Yenni Lorena LÓPEZ GALVIS

Characterization of a family of Arabidopsis trehalose biosynthesis genes and their role in root development and stress tolerance

June 2011

KATHOLIEKE UNIVERSITEIT
LEUVEN

Arenberg Doctoral School of Science , Engineering & Technology
Faculty of Sciences
Department of Biology

Characterization of a family of Arabidopsis trehalose biosynthesis genes and their role in root development and stress tolerance

Yenni Lorena LÓPEZ GALVIS

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor of Biology

June 2011

KATHOLIEKE UNIVERSITEIT LEUVEN

Faculteit Wetenschappen

Department of Biology
Laboratory of Molecular Cell Biology
Kasteelpark Arenberg 31
3001 Heverlee-Leuven

Characterization of a family of *Arabidopsis* trehalose biosynthesis genes and their role in root development and stress tolerance

Yenni Lorena López Galvis

Supervisors:
Prof. Dr. P. Van Dijck, KULeuven
Prof. Dr. T. Beeckman, UGent

Members of the Examination Committee:
Prof. Dr. J. Thevelein
Prof. Dr. F. Rolland
Prof. Dr. A. Goossens
Dr. J. Lunn
Dr. N. Avonce

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor of Biology

June 2011

ISBN 978-90-8649-435-4
D/ 2011/10.705/53

a mom, dad, mamburú, tianito y gayota
los motores de mi vida.
a maito
mi compañero de viaje.

Table of Contents

Frequently used abbreviations

ABA	abscisic acid
aba	*ABA synthesis*
ABI	abscisic acid insensitive
abi	*ABA insensitive*
AGPase	adenosine diphosphate-glucose pyrophosphorylase
ARF	auxin response factor
AuxRE	auxin responsive element
Aux/IAA	auxin/indole-3-acetic acid
AXR	AUXIN RESISTANT
BD	DNA binding domain
CaMV35S	cauliflower mosaic virus 35S promoter
CDK	cyclin-dependent kinase
CK	cytokinin
CYC	cyclin
DAG	days after germination
DMSO	dimethylsulfoxide
Fruc	fructose
GFP	green fluorescent protein
GIN	glucose insensitive
Glc	glucose
Glc6P	glucose-6-phosphate
GUS	β-glucuronidase
HXK	hexokinase
HY5	long hypocotyl 5
IAA	indole-3-acetic acid
KO	knock-out
LR	lateral roots
LRIS	lateral root inducible system
LRP	lateral root primordium
NAA	naphthalene-1-acetic acid
NPA	1-naphthylphthalamic acid
OE	overexpressor
PI	propidium iodide
prom	promoter
Q-RT-PCR	semi-quantitative reverse transcriptase polymerase chain reaction
RAM	root apical meristem
SAM	shoot apical meristem

SLR	SOLITARY ROOT
SnRK1	Snf1-related kinase 1
STRE	stress responsive elements
Suc	sucrose
T-DNA	transfer DNA
TF	transcription factor
TPP	trehalose-6-phosphate phosphatase
TPS	trehalose-6-phosphate synthase
TRE	trehalose
TRE1	trehalase
Tre6P	trehalose-6-phosphate
UDPG	uridine 5’-diphosphate-glucose
Val A	Validamycin A
wt	wild- type
XPP	xylem pole pericycle

Scope of the research

Sugars are the fuel of life to plants and other organisms. They are the result of the fixed energy from the sun that feeds the plant cells to sustain growth and development. Sucrose is the main sugar transported in the plant and, as such reaches the cells where it is cleaved in glucose and fructose. Many vital processes are governed by sugars, but complex networks had to evolve allowing the plant to trigger the right program depending on its energy status. Surprisingly, one sugar phosphate is emerging to connect carbon status with the growth program. That sugar is trehalose-6-phosphate, the intermediate on the trehalose metabolism pathway. This molecule is a signal of sucrose status in the cell and it is also an inhibitor of the main regulator of starvation responses in plants. The mechanism mediating trehalose-6-phosphate balancing of energy levels is waiting to be unraveled.

The aim of this work was to scout into the possible role of the trehalose biosynthesis pathway in root development and stress tolerance. The focus was on the 10 active phosphatase members that convert the intermediate trehalose-6-phosphate into trehalose. By a forward genetics approach, we could identify two potential candidates important for lateral root development. Interestingly, they appear to function in an antagonistic manner in this organogenesis process and are regulated by auxin. Additionally, two other enzymes in the trehalose pathway were found to regulate abscisic acid responses. With these results, we open a big window of research searching on the molecular and hormonal components interacting with the trehalose pathway.

