjusted P-value of 0.001232 using Babelomics 4 (http://babelomics.bioinfo.cipf.es/). We thus confirm enrichment of photosynthesis genes in the genes jointly controlled by KIN10 and PIF4. DCMU, oxygen deprivation and extended darkness, however, have been reported to increase SnRK1 activity and the expression of photosynthetic genes (Baena-Gonzalez et al., 2007). In continuous darkness when PIF are generally present, T6P accumulation inhibited SnRK1 yet photosynthetic genes CAB2 and PC1 were induced and hypocotyl elongation is repressed. Over-expressing KIN10 and thus increasing SnRK1 activity, furthermore maintained etiolation and suppressed photomorphogenesis. In continuous darkness therefore the SnRK1 control over genes in photosynthesis were opposite. The 7 photosynthesis genes commonly regulated by PIF and KIN10 were much induced (3 to 10-fold) in the *pif1,3,4,5* mutant compared to WT. PIF thus repressed these common photosynthesis targets, as expected from previous analyses (Leivar, et al., 2009). PIF4 suppression of T6P dependent deetiolation suggested that when SnRK1 was inhibited by T6P these common photosynthesis targets may have been repressed by PIF4.

Among the 120 genes commonly regulated by PIF and KIN10, genes associated with growth were absent, this could be due to the light, the time of the day and protoplasts that had been used to determine the KIN10 targets by Baena-Gonzalez et al. (2007). ASN1 (AT3G47340) and DIN4 (3-methyl-2-oxobutanoate dehydrogenase, AT3G13450) were present however as 2-fold induced in *pif1,3,4,5* over WT. Recently, PIF along with the BRZ1 transcription factor have been reported to mediate hypocotyl elongation during etiolated growth in the dark by inducing the expression of PRE proteins (Oh, et al., 2012). Intersection of the lists of BRZ1 responsive genes (3620 genes, seedlings in the dark;(Oh, et al., 2012)) with KIN10 responsive genes (1021 genes, protoplasts in light; Baena-Gonzalez, et al., 2007) revealed a minor amount of overlap in target genes (164 BRZ1 repressed and KIN10 controlled and 10 BRZ1 induced and also KIN10

controlled). A comprehensive analysis of genes under SnRK1 regulation in the dark is missing to allow definitive comparisons.

We thus conclude that T6P dependent deetiolation in seedlings grown in continuous darkness is mediated by inhibition of SnRK1 when T6P accumulates. The data further is consistent with the involvement of the DELLA/PIF pathway downstream of SnRK1 but does not rule out that the pathways are separate. Over-expression of KIN10 in the well documented *pif1,3,4,5* mutant will tell whether KIN10 can suppress the deetiolation phenotype in the absence of PIF. Suppression of SnRK1 in the well documented PIF4 over-expressor lines would tell whether SnRK1 is required to influence the activity of PIF4. The data obtained from a genetical approach should be supplemented with data from gene expression profiling of the effect of KIN10/11 expression in the dark and its comparison to profiles obtained using PIF over-expressors and mutants.

T6P dependent deetiolation in the dark and the ethylene pathway

SnRK1 was shown to directly phosphorylate the FUS3 transcription factor and thus lead to the stabilization of FUS3 (Lumba, et al., 2012). SnRK1 could similarly affect PIF transcription factors, which would result in PIF destabilization when T6P accumulates and inhibits SnRK1. The results obtained above are consistent with such a hypothesis. Alternatively, lacking FUS3 may cause T6P dependent deetiolation. *fus3-3* mutants were indeed reported to undergo a triple response when grown in continuous darkness and exhibit short hypocotyls (Tsai and Gazzarrini, 2012a). We therefore studied the effect of the ethylene pathway and of FUS3 on T6P dependent deetiolation.

Effects of the ethylene biosynthesis inhibitor AVG and the ethylene perception inhibitor $AgNO_3$ on the inhibition of hypocotyl elongation in seedlings of differing mutants and WT grown on trehalose are shown in Fig. 4a. *eto1-1* seedlings are producing ethylene constitutively and therefore had short hypocotyls on sorbitol medium (Guzman and Ecker, 1990). Whilst AVG restored as predicted hypocotyl length in *eto1-1* seedlings on sorbitol, AVG did not suppress T6P dependent deetiolation in WT or eto1-1 seedlings on trehalose medium. Trehalose and ethylene inhibited hypocotyl growth additively as seen by the shorter hypocotyls of *eto1-1* seedlings on trehalose compared to sorbitol. We conclude that ethylene accumulation did not cause the short hypocotyls observed in WT seedlings grown on trehalose in continuous darkness.

Inhibition of ethylene perception by $AgNO_3$ furthermore did not interfere with deetiolation phenotypes on trehalose medium, ruling out a role for the ETR1 ethylene receptor in the response to trehalose feeding. Mutants in ethylene signal transduction

components including *ein2-1* (Fig. 4a) and eil/ein3 (not shown) were equally affected by trehalose as the WT. Because *ein2-1* was reported to suppress the triple response of seedlings lacking FUS3, wild type response of *ein2-1* suggested that FUS3 was not involved in T6P-dependent deetiolation. Suppression of T6P dependent etiolation in KIN10 over-expressing seedlings or the quadruple and quintuple DELLA mutants was not inhibited when the ethylene pathway was inhibited by either AVG or $AgNO_3$ (Fig. 4a) indicating that suppression of T6P-dependent deetiolation by SnRK1 and the DELLAs was entirely independent of ethylene.

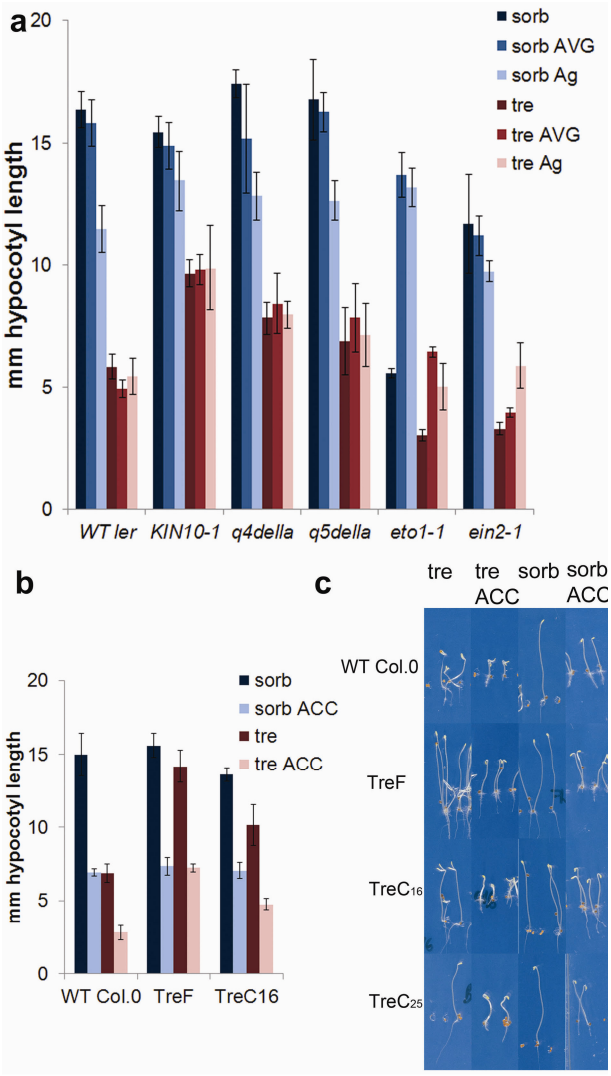

Figure 4. T6P dependent deetiolation and the ethylene pathway.

a Hypocotyl lengths of seedlings after 4 days in the dark on medium with 100 mM of either trehalose (tre) or sorbitol (sorb) alone or combined with 10 µM Aminoethoxyvinylglycine (AVG) or 100 µM $AgNO_3$ (Ag). Wild type Landsberg erecta (*WT ler*), KIN10 overexpressor line 01 (*KIN10-1*), quadruple della mutant (*q4della*), quintuple della mutant (*q5della), eto1-1* and *ein2-1* mutants.

b, c Hypocotyl lengths and images of seedlings after 4 days in the dark on medium with 100 mM of either trehalose (tre) or sorbitol (sorb) without or with 50 uM 1-aminocyclopropane-1-carboxylic acid (ACC). Wild type Col.0 (WT Col.0), *E.coli* trehalase expressing line 42.1 (TreF), *E.coli* treC expressing lines 16 and 25 (TreC16 and TreC 25).

Conversely, we established that ethylene had an additive effect on hypocotyl elongation when adding the ethylene precursor ACC (50 uM) to seedlings with altered

T6P metabolism (Fig. 4b,c). Hypocotyls of WT on trehalose medium with ACC were much shorter than those on trehalose alone. Addition of ACC inhibited hypocotyl extension of seedlings expressing *E.coli* trehalase (TreF) on sorbitol equally as on trehalose suggesting again that the effects of trehalose and ACC on hypocotyl elongation were independent (Fig. 4b TreF). Seedlings from lines expressing the *E.coli* T6P hydrolase (TreC) were less etiolated and cotyledons shorter than the TreF lines on trehalose, in part because TreF cleavage of trehalose yielded metabolizable glucose. However, the shorter hypocotyls of the TreC seedlings on the combination of trehalose medium and ACC further confirmed that the ethylene and T6P dependent inhibition of hypocotyl extension were entirely distinct pathways (Fig. 4b TreC16 and TreC25).

DISCUSSION

Results presented established that when T6P accumulates to high levels in seedlings grown in continuous darkness on trehalose without additional metabolizable carbon, seedlings switch to photomorphogenic growth in the dark; the mechanism involves T6P inhibition of SnRK1. In other words: T6P metabolite levels signal by way of the AMP dependent kinase to control development otherwise mediated by DELLA/PIF interactions. Animal physiology and light-responses are coupled to metabolism also by way of the central integrator of energy metabolism AMP-activated protein kinase: AMP-activated protein kinase controls the protein stability of CRY, a component of the cellular clock that in turn controls the circadian expression of a majority of genes (Lamia, et al., 2009). The mechanism mediates physiological adaptation but does not alter animal development. By contrast in plants, T6P inhibition of SnRK1 caused seedling deetiolation in darkness and therefore altered development.

T6P accumulation caused the developmental switch to deetiolation. Heterologous expression of *E.coli* TPS and the differential T6P accumulation in seedlings growing on trehalose established that accumulation of T6P caused inhibition of hypocotyl growth, expansion of the cotyledons and their opening, and the increased expression of CAB and PC (Fig.3). T6P is thought to increase when feeding trehalose at high concentrations (100 mM) because trehalose is transported into cells and the high intracellular trehalose slows down the T6P (100 nM) de-phosphorylation reaction by T6P phosphatase (Schluepmann, et al., 2004). It follows then that the degree of T6P accumulation on trehalose medium is a read-out of T6P biosynthesis rates. Expression of *E.coli* treC removes T6P and at the same time provides carbon. We reason, however, that the carbon provided from T6P cleavage is minimal since T6P concentrations are so low and the flux through T6P unlikely very high since trehalase inhibition by Validamycin does not lead to quantitative accumulation of trehalose (Schluepmann

and Paul, 2009). The increased expression of photosynthesis genes when T6P accumulates is consistent with earlier results showing that when T6P accumulates photosynthetic capacity of plants increased (Pellny, et al., 2004) and when T6P was much decreased cotyledons were bleached and light green (Schluepmann, et al., 2003).

