Analysis of Trehalose-6-Phosphate Control over Carbon Allocation and Growth in Plants

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Analyse van de rol van trehalose-6-fosfaat bij koolstof allocatie en groei in planten

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Contents

CHAPTER 1

General introduction

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Carbon assimilation and allocation in plants

Plants along with algae and a number of bacteria convert CO_2 from the atmosphere into carbohydrate. To do so they require energy that can be supplied by light or other energy sources. For photosynthesis, light is harvested by protein complexes of the photosynthetic machinery to generate a redox potential sufficient for CO_2 reduction. Fixed carbohydrates may be used for respiration, thus releasing energy and CO_2. Carbohydrates are also used for biosynthesis of the cellular system or transport to other tissues. Particularly in multi-cellular organisms such as plants, carbohydrate is transported from leaf mesophyll cells to tissues that do not fix carbon, the carbon sinks, where it can be used for growth. Carbon sinks can be meristems, flowers, fruits and roots. Carbon sources are generally mature leaves with active photosynthesis or reserves in germinating seeds. Distribution of the carbohydrate to the different plant organs is named carbon allocation.

Reactions involved in photosynthesis have been extensively studied. Reactions involved in carbon storage in chloroplasts and vacuoles are also increasingly understood. Genes have been characterized that mediate carbon transport away from the chloroplast and the source cell. However, the regulation and coordination of carbon assimilation and allocation are poorly understood. These processes constitute networks of regulators and are complex since they necessarily coordinate available carbon with other nutrients, such as nitrogen and phosphate, and with demand and supply. These processes necessarily also integrate nutrient stress signals with carbon supply and demand (Paul, M. J. and Foyer, C. H. 2001). Little is known about the precise structure and components involved in regulation of carbon allocation. Systems biology techniques that can study network topologies are beginning to be developed (Gutierrez, R. A. et al. 2007).

This thesis deals with trehalose metabolism that constitutes a ubiquitous ancient regulatory metabolic link between primary carbon metabolism and stress responses in microbes, nematodes insects and plants (Behm, C. A. 1997, Elbein, A. D. et al. 2003, Garg, A. K. et al. 2002, Holmstrom, K. O. et al. 1996, Hottiger, T. et al. 1989, Kaasen, I. et al. 1994, Wiemken, A. 1990). Trehalose is the alpha, alpha-1,1-linked glucose disaccharide. Research presented in this thesis analyses the control of the intermediate trehalose-6-phosphate over plant growth and carbon allocation. Results uncover components of a further role of this metabolite in the regulation of photosynthesis, photorespiration and stress responses.

Regulation of carbon assimilation for growth under stress

To understand carbon assimilation and allocation fully and so break through the yield ceiling that has developed for many crops in the late twentieth century,

an understanding is required of the regulation of carbon assimilation and allocation at the whole plant level (Bovensmann, H. et al. 1999, Pellny, T. K. et al. 2004). Work carried out during the last century has uncovered links between nutrients provided from the soil and photosynthesis and much has been done to optimize yields by optimizing nutrient supply in the soil. However, breeding tendencies have changed because exploding population and urbanization encroach on arable lands and thus agricultural lands are moving to less favorable soils and conditions. In addition, plant productivity is now also optimized to minimize leaching of nutrients from the soils.

Understanding carbon assimilation and allocation to improve yield under stress, including low mineral nutrition, will require understanding the role of the products of photosynthesis in protecting or inhibiting plant growth under stress (Noctor, G. and Foyer, C. H. 2000). These products include not only carbohydrate, but also reactive oxygen species such as H_2O_2 known as mediators of stress responses (Kovtun, Y. et al. 2000, Nakagami, H. et al. 2004) and redox systems that provide redox equivalent and regulate many reactions in different sub-cellular compartments (Baxter, C. J. et al. 2007). H_2O_2 furthermore represses auxin signaling (Kovtun, Y. et al. 2000) and is involved in signaling a systemic response to light patches in low-light adapted Arabidopsis (Karpinski, S. et al. 1999).

Carbon fixation is inherently linked with stress signaling

Photorespiration is inherent to photosynthesis because Rubisco accepts both CO_2 and O_2 at its reactive center. Photorespiration spans reactions in chloroplasts, mitochondrion and glyoxysome. One O_2 molecule is fixed into 2-phosphoglycolate that is then de-phosphorylated and transported into peroxisomes where another O_2 molecule is used to form glyoxylate and H_2O_2. For every carbon atom fixed and under non-stressful conditions in well watered C3 plants at 25-30°C, 0.67-1 carbon passes through photorespiration, generating stoichiometric amounts of H_2O_2 (de Veau, E. J. and Burris, J. E. 1989, Schleucher, J. et al. 1998). Under stress conditions, particularly under drought when stomata close and O_2 accumulates, photorespiration is increased, generating even more H_2O_2 (Igamberdiev, A. U. et al. 2001). The glyoxylate produced is converted into glycine, linking carbon assimilation with nitrogen assimilation. Glycin is then transported to mitochondria and generates redox equivalents. It has been possible to engineer Rubisco with an improved ratio of acceptance of CO_2 compared to O_2, but it is uncertain whether mutagenesis will ever yield an enzyme that efficiently discriminates between CO_2 and O_2 (Cheng, Z. Q. and McFadden, B. A. 1998). Mutants are available for most enzyme reactions in the photorespiratory pathway; some of these mutants are unable to grow in air but grow in reduced partial O_2 pressure, suggesting that the

pathway downstream of Rubisco may be essential to recycle the carbon in glycolate (Boldt, R. et al. 2005). One major aspect of the photorespiratory pathway is that it links metabolisms in the three sub-cellular compartments. Important for this thesis is that it relates carbon assimilation and stress signaling. Cross-compartment regulation is revealed for example by *ucp1*, a mutant in mitochondrial uncoupling factor 1 (Sweetlove, L. J. et al. 2006). *ucp1* reveals that uncoupling of the respiratory chain is necessary to absorb redox equivalent coming from photorespiration during the day time. Mutants in this UCP1 are also inhibited in photosynthesis suggesting a feed back regulatory signal from the mitochondrion to photosynthesis in the chloroplast. One of the roles of photorespiration perhaps could be the production of H_2O_2 regulating stress and developmental responses in addition to guard cell closure (Desikan, R. et al. 2006, Gudesblat, G. E. et al. 2007, Kovtun, Y. et al. 2000, Nakagami, H. et al. 2004).

Regulation of carbon assimilation and allocation

Environmental variables such as light, temperature and nutrition determine the rate of photosynthesis for consumption of the carbon fixed in the Calvin cycle. This has been named the feedforward regulation of photosynthesis (Paul, M. et al. 2001). Much work has been done to characterize the chain of events from light to, for example, the increased expression of light-harvesting proteins such as *LHCB1* (Mochizuki, N. et al. 2001). Photosynthesis is also regulated by the needs of its products and this has been termed feedbackward metabolic regulation. Accumulation of sugars such as glucose and sucrose typically down regulates the expression of a number of genes encoding components of the photosynthetic machinery, including LHCB1 expression (Vinti, G. et al. 2005). Hexokinase1 in Arabidopsis is thought to partly mediate this regulation (Cho, Y. H. et al. 2006, Moore, B. et al. 2003). Also the redox-poise and reactive oxygen species, including H_2O_2 have been shown to mediate regulation of photosynthesis and carbon assimilation at the cellular level (Geigenberger, P. et al. 2005, Heiber, I. et al. 2007, Noctor, G. and Foyer, C. H. 2000).

Regulation of carbon assimilation and allocation at the whole plant level is poorly understood. A number of transgenic plants have been made with increased yield by way of increased carbon allocation to a specific organ. For example, potato yields were increased by increasing the level of the metabolite precursor for starch synthesis, e.g. adenosine-diphosphate, in the chloroplasts by over-expressing the plastidial ATP/ADP transporter (Tjaden, J. et al. 1998). Potato yields were also increased by anti-sense repression of chloroplastic adenylate kinase. Reduced chloroplastic adenylate kinase increased levels of adenosine diphosphate and adenosine diphosphate glucose, the immediate substrate for starch synthesis (Regierer, B. et al. 2002). These strategies aimed at increasing the rate of utilization

of the carbon supplied to tubers and thus suggest that increasing the sink-strength of an organ can alter carbon allocation.

Carbon allocation can be stopped by expression of *E.coli* inorganic pyrophosphatase specifically in the phloem (Lerchl, J. et al. 1995). This results in sugar accumulation in source leaves and stunted growth. Expression of yeast cytosolic invertase in the phloem complements the phenotype and reveals that carbon allocation is dependent on sucrose cleavage in the phloem cells. Pyrophosphate is required for sucrose cleavage via sucrose synthase and thus carbon allocation. Conceivably, sucrose cleavage in the phloem might be the target of the regulation of carbon allocation, but further work is required to identify components that mediate such regulation at the whole plant level. Examples of engineering carbon allocation with regulators such as SNF1-related kinases have not yet led to crops with increased yield (McKibbin, R. S. et al. 2006). It would seem, however, that SNF1-realated kinases are important for carbon allocation to roots (Schwachtje, J. et al. 2006). The SNF1-related kinases of plants have also been implicated in mediating drought resistance in Arabidopsis (Umezawa, T. et al. 2004), showing again that stress signaling and allocation are intimately linked, in this case by regulating the SNF1-kinase.

The most striking example of altered allocation control is that observed when Arabidopsis seedlings are grown on trehalose (Wingler, A. et al. 2000). When this sugar is supplied to medium it spurs massive accumulation of starch in source tissue and traditional sinks such as meristems and roots lack carbon and are growth inhibited.

Trehalose metabolism in plants

Trehalose metabolism is found in a wide variety of organisms and is seen as evolutionary old (Elbein, A. D. 1974). Trehalose was shown to hold several biological functions. Trehalose is a carbon reserve. In fungal spores, trehalose hydrolysis occurs during early germination and presumably provides glucose for energy and biosynthesis (Thevelein, J. M. 1984). In insects, trehalose is a major sugar in the haemolymph and thorax muscles supplying glucose consumed during flight (Becker, A. et al. 1996). Trehalose is a stress protectant and protects proteins and membranes from denaturation by replacing water as it makes hydrogen bonds to polar residues. In addition, it forms a "glass" in the dry state, a process required to stabilize dry macromolecules or blood (Brumfiel, G. 2004, Crowe, J. H. et al. 1998, Wolkers, W. F. et al. 2003). Finally, trehalose is also a precursor for synthesis of several cell wall components in certain microbes. *M. tuberculosis* contains the unusual fatty acid mycolic acid esterified to the C6-hydroxyl group of each glucose to give trehalose-dimicolate. This lipid appears responsible for the low permeability of the

mycobacterial cell wall and thus the considerable drug resistance of these organisms (Brennan, P. J. and Nikaido, H. 1995).

Plants generally contain only trace amounts of trehalose (Muller, J. et al. 1995, Zentella, R. et al. 1999). Exceptions to this exist and these are plants with extreme drought stress resistance such as *Selaginella lepidophylla* that accumulate quantitative amounts of trehalose. All plants seem to contain genes for trehalose metabolism (Blazquez, M. A. et al. 1998, Leyman, B. et al. 2001, Shima, S. et al. 2007, Vogel, G. et al. 1998). Synthesis of trehalose in plants is typically via its phosphorylated intermediate, trehalose-6-phosphate (T6P). Trehalose-6-phosphate synthase (TPS) converts UDP-Glucose and Glucose-6-phosphate to T6P. Trehalose phosphate phosphatase (TPP) de-phosphorylates T6P to trehalose. Trehalase cleaves trehalose to two glucose molecules (Fig.1). In all plants for which sufficient sequence data is available to draw conclusions, extensive radiation of genes associated with T6P metabolism has occured. In Arabidopsis, for example, 11 TPS and 10 TPP orthologues are found. The rice genome likely contains even more numerous gene-families of these enzymes (Pramanik, M. H. and Imai, R. 2005, Shima, S. et al. 2007). In contrast, plant genomes only contain 1 or 2 trehalases. Evidence for the function of only very few of the trehalose metabolism genes is currently available. Arabidopsis *TPS1*, *TPPA* and *TPPB* were identified by complementation of yeast deficient in these activities. Further, genes with homology to microbial or insect enzymes of trehalose metabolism (e.g. *TPH*) have not been identified in plants. None of the genes mediating transport of trehalose or T6P have been identified. *AtTPS1* is essential for Arabidopsis as *tps1* mutants are embryo lethal (Eastmond, P. J. et al. 2002). *AtTPS1* was further shown to be required for root growth and transition to flowering (van Dijken, A. J. et al. 2004). Complementation experiments with the small *E.coli TPS* suggest that the product of the enzymic activity is required rather than some other function of AtTPS1. Trehalose metabolism is thus essential in plants, likely because of a requirement of T6P or a product derived thereof (Schluepmann, H. et al. 2003, Schluepmann, H. et al. 2004).

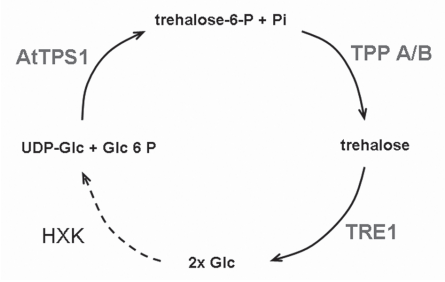

Figure 1. Trehalose metabolism in Arabidopsis. Yeast complementation experiments confirmed the functions of several genes of the Arabidopsis trehalose in metabolism, AtTPS1 encodes trehalose phosphate synthase (Blazquez, M. A. et al. 1998), AtTPPA/B encode trehalose phosphate phosphatases (Vogel, G. et al. 1998), and TRE1 encodes a trehalase (Aeschbacher, R. A. et al. 1999, Muller, J. et al. 1995).

Trehalose-6-phosphate, carbon assimilation and allocation

To study the role of T6P in plants, a set of transgenic Arabidopsis lines was engineered with altered levels of T6P by way of *E.coli TPS* and *TPP* expression (Schluepmann, H. et al. 2003). In addition, lines were made using *E.coli* trehalose-6-phosphate hydrolase (TPH) that converts T6P to glucose-6-phosphate and glucose. Lines expressing *TPS* had increased T6P, whilst lines expressing *TPP* or *TPH* had decreased T6P. Lines with altered T6P exhibited strong pleiotropic phenotypes in spite of relatively small changes of T6P steady states. *E.coli* trehalase over-expressors had WT T6P levels and were indistinguishable from WT. On medium with metabolisable sugar, growth as defined by the increase in dry or fresh weight was increased in seedlings of lines with increased T6P. On metabolisable sugars, seedlings with decreased T6P were growth inhibited. Here, T6P therefore determines carbon utilization in these seedlings. Growth inhibition also occurs in yeast cells deficient in the yeast TPS. T6P was shown to inhibit yeast hexokinase and uncontrolled flux into glucose-6-phosphate is thought to cause the growth inhibition of yeast that do not synthesize T6P (Blazquez, M. A. et al. 1993, Bonini, B. M. et al. 2003, Hohmann, S. et al. 1993). T6P does not appear to inhibit plant hexokinases (Eastmond, P. J. et al. 2002, Harthill, J. E. et al. 2006).

Paradoxically, accumulation of T6P in the absence of sufficient metabolisable carbon also limits growth (Schluepmann, H. et al. 2004). This is the case when seedlings are grown on medium containing 100 mM trehalose. On this medium, seedlings accumulated T6P and seedling expressing *TPH* resist the growth inhibition. Thus seedlings stop growing as a result of T6P accumulation. Trehalose is readily taken up into the plant cells and probably, the high intracellular amounts of trehalose are though to make the TPP reaction unfavorable, resulting in T6P accumulation in the cells. Combining trehalose in the medium with metabolisable sugar allows growth (Schluepmann, H. et al. 2004, Wingler, A. et al. 2000). Feeding only 30 mM trehalose in combination with 10 μM of the trehalase inhibitor Validamycine A also leads to seedling growth arrest but it is uncertain if this is due to T6P accumulation. Under these conditions, expression of *ABI4*, an important mediator of sugar signaling, is increased and *abi4* mutants appear more resistant to the trehalose and Validamycine A combination than WT (Arenas-Huertero, F. et al. 2000, Huijser, C. et al. 2000, Laby, R. J. et al. 2000, Ramon, M. et al. 2007). ABI4 is a transcription factor and was reported to bind directly to the sugar and ABA responsive element to mediate transcriptional repression in the promoter of the small subunit of ribulose-1,5-bisphosphate carboxylase, *RBCS* (Acevedo-Hernandez, G. J. et al. 2005). *RBCS* is but one nuclear gene encoding a component of the photosynthetic machinery that is subjected to sugar repression (Jang, J. C. and Sheen, J. 1994, Krapp, A. et al. 1991). Expression of *ABI4* was also increased in plants over-expressing *AtTPS1*

and displaying resistance to glucose and ABA in the medium (Avonce, N. et al. 2004). These results suggest the interesting possibility that known sugar-signaling components interact with T6P control over growth and carbon utilization.

However, control of T6P over carbon assimilation does not fully explain the pleiotropic phenotypes observed in plants with altered T6P steady states. Sugar accumulation in the leaves of TPP over-expressor does explain the pale green color of these plants but not, for example, the larger leaves observed. Accumulation of free sugars in the compartments of leaf cells was shown to cause stunted growth (Dickinson, C. D. et al. 1991, Heineke, D. et al. 1992, Jang, J. C. et al. 1997, Sonnewald, U. et al. 1997).

Trehalose-6-phosphate and photosynthetic capacity

Tobacco plants engineered with the *E.coli TPS* and *TPP* genes are also altered in their T6P steady state and exhibit the same pleiotropic phenotypes observed in Arabidopsis (Pellny, T. K. et al. 2004). Plants with high T6P have darker green and smaller leaves, while plants with low T6P have pale green and larger leaves. The pale leaves in plants with low T6P could be explained by accumulation of free sugar in these leaves and associated photosynthesis gene-repression as in the case of plants over-expressing inorganic phosphatase in the phloem (Lerchl, J. et al. 1995). Yet gene-expression profiling in Arabidopsis does not support this hypothesis as none of the photosynthesis genes are altered in their expression in these plants (Schluepmann, H. et al. 2004). The plants are also not stunted in their growth, like plants unable to transport assimilate by way of pyrophosphatase expression in the phloem (Lerchl, J. et al. 1995). Arabidopsis with low T6P and pale leaves have pronounced apical dominance and the plants are generally taller than the WT (Schluepmann, H. et al. 2003). Furthermore, in tobacco, growth correlates with leaf area and thus the relative growth rate in plants with pale leaves and lower T6P is higher than in WT (Pellny, T. K. et al. 2004). T6P levels in tobacco are shown to correlate with photosynthetic capacity per leaf area. Altering T6P also alters Rubisco activity under both low light and saturating light conditions. T6P levels further correlate with the amount of carboxyarabinitol 1-phosphate, the naturally occurring inhibitor of Rubisco (Pellny, T. K. et al. 2004). Therefore, results suggest that although T6P appears to be synthesized in the cytosol, it controls targets in chloroplasts involved in control of photosynthetic capacity. T6P also controls the redox–activation of AGPase, another chloroplastic enzyme (Kolbe, A. et al. 2005). Redox activation of this enzyme is mediated by ferredoxin which in turn is reduced by photosynthesis (Balmer, Y. et al. 2006). Taken together, T6P not only has control over carbon assimilation but it also controls steps of chloroplastic metabolism.

Trehalose metabolism and stress

Attempts to produce trehalose in plants by over-expressing yeast *TPS* in tobacco yielded drought resistant plants (Holmstrom, K. O. et al. 1996, Romero, C. et al. 1997). Expression of *E.coli TPS-TPP* fusions in rice also yielded drought tolerance and in addition salt tolerance (Garg, A. K. et al. 2002, Jang, I. C. et al. 2003). Levels of trehalose in the plants obtained, however, failed to correlate with drought tolerance. Lack of correlation could be due to the fact that trehalose is not the active compound but rather T6P, or other derived component. Trehalose feeding to Arabidopsis seedlings in the presence of metabolisable carbon induces chemical detoxification proteins and stress response proteins (Bae, H. et al. 2005). Apparently, trehalose metabolism also mediates abiotic stress tolerance in plants, but we have yet to find out whether this is due to trehalose or to T6P, or to some metabolite derived from either two compounds.

Trehalose metabolism has been implicated in biotic stress resistance as well. Spraying wheat with trehalose solution confers resistance to *Blumeria graminis* infection. Trehalose appears to activate plant defense responses e.g. papilla deposition, phenylamonia-lyase and peroxidase activities (Reignault, P. et al. 2001). Validamycin A is a compound that inhibits trehalase and leads to accumulation of trehalose in plants (Goddijn, O. J. et al. 1997). Spray application of Validamycin A controls infection of tomato by *Fusarium oxysporum*, the tomato wilt, and *Phytophtora infestans*, the tomato late blight (Ishikawa, R. et al. 2005). Trehalose feeding also effectively protects Arabidopsis from infection with *Hyaloperonospora parasitica* (Schluepmann, H. and Smeekens, S. 2002). Arabidopsis lines over-expressing *E.coli TPS* show protection against infection. Arabidopsis expressing *TPP*, on the other hand, appear to allow more rapid cycling of the oomycete than the WT. In conclusion, the data suggests that trehalose and/or T6P may be a key component in regulating the host defense responses against important pathogens. The underlying mechanism is unclear, so far.

Relationship between carbon metabolism and stress responses

The effective induction of stress responses requires a sufficient supply of metabolites, energy and reducing equivalents. This contribution of primary metabolism to plant stress responses and senescence is still poorly understood (Rolland, F. et al. 2002, Rolland, F. et al. 2006). There are also intimate connections between carbon assimilation reactions and release of the stress-signaling molecule H_2O_2 as presented earlier.

Abiotic stress resistance is often associated with sucrose accumulation and sucrose is though to act as a protective solute. In Arabidopsis subjected to 5°C,

sucrose accumulates in the developing leaves and this accumulation no longer inhibits photosynthesis and photosynthetic gene expression (Strand, A. et al. 1997). Stress responses thus affect carbon metabolism and signaling. Conversely, supply of sugars and manipulation of carbon metabolism also can trigger stress responses. These specific stress responses are different from carbon starvation responses and also occur in the presence of available metabolisable carbon. For example the "high sugar resistance" phenotype is characterized by an enhanced resistance against pathogens mediated through elevated levels of soluble carbohydrates and corresponding alterations in primary metabolism (Horsfall, J. G. and Diamond, A. E. 1957). This observation is consistent with other studies demonstrating that feeding sugar to tissues or causing an accumulation of sugar in transgenic plants can activate various *PR* genes (Conrath, U. et al. 2003, Herbers, K. et al. 1996, Johnson, R. and Ryan, C. A. 1990). Bae et al. (2005) showed that trehalose induction of chemical detoxification and stress proteins occurs in the presence of 30 mM sucrose in the medium and thus is independent of carbon starvation of tissues that accumulate T6P as a result of trehalose feeding. These results from studies on trehalose metabolism further establish the existence of direct links between carbon utilization and stress responses. The nature of these links may stem from evolutionally old connections (Fig. 2) and results presented in this thesis further support this.

Figure 2. Evolutionary old connections between trehalose metabolism and stress responses may have been retained in plants.
In microbes, stress triggers changes in primary metabolism that channel carbon into T6P for quantitative accumulation of trehalose. In most plants, trehalose is not accumulated upon stress. Stress does, however, much influence the expression of TPS and TPP genes (Genevestigator.org) and may therefore influence T6P levels. Radiation in the TPS and TPP gene families suggests that these genes have taken up increasingly differential roles in regulating T6P accumulation and possibly the varied downstream responses. These responses include altered photosynthesis (Pellny, T. K. et al. 2004), growth (Schluepmann, H. et al. 2003, Schluepmann, H. et al. 2004), disease resistance (Schluepmann, H. and Smeekens, S. 2002) and development (van Dijken, A. J. et al. 2004). This thesis describes a genetic analysis of these responses.

Outline of this thesis

The general aim of this thesis is to dissect the pleiotropic effects of T6P using a genetic approach. T6P accumulation on 100 mM trehalose causes growth inhibition. Therefore, mutants were obtained that grow and accumulate T6P on 100 mM trehalose medium. Trehalose feeding to seedlings of *Arabidopsis thaliana* is characterized in **Chapter 2**. Results obtained from screening the LeClere and Bartels cDNA over-expression collection on 100 mM trehalose are presented in **Chapter 3**. These mutants express randomly cloned cDNA's and 13 trehalose resistant (*trr*) lines are identified with trehalose resistance segregating as a dominant trait. Expression analysis and re-transformation with the CaMV35S/cDNA constructs were carried out to confirm the link between cDNA expression and resistance to trehalose. *trr* mutant physiology is further characterized in **Chapter 4**. Characterization includes measurements of T6P, starch, chlorophyll, anthocyanin and photosynthesis. Starch accumulation and growth inhibition are found to be two independent effects of T6P accumulation. *trr* mutants with *LHCB1* suppression and over-expression of *GR-RBP2* no longer respond to the growth inhibition but still accumulate starch. *trr* mutants over-expressing *TRR14* no longer respond to growth inhibition and starch accumulation. Studies to further characterize the function of the GR-RBP2 and TRR14 proteins are presented in **Chapter 5**. A summarizing discussion of the results obtained is the subject of **Chapter 6**.

This thesis initially set out to study targets of T6P that relate to growth control and carbon allocation. Results obtained have uncovered links between carbon allocation and growth, and proteins such as LHCB1 involved in light harvesting and regulation of photo-oxidative damage and GR-RBP2 known to mediate resistance to cold stress.

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CHAPTER 2

The trehalose pathway regulates carbon partitioning in light and dark

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(Results presented in Figure 2 are part of Schluepmann et al. 2004)

Abstract

The effects of 100 mM trehalose on growth, carbon allocation and gene-expression in seedlings are characterized in this chapter. In light, growth arrest on 100 mM trehalose is due to T6P accumulation and can be rescued by exogenous supply of metabolisable sugar (Schluepmann et al. 2004). This growth arrest is not mediated by the known sugar signaling proteins ABI4, ABI3, ABA2 and HXK1. This growth arrest further occurs in all accessions of Arabidopsis tested but is significantly less in seedlings of Cvi. Partial resistance of Cvi seedlings is not due to the altered *MYB75/PAP1* allele found in this accession. Gene expression profiling after 24h reveals that 100 mM trehalose affects 5% of the genes controlled by 100 mM sucrose. After 24h, trehalose does not cause carbon catabolite repression of gene-expression, but induces a specific combination of genes known from biotic stress responses. After 14d, trehalose results in reduced chlorophyll contents, in anthocyanin accumulation, and causes cells of the root extension zone to swell and lyse. In the dark, 100 mM trehalose leads to sugar-induced skotomorphogenesis in seedlings yet it inhibits hypocotyl elongation without altering root growth.

Introduction

Trehalose metabolism has recently been recognized to play an important role in carbon signalling in plants (Rolland, F. et al. 2002, Rolland, F. et al. 2006). Trehalose is the alpha, alpha-1,1-linked glucose disaccharide, which is found ubiquitously and is therefore thought to be evolutionary ancient (Elbein, A. D. et al. 2003). The first mutant that implied a role of trehalose metabolism in carbon signaling was found in *S.cerevisiae*. The *S.cerevisiae* tps1Δ stops growing on full medium and was called the General Glucose Sensor1 mutant until it was found to be a mutant of the enzyme Trehalose Phosphate Synthase (TPS). TPS catalyses the conversion of glucose-6-phosphate and UDP-glucose into trehalose-6-phosphate (T6P), the phosphorylated intermediate of trehalose biosynthesis. The defect in *ScTPS1* can be suppressed by restricting the influx of sugars into glycolysis and by mutations in hexokinase II (Blazquez, M. A. et al. 1993, Blazquez, M. A. et al. 1994a, Blazquez, M. A. and Gancedo, C. 1995). T6P is also a direct inhibitor of *S.cerevisiae* HXKII *in vitro*, yet this is not the only target of T6P regulation in yeasts (Blazquez, M. A. et al. 1994b, Bonini, B. M. et al. 2003).

In the model plant Arabidopsis two independent *tps1* mutants (*tps1-1 and tps1-2*) were isolated from transposon mutagenised plants (Eastmond, P. J. et al. 2002). *tps1* is disrupted in the *AtTPS1* gene encoding a functional TPS (Blazquez, M. A. et al. 1998). *tps1* embryo morphogenesis is normal but development is retarded and stalls early in the phase of cell expansion and storage accumulation. The embryo-lethal phenotype of *tps1* can be partially rescued *in vitro* by reducing sucrose levels in

the medium (Eastmond, P. J. et al. 2002). Absence of *AtTPS1* in Arabidopsis plants precludes root growth and transition to flowering (van Dijken, A. J. et al. 2004). *tps1* mutants thus establish the absolute requirement for trehalose metabolism in plants. Arabidopsis over-expressing several different enzymes of *E.coli* trehalose metabolism were subsequently generated and used to show that T6P is essential for carbon utilization and affects sugar phosphate steady states (Schluepmann, H. et al. 2003). Unlike in yeast, a link between T6P and hexokinases could not be established for plants (Eastmond, P. J. et al. 2002, Harthill, J. E. et al. 2006).

Arabidopsis, seedlings over-expressing *AtTPS1* exhibit glucose and abscisic acid (ABA)-insensitive phenotypes. These insensitive phenotypes are at least partly due to an altered regulation of genes involved in glucose and ABA signaling during seedling vegetative growth suggesting that plant trehalose metabolism is linked to sugar signaling pathways (Avonce, N. et al. 2004). The ABSISIC ACID INSENSITIVE 4 (ABI4) transcription factor mediates in part the growth inhibition resulting from 25 mM trehalose combined with 10 μM Validamycin A (Ramon, M. et al. 2007). Results thus imply common paths between the control of trehalose metabolism over carbon utilization and the signal transduction pathway surrounding ABI4. Further research is needed to relate effects of trehalose feeding to other sugar signaling responses and T6P, and to identify components of the signal transduction pathway surrounding AtTPS1 and T6P.

Sugars are signaling molecules and mutant approaches have been used to identify signal transduction components. Different screens have delivered: *gin* (Zhou, L. et al. 1998), *sis* (Laby, R. J. et al. 2000), *sun* (Dijkwel, P. P. et al. 1997), *isi* (Rook, F. et al. 2001) and *cai* (Boxall, S. F et al. 1997). *gin* mutants were isolated at the seedling stage and identified the role of a hexokinase, AtHXK2, as one mediator of glucose signaling to achieve glucose regulation of gene expression. AtHXK2 signaling was independent of enzyme activity. Signaling occurred in the nucleus and required complexing of AtHXK2 with other metabolic enzymes of previously unrecognized nuclear functions (Cho, Y. H. et al. 2006, Harrington, G. N. and Bush, D. R. 2003, Moore, B. et al. 2003, Rolland, F. et al. 2006). Mutants obtained by screens on sugars also uncovered strong links between hormone and sugar signaling. For example, the ABI4 transcription factor mediates both sugar and ABA responses and mutants are resistant to glucose and ABA in the medium. Similarly, *abi3* mutants reveal that ABA signal transduction and glucose signal transduction have common parts (Arenas-Huertero, F. et al. 2000, Huijser, C. et al. 2000, Laby, R. J. et al. 2000). Another hormone that clearly interacts with sugar signals in controlling seedling development is ethylene. *eto1* (ethylene over-producer) and *ctr1,* mutants are glucose insensitive, whereas *etr1* (ethylene resistant) or *ein2* (ethylene insensitive) mutants are hypersensitive to glucose (Zhou, L. et al. 1998). A similar mutant approach could also be employed to identify components of the regulatory role of the trehalose pathway in plants.

Supplied trehalose is transported through plant tissue and enters plant cells since plants expressing trehalase in the cytosol thrive on medium with trehalose (Schluepmann et al. 2003, 2004). Exogenously applied trehalose strongly reduces elongation of Arabidopsis roots and, concomitantly, induces a strong accumulation of starch in the shoots, an increased activity of ADP-glc pyrophosphorylase (AGPase) and an induction of the expression of the AGPase subunit APL3 (Wingler, A. et al. 2000). In seedlings grown on 100 mM trehalose, rapid accumulation of T6P is detected, possibly due to product inhibition of the T6P dephosphorylation reaction. If T6P concentrations increase in the absence of externally supplied metabolisable sugar, seedlings fail to grow. Seedlings expressing the *E.coli* T6P phosphorylase, which cleaves T6P to glucose and glucose-6-phosphate, grow on 100 mM trehalose (Schluepmann, H. et al. 2004). Growth arrest on 100 mM trehalose is therefore due to T6P accumulation. Accumulation of starch on medium with trehalose may also stem from T6P accumulation: T6P levels in the cell have been shown to affect the redox status and thereby the activity of AGPase as well as starch accumulation rates (Kolbe, A. et al. 2005). It is not entirely clear if control over starch deposition is causing growth arrest of sink tissues in seedlings grown on trehalose. Results suggest that this might not be the only cause of growth arrest on trehalose because seedlings impaired in starch synthesis such as the *pgm1* mutants fail to grow under these conditions (Fritzius, T. et al. 2001). Increased supply of metabolisable sugars in plants is accompanied by increases in the level of T6P, redox activation of AGPase and stimulation of starch synthesis *in vivo* (Kolbe, A. et al. 2005, Schluepmann, H. et al. 2004). Mutants resistant to exogenous trehalose at 100 mM might reveal the processes by which T6P regulates growth.

In this chapter, growth inhibition due to T6P accumulation on 100 mM trehalose is characterized further. This characterization is necessary since the effects of 100 mM trehalose may be different from the effects of 25 mM trehalose combined with 10 μM Validamycine A that were used previously to describe the effect of trehalose (Wingler et al. 2000; Fritzius et al. 2002; Ramon et al 2007). Growth inhibition on 100 mM trehalose was analysed using various sugar additives, sugar signaling mutants and accessions of Arabidopsis. The effect of 100 mM trehalose was compared to that of 100 mM sorbitol or sucrose by way of gene-expression profiling. Growth inhibition on 100 mM trehalose was also studied in seedlings grown in the dark where this sugar induces skotomorphogenesis but not hypocotyl elongation.

Results

Trehalose-6-phosphate mediated growth arrest on trehalose is due to altered carbon allocation

Trehalose in the medium leads to accumulation of large amounts of starch in seedling source tissue, the cotyledon, and to a depletion of starch in the columnella cells of the root cap, a sink tissue (Fig. 1a-c; Ramon, M. et al. 2007, Wingler, A. et al. 2000). Confocal microscopy of seedling roots stained with propidium iodide reveals swelling as well as lysis of cells in the extension zone of roots grown on 100 mM trehalose but not on 100 mM sorbitol (Fig. 1d-f). In addition to altered starch distribution and reduced root growth, trehalose appears to alter cell wall elasticity compared with sorbitol. By 100 mM trehalose feeding chlorophyll levels were decreased but anthocyanin amounts were increased (Fig. 1g, h). Root growth inhibition can be overcome by simultaneous supply of a range of metabolisable sugars (Schluepmann, H. et al. 2004, Wingler, A. et al. 2000). Growth inhibition by trehalose can also be overcome by expression of *E.coli* trehalase, TreF a soluble enzyme targeted to the cytosol, suggesting that trehalose supplied exogenously is imported into plant cells, then cleaved by expressed trehalase and the released carbon is utilized for growth (Schluepmann, H. et al. 2003). Growth inhibition on trehalose can be overcome by expression of *E.coli* trehalose phosphate hydrolase, an enzyme that cleaves T6P into Glucose-6-phosphate and glucose, suggesting that T6P accumulation is causing growth arrest (Schluepmann, H. et al. 2004). Studying the effect of sugars on T6P mediated growth arrest may therefore reveal interactions between T6P and sugar signaling pathways that control carbon utilization in the source tissues or that control carbon allocation and transport.

The seedling response to sugars without added trehalose was analyzed at 50 mM concentrations except for the toxic mannose where 6 mM was used (Fig. 2-without tre). Growth on 50 mM sorbitol, the osmoticum control, equals that on half strength MS medium and thus suggests that osmoticum has little effect on growth at these sugar concentrations. Growth is enhanced when seedlings are supplied 50 mM of either glucose, fructose, sucrose or maltose compared to seedlings supplied with sorbitol; seedlings therefore utilize the supplied metabolisable carbon for growth. Interestingly, growth on 100 mM of either fructose or glucose is not as vigorous as growth on 50 mM of each glucose and fructose or 50 mM of glucose or fructose. This is the case for both WT and trehalase expressing seedlings. Growth on palatinose equals that on sorbitol suggesting that this sugar, like sorbitol is not utilized. Trehalose at 50 mM inhibits root growth significantly to 30% of control levels. Seedlings do not germinate on 50 mM 2-deoxy glucose or 6 mM mannose.