Chapter I

Introduction

Part 1. Trehalose and Trehalose-6-phosphate: a sweet molecule and a sugar signal

Trehalose metabolism is widespread in living organisms. It is a sugar present in bacteria, fungi, nematodes and it is the main sugar in the blood of insects. In plants is present as well but only in minute amounts, with the exception of some resurrection plants (Bianchi et al., 1993). Trehalose as well as sucrose are non-reducing disaccharides being important sources of soluble energy. Trehalose is composed of two units of glucose linked in a stable α,α-1,1 conformation and has particular properties that make it unique. It is known as a protectant of proteins and membranes from denaturation in cases of dessication, freezing and heat stress in yeast, bacteria and other organisms (Wiemken, 1990). In those cases, trehalose replaces water and during dessication forms a glass structure that restricts the formation of protein aggregates and free radical diffusion. However, it has also been reported as a carbon source, structural component and stored compatible solute in prokaryotic organisms (Arguelles, 2000). The function of this sweet molecule in plants, where minute amounts are accumulated but a large family of putative trehalose biosynthesis enzyme exists, is still under research. Particular attention to the intermediate in the synthesis of trehalose, trehalose-6-phosphate (Tre6P), is rising since it was demonstrated that important growth and developmental phenotypes in plants were attributed to changes in the levels of Tre6P rather than of trehalose.

1. SYNTHESIS OF TREHALOSE

1.1 Different pathways for trehalose metabolism: from archaea to higher plants

There are five known trehalose biosynthetic routes (Elbein et al., 2003; Avonce et al., 2006) commonly denoted by the name of the enzymes involved (Figure 1). The most studied one is the TPS/TPP pathway, since it is the most widely spread in prokaryotic and eukaryotic organisms. This pathway involves the intermediate Tre6P and is the only one present in plants. The glucose from Uridine-diphosphate-Glucose (UDPG) is transfered to Glucose-6-Phosphate (Glc6P) by trehalose-6-phosphate synthase (TPS) producing Tre6P and UDP; then Tre6P is dephosphorylated by Tre6 phosphatases (TPP) producing trehalose and inorganic phosphate. The TreP pathway involves trehalose phosphorylase (TreP) catalyzing the reaction between Glucose-1-phosphate and glucose, and is found in fungi. In some bacteria, the TreS pathway functions by maltose being isomerized to trehalose by trehalose synthase. In other prokaryotes, malto-oligosaccharides are converted to malto-oligosyltrehalose by maltooligosyl-trehalose synthase (TreY), and then

the synthesis of trehalose is catalyzed by maltoologosyl-trehalose trehalohydrolase (TreZ) producing also glucose. In some archaea and bacteria, trehalose is formed from ADP-glucose and glucose by means of trehalose glycosyltransferring synthase; this is the TreT pathway. In *E. coli*, Tre6P can also be converted to glucose and Glc6P by Tre6P hydrolase (TreH). In all organisms, the enzyme trehalase hydrolyzes trehalose in two molecules of glucose.