The underlying mechanism for T6P dependent deetiolation in the dark was likely T6P inhibition of SnRK1 because over-expression of KIN10 in the seedlings suppressed the phenotype. This is consistent with earlier work reporting T6P accumulation in seedlings growing in day/night cycles on trehalose and with biochemical and genetic evidence for the inhibition of SnRK1 by T6P (Zhang, et al., 2009; Delatte, et al., 2011). The photomorphogenic effect of T6P accumulation was however surprising given previous reports characterizing SnRK1 targets in relation to nutrient and energy supply (Baena-Gonzalez, et al., 2007). Generally, when metabolizable sugar such as sucrose was fed to plant tissue, T6P accumulated (Schluepmann, et al., 2004; Lunn, et al., 2006), and consistent with T6P inactivation of SnRK1 (Zhang et al., 2009), the expression of photosynthesis genes was shown to decline (Baena-Gonzalez et al., 2007). Suppression of the expression of photosynthesis genes by inactive SnRK1 was shown in seedlings grown in the presence of 0.5% sucrose and in the light (Baena-Gonzalez, et al., 2007). In continuous darkness, however, inactivation of SnRK1 induced the expression of photosynthesis genes and deetiolation. Suppression of T6P dependent deetiolation by external supply of metabolizable carbon or cleavage of trehalose by trehalase suggests that the inhibition of SnRK1 by T6P and metabolizable carbon are required together to inhibit expression of photosynthetic genes and proceed with etiolated development. T6P accumulation and consequent SnRK1 inhibition on their own were insufficient to suppress expression of photosynthesis genes, to the contrary, in continuous darkness they promote photomorphogenesis. The system was behaving as if SnRK1 was active and mediating a starvation signal also found in DCMU treated or oxygen depleted plants (Baena-Gonzalez, et al., 2007). Because over-expression of KIN10 suppressed photomorphogenesis, we must conclude that SnRK1 activity was lacking and causing starvation resulting in photomorphogenesis.

In the day/night cycle and in continuous darkness, T6P accumulation caused arrest of growth in different tissues. In the day/night cycle root growth and emergence of primary leaves were stopped, whilst in the dark hypocotyl elongation was stopped. These effects on growth could all be suppressed by supply of metabolizable carbon or increasing SnRK1 activity, again pointing to the conclusion that T6P inhibition of SnRK1 caused starvation, which interfered with growth of either the hypocotyl, the root or the apical meristem. A mechanism to explain how low SnRK1 may cause starvation and thus inhibit growth is not known. (Kulma, et al., 2004) and (Nakamura, et al., 2002)

report that SnRK1-like activities control the nitrate reductase enzyme and phosphorylate the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase enzymes. Changes in the activities of both these enzymes were associated with changes in the steady state of the important allosteric regulator fructose-2,6-bisphosphate (Kulma, et al., 2004); fructose-2,6-bisphosphate was proposed to regulate synthesis of sucrose by inhibiting the cytosolic isoform of fructose-1,6-bisphosphatase (Nielsen and Stitt, 2001). If SnRK1 inactivation increased fructose-2,6-bisphosphate this then would decrease sucrose synthesis and force the accumulation of starch. Light grown seedlings that accumulate T6P on trehalose indeed accumulate large quantities of starch in their cotyledons, but they surprisingly also accumulate free sugars including sucrose (Delatte, et al., 2011; Wingler, et al., 2000); dark grown seedlings accumulating T6P in trehalose medium did not accumulate starch however (data not shown). We have yet to resolve the subcellular and organismal partitioning of the excess carbon of seedlings grown on trehalose to understand fully the potential impact of SnRK1 on enzyme activities required for growth. We already can infer from previous work, however, that T6P is required for carbon utilization, consistent with SnRK1 inactivation being required for carbon utilization and up-regulation of biosynthetic processes associated with growth (Paul, et al., 2010). The starvation in seedlings accumulating T6P without external carbon supply shown here (and in Delatte et al. 2011) further suggest that active SnRK1 is necessary for sucrose supply to cells as proposed by Bitrian et al. (2011) and is consistent with the membrane association of the enzyme complex (Bitrian, et al., 2011). Active SnRK1 apparently also reduces the rate of T6P synthesis since on trehalose medium, seedlings over-expressing KIN10 accumulated much less T6P (Fig. 3). Possibly then, SnRK1/T6P constitute a regulatory loop: when sucrose accumulates, T6P also accumulates which decreases SnRK1 and triggers biosynthetic processes until sucrose is utilized and present at low levels thus reducing T6P which triggers SnRK1 activity and sucrose import into the cells (Schluepmann, et al., 2011).

SnRK1 is furthermore an important regulator of gene expression (Baena-Gonzalez, et al., 2007). SnRK1 phosphorylation of transcription factors may therefore also be important to explain the growth inhibitory effects of T6P accumulation and thence SnRK1 inactivation. SnRK1 phosphorylation of FUSC3 was recently shown to stabilize this transcription factor and be necessary during phase transitions (Tsai and Gazzarrini, 2012a). PIFs have previously been shown to redundantly maintain etiolated growth in seedlings grown in continuous darkness as evidenced by the deetiolated phenotype of the quadruple mutant *pif1,3,4,5* (Leivar, et al., 2008). We show here that accumulation of T6P achieved a similar phenotype to *pif1,3,4,5* in hypocotyl elongation and cotyledon development. The phenotype was suppressed if PIF4 was over-expressed or when PIFs were not inhibited by DELLA as in the *quadruple and quintuple della* mutants. In addition a known PIF3 target in the dark is CAB2 (Sentandreu, et al., 2011)

and we showed that when T6P accumulated, CAB2 was de-repressed along with PLASTOCYANIN C. Together the results genetically link T6P/SnRK1 signaling to growth control by the growth integrating PIF transcription factors (Figure 5). The link is such that T6P is likely upstream of PIF function. This link is confirmed by the previous finding that PIFs mediate the sucrose induced elongation of hypocotyls after seedlings exposed to sucrose are transferred into the dark (Liu, et al., 2011; Stewart, et al., 2011). SnRK1 antisense was further shown to improve growth of seedlings on sucrose ((Baena-Gonzalez, et al., 2007), supplementary data) and so did T6P accumulation (Schluepmann, et al., 2003). It would therefore seem that in the presence of both sucrose and T6P, PIF are active and permit growth, but when T6P accumulates in the absence of sucrose then PIF activity becomes limiting thus altering development. The result is significant as it shows that a primary metabolite such as T6P is not at the bottom of the hierarchy DNA-RNA-Protein-metabolite but that by its influence on kinase signaling alters the activity of transcription factors and causes developmental switches. It further shows that such metabolite signaling when energy is low does not just arrests growth and development but alters the developmental decision of a plant.

The components linking T6P/SnRK1 and growth controlled by PIF have yet to be identified, however. Recent advances have shown that multiple factors affect the PIF central integrators of growth (Fig. 5) including environmental factors such as light, nutrient and heat stress and endogenous factors such as Brassinosteroids. On trehalose when T6P accumulates PHYB was not altering PIF stability because the PHYB mutant behaved as the WT did. We thus conclude that T6P accumulation does not influence light signaling by phytochromes. To our surprise, the RGA:GFP fusion accumulated in seedlings grown in continuous darkness when the activity of PIF is essential to maintain etiolation processes including hypocotyl growth (Leivar, et al., 2008). A role for DELLA proteins in continuous darkness on trehalose was confirmed by our observation that the quadruple and quintuple *della* mutants no longer underwent full deetiolation on trehalose (Fig. 2 and 4). Yet disappearance of RGA:GFP when adding GA_3 to all media proved that T6P accumulation did not inhibit GA_3/GID1/GID2 mediated degradation of DELLA; it further proved that T6P accumulation caused deetiolation in spite of GA_3 mediated degradation of DELLA. A possible interpretation of the data could be that T6P accumulation may reduce PIF activity in a manner that competes with DELLA. Such pathway has been recently described and involves the PAR1 and PAR2 bHLH transcription factors that bind PIF4 (Hao, et al., 2012): The PAR were shown to inhibit PIF4 and PAR1 over-expressors had a decreased hypocotyl elongation in response to GA compared to WT. PAR1 and PAR2 however may not be present in seedlings grown continuously in the dark as their expression is only induced by light.

KIN10 is a Ser/Thr kinase located at the plasma membrane and in the cytosol of undifferentiated dividing cells; it is further found in the nucleus of differentiated cells (Bitrian, et al., 2011). KIN10 was recently shown to phosphorylate and thence stabilize the transcription factor FUSCA3 (FUS3) (Tsai and Gazzarrini, 2012a; Tsai and Gazzarrini, 2012b). FUS3 is known for its importance in seed maturation where it regulates ABA and GA levels. FUS3 inhibits GA biosynthesis during seed development yet the normal maturation of *qdella* mutant seed implies that GA control of DELLA stability is not of great importance during seed maturation. A role for FUS3 during etiolated growth in the dark has just very recently been described: 5 day old seedlings of *fus3-3* have much reduced hypocotyl lengths but not seedlings of *fus3-3 ein2-1* double mutants (Lumba, et al., 2012). The authors show that when FUS3 is lacking, ethylene signaling is increased in the absence of increased ethylene. Experiments with *ein2-1* on trehalose (Fig.4) suggested that EIN2 was not involved in the deetiolation in the dark but further experiments are under way to test whether KIN10 over-expression in *fus3-3* mutants still suppresses deetiolation on trehalose.

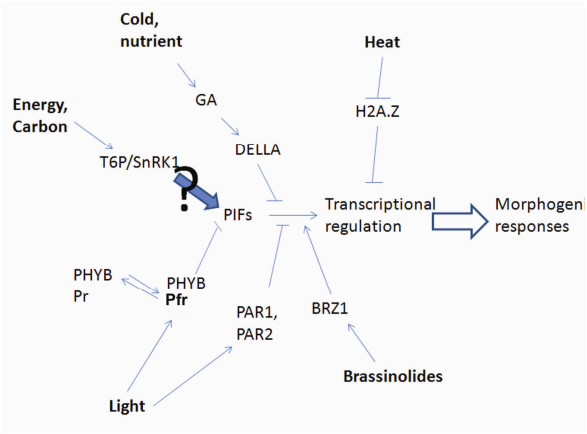

Figure 5. Metabolite control and other pathways regulating deetiolation. Simplified representation of pathways likely regulating PIF dependent etiolation. Light quality regulates PHYB levels of Pfr that in an interaction with HEMERA control the proteasomal degradation of PIF proteins (Galvao, et al., 2012). Light further stabilizes HLH transcription factors PAR1 and PAR2 that interact with PIF and inhibit PIF transcriptional regulation (Hao, et al., 2012). GA accumulation leads to the degradation of DELLA proteins; DELLA proteins otherwise interact with PIFs and interfere with DNA

We previously have shown that bZIP11, like PIF4, over-expression may suppress the effects of T6P accumulation. A link was shown between S-group bZIP bZIP11 and KIN10 (Baena-Gonzalez, et al., 2007), but bZIP11 may not be a direct target of KIN10. In addition, reports on PIF phosphorylation are revealing increasing complexity of PIF regulation by phosphorylation (Bu, et al., 2011) and PIF interaction with other transcription factors including BRZ1, PRE1 and PAR1 (Oh, et al., 2012; Hao, et al., 2012). Future work will aim to establish whether and how PIF4, bZIP11 and/or FUS3 act downstream of T6P/SnRK1 signaling.