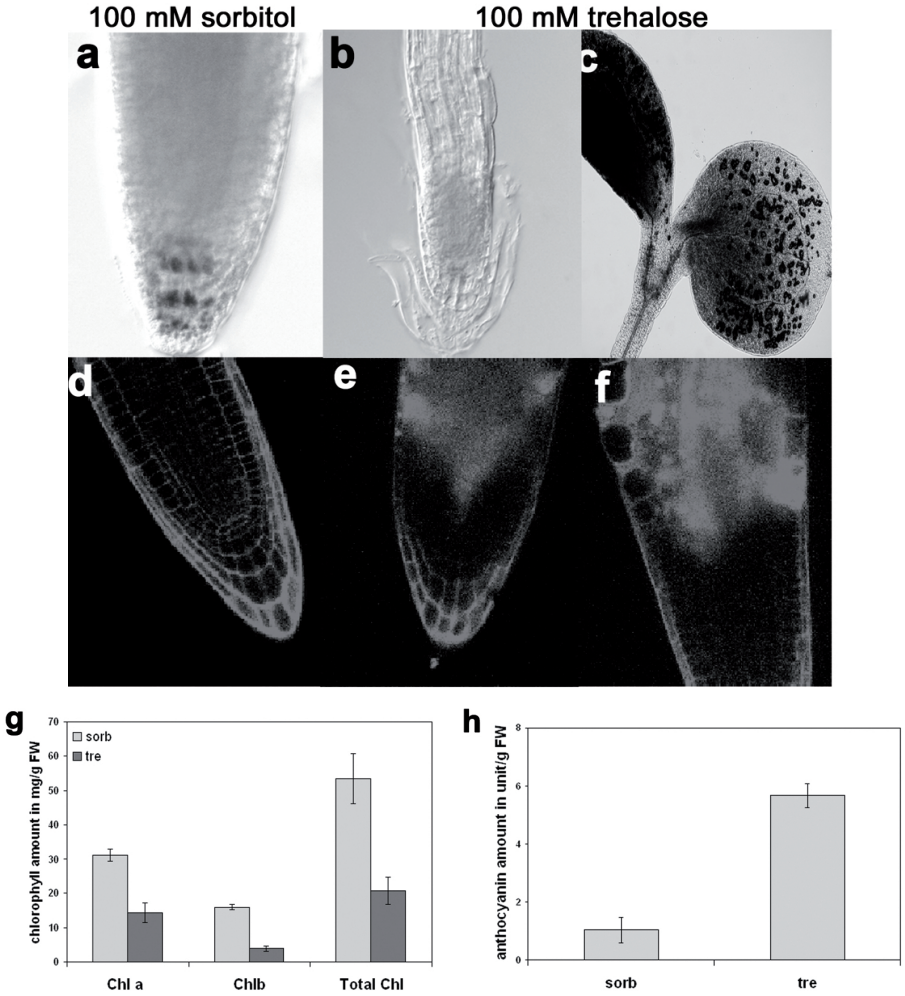

Figure 1. Description of the growth arrest on 100 mM trehalose. Altered allocation of starch. Seedlings were grown 14d in long day conditions, then stained with KI/I_2 and studied using Nomarski microscopy. **a)** Starch in the columnella of wt roots grown on 100 mM sorbitol osmoticum control, **b)** WT roots grown on 100 mM trehalose, **c)** on trehalose starch massively accumulates in the source tissues of the cotyledons. **Cell lysis in the root extension zone.** Seedlings were grown for 14d then stained with propidium iodide.**(d)** Typical root on 100 mM sorbitol osmoticum control; **(e,f)** Typical swelling and lysis of cells at the extension zone of seedling roots on 100 mM trehalose. **Altered chlorophyl content (g).** Seedlings were grown for 14d on 100 mM of either sorbitol (sorb) or trehalose (tre), then chlorophyll were analysed. Chla, chlorophyll A, Chlb, chlorophyll B, Total Chl, total chlorophyll. **Altered anthocyanin content (h).** Seedlings were grown 14 d on 100 mM of either sorbitol (sorb) or trehalose (tre), and anthocyanin contents determined.

The seedling response to 50 mM sugar combined with 50 mM trehalose was investigated (Fig. 2A, B). Sucrose, maltose and a combination of fructose and glucose completely alleviate the growth inhibitory effects of trehalose. 50 mM of fructose or glucose alleviate trehalose mediated growth inhibition partially. Sorbitol and palatinose are ineffective against growth inhibition. T6P accumulation on trehalose

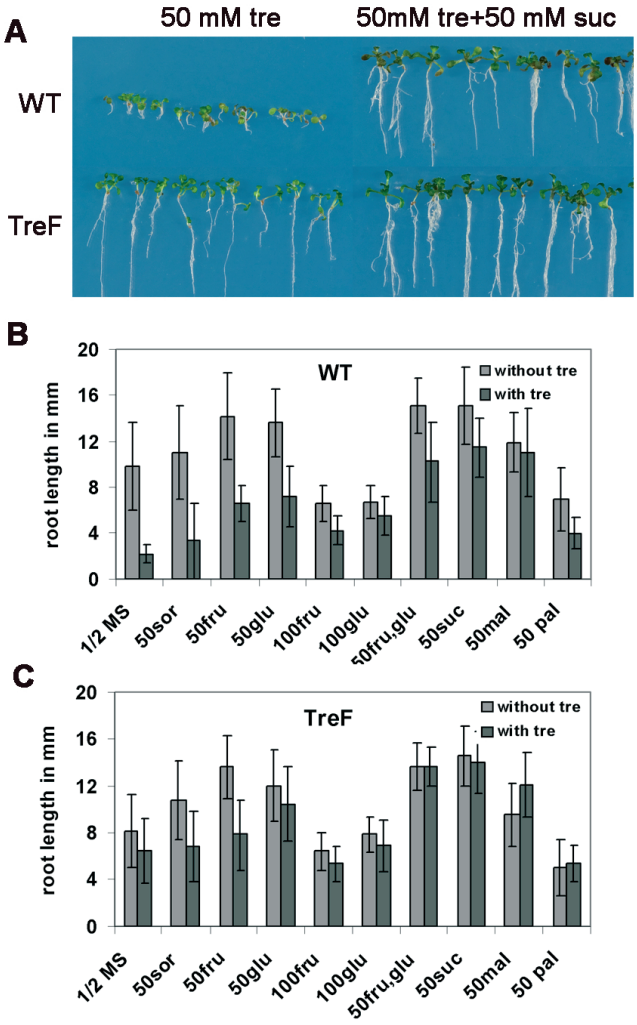

Figure 2. Effect of metabolisable sugar on the growth inhibitory effect of trehalose. Seedlings were grown for 14 days in long day conditions on half strength MS medium containing 50 mM trehalose with or without different sugars before root lengths were determined using Image J. WT, seedlings from WT; TreF, seedlings from the *E.coli* trehalase, *TreF*, expressing line 46.2; **A)** WT and the trehalase over-expressor line (TreF) on 50 mM trehalose with 50 mM sorbitol (50 mM tre) or 50 mM sucrose (50 mM tre+50 mM suc). **B)** Root lengths of WT on half strength MS medium (1/2MS) with different sugar combinations. with tre, 50 mM trehalose. Sugars were at 50 mM except 100 glc, 100 frc, 50 frc, glc and mann, where the concentrations were 100 mM glc, 100 mM fru, 50 mM fructose combined with 50 mM glucose and 6 mM mannose, respectively. **C)** Root lengths of TreF in media with different sugar combinations as in B).

does not rescue inhibition of seedling germination due to 2-deoxyglucose, and so T6P unlikely acts as an inhibitor of HXK2 mediated signaling. This is further supported by the fact that T6P accumulation does not rescue mannose inhibition of germination. After 7d, growth of TreF expressing seedlings on trehalose equals

that on sorbitol (Fig. 2A, C, with tre). If grown for longer periods of time, these seedlings thrive on trehalose with growth exceeding that on sorbitol, presumably because glucose from trehalose cleavage is used for growth. Addition of sucrose, maltose or a combination of glucose and fructose increase growth of trehalase expressors on trehalose further, their root lengths being twice as long on medium with trehalose and the metabolisable sugars.

Taken together, metabolisable sugars such as sucrose, maltose or glucose combined with fructose effectively relieve seedlings from the growth inhibitory accumulation of T6P on 100 mM trehalose medium. But trehalose in the medium does not relieve the HXK2 mediated inhibition of germination by mannose or 2-deoxy glucose suggesting that, unlike the HXK inhibitor mannoheptulose, T6P accumulation is not inhibiting this enzyme. Increased elasticity of walls in the extension zone and absence of starch accumulation in columnella cells of the root tip suggest that T6P accumulation throughout the plant tissues likely causes starvation of sink tissues important for growth, such as shoot and root apical meristems. Sink starvation is not caused by the sink's inability to metabolize carbon since carbon supplied is utilized and the effects of T6P accumulation are then overcome.

Carbohydrate signaling mutants are sensitive to 100 mM trehalose

To further characterize the effect of 100 mM trehalose on plant growth, known sugar signaling mutants were assayed for their ability to grow on medium with 100 mM trehalose. *abi3-1*, *abi4-3*, *pgm1* and *sex1* were chosen because *abi3-1* and *abi4-3* are insensitive to glucose, *pgm1* cannot synthesize starch and *sex*, a starch excess mutant, is defective in export of glucose resulting from hydrolytic starch breakdown.

On half strength MS medium supplemented with 100 mM trehalose seedlings of *abi3-1* and *abi4-3* and their respective WTs Ler and C24, developed poorly and root lengths after 14 days did not differ significantly from wild type controls (Fig. 3A). ABI3 and ABI4 are therefore not mediating growth arrest on 100 mM trehalose.

gin2-1 (Ler), *gin1*(WS), *aba2-1*(Col-0), *eto1-1*(Col-0), *etr1-1*(Col-0) had short roots on 100 mM trehalose that did not differ significantly from their respective wild type backgrounds (Fig. 3A). Starch accumulated in cotyledons but failed to accumulate in the columnella of root tips of these sugar signaling mutants (data not shown). Seedlings from *ein2-1*, an ethylene insensitive mutant, had apparently longer roots than all other mutants tested on 100 mM trehalose, but root lengths did not differ significantly in the Col-0 wild type background because of the high variation of root lengths.

pgm1 mutants grew apparently longer roots than *abi3-1* and *abi4-3*, but root lengths did not differ significantly from wild type controls (Fig. 3A, B). After 14 days

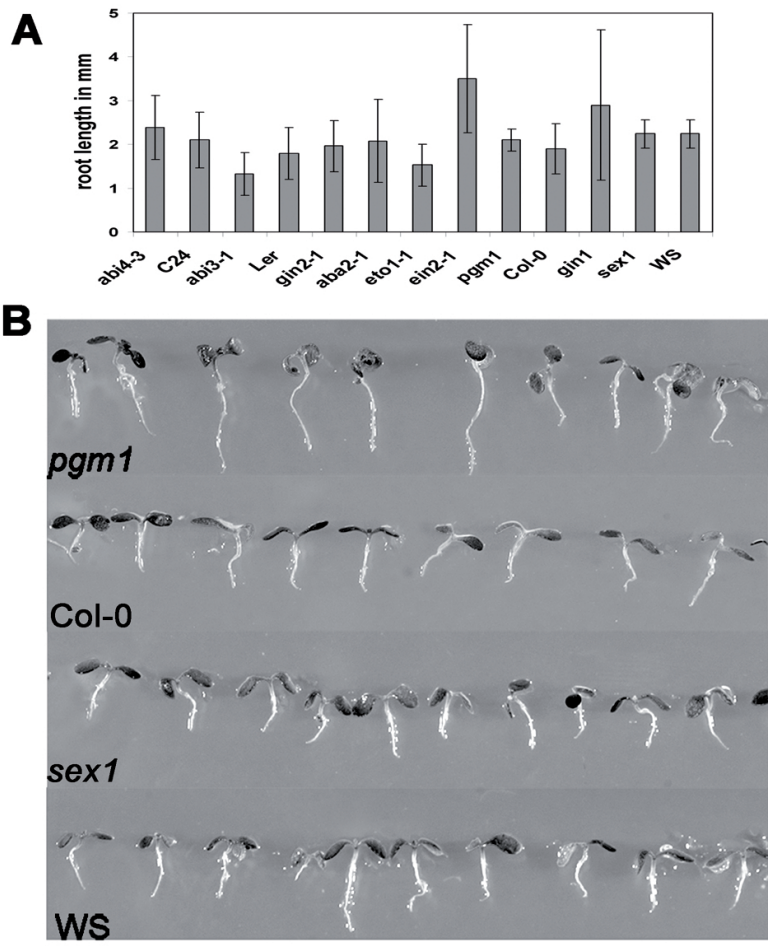

Figure 3. Growth of carbohydrate signalling mutants on 100 mM trehalose. A) Root lengths of different carbohydrate signalling mutants and their respective ecotype background on 100 mM trehalose. **B)** ***pgm, sex1*** and their respective accessions background on 100 mM trehalose. Seedlings were grown for 14 days on ½ MS medium containing 100 mM trehalose. After 14 days root lengths were measured using the image J programme.

on 100 mM trehalose, primary and secondary leaf anlagen emerged from 10% of the *pgm1* seedlings but these organs failed to extend.

The Cvi accession is partially resistant to 100 mM trehalose

Several accessions of *A. thaliana* were tested for their ability to grow on 100 mM trehalose. Growth arrest occurs in all accessions of Arabidopsis tested but is significantly less in seedlings of Cvi: seedlings from Cvi grew longer roots compared to all other tested accessions on 100 mM trehalose and displayed the same root length as other accessions on sorbitol control (not shown). Seedlings from Kas

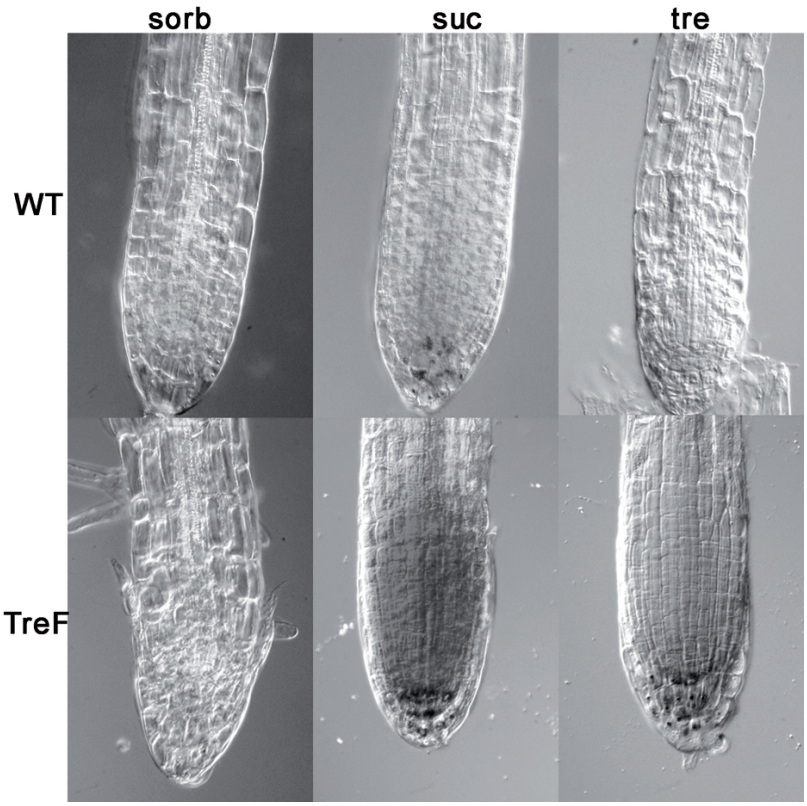

Figure 4. Effect of trehalose on starch accumulation in the dark. Seedlings WT and a trehalase expressing line (TreF) were grown for 14 d in the dark on half strength MS supplemented with 100 mM of either sorbitol (sorb), sucrose (suc) or trehalose (tre); seedlings were then stained with KI/I_2 and the roots viewed using Nomarski microscopy (x200).

appeared partially resistant to trehalose as well but already grew longer roots on 100 mM sorbitol compared to the other accessions. Increased trehalose tolerance of Kas is thus likely due to increased vigor of Kas seedlings (not shown). Cvi seedlings also accumulate less anthocyanins on 100 mM trehalose than all other accessions. Cvi seedlings synthesize less anthocyanins in response to sugars, the major locus responsible for this is SIAA, which was found to encode the *Myb75/PAP1* gene (Teng, S. et al. 2005). We therefore tested if altered *Myb75/PAP1* in Cvi was also responsible for trehalose resistance. This mutant is in the Nos background and stems from a transposon mutagenesis involving 2 parents. Growth on trehalose is equal in the parent lines, NaeAc380-16, Ds3-390-1, and the Pst16228 mutant thus excluding *Myb75/PAP1* as the locus responsible for the partial resistance of Cvi seedlings to trehalose (not shown).

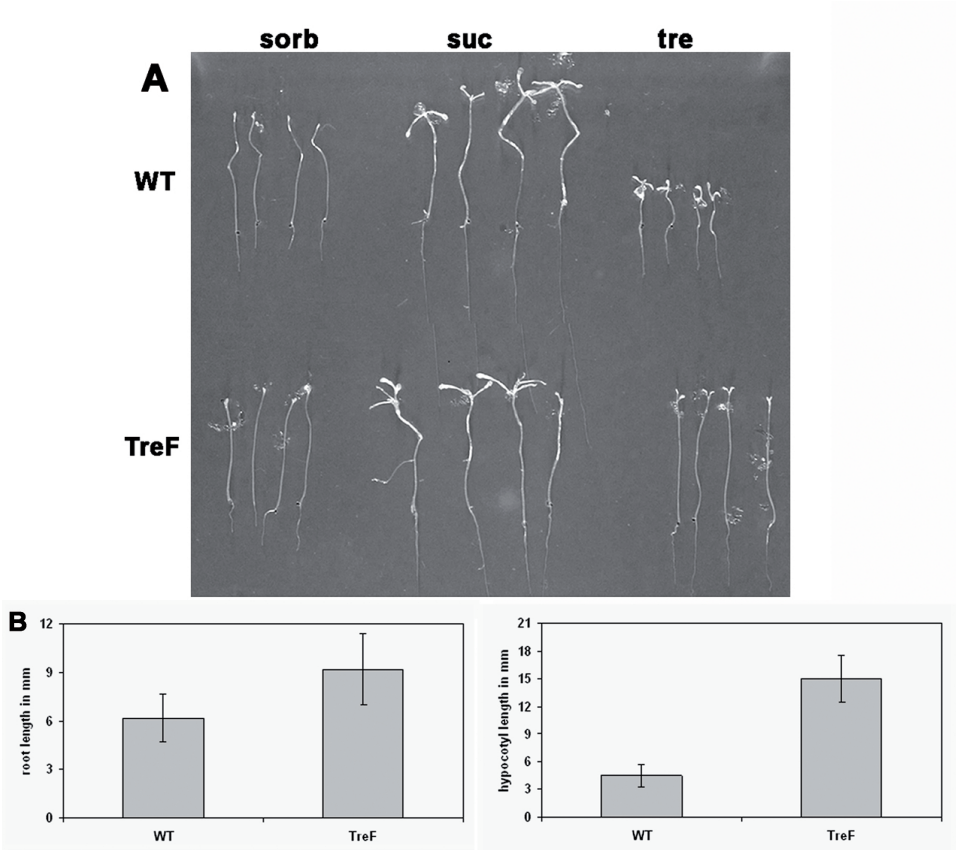

Figure 5. Effect of trehalose on growth in the dark. A) WT and TreF phenotypes on 100 mM of either sorbitol (sorb), sucrose (suc) or trehalose (tre) after 14 d in the dark. **B)** Root and hypocotyl lengths in WT and TreF seedlings growing on 100 mM trehalose for 14 d in the dark.

Dark development in seedlings growing on 100 mM trehalose

WT and TreF seedlings were grown on medium with 100 mM trehalose in continuous darkness for 14 days to study the effect of trehalose in the absence of photosynthesis. On trehalose and sorbitol, starch did not accumulate in the columnella of WT roots grown in darkness for 14d, whilst starch did accumulate in the columnella of WT roots grown on sucrose and of TreF expressing roots grown on trehalose as well as on sucrose (Fig. 4). Root lengths of WT and TreF expressing seedlings growing on 100 mM trehalose were not significantly different (Fig. 5A, B). Sorbitol at 100 mM concentration did not cause skotomorphogenesis (Fig. 5A). Trehalose, on the other hand lead to de-etiolation (not shown) and to skotomorphogenesis of WT seedlings, but the effect was not identical to the effect observed on sucrose (Fig. 5A). Hypocotyls length of WT seedlings were markedly shorter on trehalose compared with sucrose (Fig. 5B). In addition, hypocotyl lengths

of TreF expressing seedlings were restored and comparable to those obtained on sorbitol (Fig. 5A, B). Trehalose therefore triggers skotomorphogenesis like sucrose does, but trehalose does not support or inhibit hypocotyl elongation like sucrose. The data is consistent with starvation of the elongating hypocotyls on trehalose. The data also shows that photosynthesis is not required to mediate starvation effects of T6P or trehalose accumulation.

Specific changes in seedling gene-expression after trehalose feeding

In an attempt to characterize further the processes that are altered when seedlings are grown on 100 mM trehalose, expression profiling was carried out using the 8K gene chip from Affymetrix. Seedlings grown for 6d on half strength MS were then transferred for 24h to medium with 100 mM of either sorbitol, sucrose or trehalose. Three biological replicates were pooled to constitute one mRNA extract for one probe on each microarray, two microarrays were used for every sugar treatment. Results from this experiment have been published in Schluepmann et al. 2004, but genes specifically controlled by trehalose were not extracted from the data presented. Re-analysis using the LIMMA and VSN packages as described in Materials and Methods yielded the diagram shown in Figure 6. Whilst sucrose feeding leads to 1745 genes with significantly changed expression, trehalose leads to only 162 genes with significantly changed gene expression compared to sorbitol after 24h feeding. The changes after trehalose feeding range from the 4 fold repression of At3G60140, a glycosyl hydrolase family protein, up to 38 fold induction of *WAK1*. 60% of the genes affected by trehalose are also affected by sucrose: of the 113 trehalose induced genes, 57 are also induced by sucrose, and of the 49 genes repressed by trehalose 40 are also repressed by sucrose feeding. Seedlings grow on sucrose but fail to develop on trehalose medium. This could be due to trehalose induction of sucrose-induced genes during a lack of metabolisable carbon. A list of genes that respond to trehalose and sucrose in a similar way is provided in Table 1. Alternatively, induction or repression of genes important for growth supported by sucrose no longer occurs on trehalose and thus development is arrested. A list of genes differentially regulated by sucrose and trehalose is provided in Table 2. Statistical analysis failed to uncover genes that are repressed by trehalose but induced by sucrose, yet it identified 4 genes that are induced by trehalose but repressed by sucrose. These genes include *TPPF* (At4g12430), a homologue to *AtTPPB*, *PRO1* the mitochondrial proline oxidase the expression of which is stress responsive, a protein related to the vacuolar calcium binding protein encoded by At1g62480 and a protein related to the bacterial TOLB encoded by At4g01870. *TPPF* induction suggests that T6P accumulation upon trehalose feeding is sensed. Listing genes that respond to trehalose and remain unchanged by sucrose confirms this (Table 3): *TPPG* encoded by At4g22590, *TPPH* encoded by At4g39770 and

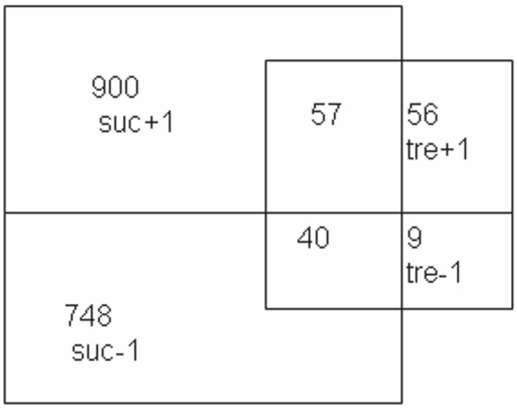

Figure 6. Genes with an expression specifically controlled by trehalose
Diagram of sugar induced gene-expression compared to sorbitol treatment; numbers refer to the number of genes with significant change; +suc, induced by sucrose; -suc, repressed by sucrose; +tre, induced by trehalose; -tre, repressed by trehalose. Affymetrix 8K gene chip as described in Schluepmann et al. 2004.

AtTPPB are also induced 5.8, 2.7 and 2.3 fold, respectively after trehalose feeding. In addition, trehalose specific gene induction reflects a specific stress response. In Table 2, *PRO1* is induced by trehalose whilst it is repressed by sucrose; in Table 3, induction of glutathione S-transferase, *TSA1*, *HSP17.7-CII* and *HSP17.4-CI*, *SAG102*, *PAD4*, anthranilate phophoribosyltransferase, sulfate adenylyltransferase3, *PDF2.3* and of several peroxidases is further indicative of oxidative stress and induction of secondary metabolite pathways. Five fold induction of the ethylene response factor *AtERF-2* and 4-fold induction of the coronatine induced protein1 further reveal likely involvement of JA and ethylene signal transduction mechanisms. Surprisingly, a number of genes that respond specifically to trehalose but not to sucrose are known from the field of biotic interactions: AvrRpt2-induced *AIG2* (4 fold induced), *EDS5* (4 fold induced), *PAD4* (4 fold induced) and *WAK2* (2.5 fold induced). In addition these are complemented by genes that are induced by both sucrose and trehalose, such as for example, *WAK1* (38 fold induced), *EDS1* (3 fold), suggestive of trehalose mediated priming for a specific disease response. Table 3B also reveals down regulation of *GASA1, MERI5B* and *EXGT-A3*, and *SEX1* on trehalose but not on sucrose. Down regulation of *SEX1* was also found by Ramon et al. 2007 in seedlings growing on the combination of 30 mM trehalose and 10 μM Validamycin. Down regulation of *GASA1, MERI5B* and *EXGT-A-3* may affect cell-elongation processes in roots and hypocotyls of seedlings grown on 100 mM trehalose. *ICL* is present on this 8K chip and the gene is seemingly catabolite repressed on sucrose but repression is not significant on trehalose (Table 4). Table 4 shows expression of genes typically repressed by carbon catabolite repression on sucrose; inspection of the behavior of genes in Table 4 on trehalose reveals that

trehalose is unable to raise catabolite repression as sucrose does. For example, from the 41 genes listed in Table 4 that show more than 5-fold repression by sucrose, only 4 genes are also repressed by trehalose: carbonic anhydrase 1 and 2 (2.5-fold each), *STP1* glucose transporter1 (3-fold) and asparagine synthetase2 (3.5-fold). *STP1* was previously found to be repressed by the combination of 30 mM trehalose and 10 μM validamycine (Ramon et al. 2007). In addition *MYB75* is 4-fold induced by sucrose but not by trehalose, again suggesting that trehalose is not just acting as a sucrose analogue on sucrose sensing pathways.

Table 1A: Genes induced in response to trehalose that are also induced by sucrose.
Seedlings were grown in shaking liquid medium in continuous light then supplied for 24h with 100 mM of either sorbitol, sucrose or trehalose before harvest and RNA extractions. The Affymetrix 8K gene chip was used for transcriptional profiling, and normalization and data analysis were as described in Materials and Methods. Probe set, probe set identity; A, average expression over the experiment (log2); suc, change after sucrose treatment with reference to sorbitol treatment (log2); tre, change after trehalose treatment with reference to sorbitol treatment (log2). P value suc, P value for the change after Sucrose; P value tre, P value for the change after trehalose; Locus, AGI number of the gene detected with the probe set; description, abbreviated TAIR annotation dating from February 2007.

Probe set NA	A	suc	tre	p.value suc	p.value tre	Locus	description
15616_s_at	9,5	5,0	5,2	9,1E-09	6,2E-09	AT1G21250	wall-associated kinase 1 (WAK1)
12879_at	8,0	2,5	4,8	4,9E-07	6,4E-09	AT1G33960	avirulence-responsive protein / avirulence induced gene (AIG1)
13217_s_at	7,4	6,8	5,1	4,9E-09	3,2E-08	AT3G50770	calmodulin-related protein, putative, similar to regulator of gene silencing calmodulin-related protein
17917_s_at	9,3	5,3	3,7	4,8E-09	5,7E-08	AT2G41090	calmodulin-like calcium-binding protein, 22 kDa (CaBP-22),
15431_at	9,0	2,2	2,0	1,6E-06	3,1E-06	AT4G27280	calcium-binding EF hand family protein
12521_at	9,2	4,0	2,4	1,0E-07	3,3E-06	AT3G51860	cation exchanger, putative (CAX3)
20323_at	6,5	2,7	2,4	1,6E-06	3,3E-06	AT2G29500	17.6 kDa class I small heat shock protein (HSP17.6B-CI)
16638_at	5,7	2,1	2,1	3,8E-06	4,5E-06	AT3G28210	zinc finger (AN1-like) family protein
18003_at	9,8	2,5	1,5	1,5E-07	4,6E-06	AT1G72930	Toll-Interleukin-Resistance (TIR) domain-containing protein
20653_s_at	6,7	1,2	1,6	5,4E-05	8,3E-06	AT3G48090	disease resistance protein (EDS1)
13177_at	8,4	1,2	2,0	2,4E-04	8,9E-06	AT4G12720	MutT/nudix family protein
16465_at	9,2	2,2	1,8	3,7E-06	1,1E-05	AT5G02490	heat shock cognate 70 kDa protein 2 (HSC70-2) (HSP70-2)
13279_at	7,1	1,4	2,2	2,6E-04	1,1E-05	AT5G12020	17.6 kDa class II heat shock protein (HSP17.6-CII)
18672_s_at	9,8	1,6	1,5	5,7E-06	1,3E-05	AT1G27770	calcium-transporting ATPase 1
20018_at	7,1	1,8	1,7	8,3E-06	1,5E-05	AT2G44300	lipid transfer protein-related
15483_s_at	5,8	2,2	2,9	1,0E-04	1,6E-05	AT2G46650	cytochrome b5
17104_s_at	10,2	2,2	1,7	2,6E-06	1,7E-05	AT4G35630	phosphoserine aminotransferase, chloroplast (PSAT)
14984_s_at	5,4	1,5	1,1	4,8E-06	3,1E-05	AT4G27560; AT4G27570	glycosyltransferase family protein
15629_s_at	8,9	4,4	2,4	5,2E-07	3,3E-05	AT1G17745	D-3-phosphoglycerate dehydrogenase / 3-PGDH,
15193_s_at	12,0	1,8	1,6	1,8E-05	4,0E-05	AT2G30870	glutathione S-transferase
13588_at	10,3	2,3	1,5	2,4E-06	4,6E-05	AT4G34200	D-3-phosphoglycerate dehydrogenase, putative / 3-PGDH
12881_s_at	11,4	1,2	1,5	1,9E-04	5,2E-05	AT5G42650	allene oxide synthase (AOS) / hydroperoxide dehydrase / cytochrome P450 74A (CYP74A),
12515_at	7,9	1,6	1,1	5,6E-06	6,2E-05	AT2G39700	expansin, putative (EXP4), similar to alpha-expansin 6 precursor
18591_at	8,3	1,5	1,6	7,7E-05	6,2E-05	AT5G08790	no apical meristem (NAM) family protein, contains Pfam
12500_s_at	8,2	2,9	1,3	3,5E-07	6,8E-05	AT1G51760	IAA-amino acid hydrolase 3 / IAA-Ala hydrolase 3 (IAR3), identical to IAA-Ala hydrolase (IAR3)
20017_at	10,8	2,6	1,6	3,2E-06	7,3E-05	AT2G44290	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein (YLS3)
17092_at	9,1	1,5	1,2	1,5E-05	7,7E-05	AT5G63980	3'(2'),5'-bisphosphate nucleotidase / inositol polyphosphate 1-phosphatase / FIERY1 protein (FRY1) (SAL1),
15211_s_at	8,3	0,8	1,0	3,3E-04	7,8E-05	AT5G27380	glutathione synthetase (GSH2)
13641_at	8,9	1,3	1,6	3,5E-04	8,6E-05	AT4G33300	disease resistance protein (CC-NBS-LRR class)
17600_s_at	6,4	1,6	1,5	6,1E-05	9,0E-05	AT5G42650	allene oxide synthase (AOS) / hydroperoxide dehydrase / cytochrome P450
18585_at	7,4	0,8	1,0	3,8E-04	9,7E-05	AT5G53130	cyclic nucleotide-regulated ion channel / cyclic nucleotide-gated channel (CNGC1),
16545_s_at	5,3	1,0	1,1	2,1E-04	1,0E-04	AT1G19850	transcription factor MONOPTEROS (MP) / auxin-responsive protein (IAA24) / auxin response factor 5 (ARF5
14686_at	8,2	1,1	1,3	2,7E-04	1,1E-04	AT1G09970	leucine-rich repeat transmembrane protein kinase, putative, Similar to A. thaliana receptor-like protein kinase
19833_s_at	9,9	0,9	1,0	3,1E-04	1,2E-04	AT5G04590	sulfite reductase / ferredoxin (SIR)
16916_s_at	7,1	2,9	2,0	1,2E-05	1,2E-04	AT5G02490	heat shock cognate 70 kDa protein 2 (HSC70-2) (HSP70-2)
14032_at	7,9	2,7	1,7	5,4E-06	1,4E-04	AT4G37370	cytochrome P450
12002_at	8,2	2,4	1,0	5,4E-07	1,5E-04	AT4G02940	oxidoreductase, 2OG-Fe(II) oxygenase family protein
14704_s_at	6,3	1,6	1,4	5,4E-05	1,5E-04	AT2G14560	expressed protein
18561_at	8,9	1,7	0,9	1,8E-06	1,7E-04	AT5G12140	cysteine protease inhibitor, putative / cystatin, putative
15196_s_at	5,6	1,5	1,2	5,2E-05	1,8E-04	AT4G04610	5'-adenylylsulfate reductase (APR1) / PAPS reductase homolog (PRH19),
18010_s_at	8,5	1,6	1,1	1,7E-05	1,9E-04	AT3G54110	plant uncoupling mitochondrial protein (PUMP)
18735_s_at	9,7	0,9	0,9	1,5E-04	2,0E-04	AT4G23100	glutamate-cysteine ligase / gamma-glutamylcysteine synthetase (GSH1)
14882_at	4,8	1,3	1,2	1,1E-04	2,1E-04	AT4G39670	expressed protein
19664_at	5,9	1,2	1,1	1,5E-04	2,5E-04	AT4G39230	isoflavone reductase
12883_at	8,2	1,8	0,8	1,3E-06	2,5E-04	AT4G08390	L-ascorbate peroxidase, stromal (sAPX)
17781_at	5,1	2,6	1,0	6,4E-07	2,6E-04	AT3G47780	ABC transporter family protein
15846_at	6,4	1,1	1,0	1,3E-04	2,6E-04	AT2G14560	expressed protein
17109_s_at	9,4	1,4	1,0	2,9E-05	3,0E-04	AT3G03640	glycosyl hydrolase family 1 protein
13834_at	6,1	1,2	1,2	2,9E-04	3,4E-04	AT4G01010	cyclic nucleotide-regulated ion channel
18594_at	9,2	1,6	0,8	4,1E-06	3,6E-04	AT1G01470	late embryogenesis abundant protein
15668_s_at	11,1	2,4	0,8	2,7E-07	3,6E-04	AT4G11010	nucleoside diphosphate kinase 3, mitochondrial (NDK3
14573_at	6,0	1,9	0,9	3,5E-06	3,9E-04	AT4G02360	expressed protein
18596_at	6,2	3,7	1,0	8,5E-08	4,0E-04	AT1G62570	flavin-containing monooxygenase family protein / FMO family protein
13282_s_at	6,7	5,8	1,2	1,5E-08	4,4E-04	AT4G25200	23.6 kDa mitochondrial small heat shock protein (HSP23.6-M)
14720_s_at	8,4	1,0	0,9	3,3E-04	5,0E-04	AT1G35720	annexin 1 (ANN1), identical to annexin (AnnAt1)
16462_s_at	8,6	1,6	0,7	2,9E-06	5,1E-04	AT2G38380	Peroxidase 22 (PER22)(P22)(PRXEA)/basic peroxidase E