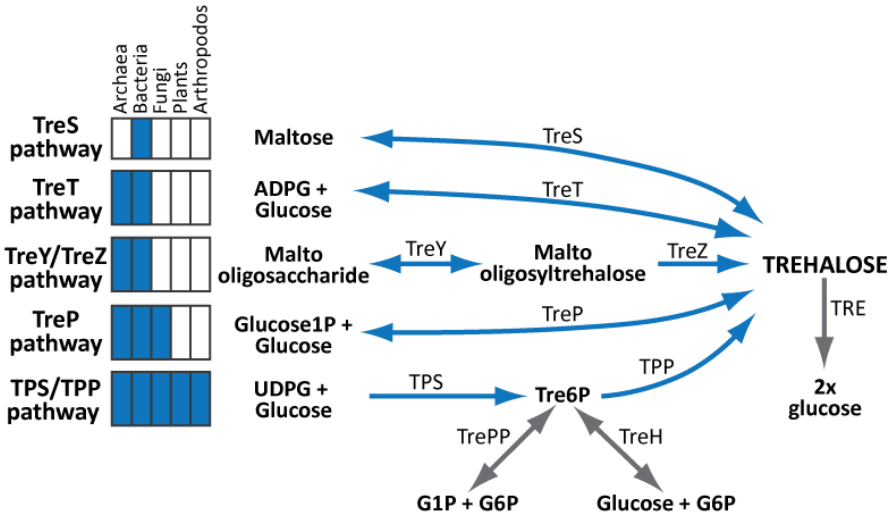


Figure 1. Known biosynthetic routes of trehalose in eukaryotes and prokaryotes. Based on Avonce et al. (2006). The pathways are denoted according to the enzymes involved in them. Blue arrows are the major trehalose pathways and, gray arrows are other biosynthetic routes. The gray boxes indicate the presence of the pathway in the corresponding phila. Tre6P= trehalose-6-phosphate, TreS=trehalose synthase, TreT=trehalose glycosyltransferring synthase, TreY=maltooligosyl-trehalose synthase, Trez=maltoologosyl-trehalose trehalohydrolase, TreP=trehalose phosphorylase, TPS=trehalose-6-phosphate synthases, TPP=trehalose-6-phosphate phosphatases, TrePP=Tre6P phosphorylase, TreH= Tre6P hydrolase, TRE=trehalase

1.2 Large families of trehalose biosynthesis genes are needed to produce a scarce sugar in *Arabidopsis*

In *Arabidopsis*, large families of trehalose biosynthesis genes can be found, opposing to what has been found in yeast and bacteria (Lunn, 2007). *S. cerevisiae* has one TPS (ScTps1) and one TPP (ScTps2) that form a complex with a regulatory subunit, encoded by TPS3 and TSL (trehalose synthase long chain) (Londesborough and Vuorio, 1991; Bell et al., 1998). *E.coli* has only one TPS (OtsA) and one TPP (OtsB) (Kaasen et al., 1992).

In *Arabidopsis*, 21 trehalose biosynthesis genes are divided into three classes according to their homology to the yeast ScTps1 and ScTps2 (Leyman et al., 2001) and the presence of conserved phosphatase sequences (Thaller et al., 1998) (Figure 2).

Class I trehalose biosynthesis enzymes have homology to the yeast ScTps1, having a N-terminal TPS-like domain, and a C-terminal part lacking the conserved phosphatase boxes. To this class belong the TPS1-TPS4 enzymes, but not all of them are functional synthases

when assayed in the *tps1Δ* yeast strain. Only TPS1 restores the growth of *tps1Δ* yeast strain in glucose medium (Blázquez et al., 1998; Vandesteene et al., 2010). Interestingly, this protein has an N-terminal extension acting as an auto-inhibitory domain and, its deletion highly increases TPS1 catalytic activity in yeast (Van Dijck et al., 2002).

Class II proteins contain a TPS-like domain at the N-terminal part and a TPP-like domain, with the highly conserved phosphatase boxes, in the C-terminal part. To class II belong TPS5-TPS11 enzymes, however, these proteins are neither synthases nor phosphatases, as they could not complement *tps1Δ* nor *tps2Δ* yeast strain growth. Possibly, they have a regulatory role and are not directly involved in trehalose metabolism (Ramon et al., 2009).

Class III trehalose enzymes only conserved the phosphatase boxes similar to the C-terminal part of ScTPS2. Ten TPPs are part of this class, TPPA-TPPJ, and phosphatase activity has been reported for all the members of this class by growth complementation of the *tps2Δ* yeast strain (Vogel et al., 1998; Vandesteene, 2009; see also Chapter II). The specificity of these enzymes for its substrate Tre6P, and not for Glc6P or Suc6P, has only been demonstrated for TPPA and TPPB (Vogel et al., 1998), or in TPPs from other plant species such as rice (Shima et al., 2007) and maize (Satoh-Nagasawa et al., 2006).

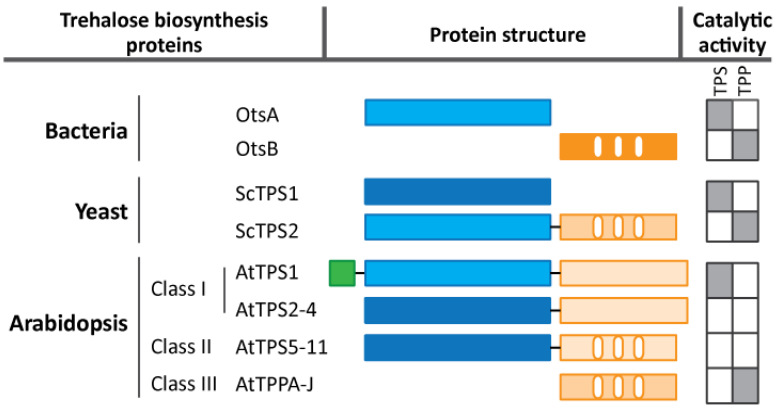


Figure 2. Similarity of trehalose biosynthesis proteins in bacteria, yeast and Arabidopsis. Based on Leyman et al. (2001), Vandesteene (2009), Ramon et al. (2009) and Vandesteene et al. (2010). Blue boxes indicate the synthase sequence, orange boxes correspond to the phosphatase sequence, white insets in the orange boxes are the phosphatase conserved domains, and the green box correspond to the N-terminal part of TPS1. The grey boxes indicate the catalytic activity of each enzyme.