METHODS

Plant materials and growth conditions

Lines in Col.0 accession were: Tref line 42, TreC lines 16 and 25 (Schluepmann et al., 2003), *pif4,5, and the two PIF4 over expression lines* (Lorrain et al. 2009*)*. Lines in Ler accession were: *phyB-5* (Koornneef Rolf and Spruit, 1980), quadruple *della* (Achard et al. 2006), quintuple *della* (Feng et al., 2008), *rga-24*, *gai* (Dill and Sun 2001), pRGA:GFP-RGA expressors (Silverstone et al. 2001), *tre1-2* (Vandesteene, 2009), KIN10 O1 and KIN10 O2 (Baena-Gonzalez et al., 2007). Seeds were generally vapor-phase sterilized (Clough and Bent, 1998), plated on medium and cold treated at 4^0C for 72 h in darkness. Medium used for growth of seedlings was generally agar-solidified (0.8 % w/v) half-strength MS (Murashigue and Skoog, 1962) with 100 mM of filter-sterilized sorbitol or trehalose. The plates were then transferred for light treatment during 6 h at 22^oC with 100 µM m^{-2} s^{-1} light intensity to induce germination, then grown at 22^oC in continuous darkness. Hypocotyl lengths were determined from images using ImageJ (http://rsbweb.nih.gov/ij/).

T6P determinations

Seedlings were grown in continuous darkness for 6 or 14 d. Five replicate samples of 50 mg each were harvested. Lactose-6-phosphate (5 nmol) was added as an internal standard. Materials were then ground frozen, extracted and analyzed as described in Delatte et al. (2009). Briefly, seedling extracts obtained by the subsequent liquid–liquid and solid-phase extractions were reconstituted in water and analyzed by anion-exchange chromatography combined with electrospray ionization mass spectrometry. The method provided baseline resolution of T6P and allowed its specific detection at m/z 421 with good linearity. T6P concentrations were inferred from a five-point calibration curve using the signal obtained for the internal standard to correct for potential recovery losses.

Confocal microscopy

The line expressed the RGA:GFP protein fusion behind the RGA promoter (Silverstone et al., 2001). Seedlings were grown for 2-14 d on MS supplemented with 100 mM of either sorbitol or trehalose in continuous darkness. Seedlings were mounted with the root only between the cover slips or the entire seedling when studying the hypocotyl, mounting was immediately before visualization under the confocal microscope (63x NA 1.4 Plan apochromat water immersion objective, Leica SP2 inverted laser confocal microscope with an Ar 488-nm laser excitation, dichroic 488/543/633 and emission settings 562-588 nm).

RNA extraction and Q-PCR

RNA was extracted from flash frozen seedlings collected at midday (50 mg FW). Samples were in triplicate biological replicates for each data point and the extraction protocol was according to the instruction manual (Spectrum Plant Total RNA Kit, Sigma-Aldrich). DNase treatment, reverse transcription and Q-PCR were as described previously (Hanson et al., 2008). Primers used for the Q-PCR reactions are described in Delatte et al. (2011). Primers for the PIF genes were from the CATMA site http://www.catma.org/.

Assays for trehalase activity

Trehalase activity was assayed as previously described with some modifications (Brodmann et al., 2002). Seedlings were grown on trehalose medium in continuous darkness. Seedlings were pooled as 70 mg FW and ground in 50 µl Buffer (0.1 M MES/KOH (pH 6.3), 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM DTT, 0.01% (vol/vol) Triton-X-100) at 4^{o}C. Subsequently, 100 ul Buffer was added, the crude extract mixed then centrifuged 5 min, 13000 rpm at 4^{o}C to remove the insoluble fraction. Sugars in the soluble supernatant were then removed by repeated dilution, then the proteins in the extract were concentrated using regenerated cellulose membrane (Amicon Ultra- 0.5 ml 10K Ultracel, Millipore), and with 3 consecutive additions of 300 µl 20 mM MES/KOH (pH 6.3). The final volume of extract was adjusted to 50 µl. To assay trehalase, extract (10 µl) was incubated in triplicate with 15 mM trehalose for 1 h at 37^{o}C, then boiled for 10 min. Alternatively control assays were boiled immediately. Glc released from trehalose was quantified (Enzytec D-Glucose kit , scil Diagnostics, Viemheim, Germany) as the difference between boiled controls and samples which were assayed for 1 h. Values were averaged from 3 biological replicates with standard deviations.

Assays for SnRK1 activity

Seedlings were grown for 14 d in 100 mM sorbitol or trehalose, snap frozen as 100 mg FW replicates. Three biological replicates for each data point were each ground frozen then extracted and T6P as well as sugars removed by the desalting procedure previously described (Zhang et al., 2009). SnRK1 activity determinations were using the AMARA peptide as substrate.

In silico analyses of shared targets for SnRK1 (KIN10) and PIF

Lists of targets for KIN10 and PIF were extracted from (Oh, et al., 2012; Leivar, et al., 2009; Baena-Gonzalez, et al., 2007) and analysed using R

(http://manuals.bioinformatics.ucr.edu/home/R_BioCondManual) to extract intersections and draw Venn diagrams. The lists obtained from R were further fed into the analysis suite of Athena for visualization of potential regulatory sequences in the promoters of targets (set at 1 kb upstream of the TATAA; http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/analysis_select.pl).
Enrichment of GO terms was analyses using Babelomics 4 (http://babelomics.bioinfo.cipf.es/).

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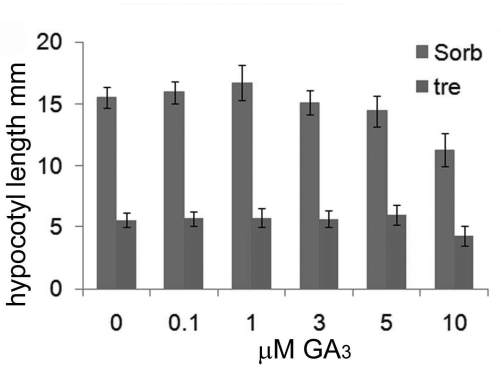
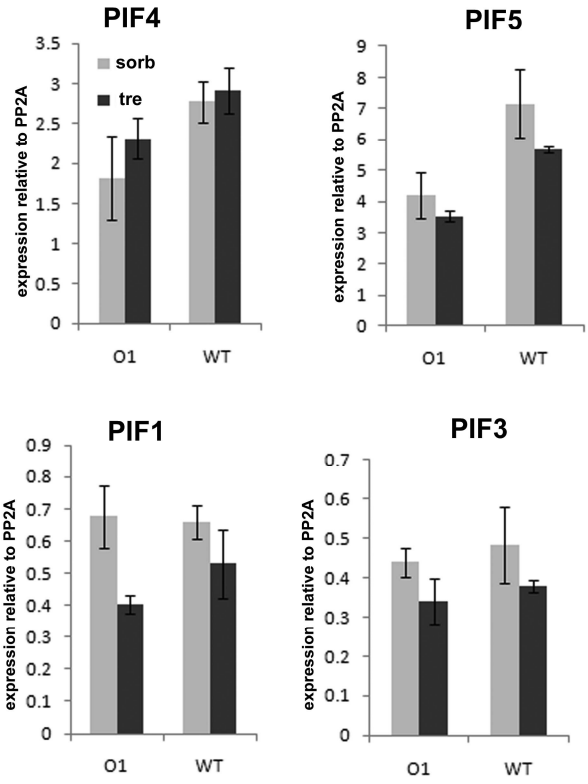


Supplemental Figure 1. GA and hypocotyl elongation on trehalose.

Seedlings were WT Col.0 grown for 14 days in continuous darkness on sorbitol (sorb) or trehalose (tre) with in addition GA_3 as indicated.

Supplemental Figure 2. Expression of PIFs in KIN10 over-expressors and WT Col.0.

Seedlings were grown for 4 days in continuous darkness on 100 mM sorbitol (sorb) or trehalose (tre).



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CHAPTER 4

ENGINEERING TREHALOSE LEVELS IN PLANTS INDEPENDENTLY OF THE INTERMEDIATE SIGNALING MOLECULE T6P

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ABSTRACT

The aim of this chapter was to generate, characterize then test transgenic plants with altered trehalose content but unchanged trehalose-6-phosphate. Trehalose synthase enzymes from bacterial trehalose biosynthetic pathways were first confirmed to be active as recombinant proteins in *E.coli* extracts. Trehalose synthase from *Thermobifida fusca* (TreS) and Trehalose glucosyl transferase (TreT) from *Rubrobater xylanophylus* were cloned into Gateway vectors for constitutive expression in plants. The gene-constructs were designed to target the enzymes either to the cytosol or the chloroplast; chloroplast targeting was achieved using the *Silene pratensis* ferredoxin plastid transit peptide (Rensink, et al., 2000). The gene-constructs were inserted in Gateway vectors with and without fusion to GFP at the C-terminus (Nakagawa, et al., 2007), then transformed into *Arabidopsis thaliana*. Both TreS:GFP and TreT:GFP were expressed and the enzyme-GFP fusions extracted from transgenic plants were active in *in vitro* assays. For easy tracking of the recombinant proteins, only lines with the enzyme-GFP fusions were further analysed. Confocal microscopy revealed that the TreS:GFP and TreT:GFP fusions were located in the cytosol. In contrast, when the ferredoxin transit peptide was present the GFP fusions were mostly in plastids. The reaction catalyzed by TreS is reversible and *Arabidopsis thaliana* seedlings expressing TreS:GFP were resistant to trehalose medium. The TreS:GFP fusion was thus active *in planta* and TreS could be used as selectable marker to select transgenic plants on medium with trehalose. Preliminary analyses revealed that plants expressing high levels of the transgenes were indistinguishable from wild types under differing growth conditions including standard growth chamber, drought stress, osmotic and salt stress, but this work needs refinement in the future.

INTRODUCTION

Trehalose is accumulated in high amounts in anhydrobiotic organisms, including in a few desert plants where it is thought to protect desiccated tissues (Crowe and Crowe, 1982; Crowe, 2007). In crop plants, however, trehalose is present at nearly undetectable levels. Genes encoding enzymes of the trehalose metabolism have been expressed in crop plants to successfully increase plant resistance to various types of stress. Trehalose phosphate synthase (TPS, OtsA) and trehalose phosphate phosphatase (TPP, OtsB) from *E.coli* or TPS from yeast have been expressed first in tobacco ((Holmström, et al., 1990; Pilon-Smits, et al., 1998), then in tomato, potato and rice as well as other crops summarized in (Iordachescu and Imai, 2008). The resulting transgenic plants were improved in their resistance to drought, heat, cold and salt.

Phenotypic abnormalities in plants with engineered trehalose metabolism, including stunted growth, were observed from the beginning. Accumulation of the biosynthetic intermediate trehalose-6-phosphate (T6P) is one of the likely reasons for the phenotypic alterations (Pellny, et al., 2004; Schluepmann, et al., 2003). Fusing both TPS with TPP enzymes to channel the trehalose biosynthesis reactions and avoid the possible accumulation of T6P as the intermediate product resulted in transgenic plants with resistance to a variety of stresses and with a phenotype that is undistinguishable from the wild type plants (Garg, et al., 2002; Jang, et al., 2003). These authors suggest that trehalose rather than T6P accumulation may be necessary to elicit stress resistance. The question however, has not yet fully been resolved, since the enzymes used to mediate stress resistance were from the TPS/TPP pathway of trehalose biosynthesis and T6P was not measured in these studies. Expressing yeast TPS targeted to chloroplasts also appeared to suppress the side effects observed when the enzyme was targeted to the cytosol (Karim, et al., 2007).

An alternative approach to studying the effects of trehalose independently of those of T6P is to engineer plants with prokaryotic pathways for trehalose biosynthesis that do not involve T6P as an intermediate. Pathways that require a single enzyme include the conversion of maltose into trehalose by trehalose synthase TreS (Wei, et al., 2004), the conversion of Glc and ADP-Glc into trehalose by trehalose glucosyl transferase TreT (Kouril, et al., 2008) or the conversion of glucose-1-phoshate and glucose into trehalose by trehalose phosphorylase TreP (Ren, et al., 2005). The TreS reaction is reversible and reported to reach an equilibrium at 60% trehalose and 40% maltose (Wei, et al., 2004). The TreT reaction was unidirectional unless the trehalose level exceeded more than 100-fold the substrate level (Kouril, et al., 2008). The reaction

catalyzed by the TreP enzyme from the protozoa Euglena was reported reversible (Marechal and Belocopitow, 1972).