Table 1 B: Genes repressed by trehalose that are also repressed by sucrose

Probe set NA	A	suc	tre	p.value suc	p.value tre	Locus	description
13645_at	8,0	-1,7	-1,8	1,3E-05	1,1E-05	AT1G05340	expressed protein
12574_at	5,6	-3,5	-2,1	4,2E-07	1,4E-05	AT3G60140	glycosyl hydrolase family 1 protein
16422_at	9,2	-5,0	-1,7	1,0E-08	1,6E-05	AT2G33830	dormancy/auxin associated family protein
16488_at	8,9	-4,3	-1,6	2,9E-08	2,2E-05	AT1G11260	glucose transporter (STP1)
15122_at	11,9	-2,1	-1,2	9,7E-07	4,2E-05	AT3G16240	delta tonoplast integral protein (delta-TIP)
15422_at	7,1	-3,6	-1,4	1,2E-07	5,0E-05	AT4G04330	expressed protein
20290_s_at	6,6	-1,4	-1,2	2,6E-05	9,3E-05	AT5G25120 AT5G25130	cytochrome P450 family protein
18755_at	7,2	-2,1	-1,3	3,7E-06	1,0E-04	AT4G25780	pathogenesis-related protein, putative, similar to gene PR-1 protein
19734_at	6,8	-1,9	-1,2	6,7E-06	1,0E-04	AT3G26630	pentatricopeptide (PPR) repeat-containing protein
20362_at	7,5	-3,8	-1,2	6,4E-08	1,2E-04	AT1G71030	myb family transcription factor
19453_at	5,1	-4,1	-1,9	9,3E-07	1,4E-04	AT2G22980	similar to serine carboxypeptidase S10 family protein
13972_at	5,8	-2,0	-1,1	2,9E-06	1,4E-04	AT4G17810	similar to zinc finger (C2H2 type) family protein
15901_at	7,4	-2,6	-0,9	2,1E-07	1,7E-04	AT1G54740	expressed protein
14737_s_at	10,1	-3,9	-1,2	6,3E-08	1,9E-04	AT2G13360	serine-glyoxylate aminotransferase-related
14386_at	7,9	-2,8	-1,2	6,5E-07	2,1E-04	AT2G47910	expressed protein
12389_at	5,9	-1,2	-1,0	4,5E-05	2,1E-04	AT1G78720	protein transport protein sec61
19759_at	8,3	-1,5	-0,8	5,4E-06	2,4E-04	AT1G23020	ferric-chelate reductase
16648_at	8,4	-2,5	-0,9	1,9E-07	2,6E-04	AT5G20240	floral homeotic protein PISTILLATA (PI)
16141_s_at	8,6	-1,0	-0,8	5,3E-05	3,0E-04	AT1G58360	amino acid permease I (AAP1)
18484_at	8,4	-1,4	-0,9	2,5E-05	3,2E-04	AT4G37760	squalene monooxygenase
14930_at	7,7	-2,3	-0,8	2,2E-07	3,3E-04	AT2G21530	forkhead-associated domain-containing protein
16490_at	8,3	-2,7	-0,9	2,0E-07	3,4E-04	AT2G18280	tubby-like protein 2 (TULP2)
20344_at	6,5	-2,7	-1,3	3,7E-06	3,7E-04	AT2G15090	fatty acid elongase
13706_at	6,1	-4,2	-1,2	1,1E-07	3,7E-04	AT2G18700	glycosyl transferase family 20 protein / trehalose-phosphatase family protein, similar to trehalose-6-phosphate synthase SL-TPS/P
12768_at	9,2	-2,9	-1,0	3,9E-07	3,8E-04	AT2G15890	expressed protein
16428_at	9,3	-5,4	-1,4	4,5E-08	3,9E-04	AT3G01500	carbonic anhydrase 1
15144_s_at	8,6	-4,3	-1,3	1,8E-07	4,0E-04	AT5G14740	carbonic anhydrase 2
13803_at	7,0	-3,0	-1,0	3,4E-07	4,1E-04	AT4G16690	esterase/lipase/thioesterase family protein
13382_at	6,6	-1,8	-0,9	3,9E-06	4,1E-04	AT2G42750	DNAJ heat shock N-terminal domain
14387_g_at	6,6	-2,8	-1,2	1,5E-06	4,2E-04	AT2G47910	expressed protein
19545_at	10,0	-3,2	-0,9	9,7E-08	4,4E-04	AT1G54500	rubredoxin family protein
17361_s_at	3,4	-1,3	-1,2	2,9E-04	4,7E-04	AT4G10120	sucrose-phosphate synthase
19451_at	4,6	-2,0	-0,9	2,7E-06	4,7E-04	AT1G61820	glycosyl hydrolase family 1 protein
18976_at	7,9	-2,2	-0,9	1,2E-06	4,7E-04	AT1G08980	amidase family protein
15154_at	8,3	-3,3	-1,8	1,1E-05	4,8E-04	AT3G47340	asparagine synthetase 1
18009_s_at	8,8	-3,2	-1,5	3,1E-06	4,8E-04	AT2G25080	phospholipid hydroperoxide glutathione peroxidase
16210_at	5,4	-2,1	-0,7	4,8E-07	4,8E-04	AT4G04850	K+ efflux antiporter
12785_at	10,5	-2,5	-1,1	3,6E-06	4,9E-04	AT4G33010	glycine dehydrogenase
15576_s_at	8,0	-4,5	-1,8	1,6E-06	5,1E-04	AT2G25900	zinc finger (CCCH-type) family protein
15519_s_at	6,4	-3,3	-1,3	1,6E-06	5,4E-04	AT1G03090	methylcrotonyl-CoA carboxylase alpha chain

Table 2. Genes induced in response to trehalose that are repressed by sucrose.
Seedlings were grown in shaking liquid medium in continuous light then supplied for 24h with 100 mM of either sorbitol, sucrose or trehalose before harvest and RNA extractions. The Affymetrix 8K gene chip was used for transcriptional profiling, and normalization and data analysis were as described in Materials and Methods. Probe set, probe set identity; A, average expression over the experiment (log2); suc, change after sucrose treatment with reference to sorbitol treatment (log2); tre, change after trehalose treatment with reference to sorbitol treatment (log2). P value suc, P value for the change after Sucrose; P value tre, P value for the change after trehalose; Locus, AGI number of the gene detected with the probe set; description, abbreviated TAIR annotation dating from February 2007.

Probe set NA	A	suc	tre	p.value suc	p.value tre	locus	description
20570_at	5,62	-1,14	2,26	6,5E-05	7,2E-07	AT4G12430	trehalose-6-phosphate phosphatase, putative, similar to trehalose-6-phosphate phosphatase (AtTPPB) (Arabidopsis thaliana)
15124_s_at	10,29	-1,62	0,85	3,0E-06	1,9E-04	AT3G30775	proline oxidase, mitochondrial / osmotic stress-responsive proline dehydrogenase (POX) (PRO1) (ERD5)
17909_at	9,36	-1,99	1,49	5,5E-05	3,4E-04	AT1G62480	vacuolar calcium-binding protein-related, contains weak similarity to vacuolar calcium binding protein (Raphanus sativus)
13656_at	8,44	-1,49	1,25	1,5E-04	4,3E-04	AT4G01870	tolB protein-related, contains weak similarity to TolB protein precursor (Swiss-Prot:P44677) (Haemophilus influenzae)

Table 3A. Genes induced in response to 100 mM trehalose and that are unchanged by sucrose.
Seedlings were grown in shaking liquid medium in continuous light then supplied for 24h with 100 mM of either sorbitol, sucrose or trehalose before harvest and RNA extractions. The Affymetrix 8K gene chip was used for transcriptional profiling, and normalization and data analysis were as described in Materials and Methods. Probe set, probe set identity; A, average expression over the experiment (log2); suc, change after sucrose treatment with reference to sorbitol treatment (log2); tre, change after trehalose treatment with reference to sorbitol treatment (log2). P value suc, P value for the change after Sucrose; P value tre, P value for the change after trehalose; Locus, AGI number of the gene detected with the probe set; description, abbreviated TAIR annotation dating from February 2007.

Probe set NA	A	suc	tre	p.value suc	p.value tre	Locus	description
19640_at	7,3	1,3	3,0	6,9E-04	3,3E-06	AT2G29460	glutathione S-transferase, putative
19883_at	7,8	0,7	2,5	6,7E-04	1,1E-07	AT4G22590	trehalose-6-phosphate phosphatase, putative, similar to trehalose-6-phosphate phosphatase (AtTPPA)
16439_at	2,7	0,8	2,4	1,8E-02	3,8E-05	AT1G31580	expressed protein, identical to ORF1 (Arabidopsis thaliana)
16609_at	5,5	1,0	2,4	7,7E-04	3,8E-06	AT5G47220	encodes a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family (ATERF-2)
14672_at	6,8	1,3	2,3	9,3E-03	4,7E-04	AT3G54640	tryptophan synthase, alpha subunit (TSA1)
18881_at	8,9	0,7	2,2	4,0E-03	2,3E-06	AT1G12080	expressed protein
12880_at	5,9	0,6	2,2	9,6E-02	1,6E-04	AT3G28930	avrRpt2-induced AIG2 protein (AIG2)
13277_i_at	7,4	1,5	2,1	5,7E-04	5,7E-05	AT5G12030	17.7 kDa class II heat shock protein 17.6A (HSP17.7-CII)
15778_at	5,3	0,0	2,0	9,1E-01	5,0E-04	AT3G46090	zinc finger (C2H2 type) family protein (ZAT7)
12916_at	4,5	-0,7	2,0	5,4E-02	2,2E-04	AT1G19670	coronatine-responsive protein / coronatine-induced protein 1 (CORI1)
18228_at	5,6	-0,6	1,8	9,9E-02	5,0E-04	AT3G15356	legume lectin family protein, contains Pfam domain, PF00139: Legume lectins beta domain
17653_at	7,0	0,5	1,8	5,3E-02	1,2E-04	AT4G39030	enhanced disease susceptibility 5 (EDS5) / salicylic acid induction deficient 1 (SID1),
17901_at	7,4	0,9	1,8	6,1E-04	9,1E-06	AT2G44670	senescence-associated protein-related
14249_i_at	7,6	0,6	1,7	8,0E-02	5,3E-04	AT3G52430	phytoalexin-deficient 4 protein (PAD4)
13275_f_at	7,3	1,2	1,7	1,1E-03	1,1E-04	AT3G46230	17.4 kDa class I heat shock protein (HSP17.4-CI)
20547_at	5,7	-0,4	1,7	4,5E-02	1,6E-05	AT5G04950	nicotianamine synthase, putative, similar to nicotianamine synthase (Lycopersicon esculentum)
14620_at	6,4	0,7	1,7	2,4E-02	3,1E-04	AT5G17990	anthranilate phosphoribosyltransferase
17338_at	4,7	0,7	1,7	5,4E-03	2,1E-05	AT2G47550	pectinesterase family protein, contains Pfam profile: PF01095 pectinesterase
20429_at	8,5	-1,3	1,7	5,5E-04	1,5E-04	AT4G14400	ankyrin repeat family protein, contains ankyrin repeats, Pfam domain PF00023
13278_f_at	7,4	1,2	1,7	2,0E-03	3,6E-04	AT5G12030	17.7 kDa class II heat shock protein 17.6A (HSP17.7-CII)
14250_r_at	5,8	0,6	1,7	5,2E-02	4,3E-04	AT3G52430	phytoalexin-deficient 4 protein (PAD4)
20259_at	3,3	0,7	1,5	7,9E-04	9,3E-06	AT4G23200	protein kinase family protein, contains Pfam PF00069: Protein kinase domain
15647_s_at	5,9	0,4	1,4	1,1E-02	5,7E-06	AT4G14680	sulfate adenylyltransferase 3 / ATP-sulfurylase 3 (APS3)
15243_at	6,7	-0,5	1,4	1,1E-02	3,0E-05	AT4G39770	trehalose-6-phosphate phosphatase, putative, similar to trehalose-6-phosphate phosphatase (AtTPPB)
14781_at	6,6	-0,4	1,4	1,5E-02	8,7E-06	AT2G22850	bZIP transcription factor family protein
15859_at	7,1	0,7	1,3	1,6E-03	3,5E-05	AT2G28570	expressed protein
16140_s_at	5,9			1,9E-03	2,1E-04	AT1G21270	wall-associated kinase 2 (WAK2)
19463_s_at	7,3	1,0	1,3	2,0E-03	3,5E-04	AT4G39980	2-dehydro-3-deoxyphosphoheptonate aldolase 1 / 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase 1 / DAHP synthetase 1 (DHS1)
18314_i_at	7,4	0,3	1,3	8,3E-02	7,2E-05	AT4G27830	glycosyl hydrolase family 1 protein, contains Pfam PF00232 : Glycosyl hydrolase family 1 domain;
19713_at	7,2	-0,8	1,2	5,3E-03	3,4E-04	AT4G18340	glycosyl hydrolase family 17 protein
16416_at	8,9	0,2	1,2	2,0E-01	2,2E-05	AT2G02130	plant defensin-fusion protein, putative (PDF2.3), plant defensin protein family member
17045_at	4,8	0,4	1,2	2,1E-02	4,3E-05	AT1G78090	trehalose-6-phosphate phosphatase (TPPB)
17119_s_at	5,8	0,5	1,2	2,2E-02	2,5E-04	AT2G06050	12-oxophytodienoate reductase (OPR3) / delayed dehiscence1 (DDE1)
13236_at	7,8	0,9	1,2	2,2E-03	4,0E-04	AT4G39980	2-dehydro-3-deoxyphosphoheptonate aldolase 1 / 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase 1 / DAHP synthetase 1 (DHS1)
17464_at	8,2	0,7	1,1	5,1E-03	3,8E-04	AT1G09970	leucine-rich repeat transmembrane protein kinase
16963_at	7,2	0,4	1,1	3,1E-02	1,1E-04	AT2G38390	peroxidase, putative, similar to peroxidase isozyme (Armoracia rusticana)
16493_at	8,3	0,7	1,1	2,1E-03	1,2E-04	AT1G54010	myrosinase-associated protein, putative, similar to myrosinase-associated protein GI:1769969 from (Brassica napus)
12752_s_at	3,9	0,4	1,1	3,1E-02	1,6E-04	AT4G21960	peroxidase 42 (PER42) (P42) (PRXR1)
12744_at	6,8	0,1	1,0	2,8E-01	7,6E-05	AT3G16470	jacalin lectin family protein, contains Pfam profile: PF01419 jacalin-like lectin domain;
14697_g_at	4,7	0,2	1,0	2,2E-01	1,1E-04	AT2G21620	universal stress protein (USP) family protein / responsive to dessication protein (RD2)
17075_s_at	6,5	-0,2	1,0	2,6E-01	1,5E-04	AT5G22300	nitrilase 4 (NIT4)
12472_s_at	7,2	0,0	0,9	7,2E-01	1,5E-04	AT1G59960	aldo/keto reductase, putative, similar to NADPH-dependent codeinone reductase
15568_at	4,7	0,2	0,9	2,8E-01	5,4E-04	AT4G17910	zinc finger (C3HC4-type RING finger) family protein / pentatricopeptide (PPR) repeat-containing protein, contains Pfam domains
12202_at	5,1	0,5	0,9	1,1E-02	2,7E-04	AT3G50650	scarecrow-like transcription factor 7 (SCL7)
16902_at	6,9	0,2	0,9	1,1E-01	1,5E-04	AT2G21620	universal stress protein (USP) family protein / responsive to dessication protein (RD2),
16064_s_at	6,5	0,3	0,9	5,6E-02	2,6E-04	AT3G15210	encodes a member of the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family (ATERF-4)
17513_s_at	7,3	0,5	0,9	7,9E-03	2,7E-04	AT1G26820	ribonuclease 3 (RNS3)
16610_s_at	6,5	0,1	0,8	5,5E-01	2,2E-04	AT1G19050	two-component responsive regulator / response regulator 7 (ARR7)
16061_s_at	7,1	0,5	0,8	1,3E-02	5,3E-04	AT4G26070	mitogen-activated protein kinase kinase (MAPKK) (MKK1) (MEK1)
16362_at	7,1	0,0	0,8	7,2E-01	1,9E-04	AT2G33050	leucine-rich repeat family protein, contains leucine rich-repeat domains Pfam:PF00560,
17641_g_at	5,2	0,2	0,8	1,9E-01	4,3E-04	AT4G10310	sodium transporter (HKT1)
17341_at	4,1	0,0	0,8	6,9E-01	3,1E-04	AT4G18250	receptor serine/threonine kinase, putative, similar to to receptor serine/threonine kinase PR5K
16643_s_at	4,5	0,2	0,8	2,0E-01	5,2E-04	AT3G45610	Dof-type zinc finger domain-containing protein
18626_at	5,9	-0,1	0,7	3,8E-01	3,4E-04	AT4G00780	meprin and TRAF homology domain-containing protein / MATH domain-containing protein, contains Pfam profile PF00917: MATH domain
13172_s_at	10,5	0,0	0,7	7,6E-01	5,0E-04	AT1G20840	transporter-related, low similarity to D-xylose proton-symporter (Lactobacillus brevis)

Table 3B. Genes repressed by trehalose that are unchanged by sucrose.

Probe set NA	A	Suc	Tre	p.value suc	p.value tre	Locus	description
16014_at	12,2	0,2	-2,0	3,6E-01	1,8E-05	AT1G75750	gibberellin-regulated protein 1 (GASA1) / gibberellin-responsive protein 1
16927_s_at	11,0	0,1	-1,1	3,3E-01	2,7E-05	AT4G30270	MERI-5 protein (MERI-5) (MERI5B) / endo-xyloglucan transferase / xyloglucan endo-1,4-beta-D-glucanase (SEN4)
12532_at	8,7	-0,4	-1,0	2,2E-02	1,2E-04	AT1G10760	starch excess protein (SEX1), identical to SEX1 (Arabidopsis thaliana)
17103_s_at	9,9	-0,6	-1,1	4,6E-03	1,5E-04	AT2G01850	xyloglucan:xyloglucosyl transferase / xyloglucan endotransglycosylase / endo-xyloglucan transferase (EXGT-A3)
19877_at	5,0	-0,7	-0,9	9,6E-04	3,2E-04	AT3G48390	MA3 domain-containing protein, similar to programmed cell death 4 protein (Gallus gallus)
18270_at	8,6	-0,3	-0,7	2,2E-02	3,3E-04	AT2G30930	expressed protein
20015_at	7,6	-0,8	-1,0	1,8E-03	3,9E-04	AT1G10020	expressed protein
20066_at	7,6	-0,5	-0,8	9,0E-03	5,2E-04	AT2G35840	sucrose-phosphatase 1 (SPP1), identical to sucrose-phosphatase (SPP1) (Arabidopsis thaliana)

Table 4: Genes typically affected by catabolic repression.

Seedlings were grown in shaking liquid medium in continuous light then supplied for 24h with 100 mM of either sorbitol, sucrose or trehalose before harvest and RNA extractions. The Affymetrix 8K gene chip was used for transcriptional profiling, and normalization and data analysis were as described in Materials and Methods. Probe set, probe set identity; A, average expression over the experiment (log2); suc, change after sucrose treatment with reference to sorbitol treatment (log2); tre, change after trehalose treatment with reference to sorbitol treatment (log2). P value suc, P value for the change after Sucrose; P value tre, P value for the change after trehalose; Locus, AGI number of the gene detected with the probe set; description, abbreviated TAIR annotation dating from February 2007.

Probe set NA	A	suc	tre	p.value suc	p.value tre	Locus	description
16428_at	9,3	-5,4	-1,4	4,5E-08	3,9E-04	AT3G01500	carbonic anhydrase 1, chloroplast / carbonate dehydratase 1 (CA1
18087_s_at	9,6	-4,7	-0,6	3,1E-08	9,3E-03	AT1G03130	photosystem I reaction center subunit II, chloroplast, putative / photosystem I 20 kDa subunit, putative / PSI-D, putative (PSAD2)
16488_at	8,9	-4,3	-1,6	2,9E-08	2,2E-05	AT1G11260	glucose transporter (STP1), nearly identical to glucose transporter GB:P23586 SP:P23586 from (Arabidopsis thaliana)
15144_s_at	8,6	-4,3	-1,3	1,8E-07	4,0E-04	AT5G14740	carbonic anhydrase 2 / carbonate dehydratase 2 (CA2) (CA18)
18276_at	10,0	-4,2	-0,6	2,2E-07	1,9E-02	AT1G03600	photosystem II family protein, similar to SP:P74367 {Synechocystis sp.}
11994_at	5,7	-4,0	-1,1	1,4E-06	3,7E-03	AT2G39470	photosystem II reaction center PsbP family protein
16034_at	9,4	-3,7	-0,8	2,2E-07	2,7E-03	AT1G32060	phosphoribulokinase (PRK) / phosphopentokinase,
13678_at	10,1	-3,7	-1,0	1,4E-07	7,5E-04	AT1G19150	chlorophyll A-B binding protein, putative / LHCI type II, putative, very strong similarity to PSI type II chlorophyll a/b-binding protein Lhca2*1 GI:541565
16004_s_at	11,4	-3,6	-0,1	2,0E-07	6,7E-01	AT3G54890	chlorophyll A-B binding protein / LHCI type I (CAB), identical to chlorophyll A/B-binding protein (Arabidopsis thaliana)
16673_at	8,5	-3,6	-1,0	1,1E-07	5,7E-04	AT5G23120	photosystem II stability/assembly factor, chloroplast (HCF136
16485_s_at	10,1	-3,5	-0,8	4,7E-07	4,9E-03	AT3G50820	Encodes a protein which is an extrinsic subunit of photosystem II and which has been proposed to play a central role in stabilization of the catalytic manganese cluster.
16899_at	9,6	-3,5	-0,5	6,5E-07	4,2E-02	AT1G51400	photosystem II 5 kD protein, 100% identical to GI:4836947 (F5D21.10)
13213_s_at	11,6	-3,5	0,1	6,9E-07	6,8E-01	AT3G54890	chlorophyll A-B binding protein / LHCI type I (CAB
15133_at	11,8	-3,4	-0,2	4,0E-08	2,9E-01	AT3G61470	chlorophyll A-B binding protein (LHCA2), identical to Lhca2 protein (Arabidopsis thaliana) GI:4741940
16497_at	10,4	-3,4	-0,2	4,4E-07	2,5E-01	AT3G21055	photosystem II 5 kD protein, putative
17087_at	10,4	-3,4	-0,5	1,5E-06	7,0E-02	AT5G64040	photosystem I reaction center subunit PSI-N, chloroplast, putative / PSI-N, putative (PSAN), SP:P49107
15154_at	8,3	-3,3	-1,8	1,1E-05	4,8E-04	AT3G47340	asparagine synthetase 1 (glutamine-hydrolyzing) / glutamine-dependent asparagine synthetase 1 (ASN1)
15153_at	7,1	-3,2	-1,5	1,0E-04	9,5E-03	AT3G27690	chlorophyll A-B binding protein (LHCB2:4)
12610_at	7,5	-3,2	-0,7	3,1E-06	2,2E-02	AT3G51820	chlorophyll synthetase, putative
16449_s_at	11,8	-3,1	-0,2	2,5E-07	1,6E-01	AT5G66570	Encodes a protein which is an extrinsic subunit of photosystem II and which has been proposed to play a central role in stabilization of the catalytic manganese cluster
16018_s_at	9,4	-3,1	-0,6	4,9E-07	1,1E-02	AT3G26650	glyceraldehyde 3-phosphate dehydrogenase A, chloroplast (GAPA) / NADP-dependent glyceraldehydephosphate dehydrogenase subunit A
16424_g_at	11,5	-3,0	-0,4	4,9E-07	5,8E-02	AT2G30570	photosystem II reaction center W (PsbW) protein-related
18077_at	10,8	-3,0	-0,2	5,8E-07	2,4E-01	AT1G31330	photosystem I reaction center subunit III family protein, contains Pfam profile: PF02507: photosystem I reaction center subunit III
12611_g_at	7,6	-3,0	-0,7	1,7E-06	8,9E-03	AT3G51820	chlorophyll synthetase, putative
18081_at	10,4	-3,0	-0,4	3,1E-07	2,7E-02	AT1G52230	photosystem I reaction center subunit VI, chloroplast, putative / PSI-H, putative (PSAH2)
17411_s_at	7,0	-3,0	-0,8	4,1E-07	2,0E-03	AT1G77490	L-ascorbate peroxidase, thylakoid-bound (tAPX), identical to thylakoid-bound ascorbate peroxidase GB:CAA67426 (Arabidopsis thaliana)
18082_at	11,6	-2,8	-0,3	5,8E-07	7,0E-02	AT1G30380	photosystem I reaction center subunit psaK, chloroplast, putative / photosystem I subunit X, putative / PSI-K, putative (PSAK)
15102_s_at	11,4	-2,8	-0,2	2,9E-07	3,4E-01	AT4G10340	chlorophyll A-B binding protein CP26, chloroplast / light-harvesting complex II protein 5 / LHCIIc (LHCB5)
15182_at	10,2	-2,8	-0,9	8,2E-07	9,5E-04	AT1G68010	glycerate dehydrogenase / NADH-dependent hydroxypyruvate reductase
18088_i_at	10,7	-2,8	-0,2	7,2E-07	3,0E-01	AT4G28750	photosystem I reaction center subunit IV, chloroplast, putative / PSI-E, putative (PSAE1)
14729_s_at	11,4	-2,7	-0,5	3,9E-07	9,5E-03	AT3G26650	glyceraldehyde 3-phosphate dehydrogenase A, chloroplast (GAPA) / NADP-dependent glyceraldehydephosphate dehydrogenase subunit A
18084_s_at	11,8	-2,6	-0,3	4,8E-07	1,1E-01	AT4G12800	photosystem I reaction center subunit XI, chloroplast (PSI-L) / PSI subunit V,
13234_s_at	11,0	-2,6	-0,2	7,9E-07	1,9E-01	AT1G20340	plastocyanin, similar to plastocyanin GI:1865683 from (Arabidopsis thaliana)
15097_at	11,9	-2,6	-0,3	3,2E-07	8,0E-02	AT1G15820	chlorophyll A-B binding protein, chloroplast (LHCB6)
18665_r_at	5,5	-2,6	-0,4	3,2E-05	1,6E-01	AT2G20260	photosystem I reaction center subunit IV, chloroplast, putative / PSI-E, putative (PSAE2)
18666_s_at	10,3	-2,6	-0,2	5,8E-06	2,9E-01	AT2G20260	photosystem I reaction center subunit IV, chloroplast, putative / PSI-E, putative (PSAE2)
15995_at	12,2	-2,6	-0,1	5,3E-07	4,0E-01	AT5G01530	chlorophyll A-B binding protein CP29 (LHCB4)
15373_g_at	9,2	-2,5	-0,8	1,1E-05	1,3E-02	AT1G76100	plastocyanin, identical to plastocyanin GI:1865683 from (Arabidopsis thaliana)
16423_at	11,8	-2,5	-0,3	3,2E-07	7,7E-02	AT2G30570	photosystem II reaction center W (PsbW) protein-related
18086_s_at	10,5	-2,5	-0,3	3,3E-06	1,6E-01	AT4G02770	photosystem I reaction center subunit II, chloroplast, putative / photosystem I 20 kDa subunit, putative / PSI-D, putative (PSAD1)
16417_s_at	11,9	-2,5	-0,1	1,6E-06	4,5E-01	AT4G12800	photosystem I reaction center subunit XI, chloroplast (PSI-L) / PSI subunit V,

Discussion

In the light, growth arrest on 100 mM trehalose is due to T6P accumulation and can be rescued by exogenous supply of metabolisable sugar (Schluepmann et al. 2004). This growth arrest is not mediated by the known sugar signaling proteins ABI4, ABI3, ABA2 and HXK1. This growth arrest further occurs in all accessions of Arabidopsis tested but is significantly less in seedlings of Cvi. Partial resistance of Cvi seedlings is not due to the altered MYB75/PAP1 allele found in this accession. Gene expression profiling after 24h reveals that 100 mM trehalose alters 5% of the genes controlled by 100 mM sucrose. After 24h, trehalose does not cause carbon catabolite repression of gene-expression, but induces a specific combination of genes known from biotic stress responses. After 14d, trehalose results in reduced chlorophyll contents, in anthocyanin accumulation, and causes cells of the root extension zone to swell and lyse. In the dark, 100 mM trehalose leads to sugar-induced skotomorphogenesis in seedlings yet it inhibits hypocotyl elongation without altering root growth.

T6P control over growth is mediated by a novel pathway distinct from known sugar signaling pathways

To study genetic interactions between the control of T6P over growth and that from known pathways in sugar signaling, characteristic sugar signaling mutants were used. Growth on trehalose of the *abi3-1, abi4-3, aba2-1* mutants and WT did not differ significantly suggesting that ABA is not involved in mediating growth arrest due to T6P accumulation. This contrasts with previously published results where ABI4 was found to "partially" mediate the effects of 25 mM exogenous trehalose (Ramon, M. et al. 2007). A possible explanation for this discrepancy might be the different assay condition used by Ramon et al. 2007: lower trehalose levels combined with Validamycine A. The *gin2* mutant (Hexokinase1) was also sensitive to 100 mM trehalose suggesting that T6P mediated growth arrest on trehalose is not via AtHXK1, as previously concluded by (Ramon, M. et al. 2007). Similarly, the glucose insensitive (*eto1, ctr1)* and glucose hypersensitive mutants (*etr1, ein2*) were sensitive to 100 mM trehalose suggesting that the ethylene pathway does not interact with T6P's control over growth.

pgm1 and *sex1* susceptibility to 100 mM trehalose confirms previous work on 25 mM trehalose suggesting that starch deposition in the cotyledon is not the cause for the developmental arrest of sink meristems (Fritzius, T. et al. 2001). Interestingly, exogenous trehalose mediates some form of stress signaling that results in anthocyanin accumulation and chlorophyll decrease.

Growth arrest by T6P accumulation is found both in the light and in the dark

Initial screening for EMS mutants resistant to 100 mM trehalose yielded 27 mutants per 2g M2 seed. A subtype of bleached EMS M2 seedlings was found resistant to trehalose and this suggested that growth arrest in the light be dependent on a light generated process in the chloroplast (not shown). Growth experiments in the dark however refute this: whilst skotomorphogenesis is induced by exogenous trehalose just like by sucrose, growth and extension of the hypocotyls in the dark no longer occurs when trehalose is supplied exogenously. Hypocotyl elongation during etiolation has previously been shown to depend on gluconeogenesis from lipids stored in both the embryo and the endosperm of Arabidopsis (Cornah, J. E. et al. 2004, Eastmond, P. J. et al. 2000, Penfield, S. et al. 2004, Penfield, S. et al. 2005b, Pracharoenwattana, I. et al. 2005). Yet some of the enzymes of the glyoxylate cycle are also repressed by carbon catabolite repression (Graham, I. A. et al. 1994). Gluconeogenesis may be stopped on trehalose because of catabolite repression in the source tissue, the cotyledon, which is also the place of most lipid storage. Further lipids are stored in the endosperm and have been shown to contribute to hypocotyls extension in the dark (Penfield, S. et al. 2005a), and so it is likely that T6P accumulation controls lipid remobilization from the endosperm as well.

Genes with an expression specifically controlled by T6P

Microarray expression profiles revealed that carbon catabolite repression is not very pronounced after 24h of trehalose compared with sucrose feeding. However, long-term growth on trehalose elicits catabolite repression, chlorophyll levels and LHCB1 were considerably lower on trehalose (Fig. 1 and Chapter 3-Fig. 3, data not shown). This repression is consistent with a senescence response due to starvation rather than hexose accumulation and catabolite respression (Lim, JD. et al. 2006, Moore, B. et al. 2003). Starvation may result from reactions triggered by trehalose or T6P. Alternatively, T6P activates carbon utilization processes in the absence of metabolisable carbon. Microarray expression profiles show that fed trehalose is not just signaling like a sucrose analogue, far less genes respond to trehalose, compared to sucrose. Possibly, metabolisable sugars like sucrose are also sensed in metabolic steps downstream such as in glycolysis. This was previously found for the response to carbon and nitrogen that may depend on a metabolite product of carbon and nitrogen assimilation (Gutierrez, R. A. et al. 2007). Alternarively, trehalose is recognized specifically and elicits gene expression responses consistent with ROS and secondary metabolism activation. The profile of expression shows particularly high induction (8-fold) of a glutathione transferase (GST22) on trehalose but not on sucrose (Table 3A). The enzyme is also induced by phytoprostanes that are made upon ROS oxidation of lipids (Loeffler, C. et al. 2005); this suggests presence of oxidative stress upon trehalose feeding and is also consistent with results from Bae

at al. 2005. Induction of hypoosmotic shock inducible proline dehydrogenase may relate to the swelling of root cells in the extension zone, and may occur in response to the osmotic shock sustained by these cells, but it does not explain why these cells swell. An explanation for swollen cells of the root extension zone may be the starvation of these sink cells that may lead to reduced cell wall stability, two xyloglucan endotransglycosydases (MERI5B, EXGT-A3), enzymes known to be correlated with elongation processes are repressed by trehalose feeding (Table 3B). Interestingly, trehalose specifically induces expression of *EDS1*, *EDS5* and *PAD4* genes, part of the salicylic acid defense response whilst sucrose only elicits *EDS1*. Yet trehalose feeding does not induce *PR1* expression, possibly due to missing expression of *NDR1* (Nawrath, C. et al. 2002). Moreover trehalose feeding induces both *WAK1* and *WAK2* whilst sucrose feeding only induces *WAK1*. Results suggest biotic defense activation by trehalose or T6P.

Genes involved in carbon assimilation and allocation, except those of T6P metabolism, are conspicuous by their absence in the tables with genes specifically regulated by trehalose (Tables 2&3). Exceptions to this observation are sucrose phosphatase 1, SPP1, and the glucose transporter STP1 that are 2 and 3 fold repressed respectively by trehalose. This differs from feeding metabolisable sugars where expression of several enzymes of primary metabolism and transporters is strongly affected (Blasing, O. E. et al. 2005, Muller, R. et al. 2007, Price, J. et al. 2004). This may imply that control of carbon assimilation for growth by accumulating T6P is mainly mediated at the post-transcriptional level. A previous report revealed that sucrose starvation involves substantial translational repression: most of the mRNA appears to be translationaly repressed (183/245 genes) when starving Arabidopsis cells from sucrose (Nicolai, M. et al. 2006). In conclusion, micro-array profiling allows characterizing the physiological state of seedlings after 24 h on trehalose but it does not allow distinguishing reactions due to T6P or to trehalose. T6P steady state concentrations in plants with altered T6P metabolism were combined with microarray profiling to identify a cluster of genes that responds to T6P concentrations (Schluepmann, H. et al. 2004) and this identified some putative targets of T6P. To reveal components of T6P control over growth and allocation, identifying mutants impaired in T6P responses is a most interesting alternative approach.

A screen for altered carbon allocation

Growth arrest on trehalose is rare in M2 populations of EMS mutagenized seeds (Schluepmann and Aghdasi, unpublished). This can be exploited in a genetic screen to identify plants altered in T6P metabolism or target processes of T6P. Mutant seedlings that overcome growth arrest on 100 mM trehalose are either altered in trehalose import, trehalose catabolism, T6P synthesis or in the responses to T6P. T6P-mediated inhibition of growth is likely due to starvation of sink tissues important for growth, such as shoot and root apical meristems. Indeed starch is no

longer formed at the root tip in the columnella when WT seedlings are grown in 100 mM trehalose (Ramon, M. et al. 2007). Because *pgm1* seedlings are also growth arrested on trehalose (Fritzius, T. et al. 2001), T6P inhibition of growth is not due to carbon partitioning into starch in the cotyledons. Inhibition therefore is more likely due to carbon loading/unloading or transport. Thus a screen for suppressors of growth inhibition on 100 mM trehalose will uncover mutants in trehalose metabolism or the control thereof but also mutants that overcome T6P mediated changes in carbon allocation. The characterization of the physiological effects of 100 mM trehalose on seedlings presented in this chapter will help interpreting the mutants obtained from the proposed suppressor screen.

Materials and Methods

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* accession Columbia and TreF 46.2, a line expressing *E.coli* cytosolic trehalase behind the CaMV35S promoter (Schluepmann, H. et al. 2003), were used in this study. Seeds were sterilized 5 minutes with 70% Ethanol followed by 10 minutes in 20 % commercial bleach (4% w/v chlorine) and washed 5 times in sterile milli-Q water. Sterilized seeds were plated on agar solidified half strength MS medium supplemented with 50 mM trehalose with or without 50 mM sugars and stratified in darkness at 4°C for 2 days before the plates were transferred to a growth chamber at 25°C under a 16-h-light /8-h-dark photoperiod. In this experiment seedlings were grown vertically for 14 days. After 7 days pictures were taken and root length measured with the Image J program (Wayne Rasband, NIH Maryland, USA).