Based on phylogenetic analysis Avonce et al. (2010) proposed that TPS/TPP fused genes from prokaryotes, such as *Cytophaga hutchinsonii*, are the ancestors of eukaryotic trehalose biosynthesis genes. During evolution, plants have recruited a new type of small protein with TPP activity (Class III), probably because the Class II proteins lost their activities. A N-terminal domain in the Class I proteins, which is important for its activity, was recruited as well. Importantly, this process was initiated very early in eukaryote

evolution, because primitive algae already have the three classes of trehalose metabolic proteins.

Additionally, trehalose can be hydrolyzed in two glucose units by the trehalase enzyme. Plants encode only one trehalase enzyme, opposite to yeast and bacteria that have different trehalases. In *S. cerevisiae* there are two types of trehalases, the acid trehalase (Ath1p) and the neutral ones (Nth1, Nth2). Ath1p is localized at cell surface and degrades extracellular trehalose, but it has also been implicated in the degradation of endogenous trehalose, so the cell can use that external glucose as well (Jules et al., 2004; Parrou et al., 2005). Ath1p activity is necessary for yeast cells to grow on exogenous trehalose as a sole carbon source (Nwaka et al., 1996). Neutral trehalases are cytosolic. Nth1 breaks down the trehalose transported via the trehalose/H^+ symporter (Agt1) (Jules et al., 2004), and Nth2 has been implicated in trehalose mobilization (Jules et al., 2008). In *E. coli*, a cytoplasmic trehalase enzyme (TreF) is present (Horlacher et al., 1996), but there is also a peryplasmic trehalase (TreA). Additionally, a phosphotransferase system II operates for the degradation of trehalose, a phosphotrehalase (TreB) transports trehalose as Tre6P to the cytosol where a cytoplasmic hydrolase (TreC) breaks Tre6P into Glc6P and glucose (Arguelles, 2000; Elbein et al., 2003).

2. Importance of trehalose metabolism in yeast: Control of glycolysis by Tps1

Screen for mutants able to grow on rapidly fermentable sugars such as glucose or fructose, led to the finding of multiple alleles of the *TPS1* gene in *S. cerevisiae*. A *tps1Δ* strain can not grow on glucose or fructose because of a deregulation in glycolysis leading to hyperaccumulation of sugar phosphates, depletion of ATP, phosphate and downstream metabolites. This suggested that Tps1 was involved in the regulation of carbon flux for glycolysis, or in the balance of ATP consuming rates at the initial steps of glycolysis and/or in the steps where ATP is regenerated (review by Gancedo and Flores, 2004).

Glucose is metabolized by hexokinases in an ATP-dependent step. Interestingly, when hexokinase2 (HXK2), QRC9 or CAT3/SNF4 is deleted, *tps1Δ* growth in glucose is recovered (Hohmann et al., 1993; Blazquez and Gancedo, 1995). Thus, reducing glucose influx restores the balance of ATP in the cells of *tps1Δ* strain. Tre6P inhibits *in vitro* activity of HXK1 and HXK2 (Blazquez et al., 1993). The reduced Tre6P in the *tps1Δ* leads to HXK hyperactivity, however yeast strains overexpressing HXK do not have the same phenotype as *tps1Δ* (Ernandes et al., 1998). This suggests that Tre6P control over HXK is not the only mechanism responsible for the deregulated glycolysis in *tps1Δ*. In fact, Tps1 could have a direct regulatory role in hexokinase activity that is not totally dependent on Tre6P. Tps1 proteins from *E.coli*, *Selaginella lepidophylla* and *Arabidopsis thaliana* fully restore the growth of *tps1Δ* in glucose, but the initial accumulation of hexoses and depletion of ATP and phosphate did not change in the *tps1Δtps2Δ* mutant in comparison with the *tps1Δ*

mutant (Zentella et al., 1999; Bonini et al., 2000). This indicates that ScTps1 has a specific regulatory function in controlling glucose flux into glycolysis in yeast. On the other hand, trehalose synthesis could divert sugar phosphates away from glycolysis to recover phosphates needed for glyceraldehyde-3-phosphate dehydrogenase function (Hohmann et al., 1993). This reflects that the control of Tps1 and Tre6P in glucose metabolism is complex and that more elements might be involved.