Trehalose steady state in Arabidopsis may possibly also be increased when trehalase activity is reduced. Arabidopsis has a single known gene encoding a trehalase, AtTRE1 (Muller, et al., 2001). AtTRE1 knock down mutants have been described but not fully characterized with respect to trehalose and T6P accumulation and their stress response characteristics (Vandesteene PhD thesis Feb. 2009, KU-Leuven; (Delatte, et al., 2011a)). Applications of the trehalase inhibitor validamycin A (called jinggangmycin in China) have been used for years with a variety of crops against fungal pathogens (Zhou, et al., 2012). Validamycin A inhibits both fungal and plant trehalases, in plants it leads to trehalose accumulation (Goddijn, et al., 1997). It is therefore unclear whether the efficacy of validamycin A solely results from inhibition of the fungal trehalose metabolism or from changes in plant trehalose metabolism as well. In contrast, decrease of trehalose in Arabidopsis may be achieved by expression of *E.coli* cytosolic trehalase (TreF), an enzyme known to be active when expressed in these plants (Schluepmann, et al., 2003; Schluepmann, et al., 2004). Yet, again TreF expressing plants have not been studied with respect to trehalose content and resistance to stress.

Finally trehalose steady state has been successfully manipulated in plants simply by feeding trehalose and in this way resistance to biotic stress was obtained. Stress resistance was obtained by feeding 13 mM trehalose to wheat prior to infection with wheat powdery mildew (Reignault, et al., 2001; Renard-Merlier, et al., 2007). Feeding wild type Arabidopsis seedlings with 100 mM trehalose leads to high T6P accumulation and concomitant growth arrest as seen in the previous two chapters of this thesis. The feeding of much lower levels to wheat elicited very different responses uncoupled from T6P. The low trehalose levels fed to wheat enhanced ROS production upon infection of wheat with wheat rust (Renard-Merlier, et al., 2007). Similarly, adding ascorbic acid in the medium reduces the growth inhibitory effect of trehalose on Arabidopsis (Aghdasi, PhD thesis Utrecht Univerity, 2007). Ascorbate peroxidases catalyse the reduction of hydrogen peroxide to water by using ascorbic acid as a specific electron donor, therefore ascorbic acid suppression of the growth inhibitory effect of trehalose possibly occurs by way of scavenging hydrogen peroxide. ROS are produced partly in chloroplasts due to photooxidation at photosystem II during stress (Murata, et al., 2007), it could therefore be that trehalose affects chloroplast metabolism. Effects in chloroplast metabolism are further supported by results from Garg et al. (2002). Targeting trehalose biosynthetic enzymes to chloroplasts would be an avenue to study the role of trehalose specifically in these organelles.

In this chapter we report on the cloning and expression of prokaryotic trehalose synthase TreS from *T. fusca* (TfTreS) and *M. tuberculosis* (MtTreS) and trehalose glucosyl transferase TreT from *R. xylophillum* (RxTreT); expression of the recombinant proteins was in *E.coli* and *Arabidopsis thaliana*. Recombinant TfTreS expressed in *E.coli* was confirmed to convert trehalose into maltose but proved susceptible to validamycin A, an inhibitor thought to specifically inhibit trehalases. Recombinant enzymes fused to GFP proved active when expressed in Arabidopsis whether targeted to the cytosol or chloroplasts. The GFP fluorescence of the fusion proteins was used to facilitate screening of homozygous transgenic plants for high levels of enzyme expression over four generations. Arabidopsis expressing TreS were resistant to medium containing trehalose. TreS could therefore be used as selectable marker in Arabidospis. Initial screening for enhanced resistance to a variety of stresses of plants engineered in the trehalose metabolism was not conclusive.

RESULTS

Trehalose synthases TfTreS and MtTreS for selection of transgenic plants on trehalose

Previous research has shown that trehalose fed to plant tissues arrests growth except in tissues with high trehalase activity (Wingler, et al., 2012; Veluthambi, et al., 1982a; Veluthambi, et al., 1982b). Trehalase enzymes are inhibited by validamycin A (Asano, et al., 1987; Gibson, et al., 2007); validamycin A in combination with trehalose has been successfully employed to arrest growth of plant tissues with high trehalase activity (Wingler, et al., 2000). A selectable marker overcoming the toxicity of the combination of trehalose and validamycin A could be a convenient and novel way to select transgenic plant tissue. The trehalose synthases TfTreS and MtTreS have not previously been reported to be sensitive to validamycin A and may be good candidates as selectable markers in plant tissue on the combination trehalose/validamycin A. Trehalose would be converted to maltose by these enzymes and maltose would then be further dissimilated by the plants'disproportionating enzymes thus releasing glucose. Since metabolizable sugars suppress the effect of trehalose accumulation in plants as seen in Chapters 2 and 3 of this thesis, plants would be able to grow on trehalose medium.

The trehalose synthase enzymes TfTreS and MtTreS were tested for their susceptibility to trehalase inhibitor. TfTreS was cloned by PCR amplification from a *Thermobifida*

fusca colony, *T.fusca* is a gram-positive bacterium member of the Actinobacteria. MtTreS cDNA was from *Mycobacterium tuberculosis* (Pan, et al., 2004). TfTreS and MtTreS were expressed in *E.coli* using the nopaline synthase promoter permitting constitutive expression of the recombinant proteins. For easy purification, the proteins were fused in frame at the C-terminus of the proteins with the myc-tag followed by 6 histidines (myc-His). For convenient assay of the enzymes, expression was carried out in the *E.coli* mutant KU101 (Uhland, et al., 2000). KU101 has no periplasmic trehalase (*treA*::SpecR) and is not able to import maltose or trehalose from the medium into the cytosol (*ΔtreRBC)*. Consequently, when grown on LB-medium supplemented with either trehalose or maltose, KU101 does not acidify the medium due to glucose-induced switch to fermentation and lactic acid release into the medium as does *E.coli* DH5α (Fig.1 a DH5α and KU101). Medium pH was monitored by adding bromothymol blue pH indicator dye to the media; upon acidification, these media turn from blue to yellow.

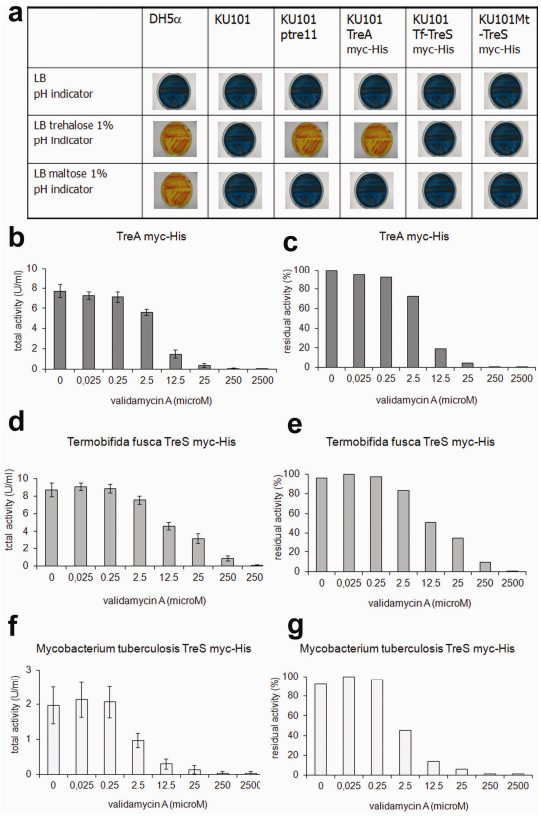

Figure 1. Activities of recombinant TreA, TreS from *Thermobifida fusca* (TfTreS) and TreS from *Mycobacterium tuberculosis* (MtTreS). **a** The TreA precursor contains a signal peptide targeting the protein to the periplasm whilst both TreS are cytosolic. *Ecoli* DH5α and the mutant KU101: (DHB3, *treA*::SpecR, *ΔtreRBC, leu*::Tn10 TetR) (JBC 275:23439-23445,2000) were grown on LB medium with pH indicator and either without or with 1% w/v trehalose or maltose. KU101 (*ΔtreRBC*) cannot import trehalose or maltose into the cytosol. ptre11 contains TreA under its endogenous promoter. **b** Activity of TreA myc–His in crude extracts of *E.coli* KU101 with increasing trehalase inhibitor validamycin A. One unit of TreA is defined as the amount of enzyme converting one micromole of trehalose into glucose per minute. **c** Residual acitivity of TreA myc-His with increasing validamycin A in % of the activity without inhibitor. **d** Activity of TfTreS myc-His in crude extracts of *E.coli* KU101 with increasing validamycin A. One unit of TreS is defined as the amount of enzyme converting one micromole of trehalose into maltose per minute. **e** Residual activity of TfTreS with increasing validamycin A. **f** Activity of MtTreS myc-His. **g** Residual activity of MtTreS with increasing validamycin A.

Expression of the unmodified or myc-His tagged TreA, *E.coli* periplasmic trehalase (Gutierrez, et al., 1989) in KU101 resulted in active enzyme releasing glucose which is then imported into the cells and fermented as evidenced by acidification of the medium with trehalose (Fig1. a KU101 ptre11 and KU101 TreA myc-His). TreA specifically cleaves trehalose and does not cleave maltose as no acidification is seen on medium with maltose. In contrast, KU101 expressing TfTreS myc-His and MtTreS myc-His did not acidify the medium (Fig. 1 a) suggesting that the recombinant proteins were in the cytosol or inactive. Furthermore, constitutive expression of the enzymes did not lead to *E.coli* toxicity as the cells grew as rapidly as the WT.

KU101 is derived from *E.coli* DHB3 which is also devoid of TreF, the cytosolic trehalase from *E.coli* (Horlacher, et al., 1996). Absence of contaminating trehalases allowed assaying TreS in crude cell extracts of KU101 using trehalose as substrate for the formation of maltose. Maltose is a reducing sugar whilst trehalose is not. Maltose produced from trehalose could thus be detected after reduction of 3,5-dinitrosalicylic acid (DNS) by absorption at 537 nm (red coloring; (Miller, 1959)). As control for an active reducing sugar producing enzyme, we first tested extracts from KU101 expressing *E.coli* periplasmic trehalase fused to myc-His (TreA myc-His; Fig. 1 b). One unit of TreA is defined as the amount of enzyme converting one micromole of trehalose into glucose per minute. TreA activity reached 8 U/ml and was completely inhibited by the trehalase inhibitor validamycin A at 250 µM. Results thus suggested that expression in KU101 allowed for assay of trehalose conversion by reducing sugar assay with no contaminating activities. Half maximum inhibition of the enzyme was at about 5 µM validamycin A (Fig 1 c). Assay of crude extracts from KU101 expressing TfTreS myc-His revealed that the enzyme was very active reaching 8.5 U/ml (Fig. 1 d). One unit of TreS was defined as the amount of enzyme converting one micromole of trehalose into maltose per minute. TfTreS myc-His is also inhibited by validamycin A with half maximal inhibition at 12.5 µM validamycin A (Fig. 1e). Activity of MtTreS myc-His in crude extracts was lower than that of TfTreS (Fig.1 f); MtTreS myc-His was more susceptible to validamycin than TreA with half maximum inhibition at about 1 µM validamycin A. MtTreS myc-His enzyme was also less stable in the extracts which could explain the lower activity detected.