Plant material and growth conditions to assess relative growth of sugar signaling mutants

Different sugar signaling mutants, *pgm* and *sex1* were used in this study. Seeds were sterilized by the same method as described above. Sterilized seeds were plated on ½ Murashige and Skoog (MS) medium (1962) supplemented with 100 mM trehalose and solidified with 0.8 % agar. Seeds were stratified in darkness at 4°C for 2 days, before transferring to 25°C and a 16-h-light/8-h-dark photoperiod. After 14 days pictures were taken and root length measured with the Image J program (Wayne Rasband, NIH Maryland, USA).

Starch staining and confocal microscopy

For analysis of starch distribution, whole seedlings were taken and destained in 70% and then 90% (V/V) ethanol. Staining was done with KI/I_2 solution and then washed with milli Q water. Pictures were taken using a Normarski microscope (Jena, Germany). For confocal Laser scanning microscopy (Zeiss, Germany) roots were stained with propidium iodine (1μg/ml).

Chlorophyll and anthocyanin measurements

Chlorophyll a, b and total chlorophyll were measured spectrophotometrically as described (Jeffrey, SW and Humphrey, GF. 1975). In brief, 14 days seedlings were ground in liquid nitrogen and extracted with 80% (V/V) acetone. Then A647, A652 and A664 nm was determined and used to calculate chlorophyll content.

Anthocyanin content of seedlings was determined using the protocol of Mita et al. (Mita, S. et al. 1997). Frozen, homogenized seedlings (20 mg) were extracted for 1 d at 4°C in 1 mL of 1% (v/v) hydrochloric acid in methanol. The mixture was centrifuged at 13,000 rpm for 15 min and the absorbance of the supernatant was measured at 530 and 657 nm. Relative anthocyanin concentrations were calculated with the formula $[A_{530} - (1/4 \times A_{657})]$. The relative anthocyanin amount was defined as the product of relative anthocyanin concentration and extraction solution volume. One anthocyanin unit equals one absorbance unit $[A_{530} - (1/4 \times A_{657})]$ in 1 mL of extraction solution.

Re-analysis of the sugar response profiling previously published in Schluepmann et al. 2004

Seedlings were grown for 6d in liquid half strength MS medium, shaking (140 rpm). Sugars were added to a final concentration of 100 mM for 24h before harvest, RNA extractions and hybridization to the Affymetrix 8K gene chip as described in Schluepmann et al. 2004. All data was analyzed statistically using the R language environment for statistical computing (http://www.r-project.org) version 2.2 and Bioconductor release 1.7 (http://www.bioconductor.org). Differential expressed genes were identified using the LIMMA package (Smyth, G. K. 2004). Data was normalized using the VSN package (Huber, W. et al. 2002) and linearly fitted using the recommendations of the LIMMA vignette. The data was also normalized using the Loess method and this gave similar results (data not shown), however as the variances were not equal across hybridizations (sucrose had a major effect compared to trehalose that had a more limited effect when compared to sorbitol in the experiment), VSN normalization will reflect the *in planta* situation better (Freudenberg, J. et al. 2004). Visual examination of raw and normalized distributions by diagnostic plots confirmed that conclusion. A posterior residual standard deviation was employed (Smyth, G. K. 2004) independently for each treatment and the interesting contrasts (change in the trehalose treatment compared to the change in sucrose). The obtained P-values were corrected for multiple testing errors using the BH procedure (Benjamini, Y and Hochberg, Y. 1995), yielding q values. Lists of q-values were transferred to Microsoft Excel™ and sorted for maximal trehalose effect. The GST sequences were aligned to the Tair6 gene model database of transcripts. Genes were classified as differentially expressed if they were significantly changed (q V 0.05) in trehalose or sucrose compared to sorbitol. To compare the list of differentially expressed genes with other gene lists Microsoft Excel™ was used.

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CHAPTER 3

Trehalose resistant *Arabidopsis* mutants

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Abstract

To gain insight into trehalose-6-phosphate control over growth, mutants were identified that grow on trehalose. The Leclere and Bartel mutant collection was used for this screen. In this collection randomly cloned cDNAs are expressed from the CaMV35S promoter. We identified 13 lines where trehalose resistance segregated as a dominant trait. The cDNA expressed in the trehalose resistant *(trr)* lines encoded different proteins. Interestingly, four of the *trr* mutants are affected in two genes connected with photosynthesis, *LHCB1* and *PSI-H*. Three of the *trr* mutants express TRR14, a protein with unknown function. Another two of the *trr* mutants express Glycin-Rich RNA-Binding protein2 (GR-RBP2). Results from expression analysis and re-transformation with the CaMV35S/cDNA constructs showed that silencing of *LHCB1* and over-expression of *PSI-H, GR-RBP2* or *TRR14* suppress trehalose-*6*-phosphate mediated growth arrest.

Introduction

Arabidopsis is the plant of choice for a genetic approach to dissect mechanisms controlling plant growth. Seedlings in particular are ideal for genetic screens since they can be grown under a multitude of different conditions on agar plates. Various genetic screens have been developed to isolate mutants in the signal transduction of sugar-mediated gene expression (Martin, T. et al. 1997, Smeekens, S. 2000). Very few screens have been undertaken to study the control over carbon utilization within the cell and within the whole plant. Screens of mutants with altered cell wall composition (Mouille, G. et al. 2003) and with altered starch content (Zeeman, S. C. et al. 1998a) have been performed. Analysis of natural variation in accumulation of free sugars and enzyme activities amongst the Arabidopsis accessions, has also been described (Calenge, F. et al. 2006, Sergeeva, L. I. et al. 2006). Several Arabidopsis starch excess *sex* mutants (e.g., *sex1, sex4*, and *dbe1*) have been isolated by screening for plants that retained high levels of leaf starch after a period of extended darkness (Carpenter, C. D. et al. 1994, Critchley, J. H. et al. 2001, Eimert, K. et al. 1995, Zeeman, S. C. et al. 1998b). *Sex4* is deficient in a distinct endo-amylase that is involved in starch degradation (Zeeman, S. C. et al. 1998b), *sex1* is defective in the Arabidopsis homolog of the R1 protein (Yu, T. S. et al. 2001) and *dbe1* is an isoamylase-deficient mutant (Zeeman, S. C. et al. 1998b).

Gene products known to alter allocation of carbon include invertase (Sonnewald, U. et al. 1997), sucrose phosphate synthase (Lundmark, M. et al. 2006, Strand, A. et al. 2003), and FBPase (Strand, A. et al. 2000, Strand, A. et al. 2003). Mutants in the major sucrose transporter of Arabidopsis, *suc2*, are greatly impaired in sucrose transport and growth (Gottwald, J. R. et al. 2000). An interesting mutant

that regulates inflorescence branching in corn, *ramosa3,* is a mutation of a trehalose-6-phosphate phosphatase. It was proposed that RAMOSA3 regulates inflorescence branching by modification of a sugar signal that moves into axillary meristems (Satoh-Nagasawa, N. et al. 2006).

The disruption of genes via chemical mutagenesis, irradiation, and insertion of T-DNA or transposable elements has been invaluable for dissecting biological pathways. However, many genes remain difficult to uncover as loss-of-function mutations (LeClere, S. and Bartel, B. 2001). This likely is the case for mutants in genes important for carbon allocation and growth. Gain of function mutations, which are generally dominant or semi dominant can access these types of genes but often require very specific alterations in the protein. Gain of function phenotypes can be caused either by mutations in the coding region that lead to constitutive activation of the resulting protein as in dominant ethylene response mutants (Chang, C. et al. 1993). Gain of function may be obtained by mutations altering levels or patterns of gene expression, as in dominant *Antp* mutants (Schneuwly, S. et al. 1987). The traditional way to induce the latter type of mutation has been through chromosomal rearrangement or transposon activation, this activation brings genes under the control of new promoters or enhancers (Brunner, E. et al. 1999, Chadwick, R. et al. 1990, Miller, M. W. et al. 1993, Smith, A. M. et al. 2005). A more directed way to induce such mutations was developed by Walden and colleagues (Hayashi, H. et al. 1992) who constructed a T-DNA vector with four copies of an enhancer element from the constitutively active promoter of the Cauliflower Mosaic Virus (CaMV) 35S transcript; this T-DNA was then used to enhance expression of genes close to the T-DNA insertion site (Odell, J. T. et al. 1985). Over-expression may offer a general route to dominant gain-of-function phenotypes useful for analyzing redundant members of gene families. Essential genes may be more easily studied with such systems because an increase in function may not cause lethality. LeClere and Bartel have developed a system designed to co-suppress or over-express cDNA in Arabidopsis (Fig.1). They constructed a binary vector containing a complex Arabidopsis cDNA library driven by the CaMV35S promoter. The T-DNA in this vector contains a bar-gene cassette for phosphinotricine selection of the transgenic

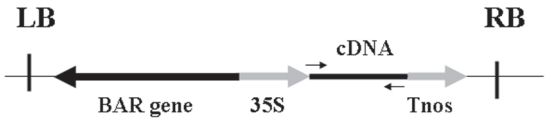

Figure 1. Structure of the T-DNA insertion in plants from the LeClere and Bartel collection. Arrows indicate the direction of transcription. The Bar gene conferrs BASTA resistance to plants transgenic with the T-DNA. The randomly cloned cDNA is expressed under the control of the CaMV35S promoter and followed by the NOS terminator sequence. Primers annealing to a specific sequence in the CaMV35S promoter and to the opposite strand of the NOS terminator sequence therefore can be used to amplify the unknown cDNA.

plants and a cassette with a randomly cloned cDNA inserted between CaM35S promoter and nopaline synthase (NOS) polyadenylation (polyA) sequences. Inserted cDNA can be amplified using PCR with primers in the promoter and polyA sequences (LeClere, S. and Bartel, B. 2001, Fig.1). The cDNA insert is likely responsible for the observed phenotype if phenotype and cDNA co-segregate as a dominant trait. To definitively relate over-expression and phenotype in plants exhibiting dominant traits, the cDNA expression cassette can be transformed again into wild type plants.

To identify mutants in trehalose metabolism, we used the LeClere and Bartel mutant seed collection and screened the 331 pools of T4 seed from this collection on 100 mM trehalose in a primary screen. Seedlings from plants identified during the primary screen were subjected to a secondary screen: resistance to trehalose and to phosphinotricine (PPT) to test segregation of the T-DNA insertion. *trr* mutants isolated during the secondary screen were then backcrossed to WT to determine if trehalose resistance segregates as a dominant trait. cDNA sequences in the T-DNA constructs of the *trr* mutants were characterized and re-transformed into WT to confirm their role in trehalose resistance.

Results

Screening for trehalose resistant mutants in a primary screen on 100 mM trehalose

Seedling growth on 100mM trehalose was monitored over a period of 5 weeks. Seeds from all 331 pools from the LeClere and Bartels collection were screened twice. Seedling resistance to trehalose could be assigned to 2 different groups. Firstly, a group of seedlings that develop much longer roots on trehalose than WT seedlings after 10 days growth. Secondly, a much larger group of seedlings that developed extensive short secondary roots and primary leaves at much later stages during selection (Fig. 2A). We choose to focus on the early resistance type of seedlings. In total 178 seedlings with roots much longer than wt after 10 days growth on 100 mM trehalose were transferred to ½ MS medium, then to soil for further growth and characterization. Each plate containing 100 mM trehalose used in the screen also contained TreF seeds, expressing, trehalase, and WT seedlings as positive and negative controls respectively; trehalase expressing seedlings (line 46.2) had 12 times longer roots than wt after 10 days growth. None of the selected seedlings grew as well as the trehalase expressing positive control. In some plant pools, there were many seedlings with a long root. All of the 178 mutant seedlings, except one, yielded fertile plants.

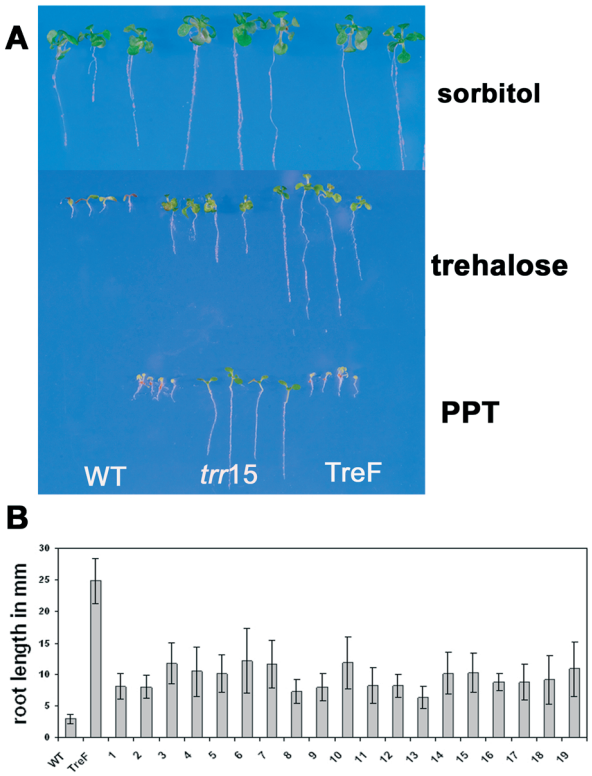

Figure 2. The primary and secondary screen on 100 mM trehalose.
Seed was sterilized and sown onto agar solidified half strength MS supplemented with 100 mM trehalose, sorbitol or 12.5 mg/l PPT. After stratification, seedlings were grown for 14 d at 22°C in long-day conditions as described in materials and methods. A) Typical results from wild type (WT), trehalose resistant (line *trr15*) and *E.coli* trehalase expressing (TreF) seedlings on the three media indicated during the secondary screen. B) Root lengths of seedlings from WT, the *E.coli* trehalase expressing TreF line and the trehalose resistant lines, *trr 1-19*.

Re-screening of mutant lines in a secondary screen

To confirm resistance to trehalose and to ensure presence of T-DNA insertions in the plants selected during the primary screening, a more refined secondary screening was applied to seed obtained from plants selected in the primary screen. Seeds were germinated on 100 mM trehalose and on 100 mM sorbitol for comparison. Growth on sorbitol reveals if enhanced root growth on trehalose resulted from a general enhanced root growth in any one of the lines compared to WT. In addition, seeds were germinated on medium with 12.5 mg/l PPT to analyze segregation of the T-DNA insertion carrying the CaMV35 promoter driven cDNA expression cassette (Fig. 1). After day 7 and 14, pictures were taken from the vertically grown seedlings on solidified media (Fig. 2A). This allowed determining germination rates, presence of emerging primary or secondary leaves, root length and resistance to PPT in the 175 lines obtained in the primary screen and in WT and trehalase expressing controls.

A total of 19 lines were obtained with an average root length minimally 3 times as long as wt and with PPT resistance in at least ¾ of the seedlings (Fig. 2B). After 14 days, the average root length of WT and the trehalase expressor line Tre F42.6 was 2 mm, and 25 mm, respectively. Root length from the trehalose resistant lines ranged between 6.3 and 12 mm and therefore did not reach the length of roots from TreF 46.2 seedlings. On 100 mM sorbitol, root length of the seedlings from the different lines did not differ significantly from WT. Therefore, the apparent resistance to trehalose of any one of these lines is not simply due to enhanced commitment of the mutants to root growth.

Segregation analysis on PPT showed that the lines were all homozygous for the T-DNA insertion or/and that they contained T-DNA insertions at multiple loci. WT and TreF seedlings had short roots and yellow cotyledons after 14 days on PPT, an observation consistent with the fact that these seedlings do not express the Bar gene. Analysis of the cDNA insertion in the **tr**ehalose **r**esistant (***trr–***) lines was restricted to the 19 lines with root lengths minimally 3 fold that of wt on trehalose, Table 1.

Table 1: Molecular characteristics of *trr* mutants
trr mutant indicates mutant number; **Plant pool** is the pool number from the LeClere and Bartel collection in which the mutant was found; **PCR fragment** gives the length(s) in base pairs **(bp)** of fragments that were amplified by PCR in the *trr* line; **Gene model** gives the gene from **A.thaliana** to which the cDNA fragment is homologous; **Encoded protein** is the name of the protein encoded by the gene model; **cDNA structure** indicates whether the cDNA fragment in the *trr* mutant is in sense orientation, whether it contains the full length coding sequence of the protein including ATG start and TAA or TGA stop codons; **Location** is predicted subcellular localization of the protein encoded by the Gene model; **Recessive or Dominant** relates to segration of trehalose resistance after backcrossing of the homozygous *trr* line with WT Col-0, **R** and **D** means that trehalose resistance segregates as a recessive and dominant trait, respectively, in this line.

trr mutant	Plant pool	PCR fragment (bp)	Gene model	Encoded protein	cDNA structure	Location	R/D
trr 1	193	800	At4g38970.1	Fructose bis-phosphate aldolase	sense, without ATG, with TGA	Chloroplast	R
trr 2	268	650	At3g16140.1	Subunit H of Photosystem 1 reaction center subunitVI	sense, without ATG, with TGA	Chloroplast	R
trr 3	2372	1700, 800	At1g52230.1	(PS1-H) Putative Photosystem 1 reaction center subunitVI	sense, **full length**, with ATG and TAA	Chloroplast	D
trr 4	298	1000	At1g52230.1	(PS1-H) Putative Photosystem 1 reaction center subunitVI	sense, **full length**, with ATG and TAA	Chloroplast	D
trr 5	2681	1000	At1g29920	LHCB1 (CAB2)	sense, **full length**, with ATG, TGA	Chloroplast	D
trr 6	325	800	At4g13850.1	Glycin-rich RNA binding protein (GR-RBP2)	sense, internal deletion, with ATG and TAA	Mitochondria	R
trr 7	311	1000	At4g13850.1	Glycin-rich RNA binding protein (GR-RBP2)	sense, **full length**, with ATG and TAA	Mitochondria	D

trr 8	392	1000	At1g73480.1	Hydrolase, alfa/beta family protein	sense, without ATG, with TGA	Chloroplast, cytoplasm	D
trr 9	5171	850	At1g52890.1	No apical meristem	sense, without ATG, with TGA	Unknown	R
trr 10	2371	1000	At1g23310.1	Glutamate: glyoxalate aminotransferase 1 (GOGAT)	Sense, without ATG and TAA or TGA	Peroxisome	D
trr 11	198	1000	At3g59970.3	Methylentetrahydrofolate reductase1 (MTHFR1)	sense, without ATG and TAA	Unknown	D
trr 12	5542	1700	At3g18080.1	Glycosylhydrolase family 1	sense, without ATG, with TGA	Endomembrane system	R
trr 13	5621	850	At4g00860.1	Stress-related ozon-induced protein (OZI1)	sense, without ATG, with TGA	Mitochondria	R
trr 14	3731	850	At4g10300.1	Expressed protein (TRR14)	sense, **full length**, with ATG and TGA	Chloroplast	D
trr 15	3992	500	At4g10300.1	Expressed protein (TRR14)	Sense, with ATG, without TAA or TGA	Chloroplast	D
trr 16	231	800	At4g10300.1	Expressed protein (TRR14)	sense, **full length**, with ATG and TGA	Chloroplast	D
trr 17	3775	1000, 1700	At5g19230.1	Expressed protein (TRR17)	sense, with ATG, without TGA or TAA	Endomembrane system	D
trr 18	3994	850	At1g60870.1	Expressed protein (TRR18)	sense, without ATG, with TGA	Unknown	D
trr 19	3991	ND*	ND	ND	ND	ND	D

***ND: not determined**

cDNA insertions in the *trr* lines range from 650 to 1700 bp

To find out if seedlings from the 19 selected *trr* lines contain cDNA fragments, we performed PCR using a forward primer on the CaMV35S promoter and a reverse primer on the nopaline synthase poly-adenylation sequence, as described in LeClere and Bartel 2001. Control PCR reactions were on DNA extracted from WT. No fragment was amplified from WT DNA. PCR analysis revealed that *trr3* and *trr17* had at least 2 insertions (Table 1). PCR reactions in the remaining lines yielded one fragment only. Fragments amplified by PCR were of different lengths and ranged from 650 to 1700 bp. Because PCR was not able to amplify any fragment in *trr15, trr16* and *trr19*, we performed Tail-PCR to identify fragments in these *trr* and this allowed characterization of fragments in *trr15* and *trr16* but not in *trr19*.

cDNA fragments in the *trr* lines encode different proteins

Sequence analysis of PCR fragments identified different cDNA fragments of several genes in the *trr* mutants. We determined whether these fragments were full length, with ATG start codon and in sense or antisense orientation (Table 1).

All cDNA fragments were in the sense orientation. Six out of 18 encode full-length cDNAs (with ATG start and TGA or TAA stop codons). For example, the

cDNA fragment in *trr5* is full length, with ATG, in sense orientation and it encodes LHCB1 (At1g29920). However, the cDNA fragment in *trr1* lacks the ATG-start codon and encodes a part of the fructose bis-phosphate aldolase (At4g38970).

Three genes were recovered independently as different cDNA entities originating from different pools. The cDNA fragments of *trr3* and *trr4* (LeClere and Bartel collection pool numbers 237 and 298) differ but encode the coding sequence of PSI-H precursor (At1g52230). Similarly, cDNA fragments in *trr6* and *trr*7 (LeClere and Bartel collection pool numbers 325 and 311) encode the full length **G**lycin-**R**ich **R**NA **B**inding **P**rotein **2** (**GR-RBP2**, At4g13850). cDNA from *trr14, trr15* and *trr16* (LeClere and Bartel collection pool numbers 3731, 3992 and 231) was also recovered from different pools and encodes an expressed protein with unknown function, TRR14, (AT4g10300).

Trehalose resistance as a dominant trait in the *trr* lines

To find out whether the trr trait is dominant or recessive, backcrossing between the 19 *trr* lines and WT was performed. Segregation of resistance to PPT was studied and revealed that in all lines PPT resistance segregates as a single locus. Segregation of resistance to trehalose was then followed in the heterozygous material obtained. Segregation of 3:1 corresponds to dominance of the trehalose resistance and segregation close to 1:3 corresponds to a recessive trait. In total 6 out of 19 lines were recessive and the remainder 13 lines were dominant (results are summarized in Table 1).

When trehalose resistance segregates as a dominant trait, this is likely due to expression of the randomly cloned cDNA, and thus 8 genes were identified whose expression influences trehalose resistance: *PSI-H, LHCB1, GR-RBP2, GOGAT1*, *alfa/beta hydrolase, MTHFR1, TRR14, TRR17* and *TRR18*. Dominance patterns coincide with identical gene in the cDNA for *PSI-H* (At1g52230) and for the expressed protein with unknown function, TRR14 encoded by At4g10300. The cDNA in *trr19* could not be amplified and therefore the identity of *trr19* remains unknown.

Expression analysis of the *trr* mutants

Expression levels of endogenous genes that are homologous to the cDNA transgenes were determined in seedlings of Col-0 WT and 8 *trr* mutants for which trehalose resistance is a dominant trait. In case of multiple recovery of cDNA encoding the same gene, the *trr* line selected for the study is that with the strongest resistance to trehalose. Expression of endogenous genes and cDNA-transgene homologues was analysed in leaves of 10 day old seedlings grown in 16h days at 22°C on half strength MS medium, using Q-PCR as described in materials and methods. Levels of gene expression were determined with reference to *AtACTIN2*.

cDNA fragments in *trr3, 5, 7 and 14* were full length with ATG and TGA or

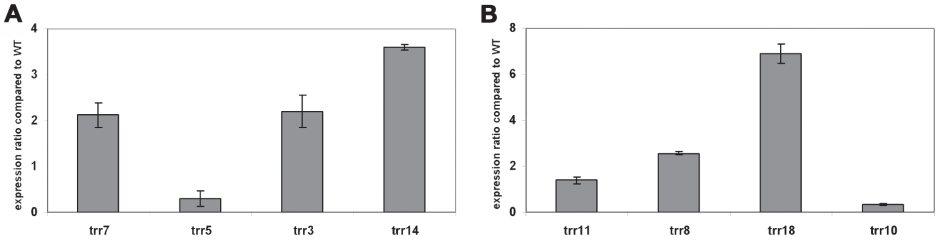

Figure 3. Gene expression in *trr* mutants with dominant segregation of trehalose resistance. A) *trr* mutants with full-length CDS in the cDNA, B) *trr* mutants with truncated CDS in the cDNA. Seedlings were grown on agar solidified half strength MS for 10 days before RNA extraction and Q-PCR analysis of gene-expression. The PCR-primers chosen (Table 2) detect expression of the endogenous gene the transgene cDNA. Expression in *trr* mutants is plotted as a ratio to expression in WT and standard deviation is calculated (n=3).

TAA, allowing detection of the sum of transcript with just a single set of primers (Table 2). Expression in *trr* lines is presented as a ratio over WT levels in Fig. 3A. Expression ratios of *GR-RBP2*, *PSI*-H and *TRR14* (At4g10300) are well above 2 suggesting that resistance to trehalose in these *trr* is due to over-expression. The expression ratio for *LHCB1* is at approx. 0.3, indicating that in *trr5* trehalose resistance is likely due to co-suppression of *LHCB1*expression and this is consistent with the pale green color of the leaves of *trr5* plants.

In cDNA fragments of *trr11* (*MTHFR*), *trr8* (alfa/beta hydrolase), *trr18* (At1g60870) and *trr10* (GOGAT1) only parts of the cDNA are expressed yet primers were chosen to detect the sum of transcript (Table 2). Results showed that, except for *trr10,* transcripts are more abundant than in WT (Fig. 3B). Most likely, expression of the transcript causes the *trr* phenotype. However, in these cases the protein synthesized from the truncated fragments differ from WT protein. The cDNA in *trr11 (MTHFR,* At3g59970*)* encodes the 130 aa C-terminal peptide from aa 276 until 386 without stop codon. The cDNA of *trr8 (alfa/beta hydrolase,* At1g73480*)* encodes the C-terminal peptide from aa 274 until 422 that includes the stop codon. The cDNA in *trr18* (At1g60870) is lacking the first 17 aa; full length expressed TRR18 is 148 aa long. The cDNA in *trr10* (*GOGAT*, At1g23310) encodes a truncated protein that lacks aa 1-213 and 478-482. Lacking start codons and N-terminal parts of the cDNA suggest that these mutations stem from incomplete cDNA synthesis before cloning into the plant expression vector. It remains unknown if and what protein is synthesized from the truncated transcripts as an internal start codon could be found in alternative reading frames.

Trehalase expression in *trr* mutants

To find out if resistance to trehalose in the *trr* is simply due to high endogenous trehalase expression and thus high trehalase activity, we analyzed the levels of expression of the *AtTRE1*, the only trehalase gene in Arabidopsis, in the *trr* mutants.

Analysis was performed using mRNA from 10d old seedlings grown on ½ MS medium with 100 mM sorbitol or trehalose and was by Q-PCR as described in Materials and Methods.

AtTRE1 expression in seedlings grown on sorbitol control showed that trehalase expression is unchanged by the constitutive cDNA expression in the *trr* is compared to WT (data not shown). Genes over-expressed in the *trr* therefore do not induce expression of trehalase. WT seedlings grown on trehalose have 10-fold induced *AtTRE1* expression compared to sorbitol. *AtTRE1* expression in seedlings of the *trr* lines grown on trehalose is similarly induced compared to WT (data not shown). Genes expressed in the *trr* thus do not induce processes that control trehalase expression on trehalose.

Re-transformation of cDNA constructs into WT Col-0

For the cDNA sequences that were identified only once during the *trr* screen, independent transgenics with the construct containing the cDNA must be tested to confirm that the sequence expressed is indeed responsible for resistance to trehalose. Such experiments were performed with cDNA from *trr7*, *trr11* and *trr14* and, in addition, with cDNA from *trr3*, *trr5*, to also obtain lines with improved resistance to trehalose. cDNA constructs from *trr17*, *trr18* and *trr19* would have been good candidates too because resistance to trehalose in these lines is a dominant trait but these were not available at the time of experimentation. cDNA constructs were thus cloned into the CaMV35S expression cassette from pBin19, and Col-0 was transformed with these constructs. Transgenic lines obtained were then analysed for resistance to trehalose in the second and third generation.

Transformations with full length cDNAs of *PSI-H* (*trr3*), *LHCB1* (*trr5*), *GR-RBP2* (*trr7*), *TRR14* (*trr14*) yielded 8 or more independent lines per construct with resistance to trehalose, showing that expression of these cDNAs is indeed causing trehalose resistance. The degree of resistance typically obtained after re-transformation of cDNA encoding LHCB1, GR-RBP2 and TRR14 is shown in Fig. 4. After 14 days, resistant seedlings have long roots and expanded primary leaves. Evidence for trehalose resistance is less convincing in the case of expression of the *MTHFR1* cDNA that lacks its ATG with 2 out of 70 lines generated exhibiting trehalose resistance. The low frequency of trehalose resistance may be due to the absence of the start codon in this cDNA. Alternatively, the cDNA expression level is not the cause of resistance to trehalose. These hypotheses remain to be tested. Resistance in the second generation after transformation is sometimes lost, as in the case *PSI-H*, suggesting that expression is not stable. Possibly, concatemeric integration of the T-DNA leads to silencing. An alternative explanation is that over-expression of the gene affects seed production.

Expression levels were determined in different re-transformed lines and Col-0 seedlings. The mRNA level in re-transformed *GR-RBP2* lines was 2 times higher

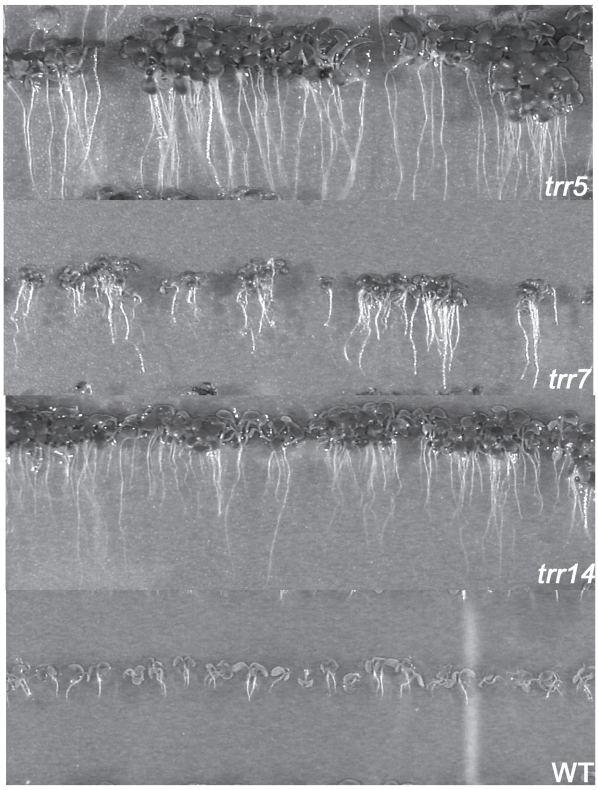

Figure 4. Trehalose resistance of seedlings from re-transformed lines.
Col-0 was transformed with cDNA constructs and transgenic plants were selected. Seed from the T2 generation of each line was sown on agar solidified half-strength MS supplemented with 100 mM trehalose. After 2 days stratification, seedlings were grown at 22°C for 14 d under 16h light/8h dark. Typical trehalose resistant phenotypes are shown here for cDNA constructs of *LHCB1* (*trr5*), *GR-RBP2* (*trr7*) and *TRR14* (*trr14*), and compared to wild type (WT).

than in WT (Fig. 5A). Expression of re-transformed *TRR14*, was much higher than in WT, ranged from 2-12 times more in the re-transformed *TRR14* lines (Fig. 5B). *LHCB1* repression in the re-transformed lines is not very marked and reaches maximally 70% in line 1 (Fig. 5C)

Interestingly, there is no relation between gene expression level and root length in the different re-transformed lines. For example, in re-transformed *TRR14* line 17, *TRR14* expression is 12 times higher than in WT, but the root length is the same as in line 7 where *TRR14* expression is 2 times higher than WT.

Trehalase expression levels did not differ in the re-transformed lines from WT, whether seedlings were grown on 100 mM of sorbitol or trehalose. Trehalose resistance in these lines is thus not due to increased *AtTRE1* expression. We conclude that over-expression of *PSI-H* (At1g52230), *GR-RBP2* (At4g13850) and *TRR14* (At4g10300), as well as co-suppression of *LHCBI* (At1g29920) result in trehalose resistance.

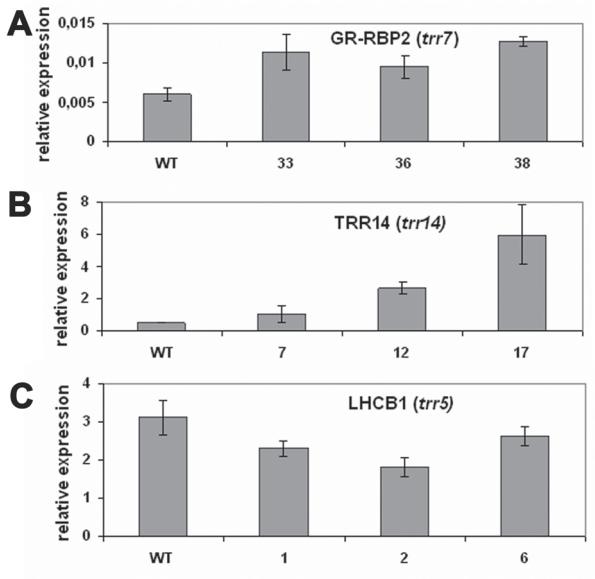

Figure 5. Expression level of genes encoded by the cDNA of *trr* mutants in trehalose resistant re-transformed lines.
Seedlings were grown and RNA extracted as in Fig.3. Expression levels were determined by Q-PCR using specific primers as described in Table 2. Expression in A) *GR-RBP2* B) *TRR14* and C) *LHCB1* is relative to *ACTIN2*.

Discussion

In Arabidopsis seedlings, exogenously supplied trehalose inhibits root growth and emergence of leaves (Wingler, A. et al. 2000). The effects of exogenous trehalose are multiple, yet growth arrest after 100 mM trehalose feeding is due to T6P accumulation because arrest is overcome in seedlings expressing *E.coli TPH*. T6P accumulation is not due to phosphorylation of trehalose as the reaction catalyzed by TPP is irreversible. Rather, T6P accumulation is likely due to ineffective dephosphorylation of the endogenously synthesized T6P in the presence of high concentrations of the product of the reaction, trehalose (Schluepmann, H. et al. 2003). Therefore, a screen for mutants capable of growth on medium with 100 mM trehalose likely will also identify plants with altered T6P metabolism as well as plants with altered responses to T6P. Previously, we observed that only 25 seedlings of 2 g EMS M2 seeds were resistant to 100 mM trehalose. Screening the LeClere and Bartel collection for resistance to trehalose identified 19 *trr* mutants. The *trr* mutation was dominant in 13 of the 19 *trr* lines suggesting that the phenotype is due to expression of the cDNA in the 13 mutants. Genetic analysis confirmed

that over-expression of *PS1-H, GR-RBP2* and *TRR14* (At4g10300) as well as co-suppression of *LHCB1* lead to seedling resistance to 100 mM trehalose. Trehalose resistance in the *trr* is not due to over-expression of *AtTRE1*, the only trehalase in Arabidopsis.

Use of the Leclere and Bartel collection

Leclere and Bartel used a complex cDNA library to generate a collection of 331 pools of 100 independent *Arabidopsis* transgenic lines that express a large number of different cDNAs. The complexity of the cDNAs in the collection is not known. Yet, generating a large number of Arabidopsis transformants, each over-expressing a cDNA insert, is an alternative to activation tagging strategies (Weigel, D. et al. 2000) and allows direct identification of the cDNA responsible for a particular phenotype. Complexity of this collection was sufficient to detect 19 *trr* lines with root lengths at least 3 fold that of WT on 100 mM trehalose after 14 days growth. In WT expression of genes encoded in cDNA's of the 19 *trr* lines range from low to high, so low abundance mRNAs are also presented in the collection. For example, the expression levels of *GR-RBP2* (0.006 fold *ACTIN2* expression levels) and *TRR14* (0.5 fold *ACTIN 2*) are low compared to those of *LHCB1* (3 fold ACTIN2 expression). Analysis of the cDNA structures in mutants selected from LeClere and Bartel revealed that 6 out of 18 cDNA encoded full length coding sequences indicating that the quality of the cDNA library used to generate the transgenics does not cover the Arabidopsis transcriptome. The seedlings selected yielded all fertile plants suggesting that the T4 seed pools obtained from the Nottingham Stock Center likely no longer contained constructs that significantly impair viability of the plants and seed set. The analysis further showed redundancy with independent transgenics expressing the same coding sequence. Expression of the cDNA behind the CaMV 35S promoter leads to constitutive expression in all tissues of the plant and therefore use of this collection does not reveal the location of T6P mediated block of growth. Using specific promoters, such as the RolC promoter that restricts expression to the phloem, for example (Lerchl, J. et al. 1995), or transforming the *TRR* genes under the control of the UAS promoter into the complete GAL4-GFP lines (Bougourd, S. et al. 2000, Kiegle, E. et al. 2000) until trehalose resistance is obtained are possible approaches to localize cells that are involved in T6P mediated growth arrest. Resistance obtained in the *trr* is not as effective as expression of *E.coli* trehalase. In addition, Arabidopsis trehalase expression was not more induced in *trr* mutants than in WT on trehalose medium suggesting that resistance to trehalose is not simply due to expression of AtTRE1 in the *trr*. Taken together, the LeClere and Bartel collection allowed the isolation of *trr* mutants even though cDNA insertions in this collection do not cover the entire transcriptome of Arabidopsis and the TRR encoded proteins are generally of low molecular weight.