Another aspect of trehalose metabolism in *S. cerevisiae* involves Tps2. In a screening for strains sensitive to grow at high temperatures in any sugar media, a Tre6P accumulating strain was found (Piper and Lockheart, 1988). A *tps2Δ* strain is sensitive to high temperatures due to the high accumulation of Tre6P, and not to the deficit in trehalose biosynthesis that is known to protect cells from heat stress. This was concluded from the fact that *tps1Δ* and *tps1Δ tps2Δ* strains do not have trehalose but are able to grow at high temperatures (Elliott et al., 1996).

3. IMPORTANCE OF TREHALOSE METABOLISM IN PLANTS: AN ARRAY OF PHENOTYPES AND LINKS WITH DIFFERENT DEVELOPMENTAL, STRESS AND SUGAR SIGNALING PROCESSES

Resurrection plants like *Selaginella lepidophylla*, were found to accumulate large amounts of trehalose under desiccation. Trehalose protects the cells from the highly osmotic environment and, upon water availability, allows the plant to rehydrate and resume its metabolic functions. Apart from some desiccation tolerant plants, higher plants do not accumulate trehalose, thus it was thought that trehalose metabolism was not relevant, or that sucrose, the major sugar in plants, had taken over its function (Paul et al., 2008). Trehalose metabolism in plants started to be valued when *otsA* and *otsB* genes from *E. coli* were overexpressed in *Nicotiana tabacum* (tobacco) and *Solanum tuberosum* (potato) (Goddijn et al., 1997). In these plants, trehalose accumulation was low, but dramatic effects in development were seen. Tobacco otsA overexpressor plants showed a short, thick, swollen root, with highly vacuolated epidermal cells in *in vitro* conditions. When these plants were moved to soil, the root phenotype was recovered, the shoot showed dark green lanceolated leaves with high photosynthetic rates, but plants were stunted with reduced apical dominance, formation of several axillary shoots and low production of seeds. Those phenotypes were not seen in transgenic potato, but accumulation of trehalose by blocking trehalase activity was inversely correlated with tuber yield. This first study was conclusive to show that trehalose function in plants was not that similar to the one of other sugars, but hides other features for plant development.

3.1 Engineering stress tolerant plants by accumulation of trehalose

Trehalose accumulation has been linked to stress resistance. In *Triticum aestivum* (wheat) cultivars subjected to drought and salt stress, trehalose was accumulated in all cultivars, but more in the stress resistant cultivars than in the sensitive ones, probably due to the high TPS activity and the reduced trehalase activity in those cultivars (El-Bashiti et al., 2005). In *Medicago sativum* (alfalfa) trehalose accumulates in roots, nodules and bacteriods upon salt stress, but the trehalose accumulation was too low to be involved in osmoregulation (Fougere et al., 1991). Rice plants transiently induce trehalose accumulation upon chilling stress, and *OsTPS1* was induced as well (Pramanik and Imai, 2005). However, far from questioning the activity of trehalose biosynthesis enzymes in crops, early work linking trehalose with stress resistance involved heterologous expression of the known microbial enzymes as a strategy to look for stress- tolerant cultivars of important crops.

Tobacco plants transformed with *otsA* and *otsB* genes from *E. coli* did not show the pleiotropic defects named above when they were subjected to low water supply. Under drought stress, these plants had a higher yield, mainly due to increased leaf production and more efficient photosynthesis than wild-type. This transgenic tobacco also had higher levels of trehalose that correlated with sucrose, glucose and fructose levels (Pilon-Smits et al., 1998). Romero et al. (1997) also transformed tobacco using the yeast *ScTPS1* gene. Drought resistant of the transgenic plants was evident as well as the effects on growth that were similar to the ones described in Goddijn's work (1997). However, these plants did not accumulate trehalose, sucrose or glucose. In another study, tobacco plants transformed with *ScTPS1* under control of the RBCS promoter were drought resistant, accumulated some trehalose and did not show morphological or developmental defects, besides a lower growth rate (Holmström et al., 1996). Other examples are *Lycopersicon esculentum* (tomato) plants resistant to salt, oxidative-stress and drought when transformed with *ScTPS1*. They accumulate chlorophyll and starch in the leaves showing dark-thick leaves and also, thick shoots and erected branches (Cortina and Culianez-Macia, 2005). Rice plants transformed with a bifunctional construct of *otsA* and *otsB* with different promoters, accumulated trehalose and enhanced tolerance to drought, salt and cold without changes in the content of other sugars and developmental defects (Garg et al., 2002; Jang et al., 2003). The same effect of a bifunctional construct was seen in *Arabidopsis* plants transformed with *ScTPS1-ScTPS2*, including freezing and heat tolerance, but low accumulation of trehalose (Miranda et al., 2007). In summary, these studies demonstrate that expression of microbial trehalose metabolism genes has important (deleterious) effects on plant development, and that trehalose accumulation does not correlate with stress resistance. Successful strategies to improve stress tolerance in plants required the use of stress-inducible or specific promoters, or the use of TPS/TPP constructs to circumvent the accumulation of Tre6P, which apparently affects sugar metabolism and signaling pathways leading to growth and developmental defects.