The His-tag allowed for purification of the recombinant enzymes using NiNTA. Active TfTreS, MtTreS but not of TreA eluted from the NiNTA columns. Activity of TfTreS eluted within 3 column volumes (Fig. 2a Activity), the enzyme was not pure however (Fig. 2a Protein SDS-page).

Testing the activity of NiNTA purified TfTreS using proton NMR to follow conversion of trehalose into maltose confirmed that the preparation converted trehalose into

maltose, yet glucose also appeared in the assay after 24 h (Fig. 2b). Addition of validamycin A at 25 µM to the assay inhibited the formation of maltose completely, it also inhibited the formation of glucose. This confirmed the susceptibility of TfTreS to validamycin A. We cannot conclude however whether the preparation was contaminated by a maltose cleaving enzyme or whether TfTreS also cleaves one of its substrates to glucose. Together the data suggest that recombinant TfTreS is more active than recombinant MtTreS and that both enzymes are susceptible to the trehalase inhibitor validamycin A. We conclude that TreS is not a suited candidate marker gene for transgene selection of plant tissues with high trehalase activity on medium with the combination of trehalose and validamycin A. TfTreS remains an interesting enzyme to promote trehalose accumulation from maltose and therefore generate plants with increased trehalose but no changes in T6P.

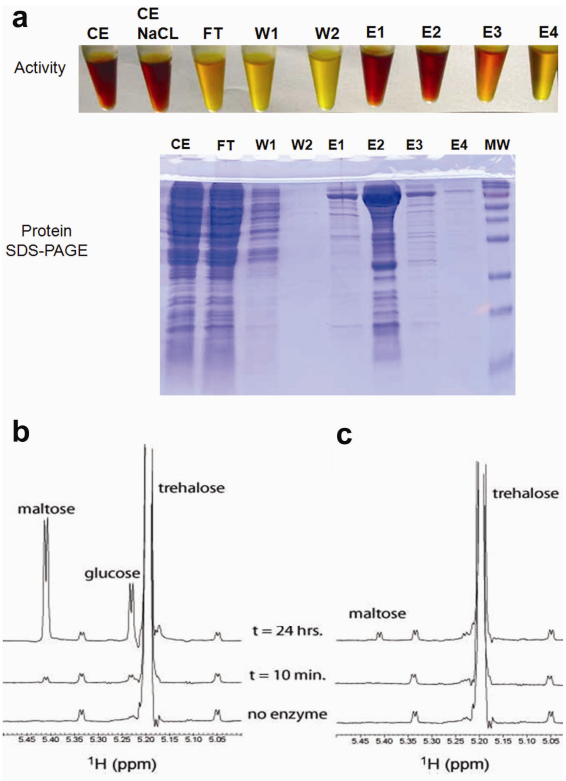

Figure 2. Purification and characterization of TfTreS myc-His expressed in *E.coli* KU101. a Purification from crude extract (CE) using NiNTA, fractions were eluted from the column then assayed for TreS activity (activity) and analysed by SDS-PAGE staining proteins with coomassie blue (protein SDS-PAGE). TreS activity assays were using the DNSA method detecting reducing sugars (red). Fractions were as following and correspond between Activity and Protein SDS-PAGE: CE, CE assayed in the presence of wash buffer 0.5 M NaCl (CENaCl), flow through after loading the column (FT), first wash (W1), second wash (W2), elution with the first column volume (E1), the second column volume (E2), third (E3) and fourth (E4). **b** Proton NMR using 100 mM trehalose substrate without and with NiNTA purified MtTreS enzyme (E2 fraction) after 10 min and 24 h. **c** As in b but with 25 mM validamycin added to the assay.

Generating Transgenic plants with trehalose synthase TfTreS and trehalose glucosyl transferase RxTreT

RxTreT cDNA encodes a trehalose phosphorylase from a gram positive bacterium also belonging to the actinobacteria: *Rubrobacter xylanophilus* (Nobre, et al., 2008). Genes encoding TfTreS and RxTreT enzymes were transferred by recombination (Gateway) to plant expression vectors pGWB502 and pGWB505 (Fig. 3; Nakagawa, et al., 2007). In addition to the cytosolic default targeting, the enzyme sequences were fused at the N-terminal end to the *Silene pratense* ferredoxin transit peptide (TP) sequence targeting the enzymes to plastids (Rensink et al. 2000). To permit tracking of the recombinant protein the gene constructs were further fused to GFP at the C-terminal end (Fig. 3) using the gateway vector Improved pGWB505 (Nakagawa, et al., 2007). After floral dip transformation, transgenic seedlings were selected on hygromycin (T0) and then transferred to soil to generate T1 and subsequently T2 seeds. T1 seedlings were selected on hygromycin to identify lines with 3:1 segregation of the hygromycin resistance consistent with single insertion of the transgene. T2 seedlings were again selected on hygromycin to identify seed batches from homozygous T1 plants. These homozygous seeds were then used as a starting material for analyses of transgenic plant lines.

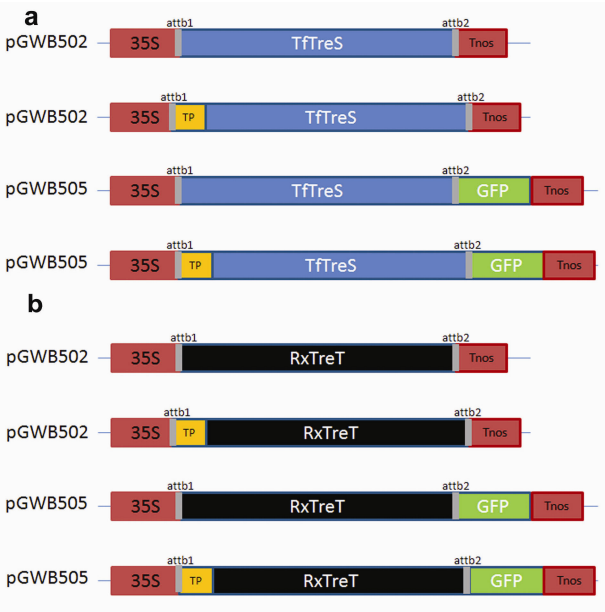

Figure 3. Summary of the constructs for transformation into plants. Four constructs were generated for TfTreS (**a**) and RxTreT (**b**). In every case the genes were expressed behind the Cauliflower Mosaic Virus 35 S promoter (35S) and with the nopaline synthase poly-adenylation signal (Tnos) provided by the two gateway vectors pGWB502 and pGWB505. The two recombination sites (attb1 and attb2) flank the TfTreS and RxTreT genes without and with the transit peptide from *Silene pratensis* (TP) fused in frame to the N-terminus of the enzyme sequence (Rensink, et al., 2000). Insertion of the attb2 site for each enzyme was such that it allowed in frame fusion of the green fluorescent protein (GFP) at the C-terminal ends.

TreS activity was first determined using desalted extracts from plants incubated with 200 mM trehalose substrate and detecting maltose or glucose released using the reducing sugar assay (Fig. 4a,b). Plants from two independent lines transformed with the empty vector control were unable to convert trehalose into reducing sugar (Fig. 4a). The assay detected very effectively the glucose released from trehalose by extracts of the line expressing *E.coli* trehalase, TreF. Reducing sugar released from trehalose was also detected in plants of the independent transgenic lines 19 and 20, expressing the fusion proteins TreS:GFP and TPTreS:GFP respectively . Levels of activity varied substantially from one plant to the next for the line 20 with TPTreS:GFP, the fusion targeted to the plastids. To interpret the results, we proceeded with detection of glc released after incubation with 200 mM of either trehalose or maltose as substrate; typical results obtained are summarized in Fig. 4b. Extracts from the transgenic lines with empty vector released little glucose from trehalose, below 0.1 nmole.mg-1 FW, and released more from maltose, 0.4 nmol.mg^{-1} FW. This suggests that whilst trehalase is little active, disproportionating enzyme converts some maltose to glucose and is more active than trehalase in control plants. Consistently, extracts from active TreS (eg.plant 20.6) incubated with 200 mM trehalose substrate released more glucose than empty vector control: maltose produced is likely further converted by disproportionating enzyme in the extract causing glucose release. Extract from TreF expressing plants release large amounts of glucose compared to those expressing TreS as expected. Growing seedlings with TreS targeted to the cytosol yielded seedlings that were able to thrive on trehalose medium (Fig. 4c). Since the TreS:GFP and TPTreS:GFP fusions were active, we decided to proceed solely with GFP-fusion lines: GFP allowed monitoring of the levels of protein and subcellular localization in each plant.

To test the subcellular targeting of the TreS:GFP and TPTreS:GFP enzymes, seedling shoots, protoplasts and roots were studied using confocal microscopy with settings to detect GFP fluorescence. Subcellular localization was most clear of interfering chlorophyll fluorescence in roots (Fig. 4d). No fluorescence was seen in the WT, whilst strong fluorescence could be detected throughout the cytosol of the line with TreS:GFP (Fig. 4d,TreSC4). In the lines expressing TPTreS:GFP with the transit peptide such as TPTreS20, fluorescence was typically associated with plastids with possibly some pro-enzyme also in the cytosol (Fig. 4d TPTreS20). When protoplasts were made from leaves of lines expressing TfTPTreS, GFP fluorescence typically overlapped with the fluorescence of the chlorophyll in chloroplasts (not shown). We conclude that recombinant TfTreS is active *in planta* when targeted to the cytosol or plastid and that it can be used as a selectable marker for transgenic Arabidopsis seedlings grown on trehalose.

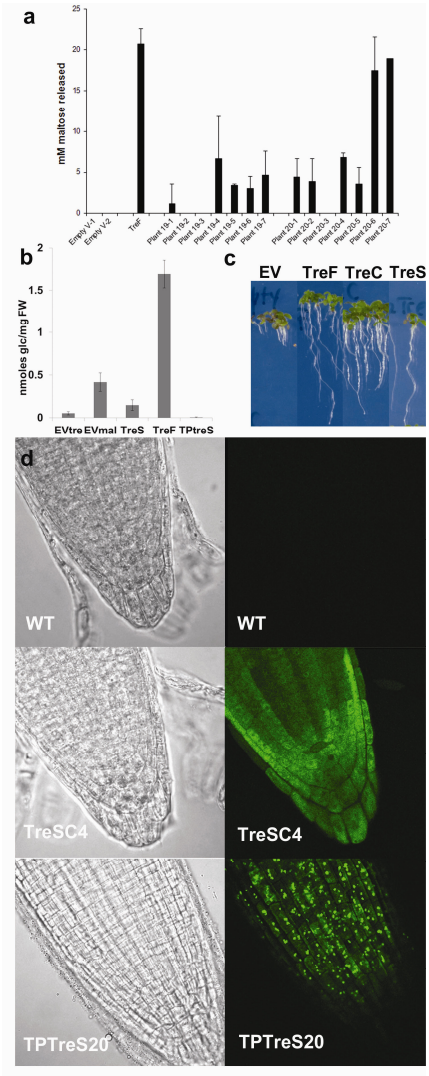

Figure 4. Characterization of plant lines expressing TfTreS:GFP and TPTfTreS:GFP.

a TreS activity in extracts of plants from line 19 expressing TreS:GFP (Plant 19-1 to Plant 19-7) and line 20 expressing TreS:GFP fused to the transit peptide TP, TPTreS:GFP (Plant 20-1 to Plant 20-7). Negative control, extracts were from 2 lines with the empty vector pGWB505 (Empty V-1; EmptyV-2). Positive control, extract was from the plant line expressing *E.coli* trehalase (TreF). TreS was detected with 200 mM trehalose substrate and sugars formed detected by reducing sugar assay.

b Glucose formed in the assays using trehalose as substrate (EVTre, TreS, TreF, TPTreS) or maltose as substrate (EVmal). Assays were with extracts from plants with the empty vector (EVtre, EVmal), the TreS:GFP (TreS), TreF or TPTreS:GFP (TPtreS).

c Seedlings after 14 day growth on medium with 100 mM trehalose. Transgenic seedlings were with the empty vector (EV), TreF (TreF), *E.coli* trehalose phosphorylase TreC (TreC) or TreS:GFP (TreS).

d Images of Nomarski and confocal fluorescence microscopy set for detection of GFP. Seedling root tips were from wild type Col.0 (WT), from the line expressing TreS:GFP (TreSC4) and from the line expressing TPTreS:GFP (TPTreS20).