Expression of the cDNA is important for *trr* phenotype

Over-expression or suppression of the cDNA determines the *trr* phenotype, as shown by re-transformation and expression level analysis. In this study such re-transformtion is needed to obtain the definitive link between expression of the cDNA and the *trr* phenotype. T-DNA constructs that allow conditional over-expression of the cDNA, for example by combining out the promoter constitute a recent improvement bypassing re-transformation (Kandel, E. S. et al. 2005). Choice of the CaMV35S promoter is appropriate to isolate mutants in T6P metabolism since the ubiquitous presence of *AtTPS1*, trehalose-phosphate synthase 1 is expressed at low levels in all tissues (van Dijken, A. J. et al. 2004). Moreover, trehalase is expressed in every tissue and since trehalose externally supplied is likely transported (Schluepmann, H. et al. 2003, Schluepmann, H. et al. 2004, van Dijken, A. J. et al. 2004). A disadvantage of the CaMV35S promoter is that ectopically expressed genes may cause trehalose resistance independently of localized processes normally controlled by T6P or trehalose metabolism *in planta*.

The identity of the genes underlying *trr* reveals processes in the chloroplasts as well as in mitochondria

Over-expression of *PSI-H, GR-RBP2* and *TRR14* (At4g10300) as well as co-suppression of *LHCB1* lead to seedling resistance to 100 mM trehalose. No genes encoding proteins involved in trehalose import, trehalose degradation were obtained. In addition, none of these genes appear to be directly involved in trehalose metabolism. Instead the genes encode organellar proteins (Table 1) targeted to either chloroplast (TRR14, LHCB1 and PSI-H) or mitochondrion (GR-RBP2). Analysis of the proteins involved in trehalose metabolism reveals that these are cytosolic (AtTPS1), extracellular (AtTRE1) and possibly also chloroplastic as in the case of AtTPPA and AtTPPB (Aeschbacher, R. A. et al. 1999, Muller, J. et al. 2001). The latter is relevant since it would imply transport of T6P to chloroplasts. Interestingly, T6P is able to affect AGPase redox activation in chloroplastic preparation (Kolbe, A. et al. 2005). T6P also controls photosynthetic capacity per leaf area without changing photosynthetic gene expression (Paul, M. et al. 2001, Pellny, T. et al. 2004). Possibly T6P somehow affects chloroplastic reactions, implying that it may be present in chloroplasts. Trehalose on the other hand is likely found extracellularly and in the cytosol because AtTRE1 is a periplasmic enzyme and because *E.coli* trehalase targeted to the cytosol successfully releases metabolisable glucose for growth (Aeschbacher, R. A. et al. 1999, Schluepmann, H. et al. 2003).

The underlying function of trehalose resistance proteins

The *LHCB1* suppressor *trr* line has pale leaves when grown under long days, similar to plants containing low T6P by way of *TPP* or *TPH* expression

(Schluepmann, H. et al. 2003). *LHCB1* encodes the LHCB1 protein. LHCB1 and LHCB2 proteins are the most abundant proteins in the LHCII trimers, the light harvesting complexes. LHCII is mostly found bound to PSII, yet this complex also docks to PSI. Interestingly, LHCII association with PSI requires the PSI-H (Naver, H. et al. 1999). Thus, it is puzzeling that LHCB1 repression and PSI-H over expression lead to growth on trehalose and since are involved in photosynthetic electron transport. PSI-H is not only required for LHCII docking to PSI, but also helps stabilizing the PSI complex under stress conditions. PSI is known to be the major detoxifier of stress induced ROS such as H_2O_2, whereby monodehydroascorbate acts as an electron acceptor of PSI (Baier, M. et al. 2000, Ivanov, B. and Khorobrykh, S. 2003, Nakano, Y. and Asada, K. 1981). Possibly, growth on trehalose requires enhanced ROS detoxification or reduced production. Also TRR14 is predicted to be located in the chloroplast (TAIR), but its function remains unknown.

According to proteome analyses, GR-RBP2 is located in mitochondria (Kruft, V. et al. 2001). A number of RNA-binding proteins have been found in plants. Some of these were identified as GR-RBPs that contain an RNA Reconition Motif on their N-terminus, and on the C-terminus a glycine-rich domain that contains repeats of the RGG amino acid sequence. Deletion analysis shows that the C-terminal glycin-rich domain is essential for RNA-binding activity (Hanano, S. et al. 1996). The Arabidopsis GR-RBP7 and GR-RBP8 are involved in the regulation of the circadian rhythm and in cold shock (Heintzen, C. et al. 1997). GR-RBP4 likely is involved in salt stress responses. The function of GR-RBP2 has recently been unraveled. It protects Arabidopsis from cold treatment and it is an RNA chaperone that stabilizes specific mitochondrial mRNAs and thus specifically alters the constitution of the mitochondrial proteome under cold stress (Kim, J.Y et al. 2007).

The mechanism of trehalose resistance

Trehalose resistance in the *trr* is not due to increased trehalase expression. None of the identified TRRs are involved in trehalose metabolism directly. Identification of the *PSI-H*, *LHCB1, TRR14* and *GR-RBP2* does not reveal the mechanisms by which trehalose resistance can be achieved in each *trr* line. The mechanisms may differ for each *trr* but PS1-H, LHCB1 and GR-RBP2 have in common that they are linked to environmental stress responses. Further work, including T6P measurements and analyses of carbon allocation in the *trr* lines will be necessary to begin to understand mechanisms of trehalose resistance in the *trr* lines. Results from this Chapter 3 nevertheless point to the importance of stress response physiology in our understanding of the suppression to T6P mediated growth arrest.

Materials and Methods

Plant materials and growth conditions in the primary screen

The collection of Arabidopsis lines containing the CaMV35S promoter cDNA transcriptional fusions described in LeClere and Bartel, 2001 was used in this study. Seeds were sterilized by the gas method sterilization. Sterilized seeds were plated on ½ Murashige and Skoog medium (1962) (MS) supplemented with 100 mM trehalose and solidified with 0.8% agar. Seeds were stratified in darkness at 4ºC for 2 days, before transfering to 25ºC and a 16-h-light/8-h-dark photoperiod. On each plate TreF 46.2 and WT seeds were used as positive and negative control, respectively. After 10 days, seedlings were screened for long root and emergence of primary leaves on 100 mM trehalose. Seedlings with long roots were transferred to ½ MS medium with 10 mM sucrose for 1 week, then transferred to soil to generate S2 seeds.

Growth conditions in the secondary screen

S2 seeds from S1 plants grown after the primary screen were surface sterilized and sown on 0.8% w/v agar with either 100 mM trehalose, 100 mM sorbitol (as osmotic control) or 12.5 mg/L PPT. Growth on trehalose allows to test tolerance to trehalose, growth on sorbitol is an osmotic control to check root growth. Growth on PPT, detects following segregation of the T-DNA insertion. Seed was stratified in darkness at 4ºC for 2 days, and then transferred to 25ºC with a 16-h-light/8-h-dark photoperiod. For this experiment seedlings were grown vertically for 14 days. After 14 days, seedlings were screened for long root and emergence of primary leaves on 100 mM trehalose compared to 100 mM sorbitol and for segregation of T-DNA inserted on 12.5 mg/L PPT.

Backcrossing

Seedlings resistant to PPT from lines resistant to trehalose in the secondary screen were transferred to soil along with wt. Upon flowering of the plants crosses with WT were carried out. Each sillique, after ripening, was collected in one bag. As for the re-screen, seeds were sown separately for each silique on ½ MS medium supplemented with 100 mM Trehalose or 12.5 mg/l PPT.

DNA extraction and PCR analysis

Three small leaves were frozen in liquid nitrogen, pulverized with glass beads for 2 minutes at 2800 rpm in a Dismembranator (Braun, Melsungen, Germany), then DNA was extracted using the Pure Gene DNA isolation kit (Amersham Pharmaciabiotec, England) following the manufacturers protocols.

To determine presence of the 35S cDNA fragment in the transgenic plants resistant to 100 mM trehalose, PCR was performed with primers 35S-F (CGCACAATCCCACTATCCTTCGCAAG) NOS-R (GATAATCATCGCAAGACCGGAACAGG). A mixture of Taq and PFU enzyme at unit

ratio of 50:2 was used. After denaturation for 2 minutes at 94°C, DNA amplification was with 35 cycles (30 sec 94°C, 30 sec 56°C and 2 min 72°C). PCR was completed with a final step at 72°C for 5 minutes. An aliquot of the PCR product was run on agarose gel and the remaining was cleaned using a DNA purification kit (Amersham Biosciences, England).

Tail-PCR was done using a set of primers. The arbitary primers were AD1: NTC GAS TWT SGW GTT, AD2: NGT CGA SWG ANA WGA A, AD3: WGT GNA GWA NCA NAG A, and AD4: NGT ASA SWG TNA WCA A. PCR reaction was in 20 μl volume. After denaturation for 2 minutes at 95°C, DNA amplification was with 5 cycles (1 min 94°C, 1 min 62°C and 2.5 min 72°C), 1 cycle (30 sec 94°C, 3 min 25°C and 3 min 72°C, 2.5 min 72°C), 15 super cycles (30 sec 94°C, 1 min 68°C and 2.5 min 72°C, 30 sec 94°C, 1 min 68°C, 2.5 min 72°C, 30 sec 94°C, 1 min 44°C, 2.5 min 72°C). PCR was completed with a final step at 72°C for 5 minutes. After diluting the primary PCR product 1:50 in water, secondary PCR was done as following: 15 supercycles (30 sec 94°C, 1 min 64 °C and 2.5 min 72°C, 30 sec 94°C, 1 min 64°C, 2.5 min 72°C, 30 sec 94°C, 1 min 44°C, 2.5 min 72°C) completing by a final step at 72°C for 5 minutes. In third step, PCR product was diluted 1:50 in water. Cycling conditions for tertiary PCR was: 25 cycles (1 min 94°C, 1 min 44°C and 2.5 min 72°C). PCR was completed with a final step at 72°C for 5 minutes. The PCR product was run on agarose gel and then specific band was cleaned using a DNA purification kit (Amersham Biosciences, England).

Cloning cDNA fragments into pGEMT

Because of the exonuclease activity of PFU polymerase, the PCR product was blunt ended, therefore an adenosine-residue was added to the 3' ends of the fragment by incubation at 72°C. The PCR fragment solution a 40 μl PCR buffer was supplemented with dNTP (5mM) and 1unit Taq polymerase. Then cleaning was repeated and fragments ligated into pGEMT. For ligation of cDNA fragment into pGEMT, DNA was concentrated to 3 μl and then was added to 5 μl 2× ligation buffer, 1 μl T4 Ligase and 1 μl pGEMT vector. The ligation mixture was incubated over night at room temperature.

100 μL of competent *E.coli* were taken from the –80°C freezer and thawed on ice for 20 minutes. 10 μl of the overnight ligation mixture was added to the cells. The mixture was left on the ice for 20 minutes. Then a heat shock for 50 seconds at 42°C was applied, followed by a 5 min cooling period on ice. Then 1 ml LB was added and cells were incubated at 37°C for 1 hour, before plating on selection medium. These LB plates contained 50 μg/mL Ampicillin for selection. IPTG and X-galactosidase were added for the blue and white screen. To check for colonies containing the plasmid with the ligated fragment, a restriction enzyme analysis was performed. Plasmid was isolated from 5 colonies using a Sigma Kit (Sigma). In digestion mixture, 2 μl plasmid, 1μl 10X buffer, 6 μl milli Q water and 1 μL EcoR1 were used. Samples were digested at 37°c for 1.5 hour. Fragments obtained were analyzed by agarose-gel electrophoresis.

Sequence analysis

For sequencing, DNA was sent to the Sequencing Facility Wageningen. Sequences obtained from analysis with forward and reverse primers were aligned and the PCR fragment structure reconstructed. The obtained sequence was mapped with regard to pGEMT vector sequences, T-DNA, CaMV 35S promoter and NOS poly A sequences. The cDNA fragment was identified by BLAST (Basic Local Alignement Search Tool) searches in TAIR (http://www.arabidopsis.org/Blast/).

RNA Isolation, RT-PCR and quantitative PCR (Q-PCR) Analysis

Different selected lines seeds were grown on ½ MS medium for 10 days. Plant material was snap frozen in liquid nitrogen, pulverized with glass beads for 2 minutes at 2800 rpm in a dismembrator (Braun, Germany). Total RNA was isolated with RNeasy plant mini kit (QIAGEN USA, Valencia, CA). RNA concentration and purity were determined by measuring absorbtion at 260 nm. Ten ng RNA was treated with 2 U DNAse I (DNA-free, Ambion, Austin, USA) to remove genomic DNA. Absence of DNA was analyzed by performing a PCR reaction (40 cycles, similar to the real–time PCR program) on the DNaseI- treated RNA using Taq-DNA polymerase. RT-PCR experiments were performed using 1 ng of total RNA extracted and used for first-strand cDNA synthesis with sixty units M-MLV Reverse Transcriptase (promega, Madison, WI), 0.5 μg of odT16v (custom oligo from invitrogen, Carlsbad, CA) and 0.5 μg random hexamer (invitrogen).

Q-PCR was carried out by ABI-prism 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA). Per reaction 12.5 μl of CYBR green PCR Master Mix (Applied Biosystems, UK) and 2.5 μl of each gene -specific primer was used (Table 2). Relative quantitation of gene expression is based on the comparative Ct method (User Bulletin No. 2: ABI PRISM 7700 Sequence Detection system, 1997) using *AtACTIN2* as a calibrator reference.

cDNA over-expression constructs and re-transformation into Col-0

For over-expression of cDNA fragments in WT, full length (for lines *trr 3*, *trr 5*, *trr 7* and *trr 14*) and truncated (line *trr11*) cDNA were isolated and purified from pGEMT clones (see "***Cloning cDNA fragments into pGEMT"***). The CaMV35S expression cassette was isolated by digestion with EcoRV from pUC-18 vector: Amp resistant. Then filling with Klenow and dNTP and subsequent ligation into pBin19 (HindIII/EcoRI) to yield pBin-35S. Purified fragments were cloned into the pBin-35S expression cassette using restriction enzymes, resulting in pBin35S/cDNA/NOS. The constructs were introduced by electroporation into *Agrobacterium tumefaciens* containing pGV2260 plasmid. The floral dip method (Clough, S. J. and Bent, A. F. 1998) was used for transformation of plant material, Col-0 WT bolting plants with *Agrobacterium tumefaciens.* Transgenic seedlings were selected on ½ MS media containing 50 mg/L Kanamycin.

Table 2. Q-PCR primers sets used in this study.
Eff means primer efficiency for each set of primers. Primer efficiencies for all primer sets were determined according to equation $E=10^{(-1/slope)}$ as described by (Rasmussen, R. 2000).

Protein	Gene model	Forward primer	Reverse primer	Eff
LHCB1	Atg29920	ggggtcagcggatagaccag	ctttcgccggaaaggctgt	2.02
PSI-H	At3g16140.1	cccacttcagagcaagttcttt	tggctcttgaggacctctctta	2.07
GR-RBP2	At4g13850.1	tcacttcggtgacgttgttg	atgtgacgaccattcagttcct	2.06
Hydrolase, alfa/beta family protein	At1g73480.1	cgacgaagagaggagatacgtt	accgatccagtcaattccatag	1.87
Expressed protein (TRR17)	At1g60870.1	aacttgatcgggatatggagtg	caactgtcgccgctttct	2.07
Methylentetrahydrofolate reductase 1 (MTHFR1)	At3g59970.3	cagagtgatcttgcttacttgaaga	gaaataggcataattccaggaaca	1.63
Expressed protein (TRR14)	At4g10300.1	atcctcctgagtccaaactaacc	catcggatccattagggtaca	1.80
Glutamate:Glyoxalate aminotransferase 1(GOGAT)	At1g23310.1	tcagtatgccgtaagaggtgaa	gctggaaatagcattccaacat	2.01
Trehalase	At4g24040	gctgcaccacgaaccagtaga	ttcttcgttctccacgttgga	1.98

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CHAPTER 4

Characterizing trehalose resistant mutants

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Abstract

In the previous chapter trehalose resistant *(trr)* mutants were identified. Here *trr* mutants are characterized in more details. T6P measurements reveal that several *trr* mutants resist trehalose by reducing the steady state levels of T6P; supporting the notion that growth arrest on trehalose is due to T6P accumulation. Six *trr* mutants on trehalose accumulate similar level of T6P to WT and are most likely altered in responses to T6P. Genetic dissection of the multiple responses to T6P reveals distinct pathways that lead to starch accumulation and growth arrest, respectively. *GR-RBP2* and *LHCB1* are involved in the growth response only. TRR14 suppresses starch accumulation and growth arrest mediated by T6P. In addition, *TRR14* over-expression renders plants resistant to T6P at the adult stage and leads to reduced photorespiration. Suppression of anthocyanin accumulation and chlorophyll reduction in all *trr* mutants that accumulate T6P implies that these typical senescence processes are likely induced secondarily, as a result of growth inhibition. Exogenous ascorbate partially suppresses T6P mediated growth and starch responses likely by lowering T6P steady states.

Introduction

Plant responses to increased T6P are multiple, as evidenced by transgenic Arabidopsis with altered T6P steady states (Schluepmann, H. et al. 2003). Plants with increased T6P levels by way of either *TPS* over-expression or trehalose feeding typically have darker green cotyledons and leaves as well as increased photosynthetic capacity per unit leaf area (Pellny, T. K. et al. 2004). Moderate increase of T6P levels by way of constitutive trehalose-6-phosphate syntase (TPS) over-expression with the CaMV35S promoter promotes growth in long day conditions (Schluepmann, H. et al. 2003). However, increase of T6P levels by way of trehalose feeding results in growth arrest possibly due to T6P accumulation in the absence of sufficient metabolizable carbon (Schluepmann, H. et al. 2004). Increased trehalose and T6P also affect starch synthesis via the control of T6P over AGPase redox activation (Kolbe, A. et al. 2005, Wingler, A. et al. 2000). The T6P effect on AGPase redox activation was reconstituted using chloroplast preparations *in vitro.* Mutants such as *pgm1* show, however, that starch accumulation and growth responses to T6P are independent (Fritzius, T. et al. 2001).

In the previous Chapter, 19 *trr* mutants that resist growth inhibition on 100 mM trehalose were isolated. To dissect the pleiotropic response to trehalose such *trr* mutants isolated must be characterized physiologically, with respect to the different effects of T6P. These include starch, chlorophyll, anthocyanin, T6P accumulation and photosynthesis on trehalose.

One of the greatest difficulties when studying T6P signalling is the accurate quantification of trehalose and T6P. Trehalose can be measured after derivatization using GC-MS, yet its concentration in most plant extracts is near the detection limit. This explains why, until 1997, trehalose was thought absent in plants, except in a few desiccation-tolerant plants (Goddijn, O. and Smeekens, S. 1998). Trehalose is now an established plant metabolite and genes for its synthesis are ubiquitously found in higher and lower plants. T6P is also an established metabolite since sequence data implies biosynthesis of trehalose via its phosphorylated intermediate in all plants for which sequences are available. Measurements of T6P levels in plant extracts have initially relied on the specific inhibition of *Yarrovinia* hexokinase by T6P levels in plant extracts (Pellny, T. K. et al. 2004). Later on, determinations were carried out using trehalase degradation or HPLC analysis in combination with pulsed amperometric detection (Schluepmann, H. et al. 2003, Schluepmann, H. et al. 2004). These methods evaluated the T6P levels in Col-0 seedlings at 2-5 $nmol \cdot g^{-1}$ FW. Detection of T6P using LC coupled to MS-Q3 allowed determining the levels of T6P in 5 d Arabidopsis seedlings more accurately at 0.3 $nmol \cdot g^{-1}$ FW (Lunn, J. E. et al. 2006) suggesting that this is the method of choice for quantification of T6P in the *trr* mutants. T6P determinations are essential to differentiate between *trr* mutants affected in T6P steady state levels and those affected in putative targets of T6P as the latter ones will accumulate T6P on trehalose medium to the same extend than WT.

Plants engineered to increase the steady state levels of trehalose were shown to have improved stress tolerance (Garg, A. K. et al. 2002). However these studies did not exclude T6P as the active principle since trehalose steady states failed to correlate with the extent of stress resistance. Trehalose feeding in the dark leads to arrest of hypocotyl elongation and we have been unable to assign this effect to either T6P or trehalose (Chapter 2). Hypocotyl elongation is a typical etiolation process yet the effect of trehalose/T6P is not seemingly related to de-etiolation since dark grown seedlings on trehalose display the typical apical hook after 5 days. Absence of hypocotyl elongation is more likely due to inhibited mobilisation of lipids, which are required for gluconeogenesis, growth and extension (Penfield, S. et al. 2005, Penfield, S. et al. 2006). However, genes uncovered in the *trr* mutants in Chapter 3 appear unrelated to known growth and gluconeogenesis processes. Instead GR-RBP2, LHCB1 and PSI-H appear involved in stress responses, reactive oxygen production and photosynthesis (Kwak, K. J. et al. 2005, Noctor, G. et al. 2000, Vermel, M. et al. 2002). Feeding seedlings 30 mM trehalose in the presence of 1% w/v sucrose consistently induced mostly detoxification and stress response proteins in the period of 6-12h after exposure (Bae, H. et al. 2005). In addition, trehalose treatment of wheat elicits a biotic response that protects the plants against powdery mildew infection (Reignault, P. et al. 2001). Therefore, some of the proteins underlying the *trr* phenotype likely will reveal processes induced by the elicitor

effects of trehalose/T6P. Probing the interaction of reactive oxygen scavengers and trehalose/T6P responses is thus of interest.

In this chapter, *trr* mutants are characterised with respect to morphological changes and the prolonged effect of trehalose feeding. In addition starch, T6P, chlorophyll and anthocyanin levels are analysed to draw a map of the metabolic responses occurring upon feeding trehalose. Growth arrest and starch accumulation are found to be unrelated effects of T6P accumulation. Ascorbic acid but not glutathione, is found to suppress effects of trehalose by suppressing T6P accumulation. The dissection of trehalose feeding effects identifies *LHCB1* and *GR-RBP2* as putative targets of T6P mediated growth control only, and *TRR14* as a more upstream target of T6P controlling both growth and starch responses.

Results

Phenotypes of adult *trr* plants

In the previous chapter, 19 *trr* mutants were identified that have roots at least 3 times longer than WT on 100 mM trehalose after 14d (Chapter 3, Table 2). On soil in long day conditions, plants from the *trr* lines are indistinguishable from WT, except for *trr3, trr4* and *trr5*. Plants from *trr3* and *trr4* over-express *PSI-H* and have somewhat larger darker green leaves compared to WT. Plants from *trr5* have lower *LHCB1* expression and have pale green leaves (Fig. 1A). *trr* lines are unchanged with regard to flowering time and are fully fertile.

Mutants were also tested for their ability to grow on 100 mM trehalose. Plants of *trr8, 14, 15* and *18* (expressing cDNAs encoding hydrolase of the alfa-beta family and two unknown proteins, respectively) typically developed mature rosettes and flowered within 4 weeks. *trr*7, *11*, *16* and *19* (expressing cDNA encoding GR-RBP2, MTHFR1, TRR14 and an unknown protein, respectively) developed and flowered within 6 weeks. Of these lines *trr*7, *11* and *19* accumulated excessive amounths of anthocyanins. *trr3*, *4* and *5* (expressing cDNA encoding PSI-H and LHCB1, respectively) changed to callus and from callus generated flowering plants within 6 weeks. *trr10* and *17* converted to callus (Fig. 1B). WT, *abi4-3*, *abi3-1* and *pgm1* failed to develop past the seedling stage (not shown).

Starch accumulation is differentially induced in the *trr* mutants on 100 mM trehalose

Trehalose induces massive starch accumulation in source tissues and inhibits starch accumulation in the columnella cells of the root tips (Fig. 2A-WT; Wingler, A. et al. 2000). The distribution of starch in *trr* mutants, TreF and WT was studied in 14d seedlings using Lugol staining. Staining revealed that the reaction to trehalose is not fully homogenous when examining a large number of WT seedlings: 72% of the seedlings respond with massive trehalose accumulation in cotyledons whilst

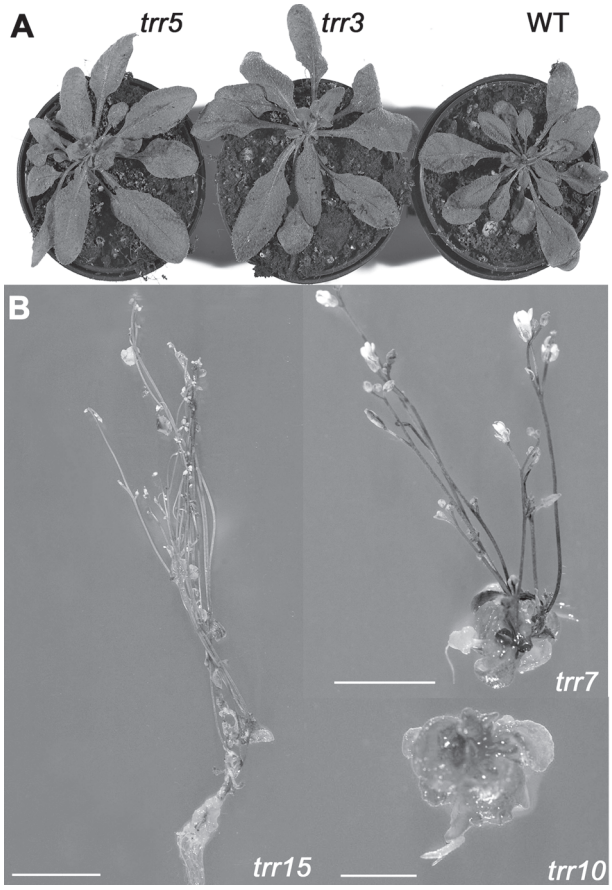

Figure 1. Growth of *trr* mutants. A) Adult plants grown in soil at 22°C under long day conditions of ***trr5, trr3*** and WT. **B)** Long-term effects of 100 mM trehalose. Plants obtained from ***trr15*** (after 4 weeks), ***trr7*** and ***trr10*** (after 6 weeks). (Bar= 1cm)

28% fail to stain. Such variation was also observed in *trr* mutants (data not shown). The response to trehalose of seedlings expressing *E.coli* trehalase (TreF line) and of seedlings from *pgm1* was homogenous, as cotyledons of these seedlings did not stain with Lugol. Seedlings of the TreF line display starch in the columnella cells of the root tips, unlike seedlings of *pgm1* (Fig. 2A-TreF, *pgm*). *trr* mutants were divided in two classes. One class shows much reduced accumulation of starch in the cotyledons and in the root tips: *trr10, 14, 15, 16, 17, 18* and *19* (Fig. 2A- *trr19*). The other class still accumulates starch in the cotyledons but has reduced starch in the columnella cells: *trr3, 4, 5, 7, 8, 11* (Fig. 2A- *trr3*). Quantification of starch in whole seedlings of the *trr* lines on trehalose is shown in Fig. 2B. WT seedlings contain 11 $mg \cdot g^{-1}$ FW starch on medium with 100 mM sorbitol (data not shown). On trehalose starch in WT increases to 52 $mg \cdot g^{-1}$ FW. TreF seedlings on trehalose contain the same amount of starch as WT on sorbitol. On trehalose, *trr* seedlings staining for starch in the cotyledons contain as much starch as WT like *trr4, 5, 7, 8* and *11*. *trr*

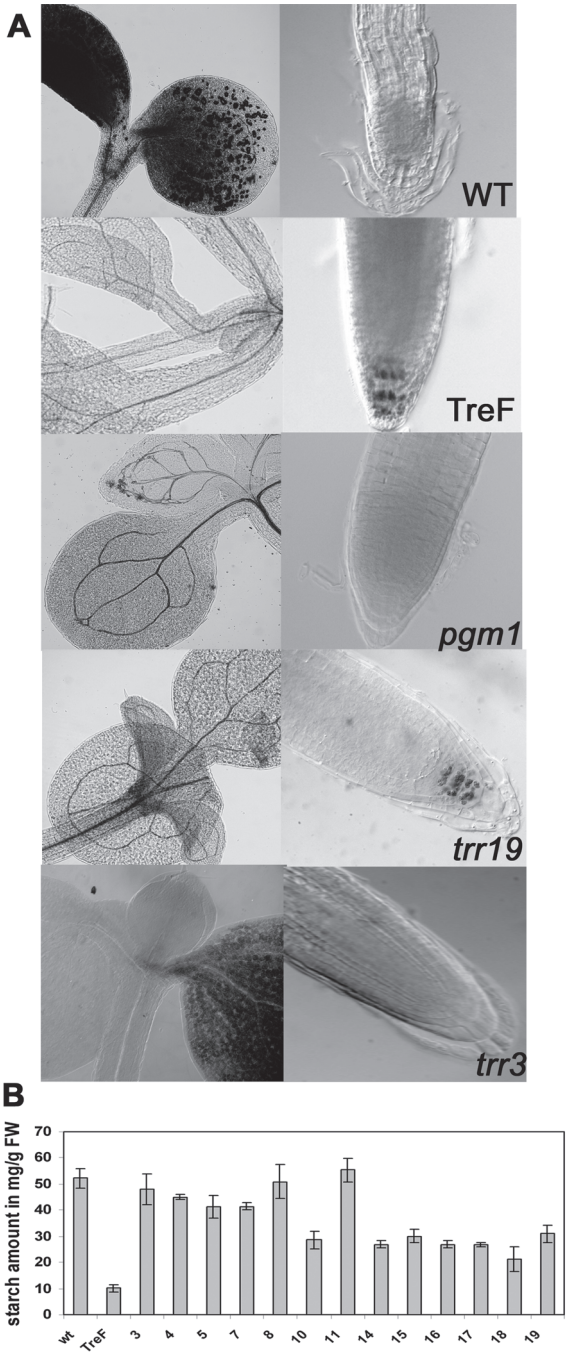

Figure 2. Starch staining and quantification in *trr* mutants. A) Typical starch staining patterns in shoot and root tip of the *trr* mutants (*trr19, trr3*) compared to staining in WT, the plastidic phosphoglucomutase 1 mutant (*pgm),* and the *E.coli* trehalase expressing line 46.2 (TreF). **B)** Starch quantifications in whole seedlings. Seedlings were grown on 100 mM trehalose for 14 days, then starch content quantified as described in materials and methods. WT, seedlings from wild type; TreF, seedlings from the *E.coli* trehalase expressing line 46.2.

seedlings that do not stain for starch in the cotyledons still contain 20-30 $mg \cdot g^{-1}$ FW starch suggesting that the mutations do not suppress starch induction entirely (*trr10, 14, 15, 16, 17, 18* and *19*). Root lengths did not correlate with starch distribution in the *trr* mutants. This lack of correlation is further confirmed by analysing starch

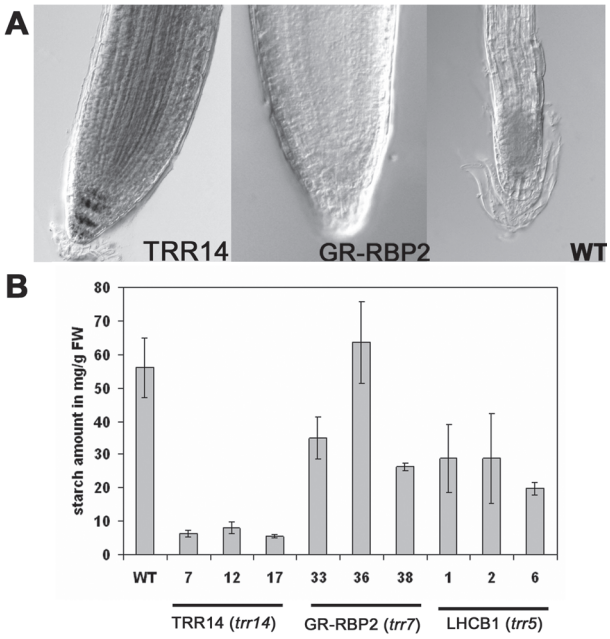

Figure 3. Starch in over-expressors of *TRR14, GR-RBP2* and suppressors of *LHCB1* on trehalose. A) Typical starch staining in the root tips of over-expressors of *TRR14* and *GR-RBP2* compared to WT grown on 100 mM trehalose. **B)** Starch contents of whole seedlings from WT and independent transgenic lines over-expressing *TRR14* (lines 7, 12 and 17), *GR-RBP2* (lines 33, 36 and 38) and *LHCB1* (lines 1, 2, and 6).

in lines with 10 fold longer roots on trehalose compared to WT, which were re-transformed with the constructs *TRR14, GR-RBP2* and *LHCB1* (Fig. 3). Seedlings from lines after re-transformation that over-express *TRR14* accumulate starch in the root tip, but those that over-express *GR-RBP2* do not (Fig. 3A). Lines over-expressing *TRR14* fail to accumulate more starch than WT on sorbitol and thus are suppressed with respect to the starch response to trehalose (Fig. 3B; lines 7, 12 and 17). Lines over-expressing *GR-RBP2* as well as those suppressing the expression of *LHCB1* accumulate starch (Fig. 3B). We thus conclude that T6P control over starch and over growth are in two independent response pathways.

Chlorophyll and anthocyanin measurements correlate with the growth response

WT seedlings growing on trehalose have dark red-rimmed cotyledons. Chlorophyll and anthocyanin contents were determined in WT and mutants. Chlorophyll content of WT seedlings are 4 fold reduced after 14d growth on trehalose compared to growth on sorbitol (Fig. 4). In mutants over-expressing *GR-RBP2* or *TRR14,* or suppressing *LHCB1,* chlorophyll content is not reduced by trehalose, compared to WT. Anthocyanin is 5 fold induced in WT seedlings after

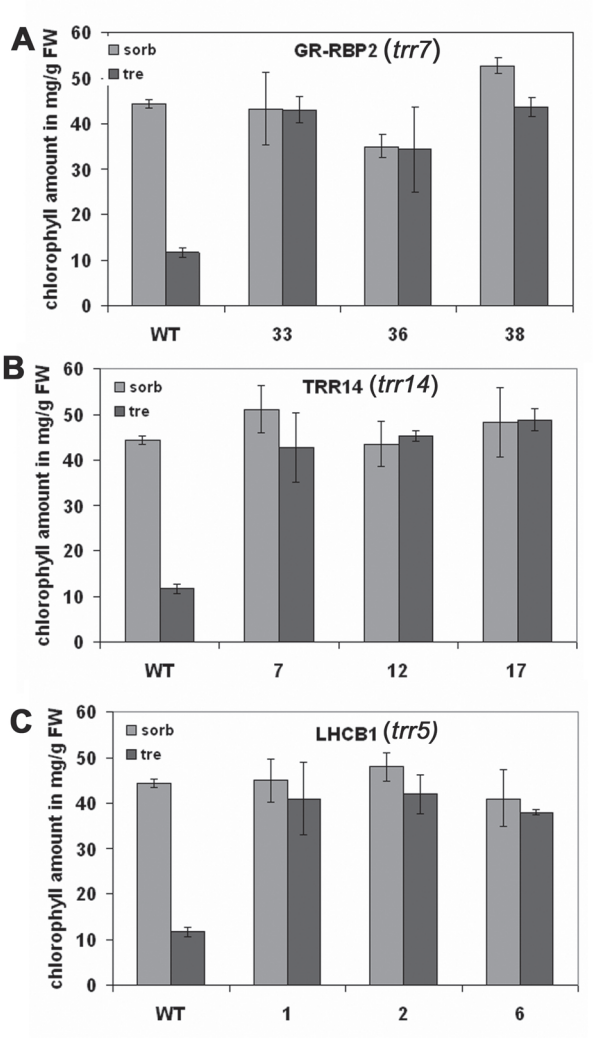

Figure 4. Chlorophyll content in re-transformed lines. A) ***GR-RBP2*** **over-expressor lines 33, 36 and 38, B)** ***TRR14*** **over-expressor lines 7, 12 and17, C)** ***LHCB1*** **suppressor lines 1, 2 and 6, compared to WT. Seedlings were grown on 100 mM sorbitol (sorb) or trehalose (tre) for 14 days before extraction and chlorophyll measurements as described in Materials and Methods. The standard variation presented is from 3 biological replicates.**

14d growth on trehalose compared to sorbitol (Fig. 5). In mutants over-expressing *GR-RBP2* or *TRR14*, or suppressed *LHCB1*, a reduced anthocyanin accumulation is observed. Altered chlorophyll and anthocyanin contents seem to correlate with growth.