In *Arabidopsis*, drought tolerance was reported in plants overexpressing their own *TPS1* gene. Interestingly, these plants had only a small increase in trehalose and Tre6P levels, and showed no developmental defects besides late flowering. Additionally, *TPS1-OE* A*rabidopsis* mutants were glucose- and ABA- insensitive (Avonce et al., 2004). Glucose-insensitivity was also reported in *Arabidopsis* plants with *ScTPS1-ScTPP2* overexpression constructs (Miranda et al., 2007). A point mutation in the *TPS6* gene also leads to drought tolerant *Arabidopsis* plants, besides other phenotypes in epidermal cell shape, plant architecture and trichomes (Chary et al., 2008). Another class II protein, TPS5, interacts with multiprotein bridging factor 1c (MBF1c), a key transcription factor regulating thermotolerance. *TPS5* was induced after 20 min of heat treatment, and its mutant is thermosensitive (Suzuki et al., 2008). What is not clear from the mutant phenotypes of Class II genes is the actual implication of Tre6P or trehalose since these proteins lack heterologous TPS/TPP activity in yeast complementation assays (Ramon et al., 2009).

3.2 Trehalose and Tre6P function in growth and development

3.2.1 The case of *AtTPS1* in embryo, vegetative and flower development

Eastmond (2002) reported the first mutant with a disrupted trehalose biosynthesis gene in *Arabidopsis*. It was a mutant in the only functional TPS in *Arabidopsis*, *TPS1*. *TPS1* deficiency is embryo lethal, seeds from heterozygous plants were wrinkled and with arrested embryos in a 3:1 ratio, showing that it was a recessive mutation. In developing seeds, embryo development is characterized by a phase of cellular division and patterning, followed by cell elongation and reserve storage and a final drying phase. *tps1* embryos grew slowly and arrested growth when cell division changed to cell expansion and accumulation of reserves, processes that are known to happen at the torpedo stage (Figure 3A). When cell elongation starts, the embryo becomes a sink, a phenomenon that is linked to the increase in sucrose, but not glucose or fructose. Accordingly, the use of low sucrose medium partially rescued the embryo lethal phenotype of *tps1* by enhancing cell expansion. Repressed expression of sucrose synthesis, sucrose degradation and starch degradation genes was found in *tps1* torpedo stage embryos compared to wild-type torpedo and cotyledon stages. However, lipid mobilization genes were upregulated in *tps1* embryos. This was correlated with higher sucrose and starch accumulation in *tps1* embryos and with the increase in cell wall thickness due to altered deposition of pectins (Gómez et al., 2006). The endosperm of *tps1* embryos develops normally, suggesting that *TPS1* directly affects embryo development but not other developmental processes in the seed.

After a long stratification period, some *tps1* embryos were able to develop further at a very slow rate producing a small rosette that stayed in vegetative stage and senesced. This phenotype suggested reduced cell division that was corroborated by following the expression of the mitotic marker CyclinB1;1 in *tps1* embryos during seed development.

Importantly, TPS1 forms complexes with CYCLIN DEPENDENT KINASE A;1 (CDKA;1), β-tubulin and CDKA;1 interacting kinesin (KCA;1) (Geelen et al., 2007). Moreover, during mitosis, TPS1 was visualized in the spindle and phragmoplast, together with KCA;1, but remained in the cytoplasm when KCA;1 was targeted to the cell plate. This may provide a link between sugar availability and control of cell division by TPS1.