RxTreT enzyme converts ADPGlc and glucose into trehalose. The reverse reaction being inefficient, we proceeded to assay the enzymes' activity in the forward reaction detecting trehalose production by HPLC/MS (Fig. 5a). Plants of the line TPTreT2-4:GFP were chosen for the assay because GFP fluorescence was particularly bright. Desalted extracts from these plants assayed after boiling revealed sucrose contamination after de-salting (Boiled GFP-tagged TreT). In contrast, desalted extracts assayed directly revealed both sucrose and trehalose (60^{o}C GFP-tagged TreT). TreT fused to GFP extracted from transgenic Arabidopsis is therefore active in *in vitro* assays. Growth assays of seedlings on trehalose revealed that the transgenic plants with the TreT constructs were not able to thrive on trehalose medium, suggesting that the reverse

reaction from trehalose to ADP-glucose and glucose was not catalyzed. It is possible that the catalysis was too slow at the temperature of 22°C, or that the reaction remained irreversible in spite of the high trehalose (100 mM) supplied to the medium. Confocal microscopy revealed that the TreT:GFP fusion was located in the cytosol in the absence of the transit peptide (Fig. 5b TreT2.2), and that the transit peptide targeted the fusion protein successfully to plastids (Fig. 5b TPTre11. 3).

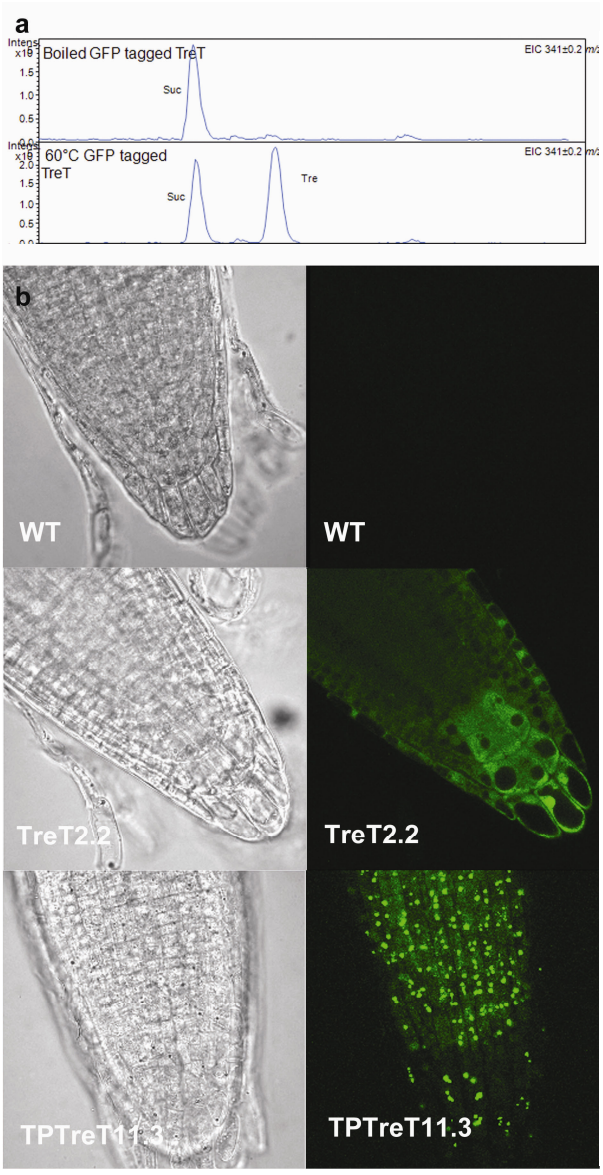

Figure 5. Characterization of plant lines expressing RxTreT and TPRxTreT.

a Sugars in the HPLC/MS elution profiles of m/z 341 obtained after TreT assay using boiled extract (Boiled GFP tagged TreT) or native extract (60°C GFP tagged TreT). Sucrose peak (suc), trehalose peak (tre).

b Images of Nomarski and confocal fluorescence microscopy set for detection of GFP. Seedling root tips were from wild type Col.0 (WT), from the line expressing TreT:GFP (TreT2.2) and from the line expressing TPTreT:GFP (TPTreT11.3).

We conclude that RxTreT is expressed and active as a GFP fusion, but its activity may be restricted to higher temperatures. This conclusion is not definitive however since only a restricted number of plants could be screened for activity of the enzyme.

Analyses will have to be carried out to determine trehalose content of the lines generated with either TfTreS or RxTreT. In the mean time, the lines were tested for resistance to a variety of abiotic stresses but results were not fully conclusive and will need confirmation.

DISCUSSION

Expression of recombinant trehalose synthase from *T. fusca* in either *E.coli* or Arabidopsis yielded active enzyme. In Arabidopsis, TfTreS retained its activity when it was targeted to chloroplasts or fused at its C-terminus with GFP. TfTreS furthermore could be used as a selectable marker to identify transgenic Arabidopsis on trehalose. TfTreS however is susceptible to inhibition by the trehalase inhibitor validamycin A. Expression of recombinant trehalose glucosyl transferase RxTreT from *R. xylanophylus* also yielded active enzyme when measured in *in vitro* assays but we have yet to show that the RxTreT is active in plant tissue. We conclude that using enzymes from prokaryotic trehalose biosynthesis pathways that do not involve the intermediate T6P may be an avenue to manipulate plant trehalose metabolism. Ultimately transgenic Arabidopsis expressing these active enzymes will be important in the dissection of the relative roles of trehalose and T6P. Dissection will be possible when the lines generated will be used along with lines previously characterized expressing TreF, OtsA, TreC, OtsB and or null mutants in the only known Arabidospis trehalase AtTRE1.

The specificity of validamycin A as a trehalase inhibitor is challenged by our observation that it also inhibits the TreS enzymes from both *T.fusca* and *M. tuberculosis*. This is consistent with a previous report of these enzymes from the genus *Bradirhizobium* (Streeter and Gomez, 2006). Streeter and Gomez further report that trehazolin specifically inhibits trehalase without affecting activities of trehalose synthase and malto-oligosyltrehalose synthase in nodules of soyabean. TfTreS may thus still prove a useful selectable marker for plant tissues with high trehalase activity when selecting on the combination of trehalose and trehazolin. This will be tested in the future when trehazolin will become available commercially (El Nemr and El Ashry el, 2011).

TreT expressing Arabidopsis seedlings did not grow on 100 mM trehalose. The enzyme was reported to be active at temperatures ranging from 20 to 80 ^{o}C (Nobre, et al., 2008). Results thus suggest that TreT did not catalyse the reverse reaction yielding ADP-glucose and glucose. Previous characterization showed that 150 mM trehalose was required for a measurable reverse reaction rate likely due to the high K_m values for trehalose (82 ± 18 mM) and for ADP (6.8 ± 0.8 mM). The high Km for ADP would indeed preclude reverse reaction in plant cells. (Nobre et al. 2008). The irreversible reaction catalyzed by RxTreT contrasts with the reversible reaction of TreS and is useful since we aim at generating Arabidopsis that accumulate trehalose in either the cytosol or the plastids.

The work in this chapter has allowed to confirm enzyme activities in the plant lines and show that they were subcellular targeted to cytosol and plastids using the *Silene pratensis* ferredoxin transit peptide studied previously (Rensink, et al., 2000). The next step will consist in determining the levels of trehalose in the plant lines. TreS targeted to the plastids may interfere with maltose metabolism at in night in these organelles (Zeeman, et al., 2007). TreS lines should be particularly studied at night. However, because the TreS reaction is reversible the potential for trehalose accumulation in these lines is less than it is for the TreT expressing lines.

The preliminary analyses comparing the stress response in the lines generated with that in lines transformed with empty vector were not conclusive. Lines expressing TreT enzyme in particular need additional screening for activity. An improved method to assay TreT activity in a higher throughput will need to be developed for a more systematic screen. Alternatively lines may be selected on the basis of accumulating trehalose before further studies on the responses to differing abiotic stresses. Given the many reports documenting that changes in trehalose steady states cause stress tolerant plants (Iordachescu and Imai, 2008), these studies are likely going to provide interesting insight. The lines should not be used in isolation but rather in combination with the complete set of Arabidopsis plants engineered in the trehalose pathway. The only limitation will be the constitutive expression of the prokaryotic genes used in our approach. Given the restricted expression of the TPP genes to specific cell files under specific conditions for example (Vandesteene, et al., 2012) the consequences of using constitutive and ubiquitous expression should also be addressed in the future.

MATERIALS AND METHODS

Materials

E.coli KU101 strain was obtained from Konstanz University and is described fully in (Uhland, et al., 2000). TreS cDNA from *Thermobifida fusca* was amplified from a culture of the CBS, The Netherlands (http://www.cbs.knaw.nl/). TreS cDNA from *Mycobaterium tuberculosis* was as described in (Pan, et al., 2004; Pan, et al., 2008). TreT cDNA from *Rubrobacter xyllanophyllus* was obtained from Anna Nobre (Nobre, et al., 2008).

Cloning for constitutive expression in *E.coli*

cDNA of TfTreS was amplified from TfTreS in pGMTeasy using the primers (Forward ATGGAGAAGTCCATGGCCACACAGCCGGC and Reverse GATTGGGAAGCTCTAGAGACCGCTGGGTC) thus inserting the Nco1 restriction site at the start and and Xba1 site allowing for in frame fusion with myc epitope and polyhistidine tag at the end of TfTreS. After digestion with Nco1 and Xba1 enzyme, the PCR product was ligated into pAS200 expression vector. The procedure was analogous for MtTreS and TreA and plasmids obtained are described in Fig. 1

Cloning for expression in plants using Gateway vectors

PCR amplification using gateway compatible primers with attb1 and attb2 recombination sites: trehalose synthase (TreS) from *T. fusca* was amplified from our existing TfTreS construct, TreS-pBin1935S. While RxTreT from *R. xyllanophyllus* was amplified from TreT in pET11a (Nobre, et al., 2008). 1821 bp TfTreS and 1251 bp RxTreT PCR products were inserted into pDONR221. The procedure was repeated with primers containing the Silene Pratensis transit peptide (Rensink, et al., 2000). The recombination reaction mixtures then were transformed into *E. coli* DH5α and selected on kanamycin containing LB medium. Restriction digests that cut at outside and inside of the gene were used for elementary check of the clones prior to sequencing confirmation. Two clones with correct sequences of each construct were inserted in plant expression vectors, Improved pGWB502 and pGWB 505 allowing C terminal GFP fusion and driven by 35S promoter (Nakagawa, et al., 2007). A summary of the constructs generated is presented in Fig. 3. After re-sequencing check in the destination vectors transformed in *E. coli*, the plasmids were transferred into *Agrobacterium tumefaciens* C54. Transformation of Arabidopsis was by the floral dip method (Clough and Bent, 1998).