T6P measurements

T6P levels were determined in 50 mg FW tissue using a pre-purification SPE procedure followed by HPLC-PAD and HPLC-MS as described in Materials and Methods. Results confirm accumulation of T6P in WT seedlings grown for 14 d on trehalose. T6P level in 14d seedlings is 10.2 ± 2.5 nmol·g^{-1} FW, compared to 2.78 ±1.3 nmol·g^{-1} FW on sorbitol. Levels of T6P in seedlings grown on sorbitol are below detection of the PAD and required LC-MS detection for quantification. T6P quantification in *trr* mutants allows discrimination of the mutants in two

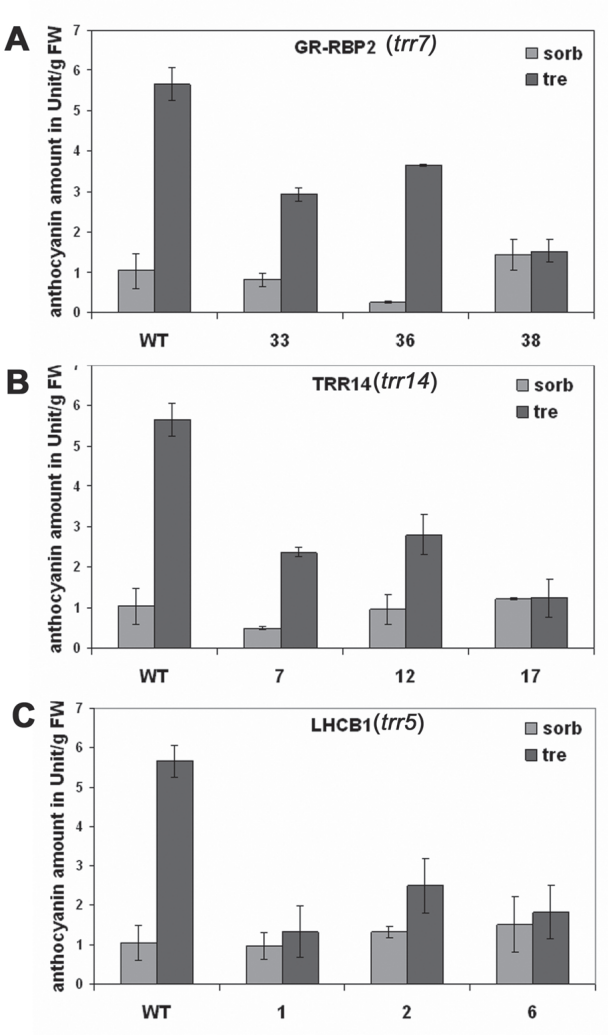

Figure 5. Anthocyanin content in re-transformed lines. A) ***GR-RBP2*** **over-expressor lines 33, 36 and 38 compared to WT, B)** ***TRR14*** **over-expressor lines 7, 12 and17, and C)** ***LHCB1*** **suppressor lines 1, 2 and 6. Seedlings were grown on 100 mM sorbitol (sorb) or trehalose (tre) for 14 days before anthocyanin measurements as described in Materials and Methods. The standard deviation presented is from 3 biological replicates.**

groups (Fig. 6). A first group of *trr* contains low T6P levels on sorbitol as well as on trehalose (*trr3, 11,* and *19*), suggesting that the screen on 100 mM trehalose effectively selects seedlings with altered T6P metabolism. Seedlings of this first group likely overcome growth arrest because of their constitutive low T6P steady state. *PSI-H* over-expressors (*trr3*) belong to this first group and therefore, *PSI-H* over-expression leads to reduced T6P steady states in seedlings on both sorbitol and trehalose. A second group contains *trr* mutants with T6P levels comparable to WT on both sorbitol and trehalose (*trr5, 7, 10, 14, 15 and 18). GOGAT, GR-RBP2, TRR14, TRR18* over expressors, and reduced *LHCB1* expressors belong to this second group (Fig. 6). Seedlings of these *trr* mutants therefore no longer respond to T6P accumulation, which implies that *GOGAT, GR-RBP2, TRR14 TRR18* and *LHCB1* act downstream of T6P. TRR14 seedlings no longer accumulate starch, when grown on trehalose implying that *TRR14* is involved in a step upstream

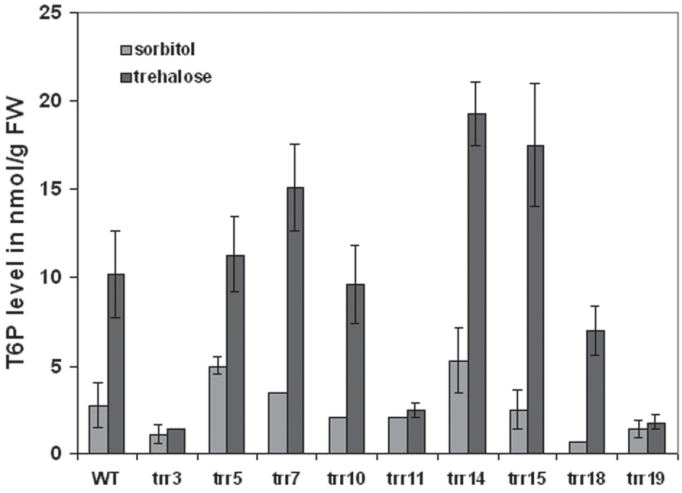

Figure 6. T6P quantification in *trr* mutants. Seedlings of WT and *trr* mutants were grown for 14d on half strength MS supplemented with 100mM of either sorbitol (sorb) or trehalose (tre). T6P was extracted and quantified as described in Materials and Methods. The standard deviation presented is from 3 biological replicates.

of both starch and growth control by T6P. Effects of *GR-RBP2* and *LHCB1* are confined to the growth-controlling pathway since the corresponding mutants still accumulate starch.

Photorespiration is altered by over-expression of *TRR14*

T6P levels are known to affect photosynthetic capacity per unit leaf area (Pellny, T. K. et al. 2004). Photosynthesis may therefore constitute a target process of T6P and we measured photosynthethic parameters in WT and *trr* plants grown in soil. Measurements of the ratio of intercellular CO_2 and ambient CO_2 concentration (Ci/Ca) revealed that plants from *trr14* and *trr16* have altered CO_2 respiration (Fig. 7A). Calculation of photorespiration using (Vo+Rp)/(Vo+Vc), where Rp is the photorespiratory CO_2 release, Vc the rate of carboxylation and Vo the rate of oxygenation (Farquhar, G. D. et al. 1980a, Farquhar, G. D. et al. 1980b), reveals that photorespiration is significantly decreased in *trr14* (Fig. 7B). *trr14* over-expresses TRR14 protein. Over-expression of TRR14 therefore results in decreased photorespiration.

Ascorbate partially suppresses the effect of trehalose

tps1 mutant has a redused T6P levels, leading to a reduced growth phenotype due to inability to utilize sugars. Metabolite profiling of *tps1* mutants grown on ½ MS at the seedling stage revealed profound changes in metabolite steady states (data not shown). *tps1*, like TPP over-expressors, are sensitive to exogenous

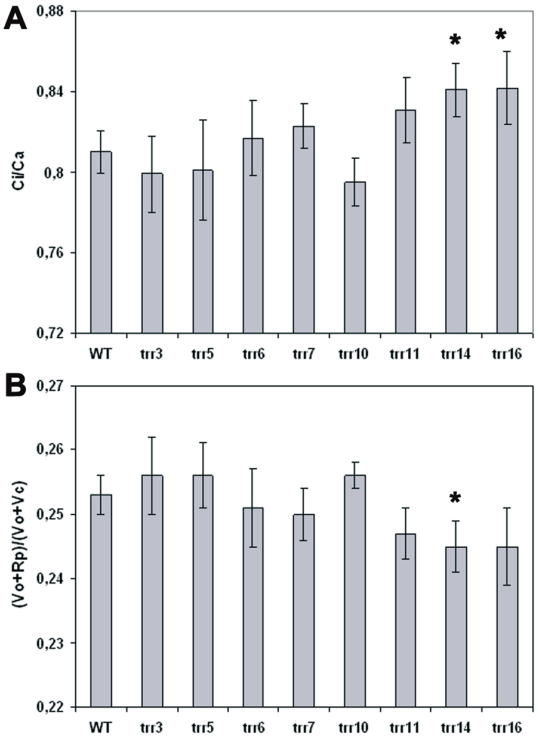

Figure 7. *TRR14* over-expression alters photosynthesis characteristics.
Plants were grown on soil under short day regimes at 22°C. A) Ratios of leaf internal versus air CO_2 concentrations were measured in WT and in the *trr* mutants with dominant segregation of mutation. B) Measurements of photorespiration using $(V_0+Rp)/(V_0+Vc)$ in WT and in the *trr* mutants. From each mutant 2 plants were measured and measurements were carried out in triplicate; * marks significantly different measures from WT measures. (P< 0.05)

supply of sucrose and on half strength MS medium accumulate fructose, sucrose and glucose (Eastmond, P. J. et al. 2002). Interestingly, *tps1* also has a dramatically changed ascorbate/dehydroascorbate balance, with ascorbate accumulation and dehydroascorbate reduction (not shown). We therefore tested the effect of ascorbic acid on the growth inhibition by trehalose (Fig. 8). At 0.1 mM ascorbic acid and lower concentrations the medium pH was at 5.5 and seedlings germination were 100%. Ascorbic acid supplied to the medium at 0.5 and 1 mM inhibits germination of WT seeds. Average root length of 14 days old WT seedlings grown on 0.1 or 0.05 mM ascorbate with 100 mM trehalose is significantly longer than on 100 mM trehalose alone (Fig. 8A). The starch content of these WT seedlings grown on 0.1 mM ascorbate and 100 mM trehalose is half that on 100 mM trehalose (Fig. 8B). Dehydroascorbate has no effect on root length or starch accumulation compared to WT.

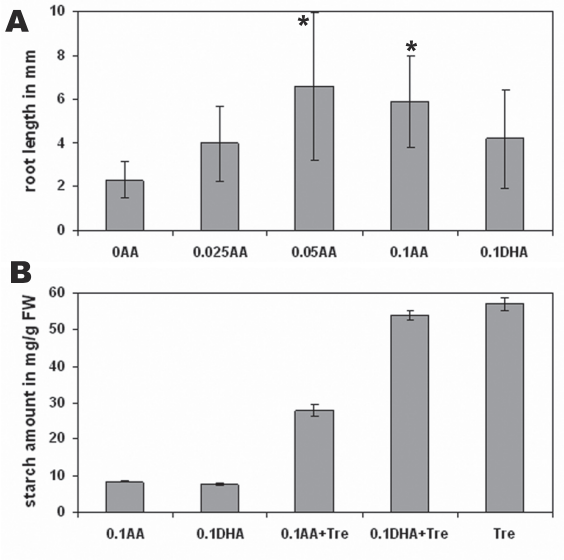

Figure 8. Effect of Ascorbic acid and dehydroascorbic acid. A) Root length of seedlings growing on half strength MS with 100 mM trehalose (0AA) supplemented with, 0.025, 0.01 and 0.1 mM ascorbic acid (0.025AA, 0.05AA, and 0.1AA, respectively), or 0.1 mM dehydroascorbic acid (0.1DHA). **B)** Starch content of 14d old seedlings growing on half strength MS supplemented with 0.1 mM ascorbic acid (0.1AA), 0.1 mM dehydroascorbic acid (0.1DHA), 0.1 mM ascorbic acid combined with 100 mM trehalose (0.1AA+Tre), 0.1 mM dehydroascorbic acid combined with 100 mM trehalose (0.1DHA+Tre) or 100 mM trehalose (Tre). The standard deviation presented is from 3 biological replicates.

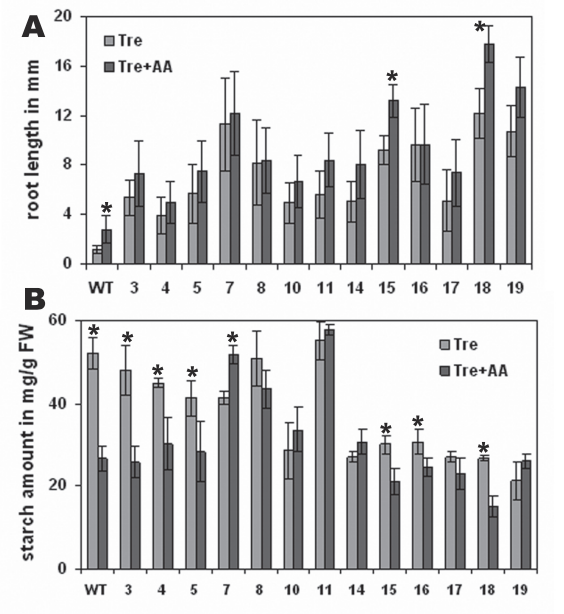

Figure 9. Effect of 0.1 mM ascorbic acid on *trr* mutants growing on 100 mM trehalose. A) Root length of *trr* mutants growing on 100 mM trehalose with or without 0.1 mM ascorbic acid. **B)** Starch amount in *trr* mutants growing on 100 mM trehalose with or without 0.1 mM ascorbic acid. Seedlings were grown on the medium for 14 days and then root length and starch were measured. The standard deviation presented is from 3 biological replicates.

Root lengths on 100 mM trehalose of the *trr* mutants were not generally affected by ascorbic acid except for *trr15* and *trr18* (Fig. 9A). Starch accumulation was reduced by ascorbate equally in WT, *trr3* and *trr4* over-expressing *PSI-H*, *trr5* with reduced *LHCB1* and in *trr15, 16, and 18*. Ascorbate increases starch accumulation in *trr7* over-expressing *GR-RBP2* (Fig. 9B).

Preliminary measurements suggest that 0.1 mM ascorbate in WT seedlings on 100 mM trehalose reduces T6P steady state (data not shown). Reduced starch and increased growth in the WT and some mutants therefore might be due to lower T6P steady state levels. An interesting interaction appears to occur in plants over-expressing *GR-GRP2* (*trr7*), encoding a mitochondrial protein. In *trr7* the presence of ascorbate elicits increased starch accumulation but growth performance on trehalose remains unchanged compared to trehalose alone.

A summarizing table including results obtained from the analyses of the *trr* lines is found in Table 1.

Table 1. Analyses of the *trr* lines with trehalose resistance segregating as a dominant trait. Line No, refers to the *trr* mutant number with WT, wild type, as control and with Re- lines obtained after re-transformation; Encoded protein, protein encoded in the cDNA expressed in the *trr* mutant; Gene expression, cDNA leads either to over-expression or to suppression of the gene (over and suppressor, respectively); Root length, length of the root on 100 mM trehalose after 14 d, the numbers of * shows how many times mutant root length is longer than WT; Starch, Chlorophyll, Anthocyanin and T6P refer to starch accumulation, chlorophyll levels, anthocyanin accumulation and T6P content on 100 mM trehalose after 14 d.

Line No	Encoded protein	Gene expression	Root length	Starch	Chlorophyll	Anthocyanin	T6P
WT	__	__	short	high	low	high	high
trr3, trr4	PSI-H	over	****	high	high	low	low
trr5	LHCB1	suppressor	****	high	high	low	high
trr7	GR-RBP2	over	****	high	high	low	high
trr14	TRR14	over	****	low	high	low	high
trr8	α/β Hydrolase	over	**	high	high	low	__
trr10	GOGAT	suppressor	****	low	high	low	high
trr11	MTHFR	over	***	high	high	low	low
trr17	TRR17	over	***	low	high	low	high
trr18	TRR18	over	***	low	high	low	high
trr19	__	__	****	low	high	low	low
Re-*PSI-H*	PSI-H	over	****	high	high	low	__
Re-*LHCB1*	LHCB1	suppressor	******	high	high	low	high
Re-*GRRBP2*	GR-RBP2	over	******	high	high	low	high
Re-*TRR14*	TRR14	over	******	low	high	low	high

Discussion

T6P measurements carried out in this chapter reveal that several *trr* mutants resist trehalose by reducing the steady state levels of T6P; supporting the notion that growth arrest on trehalose is due to T6P accumulation. Six *trr* mutants are shown to accumulate as much T6P as WT on trehalose and are likely altered in responses to T6P. Dissection of the multiple responses to T6P using the *trr* mutants reveals distinct pathways that lead to starch accumulation and growth arrest, respectively. GR-RBP2 and LHCB1 are involved in the growth response only. *TRR14* over-expression suppresses starch accumulation and growth arrest mediated by T6P. In addition, *TRR14* over-expression renders plants resistant to T6P at the adult stage and reduces photorespiration. Lack of anthocyanin accumulation and chlorophyll reduction in all *trr* mutants implies that these typical senescence processes correlate with growth inhibition and are likely induced secondarily in WT as a result of growth inhibition. Ascorbic acid suppresses T6P mediated growth inhibition and starch accumulation likely by reducing T6P steady state, but this needs confirmation.

A model of interactions surrounding T6P

PSI-H and *LHCB1* are both involved in photosynthesis. Results from T6P quantification reveal that the mechanisms by which these two proteins mediate trehalose resistance differ. *PSI-H* over-expression results in lower T6P steady states. PSI-H helps stabilizing the PSI complex under stress conditions (Naver, H. et al. 1999). PSI is known to be the major detoxifier of stress induced reactive oxygen species (ROS) such as H_2O_2. PSI activity reduces monodehydroascorbate to ascorbic acid needed for H_2O_2 removal (Baier, M. et al. 2000, Ivanov, B. and Khorobrykh, S. 2003, Nakano, Y. and Asada, K. 1981). Ascorbate feeding experiments suggest a link between T6P and ascorbic acid: high ascorbate is associated with reduced T6P levels. Therefore, increased ascorbic acid synthesis may be the mechanism by which *PSI-H* over-expression reduces T6P levels. *PSI-H* over-expressors still accumulate starch on trehalose. Redox-activation of AGPase that likely is mediated by thioredoxin F is thus intact in these plants (Geigenberger, P. et al. 2005, Kolbe, A. et al. 2005) and plastid-localized PSI-H appears not to be involved in control of the thioredoxin system.

Reduced *LHCB1* expression yields seedlings that no longer respond to accumulation of T6P on trehalose. Apparently, growth inhibition due to T6P can be overcome by reduced *LHCB1* expression. LHCB1 is mostly found in light harvesting antenna complexes where it binds pigments such as chlorophyll and carotenes. These antenna complexes absorb sunlight and transfer the excitation energy to the core complexes PSII and PSI to promote photosynthetic electron transport. Plants suppressing *LHCB1* also suppress *LHCB2* (Andersson, J. et al. 2003). The pale green plants only have a small reduction in quantum yield and

no impairment in growth under controlled conditions compared to WT plants. These plants have reduced state transitions and capacity for feedback de-excitation important to adapt to changes in light intensity (Andersson, J. et al. 2003). Reduced state transitions are likely due to the lack of substrate LHCB1&2 proteins for the kinase regulating transition state in response to the plastoquinone redox states (Horton, P. et al. 1991) and may result in changes in the plastoquinone redox pool. How this affects growth when T6P accumulates is unclear. T6P possibly enhances expression or stability of light harvesting complexes. *LHCB1* might be a target of T6P, albeit likely an indirect target because *LHCB1* repression does not affect starch accumulation whilst TRR14 affects both starch accumulation and growth. *LHCB1* antisense plants were shown to be more sensitive to environmental stress (Andersson, J. et al. 2003) Possibly, T6P's effect on *LHCB1* and *PSII* mediates the stress responses engineered by expression of *TPS* (Garg, A. K. et al. 2002, Holmstrom, K. O. et al. 1996, Jang, I. C. et al. 2003, Pilon-Smit, E. A. H. et al. 1998).

GR-RBP2 over-expression suppresses the growth inhibition caused by T6P accumulation. In these plants T6P accumulates to WT levels and starch accumulation is induced. GR-RBP2 therefore likely is an indirect target of T6P. GR-RBP2 likely affects processes down stream of T6P. GR-RBP2 is targeted to mitochondria (Kruft, V. et al. 2001), an organelle where T6P is not synthesized. None of the proteins of T6P metabolism in Arabidopsis have a mitochondrial targeting sequence (Leyman, B. et al. 2001, Schluepmann, H. et al. 2004) and none have been found associated with the mitochondrial proteome (Kruft, V. et al. 2001). *GR-RBP2* therefore is likely modulating a component down stream of T6P. *GR-RBP2* has very recently been functionally characterized as a RNA chaperone mediating cold resistance in Arabidopsis. Its over-expression leads to changes in the steady state levels of mitochondrial proteins (Kim et al. 2007). Mitochondrial and chloroplastic functions are linked. For example, uncoupling proteins are known to adjust redox production in mitochondria with that in chloroplasts (Sweetlove, L. J. et al. 2006).

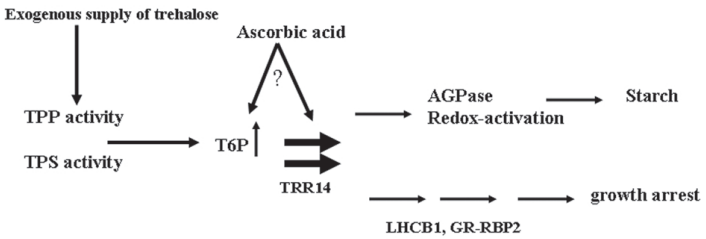

Figure 10. Summarizing model.
Exogenous supply of trehalose leads to T6P accumulation. T6P levels then affect growth and starch via separated pathways. *trr*14 has low starch and grows on trehalose and likely affect a common step in starch and growth control. *LHCB1* and GR-*RBP2* accumulate starch but do grow on trehalose. These proteins seem to affect growth control pathway only.
Ascorbic acid probably reduces T6P levels on trehalose and thereby represses starch accumulation and growth arrest.

TRR14 over-expression suppresses both the growth inhibition and starch accumulation phenotypes caused by T6P. This protein thus appears to affect steps on the controlling path down stream of T6P that are shared by starch and growth responses. The model of interactions surrounding T6P emerging from *trr* mutant analysis is presented in Fig. 10.

TRR14 plays a role in controlling starch accumulation, photorespiration and growth

TRR14 over expression provides resistance to both the growth inhibition and starch accumulation effects of T6P. Likely, *TRR14* affects an earlier step in the T6P responses than *LHCB1* and *GR-RBP2*. Remarkably over expression of *TRR14* reduces photorespiration of the plants. Photorespiration involves the conversion of phosphoglycolate to CO_2 and 3PGA in a series of reactions that involve chloroplasts, peroxisomes, and mitochondria (Kisaki, T. and Tolbert, N. E. 1969). Photorespiration results in the loss of up to 25% of the carbon that is fixed during photosynthetic carbon assimilation (Ludwig, L. J. and Canvin, D. T. 1971). Photorespiration is essential for plant growth, as mutants in enzymes of the pathway are not viable in air (~ 0.04% CO_2) and grow only in elevated CO_2 (1 to 2% CO_2), conditions under which RuBP oxygenation is suppressed (Somerville, C. R. and Ogren, W. L. 1981). All of the Arabidopsis genes encoding enzymes that catalyze the basic steps of the photorespiratory pathway have been cloned, but questions remain regarding transport of the metabolites and regulation of photorespiration (Eckardt NA 2005). Attempts to reduce photorespiration have mostly failed even though it is a major goal of for crop improvement. An increased understanding of the regulation of photorespiration is urgently needed in the context of climate change as stress-induced by drought and heat will increase photorespiration of existing crops and limit crop yield (Long, S. P. et al. 2006). This makes the effect of TRR14 on photorespiration particularly interesting. TRR14 is a protein containing the Cupin structure but with unknown function (TAIR, 2007).

Taken together, results from Chapter 4 allow the contribution of a model of interactions surrounding T6P and identifies *LHCB1, GR-RBP2* and *TRR14* as potential down stream components of T6P.

Materials and methods

Plant materials and growth conditions

Arabidopsis Columbia 0 (Col-0), TreF 46.2 (a line expressing *E.coli* cytosolic trehalse behind the CaMV35S promoter), 13 selected *trr* lines and the starch–deficient mutant *pgm1*, *abi4-3* and *abi3-1* seeds were used in this study. Seeds were sterilized 5 minutes with 70% ethanol followed by 10 minutes in 20% commercial bleach (4% W/V available chlorine) and washed 5 times in sterile miliQ water. Sterilized seeds were plated on half-strength

Murashige and Skoog (Murashige and Skoog, 1962) medium with or without 100 mM trehalose and solidified with 1% (W/V) plant agar. After seed stratification for 2 days at 4°C, plant material was transferred to 16-h-light/8-h-dark (long-day) growth conditions at 25°C. To investigate long term effect of trehalose on selected lines, 13 selected dominant lines were grown on 200 ml of ½ MS supplemented with 100 mM trehalose for 8 weeks.

Starch staining and measurement

For analysis of starch distribution, whole seedlings were taken and destained in 70% and then 90% (v/v) ethanol. Staining was done with KI/I_2 solution and then washed. Pictures were taken by Normanski microscope (Jena, Germany).

To measure starch amount, 50 mg fresh weight plant material was frozen and ground in liquid nitrogen. Sugars were extracted with 1 ml 80% ethanol for 10 minutes at 80°C in ependorf tubes. The tubes were spun for 5 minutes at 13000 rpm and the supernatant was transferred to a clean tube. The pellet was extracted again with 75 µl of milliQ for 10 minutes at 80°C and the supernatants were pooled. Starch was then extracted from the remaining pellet by incubation in 0.1 mL of 0.5 M NaOH at 60°C for 30 minutes. After addition of 6 µl acetic acid (96%), starch was digested overnight at 37°C by addition of amyloglucosidase. Starch content was measured by an enzymatic method (Boehringer Mannheim, Darmstadt, Germany) as described by the manufacturer.

T6P measurements

Fifty mg FW of 14d old seedling were snap frozen, then ground using a dismembranator (Braun, Germany) before extraction with 800ul chloroform/acetonitrile/water at a ratio of 5:7:2 for 2h at –10°C. After 5 min 6000g centrifugation at 4°C, the acetonitrile/water phase was recovered and the chlorophorm phase back-extracted with 400 ul water, the water and acetonitrile water phases were combined and dried under vacuum over night. Samples were taken up in 1ml water prior to solid phase extraction (SPE). After loading, the SPE phase was rinsed with 4 volumes 5mM NaOH, then eluted twice with 0.5 ml 2% v/v formic acid. Eluates were combined and dried under a flow of nitrogen, resuspended in 0.2 ml water, filtered and 10 µl injected onto the AS-11-HC column (2 x 250mm, Dionex) for HPLC-PAD (Dionex) or HPLC-MS (Ion trap, Agilent). The ion exchange column was eluted with a 5-100 mM gradient of NaOH. Addition of T6P (12.8 nMoles) during extraction and or immediately loading onto the HPLC allowed calculation of T6P recoveries and approximate evaluation of amounts of T6P in the extracts. Detection with HPLC-MS scanning at m/z 418-423 yielded 36.1 $pmol \cdot g^{-1}$ FW in leaves of soil-grown Arabidopsis sampled at midday. This is the same range as previous results from Lunn et al. (2006) where leaves from soil-grown plants contained 67 to 108 $pmol \cdot g^{-1}$ FW T6P recoveries during the extractions were typically around 85%.

RNA Isolation, RT-PCR and Q-PCR Analysis

Different selected re-transformed lines were grown on ½ MS medium for 10 days.

Plant material was snap frozen in liquid nitrogen, pulverized with glass beads for 2 minutes at 2800 rpm in a dismembrator (Braun, Germany). Total RNA was isolated with RNeasy plant mini kit (QIAGEN USA, Valencia, CA). RNA concentration and purity were determined by measuring absorbance at 260 nm. Ten ng RNA was treated with 2 U DNAse I (DNA-free, Ambion, Austin, USA) to remove genomic DNA. Absence of DNA was analyzed by performing a PCR reaction (40 cycles, similar to the real–time PCR program) on the DNAseI-treated RNA using Taq-DNA polymerase. RT-PCR experiments were performed using 1 ng of total RNA extracted and used for first-strand cDNA synthesis with 60 units M-MLV Reverse Transcriptase (Promega, Madison, WI), 0.5 μg of oligodT16v (custom oligo from Invitrogen, Carlsbad, CA) and 0.5 μg random hexamer (Invitrogen).

Q-PCR was carried out by ABI-prism 7700 Sequence Detection System PE-Applied Biosystems, Foster City, CA). Per reaction 12.5 μl of CYBR green PCR Master Mix (Applied Biosystems, UK) and 2.5 μl of each gene-specific primer were used. To detect expression of the *PSI-H, LHCB1, GR-RBP2, TRR14* (At4g10300) and trehalase, we used the same pair primers as in chapter 3. Relative quantitation of gene expression is based on the comparative Ct method (User Bulletin No. 2: ABI PRISM 7700 Sequence Detection system, 1997) using *AtACTIN2* as a reference.

Chlorophyll and anthocyanin measurements

Chlorophyll levels were measured spectrophotometrically as described (Jeffrey, SW and Humphrey, GF. 1975). In brief, 14 day-old seedlings were ground in liquid nitrogen and extracted with 80% (V/V) acetone. Then absorbance was determined at 647, 652 and 664 nm and the data used to calculate chlorophyll content.

Anthocyanin content of seedlings was determined using the published protocol (Mita, S. et al. 1997). Frozen, homogenized seedlings (20 mg) were extracted for 1 d at 4°C in 1 mL of 1% (v/v) hydrochloric acid in methanol. The mixture was centrifuged at 13,000 rpm for 15 min and the absorbance of the supernatant was measured at 530 and 657 nm. Relative anthocyanin concentrations were calculated using the formula $[A_{530} - (1/4 \times A_{657})]$. The relative anthocyanin amount was defined as the product of relative anthocyanin concentration and extraction solution volume. One anthocyanin unit equals one absorbance unit $[A_{530} - (1/4 \times A_{657})]$ in 1 mL of extraction solution.

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CHAPTER 5

Analysis of the suppressors of T6P mediated growth arrest, *GR-RBP2* and *TRR14*

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Abstract

Two suppressors of T6P mediated growth arrest, *GR-RBP2* and *TRR14,* are characterized in more detail. GR-RBP2 is a protein of likely prokaryotic origin that is part of a protein complex. *GR-RBP2* expression is found throughout the plant and is not responsive to long-term growth on metabolisable sugars such as glucose, fructose or sucrose. *GR-RBP2* expression also is insensitive to 100 mM trehalose. A knockout mutant of *GR-RBP2* was identified in the T-DNA insertion line SALK-059714, yet plants of this line were not altered with regard to growth on different carbon sources and on trehalose compared to WT. The data obtained did not reveal a function for GR-RBP2 with respect to growth in relation to carbon metabolism. Instead, GR-RBP2 mediates cold resistance, and its stress protective function is likely due to its RNA chaperonin activity (Kim, J.Y et al. 2007).

Unlike GR-RBP2, TRR14 affects both starch and growth phenotypes induced by T6P accumulation. TRR14 therefore seems more closely related to T6P signaling than GR-RBP2. TRR14 was found located in the chloroplast. Expression of *TRR14* is ubiquitous and not responsive to metabolisable carbon supply. Trehalose supplied at 100 mM represses *TRR14* expression specifically in the roots. The *trrcupin* knockout mutant identified in the line SALK-064435 yields seedlings and plants with root growth indistinguishable from WT, suggesting that TRR14 is not generally required for root growth under normal conditions. Repression of *TRR14* by trehalose supply is thus unlikely the direct cause for root growth arrest on trehalose. The function of TRR14, beyond its effect on photorespiration, remains unknown.

Introduction

Trehalose metabolism is involved in the control of carbon assimilation and T6P has been proposed as the critical signaling mediator (Kolbe, A. et al. 2005, Rolland, F. and Sheen, J. 2005, Schluepmann, H. et al. 2003, Schluepmann, H. et al. 2004, Wingler, A. et al. 2000). Arabidopsis Hexokinase 1 (HXK1) is a glucose sensor in plants (Cho, Y. H. et al. 2006, Moore, B. et al. 2003), but is not targeted by T6P since *gin2, hxk1,* mutants are similarly growth inhibited by trehalose in the medium as WT. In addition, during growth on trehalose, HXK1 does not mediate starch accumulation in the cotyledons since *gin, hxk1* seedlings accumulate as much starch as WT (Chapter 2). Several intermediates in hormone signaling pathways were shown to also mediate sugar signaling. These include ABI3, ABI4, ABI5 and ETR1 and CTR1 (Arenas-Huertero, F. et al. 2000, Gibon, Y. et al. 2002, Huijser, C. et al. 2000, Laby, R. J. et al. 2000, Moore, B. et al. 2003, Zhou, L. et al. 1998). Mutations in these proteins however do not restore growth on 100 mM trehalose

and it is thus unlikely that any of these proteins are intermediates of T6P signaling for growth (Chapter 2).

Combined genetic and biochemical analyses have uncovered a link between T6P and the redox activation of AGPase (Kolbe, A. et al. 2005). AGPase was previously known to be redox activated following sucrose supply to leaves. Control of this redox activation is mediated by T6P. AGPase in plants with lower T6P levels by way of TPP expression is much less activated by sucrose than in WT and, conversely, more activated by sucrose in plants accumulating T6P by way of TPS expression. T6P added to chloroplasts redox activates AGPase *in vitro* (Kolbe, A. et al. 2005). Taken together, the data suggests that AGPase is a target of T6P but an indirect one, inferring chloroplastic redox-processes as target of T6P. Since *pgm1* mutants do not suppress T6P mediated growth arrest and since *trr* (trehalose resistant) mutants were found that accumulated as much starch as WT but were capable of growth on trehalose, T6P's control over AGPase activity and starch deposition is independent of T6P's control over growth (Fritzius, T. et al. 2001). Therefore, AGPase and starch deposition are not relevant targets of T6P for growth.

In Chapter 4, six *trr* mutants of Arabidopsis were characterized that accumulate similar amounts of T6P as WT, but that grow on trehalose medium, unlike WT. Growth on trehalose of these *trr* lines is due to altered expression of three distinct proteins, two of which, TRR14 and GR-RBP2 have unknown functions. The third protein is LHCB1, an abundant and well-known protein. Research in this chapter concentrates on the two proteins with unknown function encoded by At4g13850 and At4g10300, respectively.

The protein encoded by At4g13850 is the Glycin Rich RNA binding protein, previously named GR-RBP2 (Lorkovic, Z. J. and Barta, A. 2002). Its N-terminus starts with a peptide targeting sequence that likely leads to transport into the mitochondrion. This subcellular location was verified by analysis of the Arabidopsis mitochondrial proteome (Kruft, V. et al. 2001). The GR-RBP2 protein is only 153 aa long, with aa 1-33 the likely mitochondrial targeting sequence. Residues aa 34-113 contain the RNA Recognition Motif (RRM) domain, with the consensus sequences Ribonucleoprotein (RNP)-1 and RNP-2 RNA binding domains (Hanano et al. 1996; Nomata et al. 2004). Residues 122-153 contain the Glycine rich domain thought to be involved in protein-protein interactions. The typical RRM consists of four anti-parallel beta-strands and two alpha-helices arranged in a beta-alpha, beta-beta, alpha-beta fold with side chains that stack with RNA bases. Specificity of RNA binding is determined by multiple contacts with surrounding amino acids. RRM proteins are essential for regulation of post-transcriptional processes. There are about 170 proteins in Arabidopsis with such an RRM domain. GR-RBP2 is particularly small and contains little more than one RRM domain and the short C-terminal Glycin-rich domain. There are 8 homologues of GR-RBP2 in Arabidopsis, named GR-RBP1-8 (Lorkovic, Z. J. and Barta, A. 2002). Expression

of several of these GR-RBP is induced by various stresses and unspecific RNA and DNA binding has been shown for GR-RBP3 (Carpenter, C. D. et al. 1994, Condit, C. M. and Meagher, R. B. 1987, Fang, R. X. et al. 1991). GR-RBP2 expression is induced by cold stress and suppressed by dehydration stress yet the function of GR-RBP2 remained unknown during the experimental part of this thesis (Kwak, K. J. et al. 2005,Vermel, M. et al. 2002).