In an attempt to overcome the embryo lethality of *tps1* mutants, van Dijken et al. (2004) used a dexamethasone (DEX)-inducible system to express TPS1 in *tps1* plants. After rescuing the embryo lethality of *tps1*, these seedlings were kept without DEX to check post-embryonic development. *tps1* plants showed a short root with a small root apical meristem (RAM), leaves were rounded and developed a small rosette that senescenced without flowering. Flowering transition was possible with a DEX treatment, but *tps1* plants displayed reduced apical dominance with a lot of flowering stems and small siliques with few seeds. Recently, with the same aim, Gomez and coworkers (2010) used a seed-specific promoter (Abscisic Insensitive 3, *ABI3*) to induce *TPS1* during seed development, and in a second approach, used TILLING (targeted induced local lesions in genomes) to generate weaker, non-embryo lethal alleles of *TPS1*. Absence of TPS1 after seed development led to very small plants, with an arrest in shoot and root growth (Figure 3B), and delayed flowering. In leaves, the mesophyll cells were loosely packed and lowly vacuolated, having a large chloroplast with excessive starch granules. Also, the epidermal cells looked smaller than in wild-type. Sugar measurements indicated an accumulation of sucrose, fructose and glucose, as well as sugar hexoses and starch, but reduced levels of Tre6P. Additionally, these mutants were hypersensitive to glucose and ABA, a phenotype that was expected as TPS1 overexpression plants showed resistance to the inhibition of germination by ABA, and were able to grow on high glucose concentrations (Avonce et al., 2004).

These studies on TPS1 mutants reflect the absolute requirement of TPS1 during embryo, vegetative and flowering development of *Arabidopsis*, where the key regulator, Tre6P, is linked with sugar signaling, carbohydrate metabolism and ABA signaling.

3.2.2 The case of *AtTPS6* in cell shape and plant development

In a mutant screening for vacuole-defective mutants, a *cell shape phenotype-1* (*csp-1*) mutant was found (Chary et al., 2008). *csp-1* plants have a point mutation in the TPS6 protein that produces an Arg-to-Ser substitution. These plants showed reduced lobing of epidermal cells, trichomes with two branches, epinastic leaves that were narrower and smaller than the parental line, reduced apical dominance with multiple floral stems of reduced branching and delayed flowering. Leaf and hypocotyl epidermal cells appeared smaller, and root size was reduced in comparison with that in the parental line; in general, plants were smaller. A loss-of-function mutant of TPS6 (*csp-2*) only showed the epidermal cell shape phenotype. However, the role of *TPS6* in *csp-1* phenotypes was functionally

confirmed by complementation of *csp-1* plants with the *promTPS6::TPS6* construct which restored the phenotype to wild-type. Moreover, a *TPS6* overexpressor line had phenotypes related to *csp-1*. These plants had extra branched trichomes, larger leaves and rosettes and increased apical dominance. Although the authors described TPS6 as a functional TPS/TPP enzyme, another study reported the opposite and showed that the yeast complementation assays done by Chary and coworkers (2008) could not lead to those conclusions (Ramon et al., 2009). If Class II proteins have no catalytic activity, it could be that they act by forming protein complexes, or that they are sensors of Tre6P in tissue-specific responses (Ramon et al., 2009). Cell morphogenesis is a process known to be dependent on signal-mediated cytoskeletal dynamics and reorganization (Smith and Oppenheimer, 2005). However, TPS6 showed to be a novel player in this process by sensing Tre6P, forming complexes or via another unknown mechanism.

3.2.3 A TPP in maize, RAMOSA3, modulates inflorescence architecture

Inflorescence branching in maize is controlled by the *RAMOSA* genes. *ra3* mutants have additional long branches in the male (tassel) and female (ear) structures (Figure 3C), a trait that is thought to help in the efficient packing and harvesting of maize seeds from the ear (Satoh-Nagasawa et al., 2006). Once the axillary inflorescence meristems were produced, *ra3* mutants showed a loss of determinacy and identity, and produced long branches instead of organized spikelet meristems with floral meristems. This phenotype was in agreement with RA3 expression in particular domains at the base of axillary meristems in young ear primordia, and in tassel after the initiation of the branches. RA3 was found to be a functional TPP protein. Orthologs of the RA3 protein in species such as sorghum or maize showed similar expression patterns, suggesting that TPPs might also regulate inflorescence development in these species. It was proposed that RA3's role in inflorescence branching is through the modification of a sugar signal, Tre6P, that moves into axillary meristems because RA3 is expressed in some cells subtending, but not within, the axillary meristem. Alternatively, since *RA3* acts upstream of the *RA1* transcription factor, a role of *RA3* in transcriptional regulation is suggested.