Selection of transgenic plants

Seeds harvested from the transformed plants were selected on half-strength MS with 20 mg/L hygromycin (Duchefa). Seedlings resistant to hygromycin then were transferred to soil, T1 generation seed was again germinated on hygromycin to evaluate segregation of the transgene, only lines were chosen for further evaluation where segregation was 1:3 sensitive to resistant, that is consistent with a single insertion of the transgene. Resistant seedlings from chosen lines were transferred to soil to harvest T2 seed. T2 seeds were again selected on Hygromycin to identify T2 seed batches with 100 % resistance to hygromycin from T2 plants that were homozygous for the transgene. In parallel, inspection of seedlings using fluorescence imaging allowed to identify lines with high expression of the recombinant enzyme fused to GFP over the 2 generations.

Detection of GFP fluorescence and confocal microscopy

Detection of recombinant proteins with GFP was using the Fluorescence stereomicroscope (Leica) equipped with a camera with fluorescence setting optimized for GFP visualization. Alternatively and particularly for roots the confocal microscope was used. Confocal microscopy of seedling roots was as described in Chapter 3.

Assays of recombinant enzymes in *E.coli*

Levels of trehalase are high in *E.coli* DH5*α* used for cloning, precluding direct assay of TreS in crude extracts. Expression vectors were thus transferred to *E.coli* KU101 devoid of trehalases TreA and TreF and also devoid of the transport system for trehalose and maltose (Uhland, et al., 2000). *E.coli* were then grown on LB medium supplemented with pH indicator (bromothymol blue) and with trehalose or maltose 1 % w/v to determine the location of the recombinant proteins. Having established that TreS enzymes were cytosolic, cells grown overnight in LB medium were resuspended in 50 mM K-phosphate buffer pH 6.5 and disrupted in a French Press on ice. Cell extracts were incubated with 100 mM trehalose +/- validamycin A in 50 mM K-phosphate buffer pH 6.5 at 30ºC for 2 h. Total reducing sugars released from trehalose were quantified in Miller (1959). The recombinant RxTreT enzyme was adequately described and was thus not further investigated (Nobre, et al., 2008).

Assays of recombinant enzymes in Arabidopsis plant extracts

TRES assay

Crude extract was prepared from 100 mg snap frozen leaves ground frozen twice for 1 minute at 2500 rpm using the dismembranator (Braun). 100 µL of 0.1 M phosphate

buffer pH 6.6 was added to the leaf powder, thoroughly mixed using the vortex and centrifuged at maximum speed (12,000g benchtop centrifuge) at 4^{o}C for 20 minutes. Clear supernatants were transferred to a Centrifugal Filter Unit (Amicon Ultra 0.5 mL 10K Millipore) to wash and concentrate the protein twice with 300 uL of 0.1 M phosphate buffer pH 6.6 yielding 50 µl of desalted crude extract.

In order to detect TRES activity, the reverse activity of TRES was chosen as this allowed convenient detection of the reducing sugar maltose over the nonreducing substrate trehalose using the reducing sugar assay with dinitrosalicylic acid (DNS). 15 µL of crude extract was added to 15 µL of 200 mM trehalose in phosphate buffer pH 6.6. As control, 15 µL crude extract added to 15 µL of buffer was used just to see the background sugars. The reaction mixtures then were kept at 25^{o}C for overnight to have maximum product during the assay. A 30 µL of DNS solution was added to the mixtures and incubated at 60^{o}C for 20 min. The red color developed shows the amount of maltose and glucose converted from trehalose, and was read at 570 nm wave length using a plate reader.

Note: To prepare the DNS solution, 1 g dinitrosalicylic acid was dissolved in 20 mL 2N NaOH with warming and vigorous stirring. 30 g Sodium potassium tartrate was dissolved in 50 mL distilled water. Both solutions were mixed well and adjusted to a volume of 100 mL by adding distilled water. DNS solution was then dispensed as 2 mL aliquots and kept at -80^{o}C until used.

Checking if TRES activity is contaminated with TreF was done in two ways, firstly by measuring trehalase activity in the extracts and secondly by measuring glucose content in the product of TRES. Trehalase assay was carried out as described (Delatte, et al., 2011b). This required use of the glucose determination kit (Sigma).

TRET assay

Crude enzyme was extracted from 50 mg frozen leaf powder. 300 µL of 20 mM Tris buffer pH 8.0 was added to the powder, mixed thoroughly by vortexing in the cold room followed by centrifugation (12,000g, 5 min, 4^{o}C, Eppendorf benchtop centrifuge). Supernatant was transferred to a Centrifugal Filter Unit (Amicon Ultra 0.5 mL 10K Millipore) to wash and concentrate the protein twice with 300 µL of Tris buffer, yielding 50 µl of desalted crude extract. The enzyme activity was assayed at 20 mM Tris buffer pH 8.0 containing of 15 mM glucose, 8 mM ADP-glucose, 4 mM $MgCl_2$ and incubated at 60^{o}C for 2h. The reaction was stopped by boiling the mixtures for 10 min. Trehalose produced was measured using HPLC/MS (Dionex) as previously described (Delatte, et al., 2009).

Testing the stress resistance of the transgenic plants obtained

Osmotic and salt stress assays were carried out by germinating and growing seedlings axenically on agar solidified (8 $g.l^{-1}$ agar, Duchefa) half-strength Murashige and Skoog medium supplemented with increasing concentrations of Poly Ethylene Glycol (PEG 500, Merk), 300 mM sorbitol or 200 mM NaCl in long-day conditions (16 h light, 8 h dark). Heat assays and combinations of heat and cold stresses were as above on half-strength MS.

Drought assays were carried out with 3 week old plants grown in pots filled with a mixture of sand and potting soil (1:1 by volume). Growth conditions were 100 $nmolm^{-2}.s^{-1}$ light intensity (conventional tube lights, Philips), 70 % relative humidity, long-days and 22^{o}C. Irrigation was stopped until the plants lost turgidity, then re-watered for 2 weeks.

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CHAPTER 5

SUMMARIZING DISCUSSION

This thesis started with defining plant stress resilience and its societal significance in Chapter 1. We realized that not just the absolute yield matters to farmers but rather the predictability of the yield given the agricultural practices in the socio-economic context. Climate change challenges the predictability of yield, calling for crops and agricultural practices resilient to less predictable environment. Historically crop breeding has most significantly increased crop yield, particularly in the most recent times (Evenson and Gollin, 2003). We therefore expect crop breeding to contribute significantly to climate change resilient crop production. Early breakthroughs in breeding a crop resilient to extremes in precipitation include the SCUBA rice varieties capable of tolerating submergence (Singh, et al., 2010). These are proof of concept achievements that illustrate the importance of understanding crop stress at the molecular level.

Molecular mechanisms involved in plant responses to stress include early perception of the changes in plant environment, conversion of the changes perceived into differing signals, signal integration and transduction to specific responses. We realized that signal perception mechanisms can be very diverse and also depend on the organisms as different organisms have evolved differing life strategies (Araus, et al., 2008). The downstream signal integrators can be very conserved, however. One such signal integrator that is conserved between animal, fungi and plant kingdoms is the AMP-dependent protein kinase (AMPK). AMPK relates energy metabolism to stress

responses by its action on cellular metabolism, membrane transport and nuclear gene expression (Baena-Gonzalez and Sheen, 2008). Plant AMPK is called SnRK1 and SnRK1 was shown to phosphorylate key metabolic enzymes such as nitrate reductase and sucrose synthase, and phosphorylate transcription factors (for example FUSCA3; Tsai and Gazzarrini, 2012). SnRK1 is known to regulate the expression of a large set of genes (1021 genes in protoplasts) oppositely regulated when plants experienced energy stress by way of DCMU, extended night and anoxic conditions or when plants were exposed to high CO_2 or fed with sugars (Baena-Gonzalez, et al., 2007). Another signal integrator that is conserved between animal, fungi and plant kingdoms comes from an evolutionarily conserved metabolism of the very stable sugar disaccharide: trehalose.

Trehalose metabolism is known from intensively studied fungi such as the bakers'yeast to be associated with energy storage and abiotic stress tolerance (Crowe, 2007). The sugar's stability and chemical characteristics convey protection to molecular structures, particularly to cell membranes, upon de hydration and rehydration. Trehalose is used as additive to manufacture vaccines and dry blood preparations that may be stored at room temperature for example. The sugar's biosynthetic precursor trehalose-6-phosphate (T6P) also has regulatory properties: in yeast T6P was shown to regulate the entrance into glycolysis through its inhibition of hexokinase (Blazquez, et al., 1993). A role for trehalose metabolism in plant tolerance to a variety of stresses is also known in plants: plants with engineered alterations in the trehalose pathway have been repeatedly reported to be tolerant to drought, salt, cold and heat stresses (Iordachescu and Imai, 2008). Evidence for a role of trehalose metabolism in the tolerance to biotic factors was also reported both in model plants and in crops (Reignault, et al., 2001; Singh, et al., 2011). Molecular mechanisms involved in the stress tolerances described have not been established.

In the remaining chapters of this thesis we therefore set out to further understand the role of trehalose metabolism in plant stress responses. In Chapter 2 we establish that T6P controls growth of Arabidopsis seedlings because it inhibits SnRK1 activity. This is done combining biochemical and genetic approaches: we monitored growth and T6P accumulation in seedlings with manipulated T6P levels and in seedlings over-expressing the catalytic subunit of SnRK1. Interestingly, we describe that when feeding trehalose at high levels (100 mM), this causes T6P accumulation and consequent inactivation of SnRK1 and growth arrest. The link between SnRK1 and T6P when feeding trehalose is interesting given the popular use of the trehalase inhibitor Validamycin A in crop protection (Zhou, et al., 2012). Validamycin A not only arrests fungal growth as it stops remobilization of the fungal carbon reserve trehalose, it also leads to trehalose accumulation in the plant and activates plant defense responses

(Zhou, et al., 2012; Goddijn, et al., 1997). Complementing evidence shows that feeding trehalose (5mM) to wheat protects the crop against fungal infection (Reignault, et al., 2001). Validamycin A applications or feeding trehalose at low concentrations did not alter the plant's growth, but it is possible that it altered T6P concentrations sufficiently to modulate SnRK1 and thence prime the plants'response to stress. Further research will be required to evaluate the role of T6P inhibition of SnRK1 when priming stress resistance in plants with Validamycin A or trehalose. We further show that over-expression of the transcription factor bZIP11 can suppress the growth inhibition observed when T6P accumulates on trehalose. Genes commonly regulated by SnRK1 and bZIP11 were much affected by trehalose feeding suggesting that SnRK1 inhibition leads to diminished bZIP11 stability, activity or import into the nucleus. Whilst bZIP11 has no demonstrated role in stress responses, it is known to play a role in the integration of carbon and nitrogen metabolisms (Hanson, et al., 2008). A role for bZIP11 in T6P inhibition of growth has yet to be confirmed however because the over-expression of bZIP11 may have displaced other G-box binding transcription factors important for seedling growth as we discover in Chapter 3. Chapter 2 represents a milestone in discovering mechanisms relating primary metabolism by way of the signaling metabolite T6P to growth (Delatte, et al., 2011).

In Chapter 3 we establish that T6P inhibition of SnRK1 in continuous darkness leads to deetiolation of seedlings. Previous literature had established that sugars inhibit expression of photosynthethic genes in seedlings grown in light/dark cycles because sugar feeding inhibits SnRK1 (Baena-Gonzalez, et al., 2007). In continuous darkness, therefore the SnRK1 responses can be opposite. We further show that when stabilizing PIF transcription factors in the quadruple and quintuple DELLA mutants T6P dependent deetiolation is partially suppressed; when over-expressing PIF4 T6P-depedent etiolation is completely suppressed. PIF transcription factors have been shown to integrate the growth responses of many environmental cues including heat, cold and light. PIF transcription factors further mediate the growth responses by environmental factors such as the gibberellic acid and brassinolide hormones (Oh, et al., 2012). PIF transcription factors also bind the G-box in promoters. Results are consistent with SnRK1 mediating growth responses by way of the DELLA/PIF pathway but we have yet to establish definite proof by studying the growth response in continuous darkness of seedlings with active SnRK1 lacking PIF. Chapter 3 represents a milestone towards understanding metabolite control of development: T6P inhibition of SnRK1 signals carbon availability and seedlings thus undergo deetiolation.