The TRR14 protein encoded by At4g10300 is 139aa long and contains a peptide targeting sequence that likely leads to transport into chloroplasts, followed by a sequence containing a single Cupin tertiary structure. The Cupin tertiary structure was named after its conserved β-barrel fold from 'Cupa', the Latin term for a small barrel. The characteristic Cupin domain comprises two conserved motifs, each corresponding to two β-strands, separated by a less conserved region composed of another two β-strands with an intervening variable loop (Dunwell, J. M. et al. 2001). A conserved Motif 1 with G(X) 5HXH(X) 3, 4E(X) 6G and a conserved Motif 2 with G(X) 5PXG (X) 2H (X) have been proposed. The 2 His residues and the Glu residue in Motif 1, together with the His residue in Motif 2, act as ligands for the binding of the active site metal manganese ion in the archetypal Cupin, germin (Woo, E. J. et al. 2000). Yet His residues may not always be present in the motifs (Dunwell, J. M. et al. 2001). Proteins of the Cupin family comprise both enzymatic and non-enzymatic members with one, two or many more Cupin domains. Within the conserved tertiary structure, the variety of biochemical functions is provided by minor variation of the residues in the active sites and the identity of the bound metal ion. Cupin functions vary from isomerase and epimerase activities involved in the modification of cell wall carbohydrates in bacteria, to non-enzymatic storage proteins in plant seeds, but are present in transcription factors as well. It has been estimated that there are a minimum of 18 different functional Cupin subclasses, but likely there are many more. Proteins containing a single Cupin domain and of similar size as TRR14, about 150 aa, include diverse functions: phosphomanose isomerase, polyketide synthase, dioxygenase, oxalate oxidase (germins), auxin binding protein and the somewhat larger 185 aa epimerase (Dunwell J. M. et al. 2001). Mutants over-expressing *TRR14* grow on 100 mM trehalose but no longer accumulate starch in their cotyledons, as WT does. Importantly, *TRR14* over-expressors accumulate T6P, suggesting a function of TRR14 in mediating T6P control over growth and starch deposition.

Two novel proteins suppressing T6P mediated growth arrest, GR-RBP2 and TRR14, are studied in this chapter. Phylogenetic analyses are carried out to identify close homologues, study the conservation of the characteristic active domains and reveal the origin of these proteins. Knock out mutants are identified and characterized in an attempt to reveal functions. Transcriptional fusion constructs are made to visualize expression patterns of *GR-RBP2* and *TRR14*. Translational fusions confirm the sub-cellular location predicted for TRR14. Tagged versions of

GR-RBP2 and *TRR14* are expressed to identify potential protein complexes with these proteins.

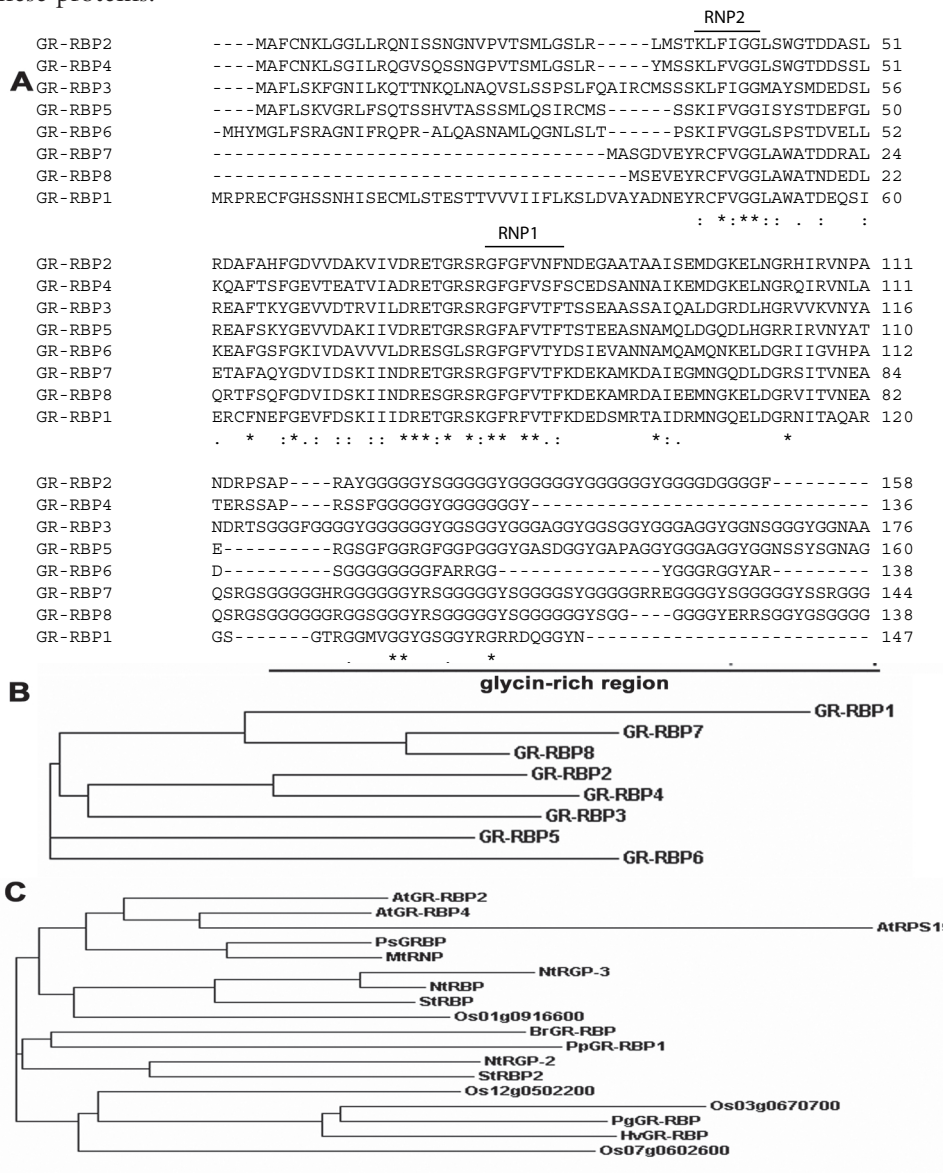

Figure 1. Arabidopsis GR-RBPs paralogs and ortologs. A) Aligned amino acid residues of GR-RBP protein in Arabidopsis. Sequences were from TAIR; the alignment was made using the Clustal W on the EBI server (http://www.ebi.ac.ul/clustalW/alignment_frame.html). The lines indicate conserved motifs in the RRM (RNP1, RNP2) and the glycin-rich domain. **B)** Phylogram derived from alignement of the eight Arabidopsis GR-RBP2's. **C)** Phylogram derived from GR-RBP2's closest homologues in NCBI. AtGR-RBP2 and AtGR-RBP4, *Arabidopsis thaliana* GR-RBP protein 2 and 4; AtRPS19, *Arabidopsis thaliana* Ribosomal protein S19; PsGR-RBP, *Pisum sativum* GR-RBP protein; MtRNP, *Medicago truncatula* RNA binding protein; StRBP, *Solanum tubersum* RNA binding protein; BrGR-RBP, *Brassica rapa* GR-RBP protein; Os01g0916660, Os07g0602600 and Os12g0502200, proteins from loci in *Oriza sativa;* PpGR-RBP, *Physcomitrella patens* GR-RBP protein; PgGR-RBP, *Picea glauca* GR-RBP protein; HvGR-RBP, *Hordeum vulgare* GR-RBP.

Results

Phylogenetic analyses of GR-RBP2 and TRR14

To identify proteins with homology to TRR14 and GR-RBP2, protein sequences were submitted to BLAST in NCBI and TAIR. BLAST analysis using the protein sequence of GR-RBP2 in TAIR revealed that the protein is identical to the cDNA product GRP2, named earlier in the "plant gene register" section of Plant Physiology by Macknight et al. 1998. AtGRP-2 has, however, also been used for the RNA binding protein encoded at the locus At4g38680 (de Oliveira, D. E. et al. 1990, Fusaro, A. F. et al. 2006). To avoid confusion we use here GR-RBP2 (At4g13850), as named by Lordovic and Bartel (2002). Alignmemt of the 8 GR-RBP homologues in Arabidopsis reveals that the GR-RBPs are conserved in their RRM domains and more divergent in the Gly rich domain (Fig. 1A). GR-RBP2, GR-RBP3, GR-RBP4, GR-RBP5 and GR-RBP6 have mitochondrial targeting sequences. The closest homologue to GR-RBP2 is GR-RBP4 (Fig. 1B). Alignment of GR-RBP2 with proteins from all organisms reveals that this protein is conserved in both monocotyledonous and dicotyledonous plants. Homologues to GR-RBP2 are further found in other eukaryotes, as well as bacteria (Fig. 1C and data not shown). One close homologue to GR-RBP2 is the ribosomal protein RPS19 (RPS19) from Arabidopsis. Homology is high in the single RRM sequence but S19 lacks the low complexity Gly rich region.

TRR14 has six homologues in Arabidopsis (Fig. 2A) and further homologues in rice and Medicago (Fig. 2C). The closest rice homologue has a higher BLAST score than the closest Arabidopsis homologue suggesting that this particular protein is conserved in monocots (Fig. 2C). Alignment with the closest Arabidopsis homologue, encoded by At3g04300, shows that the 2 proteins have 58% conserved amino acids (Fig. 2B, and data not shown). TRR14 appears nonetheless to be part of a 7-gene family in Arabidopsis (Fig. 2B). Members of this gene family have unknown subcellular locations or appear located in chloroplasts (At2g32650, At2g32180 and TRR14) and in the endomembrane system (At4g10280). TRR14's closest homologues beyond plants are found in bacteria, particularly in many cyanobacteria including *Synechococcus spp*. and *Prochlorococcus marinus* (Fig. 2C). The data thus suggests that TRR14 is of endosymbiont origin but is no longer encoded in the chloroplast genome in extant plants. The phylogeny data is consistent with the existence of a chloroplast targeting sequence at the N-terminus of the protein. TRR14 further has 61% identity with a protein in *Methylococcus capsulatus* and 57% identity with a protein in *Pelobacter propionicus* suggesting that the function of this protein is also important in these proteobacteria.

The function of even the most distant bacterial homologue, with 50% homology to TRR14, is unknown. Similarity to *AraC*, encoding a non-enzymatic transcription factor with only 20% identity to TRR14, is too low to suggest a

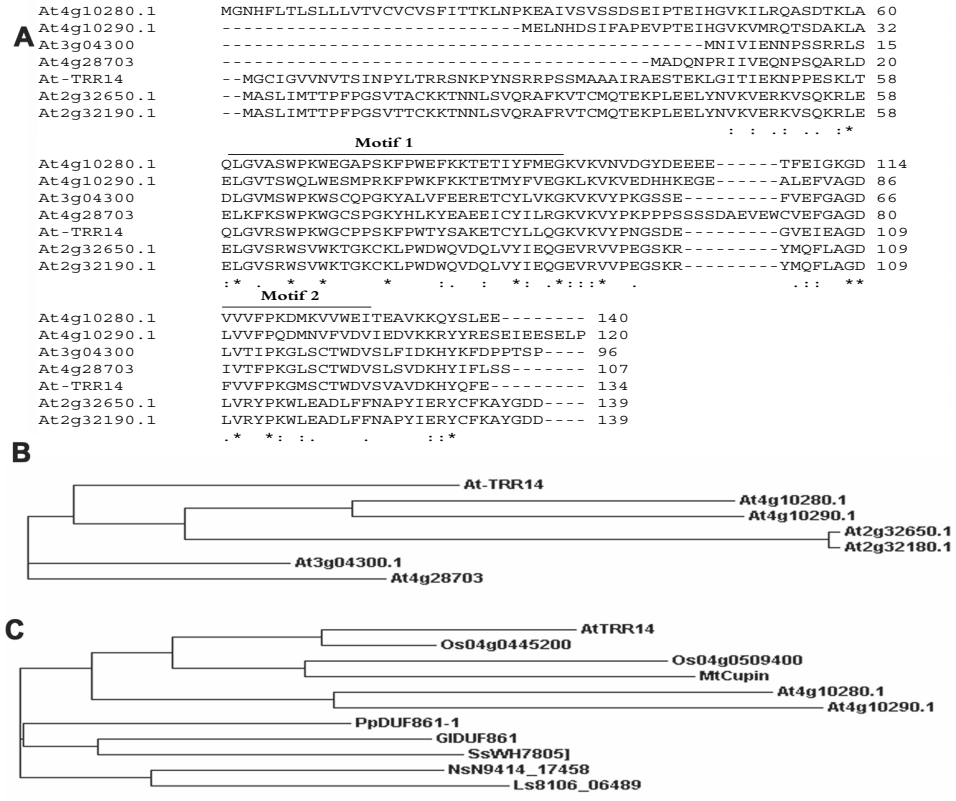

Figure 2. Arabidopsis TRR14 paralogs and ortologs. A) Aligned amino acid residues of TRR14-like proteins in Arabidopsis. Methods are as in Figure 1. Paralogs are described by their AGI numbers. **B)** Phylogram of Arabidopsis TRR14 paralogs. **C)** Phylogram TRR14 homologues. AtTRR14, *Arabidopsis thaliana* TRR14; Os04g0445200 and Os04g0509400, proteins from loci in *Oriza sativa;* MtCupin, Cupin from *Medicago truncate;* PpDUF861.1, *Pelobacter propinonicus* of the DUF861 conserved domain family*;* GlDUF861, protein from the conserved domain DUF861 from *Geobacter lolovely,* SsWH78051, protein from *Synechoccus spp;* NsN9414_17458, protein from *Nodularia spumigina;* Ls, protein from *Lyngbya sp.*

function for TRR14. TRR14 lacks the DNA binding domain of *AraC* and is possibly encoding an enzymatic function similar to the other 150 aa long single domain cupins.

Isolation and characterization of knockout mutants of GR-RBP2 and TRR14

To gain insight into the physiological functions of *GR-RBP2* and *TRR14*, knockout mutations in these genes were sought. Seed from putative insertion mutants were ordered from NASC (Nottingham, UK) and plants grown from the lines SALK-064435 and SALK-064444 for *GR-RBP2*, SALK-059714 and SALK-13850 for *TRR14*. Mutants homozygous with respect to the T-DNA insertion were isolated from segregating T3 plants. *TRR14* or *GR-RBP2* expression in plants from

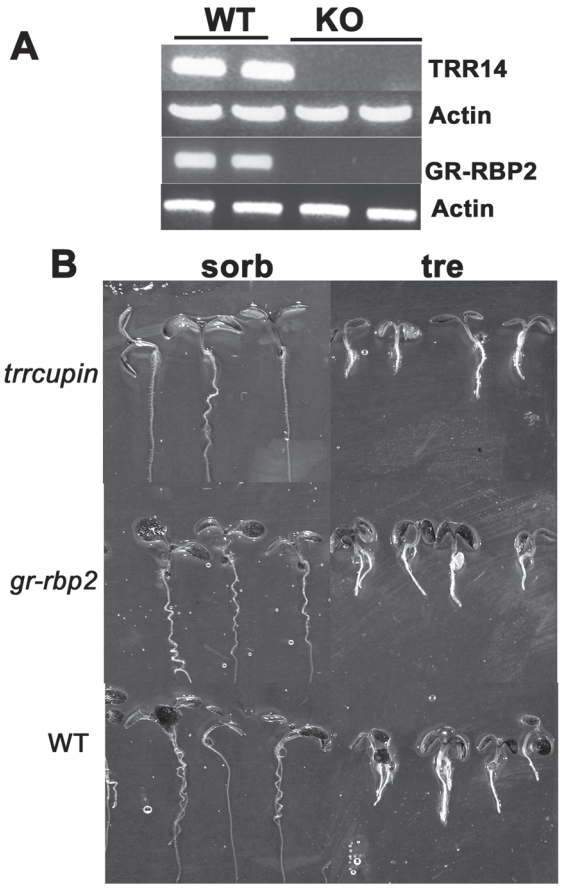

Figure 3. Knock out mutants of *TRR14* and *GR-RBP2*. A) Expression of *TRR14* and *GR-RBP2* in knockout lines of *trr14* (Salk-064435) and *gr-rbp2* (Salk-059714). Detection was by RT-PCR with 42 cycles of PCR as described in Materials and Methods. WT, WT seedlings, mutant, plant lines homozygous for insertions in *TRR14* or *GR-RBP2*. *AtACTIN2* expression levels are shown as control. B) Seedlings from WT and lines with TRR14- (*trrcupin*) or GR-RBP2-knockouts (*gr-rbp2*) after 14 d on 100 mM of either trehalose (tre) or sorbitol (sorb) under long day conditions at 22 °C.

SALK-064435 and SALK-059714, respectively, was absent (Fig, 3A). The knock out phenotype in plants from SALK-064435 is due to a T-DNA insertion in the single intron of *TRR14*. The knockout phenotype in plants from SALK-059714 is caused by T-DNA insertion in the second exon of *gr-rbp2*. The knockout plants do not have a visible seedling or mature plant growth phenotype under long day growth conditions (not shown). Seedlings from the knockout lines did also not differ from wild type when grown on 100 mM trehalose (Fig. 3B). Response of these seedlings to fructose, glucose or sucrose is similar to WT (data not shown). Taken together, knockout mutants in both *TRR14* and *GR-RBP2* were identified but failed to reveal functions of TRR14 and GR-RBP2 with respect to trehalose sensitivity or response to sugars. Functional redundancy with other members of

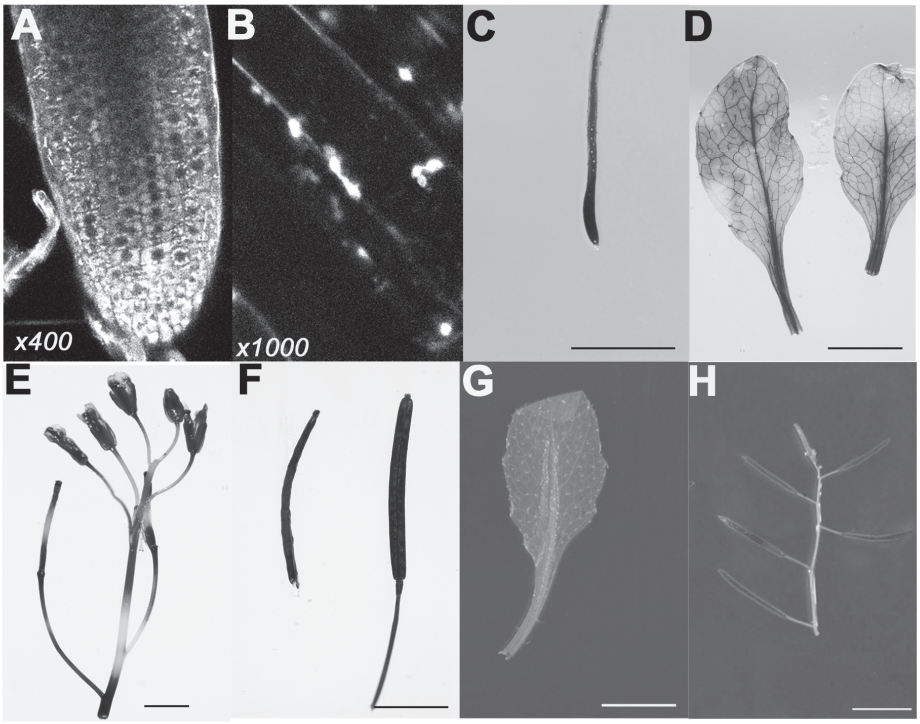

Figure 4. *GR-RBP2* gene expression and protein-localization. A) Typical fluorescence of GFP after translational fusion to the *GR-RBP2*-promoter, 5'UTR and the first exon, in root tip and **B)** in cells of the elongation zone. Typical GUS staining in plants containing the translational fusion in **C)** root tip, **D)** leaves, **E)** inflorescence, **F)** siliques, **G and H)** in a leaf and siliques of plants transformed with pMDC160 empty vector control. The typical staining pattern shown was found in all three independent transgenic lines tested from the nine lines raised. (Bar=0.5 cm)

the multi-gene families of both *TRR14* and *GR-RBP2* may be one reason for the absence of altered phenotypes.

GR-RBP2 is expressed at all stages of development

For analysis of *GR-RBP2* expression, plant lines were raised that express a transcriptional fusion of GUS using a 2kb promoter and 5'UTR of *GR-RBP2*. In addition, plant lines were raised that express translational fusions of the first exon of *GR-RBP2* to GUS or to GFP using the 2kb promotor and 5'UTR of *GR-RBP2*. As control, plant lines were raised with the T-DNAs from the empty vectors pMDC162, pMDC107 that do not contain the *GR-RBP2* sequences. In plant lines with the T-DNA of empty vector controls none of the reporter proteins were detected. Constructs with translational and transcriptional fusions lead to the same expression patterns (data not shown), and typical GUS-staining and GFP fluorescence obtained are presented in Figure 4. Plants expressing the empty vector

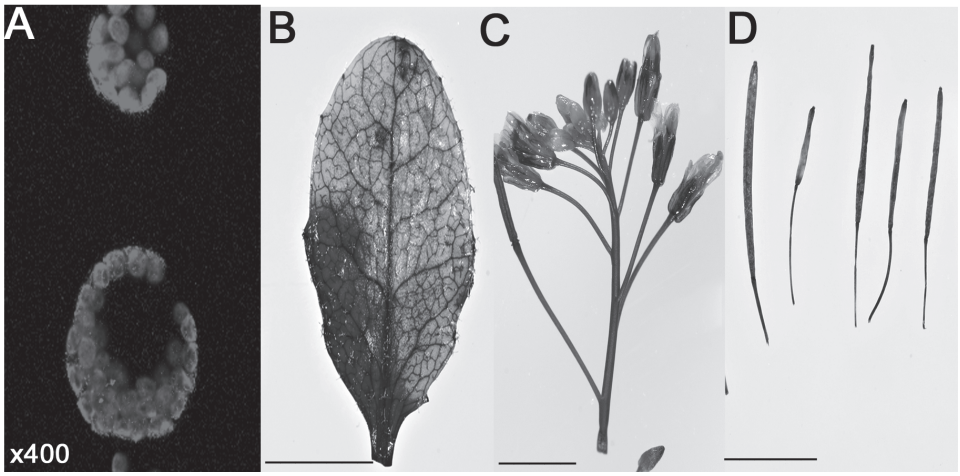

Figure 5. *TRR14* gene expression and protein-localization. Typical fluorescence of GFP after translational fusion to the promoter, 5'UTR and the first exon of *TRR14*, in protoplasts of stably transformed lines, GFP fluorescence (green) is superimposed to chlorophyll fluorescence (red) yielding yellow fluorescence. Typical GUS staining in plants containing the translational fusion of *TRR14* with GUS in *B)* leaf, *C)* inflorescence, *D)* siliques. (Bar=0.5 cm)

control did not stain (Fig. 4G,H) whilst GUS expression specific to the *GR-RBP2* promoter was detected in adult leaves (Fig. 4D), flowers (Fig. 4E) and siliques (Fig. 4F). Expression was also found in roots (Fig. 4C) and was particularly high in root tips as shown by GFP fluorescence (Fig. 4A, B). Data obtained are consistent with the data from gene expression databases such as Genevestigator (Zimmermann, P. et al. 2004), suggesting that the 2kb promoter sequence contains the regulatory sequences driving *GR-RBP2* expression *in planta*. The fusion protein with GFP was absent from nuclei but was present in small speckles in the cytosol, consistent with the mitochondrial targeting of this protein (Fig. 4A, B).

At the seedling stage, GUS-staining in lines carrying the fusion is found ubiquitously and is particularly high in the root tip. Glucose, fructose, sucrose or trehalose does not affect the expression pattern of the GUS-fusions in seedlings and therefore, the *GR-RBP2* promoter appears not to be responsive to these sugars (data not shown). Taken together, *GR-RBP2* appears expressed ubiquitously in the plants and at all stages in development; its highest expression is found in root tips. *GR-RBP2* expression seems insensitive to trehalose or metabolisable sugars.

TRR14 is located in chloroplasts

The TRR14 amino acid sequence predicts targeting of the protein to the chloroplast. The program iPsort detects a targeting signal in the N-terminal sequence from aa 1 to 37. To verify the sub-cellular localization of this protein, transgenic plants were raised with a translational GFP fusion, of this protein at aa 37, expressed under the control of the *TRR14* endogenous 2kb promoter (ref.

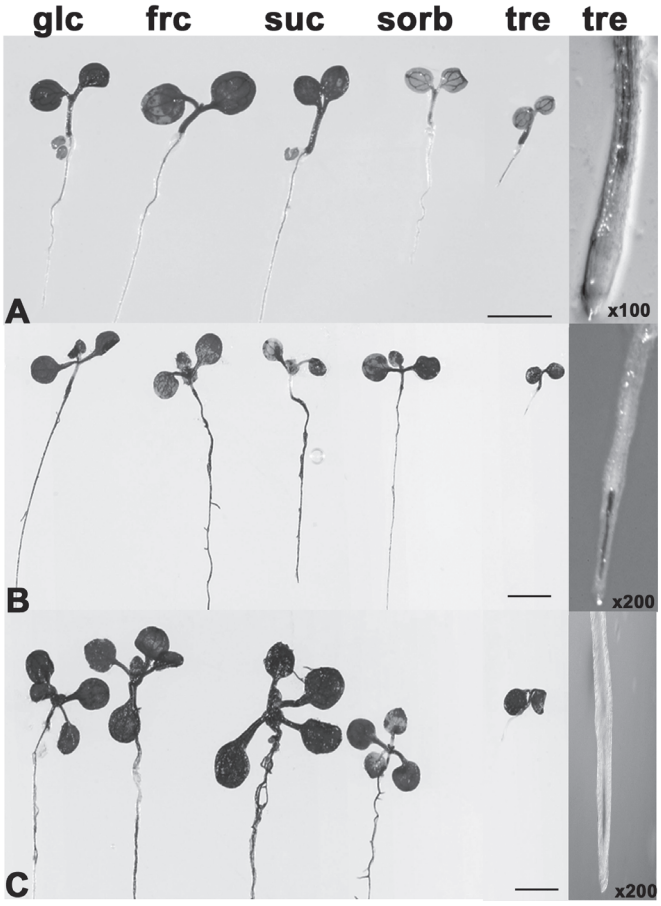

Figure 6. *TRR14* expression on sugars. A) Seedlings containing a transcriptional fusion of GUS to the 2kb *TRR14* promoter and 5'UTR were grown for 5, 10, and 15 days at 22°C, then GUS stained. Growth was on 100 mM of either trehalose (tre), sorbitol (sorb), glucose (glc), fructose (frc), or sucrose (suc). Trehalose inhibits expression of *TRR14:GUS* in roots increasingly over a 15 day growth period. Three independent transgenic lines were tested from the nine lines raised. (Bar=0.5 cm)

Material and Methods). Fifteen independent transgenic lines were obtained with GFP fluorescence. Protoplasts were isolated from leaves of 3 lines and GFP as well as chlorophyll fluorescence visualized by confocal microscopy. Over-laying fluorescence patterns from GFP (green) and chlorophyll (red) reveals that the two molecules are typically found in the same compartment, the chloroplast (Fig. 5A).

TRR14 expression in roots is sensitive to trehalose

For analysis of *TRR14* expression, plant lines were raised that express a transcriptional GUS fusion using a 2kb promoter and 5'UTR of *TRR14*. In addition, plant lines were raised with translational fusions of the first exon of *TRR14* to either GUS or GFP using the 2kb promotor, 5'UTR of *TRR14*. Control

plant lines were raised that contained T-DNA from the empty vectors as described earlier. Analysis of 4 independent transgenics for each construct revealed that GUS expression was typically found at varying intensities in leaves, flowers, stems and siliques (Fig. 5B, C, D). Staining was also found throughout seedlings grown on 100 mM of glucose, fructose, sucrose or osmotic control (Fig. 6). Expression of *TRR14* however is typically repressed in roots of seedlings grown on 100 mM trehalose. The longer the seedlings grow on trehalose, the more restricted the expression of *TRR14* (Fig. 6; tre- root tip).

GR-RBP2 is likely found in a protein complex in planta

GR-RBP2 is a small 158 aa protein with binding sites for RNA, through its RRM domain, and for proteins through its Gly rich domain. GR-RBP2 along with GR-RBP4 and GR-RBP7 were shown to bind nucleic acids *in vitro,* independently of the nucleic acid sequence (Kwak, K. J. et al. 2005). GR-RBP2 over expression leads to trehalose resistance and complex formation with other factors may underlie the observed functional specificity. To identify complexes containing GR-RBP2 and eventually the factors that interact with GR-RBP2, plants were raised that express a tagged version of *GR-RBP2*. *GR-RBP2* was fused to the Tandem Affinity Tag (TAP) at the C-terminal end (*GR-RBP2:TAP*); the TAP tag has protein A, myc- and his- epitopes (Rubio, V. et al. 2005b). To be able to test if the fusion protein is functional, the CaMV35S promoter was chosen to drive expression of the tagged protein: over-expression of a functional protein leads to trehalose resistance. In addition, control plant lines were also raised with protein N-terminally tagged and T-DNA of the empty vector control. Plant lines were screened for expression of tagged *GR-RBP2* by western blotting using Anti-myc antibody. This antibody typically detected several protein bands in plants expressing *GR-RBP2:TAP* but no protein in plants expressing the N-terminally tagged protein or plants with empty vector control (data not shown). The highest molecular weight band corresponds to a protein of 43 kD which is the expected size for the *GR-RBP2:TAP* fusion before import into the mitochondrion. A major lower molecular weight bands may correspond to the 40 kD processed protein that has been imported in the mitochondrion or to degradation products (data not shown).

Transgenic lines expressing *GR-RBP2:TAP* were also tested for their ability to grow on 100 mM trehalose. Seedling roots of the transgenic lines were longer than WT, but suppression of the growth arrest was not as marked as with *GR-RBP2*. *GR-RBP2:TAP* therefore appears functional but is likely less efficient due to the large size of the TAP tag. Extract of plants expressing *GR-RBP2:TAP* was separated by gel filtration and the fractions obtained analyzed by western blotting using anti-myc antibody (Fig. 7). Absorbtion at 280 nm reveals that the major peak of Rubisco eluted at the size expected from calibration of the system with protein standards (Fig. 7A). Anti-myc antibodies detected the dominant 43 kD

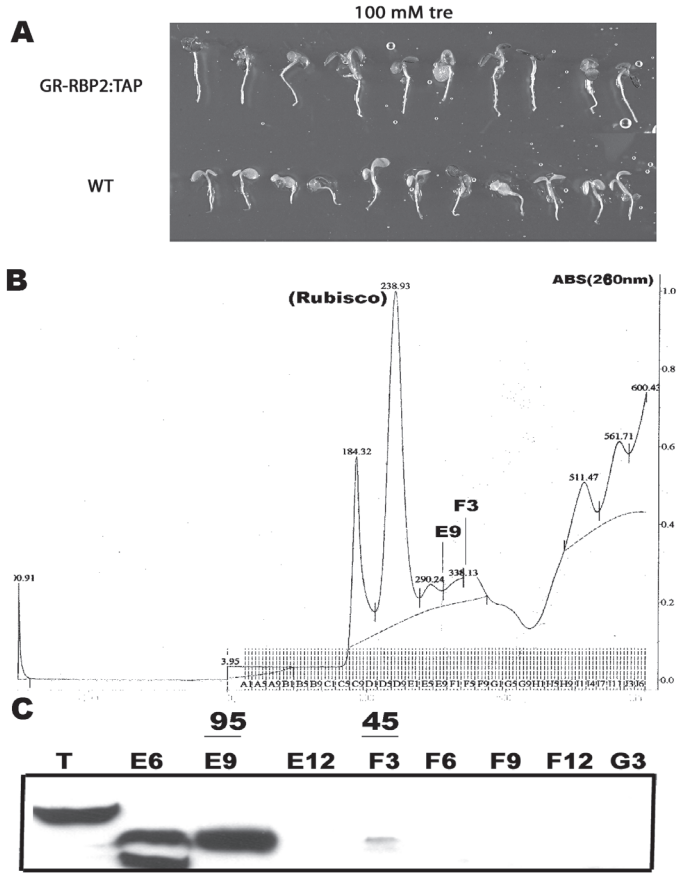

Figure 7. GR-RBP2 complex in planta. A) Over-expressors of *GR-RBP2:TAP* on 100 mM trehalose. Seedlings from a line over-expressing *GR-RBP2:TAP* and WT were grown on half strength MS medium with 100 mM trehalose for 2 weeks in long day conditions. **B)** Sepharose S-300 gel filtration of an extract of plants expressing *GR-RBP2:TAP.* Absorption at 280 nm of proteins after separation on the column: **Rubisco,** absorbance peak corresponding to rubisco protein; **E9, F3** fractions that contain the GR-RBP2 fusion as detected in C. **C)** Detection of GR-RBP2:TAP by western blotting using anti-myc antibody in fractions E6 to G3 obtained during gel filtration. **T,** total crude extract; **95** and **45,** fractions corresponding to molecular weight 95 and 45 KD, respectively.

precursor in crude extracts (Fig. 7B, lane T). Anti-myc further detected two major processed or degradation forms of GR-RBP2:TAP in fractions spanning E6 to E9 that correspond to complexes of approximately 95-100 kD (Fig. 7B, lanes E6 and E9). A very faint signal was observed in fraction F3 that corresponds to proteins of approximately 45 kD, close to the monomeric size of tagged GR-RBP2 (Fig. 7C, lane F3). Gel-filtration experiments with extracts from control plants did not reveal any components cross-reacting with anti-myc antibody (data not shown). Therefore, GR-RBP2:TAP appears to occur in a complex of at least twice its molecular weight. Tandem affinity purification of the complex was then attempted according to Rubio et al. (2005). Preliminary results show that the procedure allowed to enrich a specific set of proteins including protein corresponding to

processed GR-RBP2:TAP (data not shown). Further work is however needed to analyze proteins co-purifying with GR-RBP2:TAP. TAP-tagging of *TRR14* was also carried out because the protein is small and therefore likely does not function in isolation. Expression of C-terminally tagged *TRR14* (*TRR14:TAP*) was detected with bands corresponding to the 43.3 kD unprocessed TRR14:TAP and to the 39kD of the processed fusion protein after import into the chloroplast (data not shown). Expression of *TRR14:TAP* did not, however, yield any plants with resistance to trehalose suggesting that the fusion protein is not functional (data not shown).

Discussion

In this chapter two proteins are characterized that suppress T6P mediated growth arrest, GR-RBP2 and TRR14. GR-RBP2 is a protein of likely prokaryotic origin that is part of a protein complex. *GR-RBP2* expression is found throughout the plant and is not responsive to metabolisable sugars such as glucose, fructose or sucrose. *GR-RBP2* expression also does not respond to 100 mM trehalose. A knockout mutant of *GR-RBP2* was identified in the T-DNA insertion line SALK-059714, yet plants of this line were not altered with regard to growth on the different carbon sources compared to WT. The data obtained did not reveal a function for GR-RBP2 with respect to growth in relation to carbon metabolism.

GR-RBP2 is involved in abiotic stress and its function was recently revealed (Kim, J.Y. et al. 2007). Expression of the protein is induced in the cold and plants over-expressing *GR-RBP2* have improved cold tolerance (Kim, J. Y et al. 2007, Kwak, K. J. et al. 2005, Vermel, M. et al. 2002). The protein complements the cold sensitive *E.coli* BX04 mutant, lacking four cold shock proteins, and has transcription anti-termination activity (Kim, J. Y et al. 2007). This RNA chaperone activity affects the steady state amounts of at least 22 mitochondrial proteins (Kim, J. Y et al. 2007). Over-expressors of *GR-RBP2* increased catalase and peroxidase but decreased superoxide dismutase activities (Kim, J. Y et al. 2007). GR-RBP4 is the closest homologue to GR-RBP2 (Fig. 1). GR-RBP4 is unable to complement *E.coli* BX04 and seems involved in responses to salt stress (Kim, J. Y et al. 2007). The RNA chaperone activity of GR-RBP2, therefore, seems specific. This specificity may arise from complex formation with other factors (Fig. 7). Further characterization of the proteins in the complex with GR-RBP2 will be necessary to understand GR-RBP2 function. Possibly, *GR-RPP2* over-expression leads to growth on trehalose due to its stress protection function. T6P accumulation without addition of metabolisable carbon might generate metabolic stress that can be alleviated by GR-RBP2 mediated adaptation of mitochondrial metabolism. In *GR-RBP2* over-expressors starch accumulation on trehalose is similar to WT. Thus, altered mitochondrial metabolism that allows growth on trehalose does not affect

T6P induced starch accumulation.