In Chapter 4 we hypothesize that trehalose may have a role distinct from T6P. We therefore test the activity of enzymes from prokaryotic trehalose biosynthesis pathways that do not involve T6P as precursor (Avonce, et al., 2006). Trehalose

synthase from *T. fusca* (TfTreS) and trehalose glucosyl transferase from *R. xylophilum* (RxTreT) were expressed in Arabidopsis and enzymatically active. The plants with active enzyme were undistinguishable from the WT under normal growth conditions. Further research is needed to assess the impact of the enzymes in the transgenic lines produced with regards to trehalose accumulation and the plants' tolerance to abiotic stress.

Taken together results obtained in this thesis begin to unravel the role of trehalose metabolism in plant growth and development by way of the biosynthetic precursor T6P. It is very well possible that T6P may also be responsible for priming the plants to a variety of stress responses because plants altered in SnRK1 activity have been shown to be tolerant to a variety of stresses. In the future we will further test the role of trehalose in stress responses and attempt at dissecting that role away from T6P.

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SUMMARY IN DUTCH

Door fotosynthese in planten worden koolstofdioxide en water omgezet in suikers (sucrose, glucose en fructose) en zuurstof. Door fotosynthese geproduceerde suikers vormen de basis voor de groei van in essentie alle organismen op aarde. Suikers dienen als substraat voor alle anabole processen en voor de energieproductie die alle levende processen in stand houdt.

Suikers zijn niet alleen substraat voor het metabolisme maar zijn ook signaalmoleculen en hebben een functie vergelijkbaar met hormonen. Suikers worden door receptoren waargenomen en initiëren signalen waarop een reactie volgt, bijvoorbeeld in de vorm van groei en reproductie van een organisme of opslag van reserves in de vorm van bijvoorbeeld zetmeel of lipiden. Ook hebben suikers een belangrijke rol bij het reageren op stress signalen uit de omgeving. Een centrale regulator in eukaryote organismen is het AMP geactiveerde proteïne kinase (genaamd AMPK in animale systemen en SnRK1 in planten). Dit kinase heeft in sleutelrol in de integratie van stress signalen die de energiestatus van de plant aantasten. Als in planten suikers en daardoor energieproductie beperkend zijn, wordt SnRK1 geactiveerd met als gevolg dat het metabolisme in de spaarstand komt en de groei stopt. Bij het opnieuw beschikbaar komen van suikers, bijvoorbeeld door de fotosynthese, wordt SnRK1 geïnactiveerd en de groei gestimuleerd. Gevonden is dat SnRK1 activiteit wordt geremd door het regulatormolecuul trehalose 6-fosfaat (T6P). Dit T6P wordt aangemaakt als voldoende suikers in de vorm van sucrose aanwezig zijn en signaleert de hoog-suiker status van de cel. Gevonden is dat T6P een essentieel signaleringsmolecuul is. Zonder T6P komt groei en reproductie van de plant tot stilstand. T6P is een signaalmolecuul dat aan cellen de 'licentie' geeft om suikers te gebruiken voor groei en opslag. Het model is dat suikers de aanmaak van T6P stimuleren waardoor SnRK1 geremd wordt en de suikers gebruikt kunnen worden voor groei. In Hoofdstuk 2 van dit proefschrift worden de experimenten beschreven die de relatie tussen T6P, SnRK1 en de groei van planten aantonen. Het onderzoek suggereert dat deze regulatiemodule ook een rol speelt bij de resistentie van planten tegen ziekteverwekkers.

In Hoofdstuk 3 wordt onderzoek beschreven naar de wijze waarop ontwikkeling van de plant is gekoppeld aan beschikbare suiker en energieniveaus. Gevonden is dat T6P de de-etiolatie reactie kan onderdrukken van in het donker gekiemde zaailingen. In het donker vertonen zaailingen een etiolatie reactie, waarbij de groei van de hypocotyl sterk wordt gestimuleerd (elongatie) en de cotylen gevouwen blijven. In het licht

wordt hypocotyl elongatie geremd en ontvouwen de cotylen zich (de-etiolatie). Kunstmatige verhoging van T6P in het donker induceert de de-etiolatie reactie. Deze de-etiolatie reactie wordt onderdrukt door toevoeging van metaboliseerbare suikers. De T6P geïnduceerde de-etiolatie reactie kon worden onderdrukt door overexpressie van het PIF4 eiwit (een DNA bindend eiwit wat bindt aan de lichtsensor fytochroom) en in mutanten waarin DELLA eiwitten (componenten in de gibberellinezuur (GA) signaaltranductie) afwezig zijn. Van PIF4 is bekend dat het de-etiolatie onderdrukt, terwijl DELLA eiwitten etiolatie stimuleren. De-etiolatie kon ook worden onderdrukt door overexpressie van KIN10, de enzymatisch actieve (eiwit kinase) component van het SnRK1 complex. T6P/SnRK1 en PIF4/DELLA signaleringswegen reguleren de expressie van fotosynthesegenen en de resultaten suggereren dat T6P/SnRK1 een effect op de groei heeft door aan te grijpen op de PIF transcriptie factoren. Op deze wijze wordt de beschikbaarheid van suikers zoals gesignaleerd door T6P gekoppeld aan groei en ontwikkeling van de plant.

Allerlei stressfactoren uit de omgeving remmen de groei van planten, waarbij droogte en zout stress sterk de groei remmen. Uit onderzoek is gebleken dat het neutrale, niet-reducerende suiker trehalose het effect van omgevingsstress op de groei planten aanzienlijk kan verminderen.

RINGKASAN DALAM BAHASA INDONESIA

Seiring dengan meningkatnya penduduk dunia yang paralel dengan meningkatnya kebutuhan akan pangan, sandang dan segala perangkat yang mendukung kehidupan manusia, daya dukung lingkungan justru menurun antara lain akibat dari peningkatnya jumalah penduduk berikut pemenuhan kebutuhannya. Diantara kebutuhan tersebut, kebutuhan akan pangan adalah kebutuhan paling mendasar dan menuntut untuk terus diupayakan ketersediaannya. Dengan menurunnya kwalitas lingkungan untuk memproduksi pangan, diperlukan beberapa pebdekatan agar produksi tanaman pangan dapat memenuhi kebutuhan. Memodifikasi tanaman pangan melalui breeding program ataupun rekayasa genetika meruapakan salah satu pendekatan yang telah banyak dinikmati hasilnya guna peningkatan kwantitas maupun kwalitas hasil produksi. Mengupayakan tanaman agar tahan terhadap beberapa stres biotik maupun abiotik marak dilakukan. Memanipulasi trehalose metabolisme pada tanaman pangan telah mengubah tanaman menjadi lebih tahan terhadap garam, kekeringan dan suhu dingin (Garh et al 2002, Jang et al 2003). Namun belum banyak diketahui dengan jelas meknisme trehalose metabolisme berperan membantu tanaman bertahan menghadapi berbagai macam stres. Dalam study ini dipelajari peran trehalose metabolisme dalam menghadapi stres dengan 2 pendekatan, yakni dengan menumbuhkan tanaman pada medium yang mengandung trehalose dan melalui rekayasa genetika guna mengubah kandungan trehalose dalam tanaman. Metabolisme trehalose dalam tanaman diatur sangat ketat karena senyawa ini mempunyai peran central dalam mengatur pertumbuhan dan perkembangan tanaman. Trehalose konsentrasi tinggi bersifat toxic sehingga menyebabkan kekerdilan. Kekerdilan itu disinyalir disebabkan karena terakumulasinya T6P yang megaktifkan pembentukan pati dengan cara meningkatkan laju translasi enzyme AGPase, meningkatkan aktifitasnya serta memperlambat laju degradasi pati. Namun demikian, terakumulasinya pati pada daun bukan penyebab kekerdilan tersebut, karena mutant yang tidak mampu mensintesis pati juga mengalami kekerdilan bila ditumbuhkan pada medium mengandung trehalose. Untuk mengetahui aktivitas trehalose atas kekerdil tanaman yang di

pelajri dengan menggunakan tanaman mutant yang tahan terhadap trehalose 100 mM hasil screening dari koleksi FOX Arabidopsis yang mengekspresikan full length cDNAs. Dari hasil analysis ternyata bahwa pada tanaman tersebut mengekspresikan gene bZIP11 dan mengakumulasi T6P. SnRK1 kinase dikenal sebagai protein pengintegrasi antara sumber karbon tersedia, stres dan sinyal untuk perkembangan tanaman. T6P diketahui menghambat SnRK1 sehingga pertumbuhan terhambat. Nampaknya mutant tersebut tidak sensitive terhadap akumulasi T6P, yang kemungkinan mutant tersebut disamping mngekspresikan banyak bZIP11, juga banyak mengekspresikan SnRK1, sehingga efek dari akumulasi T6P masih dapat diatasi. Dengan demikian signaling pathway yang mengontrol pertumbuhan dalam kaitannya dengan metabolite primer melibatkan T6P, SnRK1 dan bZIP11. T6P dan SnRK1 juga terlibat dalam pengaturan pertumbuhan Arabidopsis yang ditumbuhakan dalam gelap (stres cahaya). Tanaman biasanya mengalami etiolasi dalam gelap, namun dengan adanya trehalose dalam medium mengakibatkan akumulasi T6P menyebabkan etiolasi tersebut tidak terjadi. Terakumulasinya T6P pada Arabidopsis mengubah pola pertumbuhan dalam gelap. Karena tidak terjadi mobilisasi sumber karbon dari cotyledon, maka tumbuhan mengalami kelaparan yang hebat terlebih dalam gelap. Sebagaimana sebelumnya de-etiolasi yang tergantung dari keberadaan T6P ini akan hilang bila dalam medium diberikan sumber energi. Hal ini menunjukkan bahwa SnRK1 juga terlibat dalam phenomena etiolasi-deetiolasi dalam gelap. De-etiolasi tersebut menurun pada mutant DELLA protein dan hilang sama sekali pada tumbuhan yang mengekspresikan banyak phytochrome interaction DNA binding protein (PIF4). Dengan demikian T6P, DELLA dan PIF4 terlibat dalam pola pertumbuhan tanaman dalam stres cahaya. Jelaslah bahwa T6P berperan sebagai signaling molekul, terlibat dalam pengintegration antara sumber karbon tersedia, respon terhadap stress linkungan, pertumbuhan dan perkembangan tanaman dengan bekerja sama dengan beberapa molekul lainnya terutama SnRK1.

Adapun upaya melalui rekayasa genetika guna meningkatkan trehalose pada Arabidopsis dilakukan dengan mengintroduksikan gene trehalose synthase dari Thermoifida fusca (TfTreS), Mycobacterium tuberculosis (MtTreS) dan gene glycosyl transferring enzyme (TreT) dari Rurobacter xylanophyllus, yang di design untuk diekspresikan pada cytosol dan chloroplast. TfTreS dan TreT telah

diintroduksikan ke Arabidopsis. Dengan disambungkan dengan GFP, lokalisasi dari ekspresikan gen terseut telah di verifikasi. Test in vitro terhadap kedua protein baik yang di ekstrak dari transgenik menjunjukkan aktivitasnya. Test awal atas transgenik terhadap garam tinggi dan suhu masih memerlukan verifikasi lebih lanjut untuk melihat efek keeradaan gen tersebut.

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