Kwak et. al. suggested that GR-RBPs originate from cyanobacteria where they replace the function of cold-shock proteins as found in other bacteria such as in *E.coli*. GR-RBPs are also present in mammalians, e.g. the Cold Induced RNA Binding Proteins (Nishiyama, H. et al. 1997). CIRP of the mouse is involved in growth inhibition under reduced temperature (Nishiyama, H. et al. 1997). Over-expression of CIRP leads to prolongation of the G1 phase of the cell cycle. Immuno-fluorescence studies located CIRP in the nucleus, unlike GR-RBP2 that is located in mitochondria (Fig. 4). Thus, GR-RBP2 likely exerts its effect upon growth by changing mitochondrial metabolism. Taken together, GR-RBP2 links mitochondrial stress adaptation and T6P metabolism and this link may begin to explain how trehalose metabolism mediates stress protection in plants.

TRR14 affects both starch accumulation and growth phenotypes induced by high T6P. Phylogenetic analyses reveal that TRR14 is a member of a small and divergent gene-family in Arabidopsis with a single Cupin domain. Paralogs as well as orthologs in rice, medicago or bacteria harbor the conserved domain DUF861 of unknown function (Kim, J. Y et al. 2007, Marchler-Bauer, A. and Bryant, S. H. 2004). TRR14 is located in the chloroplast. Expression of *TRR14* is ubiquitous and is not responsive to metabolisable carbon supply. Trehalose supplied at 100 mM represses *TRR14* gene expression specifically in the roots. However, the *TRR14* knockout mutant from SALK-064435 yields seedlings and plants with root growth indistinguishable from WT, suggesting that TRR14 is not required for root growth under normal growth conditions. Repression of *TRR14* by trehalose is thus unlikely the cause for root growth arrest on trehalose. TRR14 is more likely required to repress specific effects of high T6P levels, which occur in the chloroplast and affect growth. TRR14 is an unusual Cupin in that it is targeted to the chloroplasts. Single domain Cupin proteins have been found in the cytosol, extracellular matrix, in vacuoles and associated membranes (Dunwell, J. M. et al. 2001). The Cupin structure was reported to be very stable. Germin as well as germin-like proteins containing this Cupin structure have been associated with stress responses rather than carbon assimilation (Berna, A. and Bernier, F. 1997, Berna, A. and Bernier, F. 1999, Zhang, Z. G. et al. 1995). Extrapolation of the function to other Cupins for which the enzymatic functions are unknown, is difficult.

In conclusion, TRR14, a protein of the Cupin family, allows growth and inhibits starch accumulation in plants growing on trehalose. The protein is targeted to chloroplasts and this is consistent with an effect of this protein on starch accumulation and photorespiration (Chapter 4). The role of TRR14 is likely conserved in rice given the high degree of homology between TRR14 and its ortholog in rice. TRR14 suppresses several effects of T6P accumulation and further work will be required to understand the mechanism linking T6P metabolism in the cytosol and TRR14 effects in the chloroplast.

Materials and methods

Phylogenetic analyses

Protein sequences of TRR14 and GR-RBP2 were obtained from the TAIR database (12-01-2005). The BLAST searches were performed, using the TAIR and NCBI BLAST programs at http:// Arabidosis.org/ and http://ncbi.gov/, respectively. The alignment and the respective phylogenic tree were performed at http://www.ebi.ac.uk/clustalw/, using the ClustalW method, scoring matrix Blosum, gap penalty of 10.

Plant materials

Seeds from transformed plants were sterilized by the gas sterilization method. Seedlings were grown in agarose solidified (0.8%) half strength Murashige and Skoog (MS) medium (macro-, micro-salts and vitamins) supplemented with antibiotics as required for selection, 100 mM gentamycin, 100 mM spectinomycin, 12.5 μg/l hygromycin and 50 mM kanamycin. Plants were grown under long-day conditions at 22°C in soil.

DNA constructs and plant transformation

Transcriptional fusions: To detect the expression pattern of genes encoded at the loci At4g10300 and At4g13850, fragments containing 2 kb promoters and the 5' UTRs of these genes were fused to the GUS sequence in vector pMDC162 and to the GFP sequence in pMDC107 (Curtis, M. D. and Grossniklaus, U. 2003). The genomic sequences were PCR amplified from Col-0 WT DNA using a two step procedure. In step one amplification of template DNA using template specific primers (At4g10300: AAAAAGCAGGCTTCACCGCTAG GAGTGAATCCA and AGAAAGCTGGGTAAACTTAGTTGTTCTCAGCTTAC; At4g13850: AAAAAGCAGGCTTCGGTCTACTTACCTCCAGAT and AGAAAG CTGGGTATCTACAAAGTTCCAAAAACAC-3′). The PCR amplification is carried out as follows: after denaturation for 2 minutes at 95°C, DNA amplification was with 10 cycles (15 sec 94°C, 30 sec 56°C and 2 min 72°C). Then 10 μl of the first PCR product was used in the second PCR step, as a template. In this step we used attB1 and attB2 adapter primers (GGGGACAAGTTTGTACAAAAAAGCAC and GGGGACCACTTTGTACAAGAAAGCTGGGT). The PCR amplification is carried out in two phases: after denaturation for 2 minutes at 95°C, DNA amplification was with 5 cycles (15 sec 94°C, 30 sec 45°C and 2 min 72°C). The second amplification was done by 15 sec 94°C, 30 sec 55°C and 2 min 72°C. PCR was completed with a final step at 72°C for 10 min. PCR product was run on agarose gel and the specific band was cut and cleaned using a DNA purification kit (Amersham Biosciences, England).

Translational fusions with GUS and GFP: To study the subcellular location of the proteins encoded at the loci At4g10300 and At4g13850, fragments containing 2 kb promoter, the 5'UTR and the first exon were fused in frame to the GUS sequence in vector pMDC162 and to the GFP sequence in pMDC107 (Curtis, M. D. and Grossniklaus, U. 2003). The genomic sequences were amplified from Col-0 WT DNA using the two-step procedure

as described above and specific primers (At4g10300: AAAAAGCAGGCTTCACCGCTA GGAGTGAATCCA and AGAAAGCTGGGTAGATTACTTGGGCCAACTACG; At4g13850: AAAAAGCAGGCTTCGGTCTACTTACCTCCAGAT and AGAAAGCTGGGTACTCCGATGAAAAGCTTGGTA).

Cloning into plant expression vectors by way of the Gateway^R system: PCR amplified fragments were cloned into pDONR201 and sequenced. Transferring fragments from pDONR201 to pMDC162 (*GUS* reporter gene) and pMDC107 (*GFP* reporter gene) vectors was performed using the recombination reaction as described by the manufacturer (Invitrogen, Carlsbad, CA). Transcriptional and translational fusion constructs were transformed into WT Col-0 plants by the floral dip method (Clough, S. J. and Bent, A. F. 1998). Transformed seedlings were selected on ½ MS supplemented with 12.5 μg/l hygromycin then further grown on soil in long day conditions (16h light, 8h dark) at 22^0C to obtain seed.

Translational fusions with the Tandem Affinity Purification (TAPa)-tag. To generate both N- and C-terminal fusions with the tag, the cDNA's encoding full-length versions of TRR14 and GR-RBP2 were amplified by PCR and cloned into pDONR201 (Rubio, V. et al. 2005a). The transfer of genes from pDONR201 to TAPa vector was performed using the recombination reaction as described by the manufacturer (Gateway; Invitrogen, Carlsbad, CA). Constructs were transferred to wild-type plants (Col-0) by the floral dip method (Clough, S. J. and Bent, A. F. 1998). Transgenic seedlings were selected on solidified ½ MS medium supplemented with 100 mg/l gentamycin, then further grown on soil to obtain seed.

Western blot detection of TAP-tagged proteins in transgenic lines

Protein fractions separated by SDS-PAGE (12% polyacrylamide) were blotted to polyvinylidene membrane (Millipore, bedford, USA) in running buffer (3g Tris, 14.2 g glycine, 200 ml EtOH, 8 ml SDS 10% per litter) for 1.5 hour at 100V, on ice. The membranes were then blocked in phosphate buffered saline (PBS) supplemented with 5% w/v Milk (PBSM), incubated overnight with a 3000 fold dilution of Anti-myc primary antibody from mouse (Santa Cruz, biotechnology) in PBSM. Unbound antibody was washed 4 times with PBS supplemented with 0.1% v/v Tween 20 (Merk, Hohenbrunn, Germany). The blots were subsequently incubated 1.5 hour with a 3000 fold dilution of anti-mouse IgG secondary antibody conjugated to horseradish peroxidase in PBSM. Unbound antibody was washed 4 times in PBS with 0.1% v/v Tween 20, before a one minute reaction with enhanced chemi-luminescence reagent (Sigma, USA) at room temprature and exposure of the blots to X-ray film (Kodak, Sigma- Aldrich, Germany).

Gel filtration on Sepharose S-300

Protein was extracted from frozen ground leaf material (0.5g) in 500 μl ice-cold buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT, 1 mM PMSF, and 1x complete protease inhibitor cocktail (Roche,

Indianapolis, IN, USA). The resulting crude extract was filtered through four layers of cheesecloth, then centrifuged at 12000 g for 10 min at 4°C. The supernatant fraction (300 μg) was then loaded onto a Superdex S-300 column (Amersham) equilibrated in PBS and flowing at 0.1ml/min. Sixty fractions of 1 ml were collected. Proteins in each 3rd 1ml fraction were concentrated using 10 ul Strataresin (Stratagene). Resin bound proteins (5 ul) was fractionated by SDS-PAGE using 12% w/v polyacrylamide gels, that were either stained with Coomassie blue (R250, Sigma) or blotted for detection of the TAP-tag as described in the previous section.

Tandem affinity purification

TAP-tagged proteins were purified essentially as described by Rubio et al. 2005. Fifteen gram leaf fresh weight was harvested, snap frozen then ground in liquid nitrogen with mortar and pestle. Protein was then extracted in buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40) supplemented with 1 mM PMSF, and complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The crude extract was filtered through four layers of cheesecloth and centrifuged at 12 000 g for 10 min at 4°C. The protein concentration in the supernatant was determined by Bradford assay (Bio-Rad). Extracts containing similar amounts of total protein were incubated with 500 μl IgG beads (Amersham Biosciences) for 2 h at 4°C with gentle rotation. After centrifugation at 150 g for 3 min at 4°C, the IgG beads were recovered and washed three times with 10 ml of washing buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40) and once with 10 ml of cleavage buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT). Elution from the IgG beads was performed by 3h incubation with 50 μl (100 units) of 3C protease (Precission protease; Amersham Biosciences) in 2 ml of cleavage buffer at 4°C with gentle rotation. Released protein was recovered in the supernatant after centrifugation at 150 g for 3 min at 4°C and, after addition of 1 ml cleavage buffer to the beads and re-centrifugation, in the second supernatant; the supernatants were pooled and stored at 4°C. Pooled released protein was then loaded onto 0.5 ml of Co-NTA resin (Qiagen, Valencia, CA, USA) packed into a 10-ml disposable polyprep chromatography column (Bio-Rad). To increase likelyhood of binding, the flow-through was loaded again onto the Co-NTA column before washing with 5x 1 ml washing buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40). Elution from the Co-NTA was performed using 5x 0.5 ml of imidazole containing buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 0.3 M imidazole). Protein in the fractions was bound to 10 ul strataresin (Stratagene), then separated by SDS page for subsequent staining with Coomassie Blue and for blotting and detection of the TAP-tagged proteins.

Glucuronidase staining

Seedlings and different parts of transgenic GUS plants were incubated in a solution containing 1 mM X-Gluc buffer, 0.5 mM potassium ferrocyanide, 0.5 mM potassium

ferricyanide, 0.1% Triton X-100, 10 mM EDTA, and 100 mM of NaH_2PO_4 and Na_2HPO_4 pH 7 at 37°C for 10 h. To visualize the blue precipitate, chlorophyll was extracted in 70% ethanol after staining.

Protoplast isolation

Arabidopsis protoplasts were isolated essentially according to Sheen et al (2002). Leaves from plants that were 3 to 5 weeks old were collected, washed with de-ionized water, dried with a paper towel, and cut into 0.5-1 mm strips with a razor blade. Leaf sections were transferred to a Petri dish containing 20 to 25 mL of enzyme solution, 1% w/v cellulase R10 and 0.2% w/v macerozyme R10 (SERVA, Heidelberg, Germany), 0.4 M mannitol, 20 mM KCl and 20 mM MES, pH 5.7, and vacuum infiltrated for 5-30 min. Digestion continued for 3 hours in the dark at 22ºC. Released protoplasts were then filtered with a 35-75 μm nylon mesh and centrifuged at 100 g for 1-2 min. The protoplast pellet was gently re-suspended in ice-cold WI solution (0.5 M manitol, 4 mM MES pH 5.7, 20 mM KCl).

Confocal microscopy

To reveal the subcellular localization of translational fusions of TRR14 and GR-RBP2 with GFP, protoplasts were isolated from leaves as described above. Protoplast suspensions were then viewed by fluorescence microscopy using a confocal laser scanning microscope (Zeiss, Germany) equipped with a 100-mW argon laser and with settings allowing fluorescence activation and detection windows for GFP and chlorophyll (GFP: from 497 to 526 nm; Chlorophyll: from 682 to 730 nm).

Isolation and characterization of homozygous T-DNA insertion lines

Seed were obtained from insertion collections made available by the Nottingham Arabidopsis Stock Centre (Nottingham, UK). Homozygous *trr14* and *gr-rbp2* mutant plants were isolated from seeds of segregating T3 lines of the T-DNA insertion lines SALK-059714, SALK-13850 (*GR-RBP2*) and SALK-064435, SALK-064444 (*TRR14),* respectively. The insertion in the gene was identified using T-DNA left border primer LBa (TGGTTCACGTAGTGGGCCATCG) and specific primers for SALK-059714 (TTAGAATGCACCTAATGGGG and CTACTTGTTTTGGATCAGCCC), SALK-13850 (TTAGAATGCACCTAATGGGGG and CCCATCTCGTTGAATTGATG), SALK-064435 (AAATACCCGAAGATGTGGAGC and CCCCACTTGTTACCAT CATTTG) and SALK-064444 (AGGCATGAATGGATCATATATGC and TAATGCCATGAAACTTGCTTG).

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CHAPTER 6

Summarizing discussion

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Metabolism of the alpha,alpha-1,1-glucose disaccharide, trehalose, is indispensable in plants (Eastmond, P. J. et al. 2002). Trehalose metabolites are however present at only very low concentrations and their role in plants is not understood (Goddijn, O. and Smeekens, S. 1998, Paul, M. et al. 2001, Rolland, F. et al. 2006). Genes coding for trehalose metabolism have been found in all plants. Strikingly, extensive radiation is found in the genes for metabolism of the trehalose biosynthetic precursor, trehalose-6-phosphate (T6P), whilst only one or very few genes encode trehalases that hydrolyse trehalose (Leyman, B. et al. 2001, Pramanik, M. H. and Imai, R. 2005). Minor alterations of T6P steady states in plants yield dramatic and pleiotropic phenotypic changes (Pellny, T. K. et al. 2004, Pramanik, M. H. and Imai, R. 2005, Schluepmann, H. et al. 2003). Additionally, deletion of the T6P synthase (TPS), *AtTPS1*, in Arabidopsis is lethal and can be overcome by complementation with active TPS enzyme (Eastmond, P. J. et al. 2002, Schluepmann, H. et al. 2003). Evidence is thus accumulating that suggests an important regulatory role for T6P and it was shown that T6P determines carbon utilization in Arabidopsis seedlings (Schluepmann, H. et al. 2003). It is not understood however, how T6P controls carbon utilization and why changes in the steady state level of T6P yield such strong phenotypic changes. Work presented in this thesis is an attempt at identifying processes that are controlled by T6P using a genetic approach.

Trehalose supplied to the growth medium of seedlings inhibits growth and allocation of carbon to the root and shoot (Fritzius, T. et al. 2001, Schluepmann, H. et al. 2004, Wingler, A. et al. 2000). This growth inhibition was previously studied by combining 25 mM trehalose with 10 μM Validamycine, a trehalase inhibitor. In this thesis, we use 100 mM trehalose without addition of trehalase inhibitor because growth inhibition turns into growth arrest at 100 mM trehalose. That is caused by T6P accumulation (Schluepmann, H. et al. 2004). In Chapter 2 the effect of 100 mM trehalose on seedlings is further characterized. In the dark, 100 mM trehalose inhibits hypocotyl elongation but not the characteristic skotomorphogenesis induced by metabolisable sugars. Gene-expression profiling of seedlings after 24h on 100 mM trehalose reveals little changes in enzymes of primary metabolism, no induction of carbon catabolite repression but conspicuous changes in genes known to be involved in biotic stress responses. This links trehalose metabolism with a specific stress response. The stress response could result from carbon starvation of the sinks linked to T6P accumulation. Alternatively, plants may sense trehalose, a sugar quantitatively accumulating in most microbes and this may elicit the specific biotic stress expression profile observed in Chapter 2 as well as effective pathogen defense (Reignault, P. et al. 2001, Schluepmann, H. and Smeekens, S. 2002). A genetic dissection of the effects after feeding 100 mM trehalose is necessary to establish causal relations between trehalose or T6P and stress and carbon allocation.

In Chapter 3 mutants were identified that resist the growth inhibition on 100 mM trehalose. Screening delivered 19 trehalose resistant (*trr*) mutants from

the LeClere and Bartel collection. These mutants contain a T-DNA expressing randomly cloned cDNAs (LeClere, S. and Bartel, B. 2001). The *trr* phenotype segregates as a dominant trait in 13 of the 19 lines. Further analysis revealed that over-expression of *PSI-H*, *GR-RBP2* and *TRR14* and repression of *LHCB1* cause trehalose resistance. To explore the mechanisms by which trehalose resistance is obtained T6P, starch, anthocyanin and chlorophyll contents as well as photosynthesis were measured in the *trr* mutants and mutants are presented in Chapter 4. This allowed a clear separation of T6P-mediated starch accumulation and T6P-mediated growth inhibition into two independent pathways. The pathway controlling starch accumulation has partially been elucidated: T6P controls AGPase redox regulation, and it may also control starch remobilization (Kolbe, A. et al. 2005, Ramon, M. et al. 2007). The pathway controlling growth inhibition has not been studied previously except that plants with reduced T6P and pale green leaves were found slower growing and with lower photosynthetic capacity per leaf area than WT (Pellny, T. K. et al. 2004, Schluepmann, H. et al. 2003).

Over-expression of *PSI-H* reduces T6P steady states on trehalose, suggesting that resistance to T6P mediated growth arrest in these lines is due to the lower T6P. This uncovers an interesting feedback mechanism between photosynthesis and the control of T6P steady state that has been previously shown to control photosynthetic capacity per leaf area (Pellny, T. K. et al. 2004). In contrast, *GR-RBP2*, *TRR14* and *LHCB1* do not alter T6P accumulation on trehalose but alter the seedling's response to T6P. These gene products are therefore involved in processes targeted by T6P. Surprisingly the products of these genes are found in organelles, chloroplasts and mitochondria whilst T6P biosynthesis is thought to occur in the cytosol (TAIR annotation of TPS genes, March 2007). A correlative link between T6P, photosynthetic capacity and LHCB1 was previously shown (Pellny, T. K. et al. 2004). In addition, T6P controls AGPase redox-activation (Kolbe, A. et al. 2005). Together, the data from Chapter 4 and previously published results thus imply that T6P controls reactions in chloroplasts. T6P may possibly be imported into chloroplasts. Consistently, various programs predict targeting of the T6P phosphatases AtTPPA and TPPB to the chloroplast, but experimental evidence for this is lacking (TAIR annotation, March 2007; ChloroP). Alternatively, T6P control over chloroplastic reactions may occur indirectly via an effect on redox-systems that transgress organelle boundaries (Baumann, U. and Juttner, J. 2002, Noctor, G. and Foyer, C. H. 2000, Wormuth, D. et al. 2007). The link with reactions in mitochondria through GR-RBP2 is novel. The function of GR-RBP2 as an RNA chaperone (Kim, J.Y et al. 2007) suggests that it may alter transcription of mitochondrial proteins that affect T6P targets in the cytosol or chloroplast. Metabolism of these organelles is very connected by way of the photorespiratory, amino acid biosynthesis and redox pathways (Noctor, G. and Foyer, C. H. 2000). The redox poise in chloroplasts for instance is sensed and mitochondrial uncoupling proteins, such as UCP1, ensure adjustment of

mitochondrial redox activities as a function of redox in chloroplasts (Sweetlove, L. J. et al. 2006). Ascorbic acid supply is shown in Chapter 4 to partially suppress growth inhibition and starch accumulation mediated by T6P. Preliminary measurements of T6P levels in seedlings on ascorbic acid and trehalose suggest that ascorbic acid does not affect responses to T6P accumulation, but T6P steady state (Aghdasi, M., Paul, M. and Schluepmann H., unpublished). A redox-process involving ascorbic acid thus appears to regulate T6P steady state and this redox-process differs from that involved in T6P mediated thioredoxin activation of AGPase in chloroplasts. Unlike *GR-RBP2* and *LHCB1* that suppress only the effect of T6P on growth, *TRR14* suppresses both the growth inhibition and starch accumulation. TRR14 therefore may be involved in a process more directly controlled by T6P. TRR14 moreover links photorespiration with T6P's effects on carbon allocation for growth. The function of *TRR14* is unknown, except for the finding that *TRR14* over-expression leads to reduced photorespiration (Chapter 4). Reduced photorespiration implies reduced H_2O_2 production. Possibly, reactive oxygen species are made in response to T6P accumulation and suppression of photorespiration in *trr14* seedlings may suppress their production. This possibility needs to be explored further since T6P and trehalose have been shown to convey stress-resistance responses that could be mediated by specific reactive oxygen signaling (Bae, H. et al. 2005, Garg, A. K. et al. 2002, Holmstrom, K. O. et al. 1996, Jang, I. C. et al. 2003, Pilon-Smit, E. A. H. et al. 1998). *TRR14* is a novel protein important for the regulation of photorespiration, an area of research of great economic and environmental interest (Eckardt NA 2005). Importantly TRR14 restores carbon allocation for growth and therefore may represent a component regulating feedback control for photorespiration. Photorespiration imposes yield ceilings on most of the highly bred crops planted today. It is particularly limiting yields of crops grown under heat or drought stresses when O_2 accumulates in mesophyll cells due to stomata closure (Ainsworth, E. A. and Rogers, A. 2007, Long, S. P. et al. 2006). With global climate change and increasing agricultural production on marginal lands, the link between T6P and photorespiration is of interest for future studies.

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Samenvatting in het Nederlands

Trehalose is een niet-reducerend disaccharide bestaande uit twee alfa, alfa-1,1-gebonden glucose eenheden. In planten is gevonden dat een intermediair in de trehalose biosynthese, trehalose-6-fosfaat (T6P) een essentiële functie heeft in de groei en ontwikkeling. T6P deficiëntie leidt ondermeer tot embryo letaliteit. T6P stuurt ondermeer de verwerking van koolhydraten aan en reguleert mede de fotosynthetische capaciteit in bladeren. Het werkingsmechanisme van T6P in echter niet bekend. Dit onderzoek beschrijft genetische en fysiologische experimenten om het werkingsmechanisme van T6P te achterhalen in de modelplant *Arabidopsis thaliana*.

Trehalose toevoeging aan het groeimedium van Arabidopsis zaailingen resulteert in T6P ophoping. Deze T6P ophoping remt de groei en allocatie van koolhydraten naar de wortel en scheut apices. Mutanten zijn vervolgens geïdentificeerd die in meer of mindere mate ongevoelig zijn voor trehalose in het groeimedium. In totaal werden 19 *trehalose resistant* (trr) mutanten geïdentificeerd die resistant zijn voor 100 mM trehalose. Voor deze procedure is de LeClere en Bartel mutantcollectie gebruikt. Deze mutantlijnen bevatten T-DNA integraties welke willekeurig gekloneerde cDNAs tot expressie brengen door middel van de constitutieve 35S promoter. De *trr* eigenschap splitst uit als een dominante eigenschap in 13 van de 19 mutanten. Over-expressie van *PSI-H, GR-RBP2*, *TRR14* en repressie van *LHCB1* veroorzaken trehalose resistentie. Mutant analyse bevestigt dat T6P controle over zetmeelbiosynthese en groei van de zaailing afzonderlijke routes zijn. GR-RBP2 en LHCB1 onderdrukken alleen het groeiremmings effect van T6P. TRR14 overexpressie onderdrukt zowel de T6P gemedieerde ophoping van zetmeel in de cotylen als ook de groeireming. Hieruit volgt een model van het netwerk rond T6P. TRR14 beïnvloedt T6P targets die gemeenschappelijk zijn voor zetmeel en controle van groei. GR-RBP2 en LHCB1 beïnvloeden uitsluitend T6P targets in de groeicontrole route.

LHCB1 is één van de eiwitten van het lichtinvang-complex in chloroplasten en is in hoge concentraties aanwezig. Verminderde LHCB1 expressie onderdrukt het T6P gemedieerde groei effect en deze observatie sluit aan bij de hierboven beschreven verbinding tussen T6P, fotosynthese capaciteit en groei. GR-RBP2 is een mitochondriaal mRNA chaperonine waarvan zeer recent is beschreven dat het betrokken is bij kouderesistentie in Arabidopsis, waarschijnlijk door het aansturen van veranderingen in het mitochondriale metabolisme. Het onderdrukken van het T6P groei effect door GR-RBP2 overexpressie duidt op een specifieke T6P geïnduceerde stress die leidt tot groeiremming. TRR14 bevindt zich in de chloroplast en overexpressie van TRR14 laat een verminderde fotorespiratie zien.

Mogelijk is een gevolg van verhoogde T6P concentraties het ontstaan van reactieve zuurstof verbindingen (ROS, bijvoorbeeld H_2O_2) en zijn de geïdentificeerde

genen betrokken bij het reduceren van het ROS niveau, waardoor de plant weer kan groeien. Deze hypothese is interessant en zal verder worden bestudeerd omdat T6P en trehalose al eerder in verband zijn gebracht met verhoogde stress resistenties, ook van stress die samengaat met ROS productie. Vooral interessant is de observatie dat TRR14 koolhydraat allocatie en groei herstelt mogelijk in relatie met de controle van fotorespiratie.

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Finally, the last page of my thesis. The only part that everybody interested in reading it.

During these four years not only I learned many things in Molecular plant physiology, but also I learned many things about life and people and now I have new attitudes about life and science.

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Abbreviations

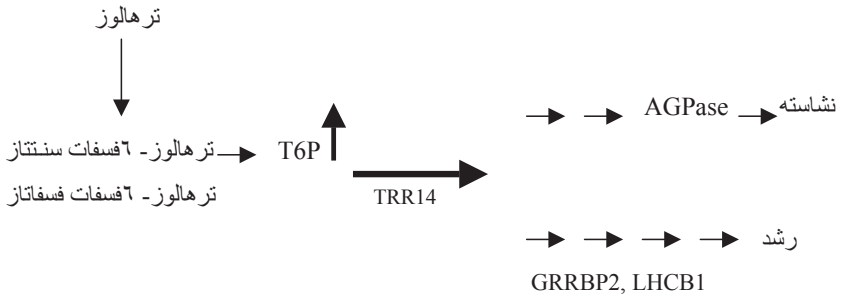
AA	Ascorbic acid
ABA	Abscisic acid
Chl	Chlorophyll
DHA	Dehydroascorbic acid
frc	Fructose
FW	Fresh weight
GFP	Green fluorescent protein
glc	Glucose
GOGAT	Glutamate:glyoxalate aminotransferase
GR-RBP	Glycin Rich RNA- Binding Protein
GUS	β-glucuronidase
HXK	Hexokinase
KO	Knock out
LHCB1	Light- harvesting complex protein of photosystem II
mal	Maltose
MTHFR	Methylentetrahydrofolate reductase
Pal	Palatinose
PPT	Phosphinotrice
PSI-H	Photosystem I subunit H
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
sorb	Sorbitol
SPE	Solid phase extraction
suc	Sucrose
T6P	Trehalose-6-phosphate
TPH	Trehalose-6-phosphate hydrolase
TPP	Trehalose phosphate phosphatase
TPS	Trehalose-6-phosphate synthetase
TreF	Trehalase expressing lines
tre	Trehalose
trr	trehalose resistant mutant

Curriculum Vitae

Mahnaz Aghdasi was born on February the 25th, 1968 in Amol, Iran. She finished her high school with a major in Natural science in 1985. In the same year, she entered the Alzahra Universtiy in Tehran to study General biology and obtained a BSc. in 1989. In the period of 1989 to1993, she continued her study at the Tarbiat Modarres University in Tehran and obtained her MSc in Plant physiology. In the same year she employed as an academic staff member of Gorgan University of Agricultural Science and Natural Resources. Five years later, she accepted in Isfahan University and obtained her M.phil. in plant physiology in 1998. In 2003, she was awarded a scholarship from the Ministry of Science, Research and technology of Iran to carry out a Ph.D in Plant Molecular Phisiology. During these four years, she has been investigating the role of trehalose-6-phosphate in carbon allocation and growth in plant at the department of Plant Molecular Physiology, Utrecht University, the Netherlands. After the defense of her thesis, she will return to Iran and continue her career in teaching and research at the department of plant biology, Gorgan University of Agricultural Science and Natural Resource.

در تنش هایی نظیر شوری و خشکی به اثبات رسیده است. نتایج حاصل از آزمایشات بتا – گلوکرونیداز نشان داد که ژن *GR-RBP2* در تمام گیاه بیان شده و میزان بیان آن در نوک ریشه بیشتر از سایر نقاط است. ضمناً بیان ژن فوق الذکر نسبت به قند های مختلف گلوکز، فروکتوز، ساکاروز، ترهالوز و سوربیتول تفاوتی از خود نشان نمیدهد. با استفاده از روش TAP-TAG ، پروتئین GR-RBP2 خالص سازی شده و با روش ژل فیلتراسیون نشان داده شد که GR-RBP2 به صورت کمپلکس در گیاه عمل میکند. با توجه به آن که هیچ یک از پروتئین های متابولیستم T6P پپتید گذر مربوط به میتوکندری را در خود ندارند، احتمالاً GR-RBP2 هدف غیر مستقیم T6P بوده و این پروتئین بر روی مراحل فرودست T6P اثر می گذارد.

TRR14 پروتئینی ناشناخته است. نتایج تحقیقات حاضر نشان داد که TRR14 در کلروپلاست جای دارد و ترهالوز سبب سرکوب بیان این ژن در ریشه می شود. ضمناً TRR14 تنفس نوری را در گیاه آرابیدوپسیس کاهش می دهد. با توجّه به آن که تنفس نوری در گیاهان C3 سبب کاهش راندمان فتوسنتز و کاهش عملکرد گیاه می شود، مطالعات بیشتر بر روی TRR14 می تواند راه کارهای جدیدی جهت افزایش راندمان فتوسنتز در گیاهان ارائه نماید.



شکل۱: افزودن ترهالوز به محیط کشت دانه رست های آرابیدوپسیس سبب انباشته شدن T6P می شود و به دنبال آن T6P از طریق مسیر های گوناگون بر روی رشد و انباشته شدن نشاسته اثر می گذارد. *Trr14* علی رغم داشتن مقادیر بالای T6P مقدار کمی از نشاسته در خود دارد و بر روی محیط کشت حاوی ترهالوز به خوبی رشد می کند. احتمالاً این پروتئین بر روی یک مرحله مشترک در کنترل رشد و نشاسته اثر می گذارد. دو لاین حاوی *GR-RBP2* و *LHCB1* با وجودی که مقادیر بالایی از نشاسته در خود دارند ولی به خوبی بر روی محیط کشت حاوی ترهالوز رشد می کنند. به نظر می رسد این دو پروتئین فقط بر روی مسیر کنترل رشد اثر می گذارند.

چکیده:

ترهالوز دی ساکاریدی است که از دو مولکول گلوکز با پیوند آلفا- آلفا ۱، ۱ ساخته شده است. اخیراً محققان دریافته اند که ترهالوز- ٦- فسفات، پیش ماده بیوسنتز ترهالوز، نقش مهمّی در رشد، نمو، فتوسنتز و مصرف کربن در گیاهان دارد؛ امّا مکانیسم عمل آن هنوز ناشناخته است. در تحقیق حاضر سعی بر آن شده است تا اثرات پلئوتروپیک ترهالوز- ٦- فسفات در گیاه مدل آرابیدوپسیس بررسی شود.

افزودن قند ترهالوز به محیط کشت سبب تجمع ترهالوز-٦- فسفات (T6P) در دانه رست های گیاه آرابیدوپسیس میشود. انباشته شدن این قند از رشد و تخصیص کربن جلوگیری می کند. ضمناً T6P سبب فعّالیت AGPase و انباشته شدن نشاسته میشود. در تحقیق حاضر با اسکرین کلیسونی ازلکلر و بارتل ، موتانت های مقاوم به ترهالوز (۱۰۰میلی مولار) یافت شده است. موتانت های یافت شده دارای cDNA ای هستند که به طور تصادفی وارد گیاه آرابیدوپسیس شده است. به دلیل مقاومت به ترهالوز (trehalose resistant) موتانت های مزبور *trr* نامگذاری شده اند. با انجام کراس بین لاین های مزبور و نمونه وحشی آرابیدوپسیس (کلمبیا- صفر) دریافتیم که صفت مقاومت به ترهالوز در۱۳ لاین از ۱۹ لاین یافت شده صفت غالب است. لاین های غالب، دارای cDNA های متفاوتی بوده که پروتئین های مختلفی را کد می کنند. از بین لاین های یافت شده سه لاین حاوی ژن هایی است که بر فتوسنتز دخالت دارند: دو لاین حاوی *PSI-H* (فتوسیستم I ساب یونیت H)، یک لاین حاوی *LHCB1* (کمپلکس جمع آوری کننده نور B1) است. یکی از لاین ها حاوی cDNA ای است که یک پروتئین غنی از گلیسین را کد می کند. این پروتئین GR-RBP2 نام دارد. سه لاین؛ یک پروتئین ناشناخته " که ما آن را *TRR14* نامگذاری کردیم" را کد می کنند. لاین های باقی مانده حاوی cDNA ای هستند که *MTHFR*, *GOGAT* , β-α هیدرولاز و پروتئین ناشناخته TRR19, TRR18 را کد می کنند.

تراریختی مجدد ژن های یافت شده و آنالیز بیان ژن نشان داد که بیان *TRR14, PSI-H, GR-RBP2* و سرکوب *LHCB1* سبب مقاومت به ترهالوز می شود. سنجش T6P و نشاسته در لاین های مزبور نشان داده که لاین حاوی *PSI-H* در روی محیط کشت حاوی ۱۰۰ mM ترهالوز، در خود T6P ذخیره نکرده امّا مقادیر قابل توجهی نشاسته در آن ذخیره شده است. ساب یونیت H فتوسیستم I نقش مهمّی در پایداری فتوسیستم I در شرایط تنش دارد.

لاین های حاوی *GR-RBP2* و *LHCB1* ضمن داشتن مقادیر بالای T6P مقادیر بالایی از نشاسته نیز در خود دارند. در مقابل لاین حاوی *TRR14* علی رغم داشتن غلظت بالای T6P مقادیر پایینی از نشاسته در خود دارد. یافته های حاضر نشان می دهد که تجمع نشاسته عامل توقف رشد نبوده و مسیر اثر T6P بر روی فرایند رشد متفاوت از نحوه اثر آن بر روی AGPase و تجمع نشاسته است (شکل۱).

آنالیز بیشتر لاین های مزبور ما را بر آن داشت تا در ادامه جزئیات بیشتری از دو لاین حاوی *GR-RBP2* و *TRR14* را مورد بررسی قرار دهیم . GR-RBP2 پروتئینی است که در میتوکندری جای دارد و اخیراً نقش آن

اساتید راهنما: پروفسور شِف اسمیکنز
دکتر هنریت اِشلوپ من

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پروفسور دکتر پاتریک ون دایک
پروفسور دکتر یوست ون دان

پژوهش های مندرج در این رساله در دپارتمان فیزیولوژی مولکولی گیاهی، دانشگاه اوترخت، کشور هلند و با حمایت مالی وزارت علوم ، تحقیقات و فناوری جمهوری اسلامی ایران انجام شده است.

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رساله

برای دریافت درجه دکتری در فیزیولوژی مولکولی گیاهی از
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