3.3 Trehalose and Tre6P function in sugar sensing and metabolism

3.3.1 Coordination of growth with carbon source availability by Tre6P

The morphological and developmental phenotypes displayed by trehalose engineered plants to tolerate stress, indicate a strong role of trehalose, but mainly Tre6P, in controlling carbon fluxes and sugar signaling. To get insight in the physiological role of trehalose metabolism in *Arabidopsis* plants, overexpressing lines with *E. coli otsA* (TPS), *otsB* (TPP), *treC* (TPH, trehalose phosphate hydrolase) and *treF* (trehalase) were constructed (Schluepmann et al., 2003). Tre6P was accumulated in plants overexpressing *otsA*, whereas lower Tre6P levels were found in *otsB* or *treC* overexpressing plants and in

treF lines. Tre6P content did not change compared to wild-type. Regarding trehalose, leaves of all lines had trehalose contents under the detection limit. Changes in Tre6P content were accompanied by changes in growth and development. Plants with high Tre6P level had smaller rosettes with dark green leaves compared to wild-type, were bushy and exhibited early flowering and low seed production (Figure 3D). In contrast, TPP and TPH plants had larger, light green leaves, late flowering and plentiful seed set. The growth of these plants was tested upon sugar feeding. TPS seedlings grew better than the wild-type in sucrose, fructose and glucose media, whereas TPP and TPH seedlings displayed a retarded growth upon sugar feeding. However, under high sucrose or glucose all lines arrested their growth.

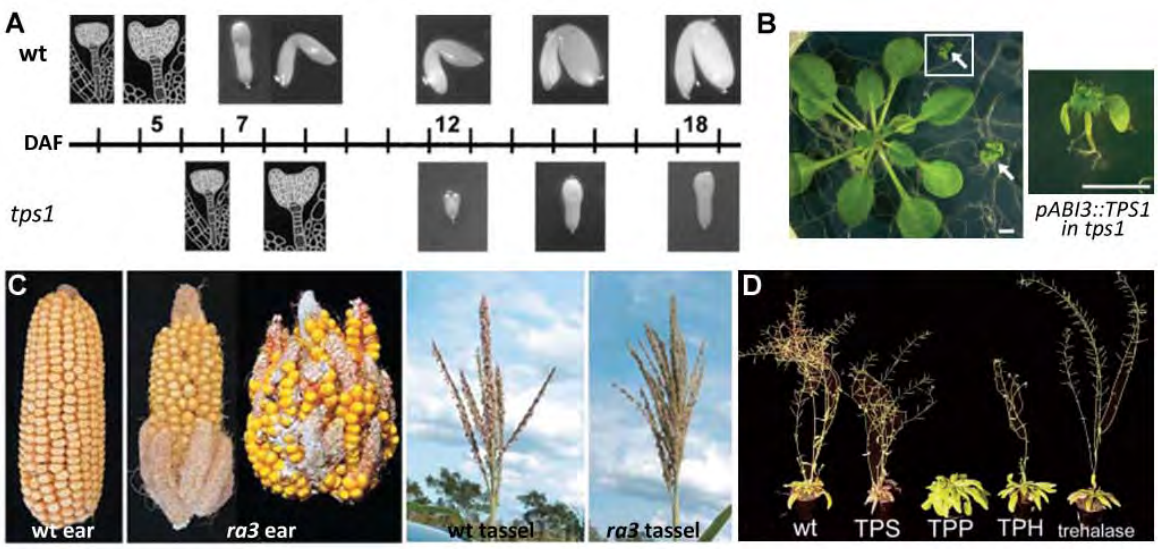


Figure 3. Growth and developmental phenotypes in plants with modified Tre6P levels. A. Progressive development of *Arabidopsis* wild-type compared with *tps1* embryo. DAF=days after flowering (Gómez et al., 2006). **B.** Growth phenotype of three weeks old *Arabipopsis tps1* plants after rescue of the embryo lethal phenotype using a seed development promoter construct (arrow) in comparison with wild-type plants (Gómez et al., 2010), reference bar=5cm. **C.** Inflorescence phenotype in maize *ra3* mutants compared to wild type. *ra3* ears have abnormal branches and irregular seed rows and, *ra3* tassels have additional long branches (Satoh-Nagasawa et al., 2006). *Arabidopsis* phenotype of plants overexpressing *E. coli* trehalose biosynthesis enzymes (Schluepmann et al., 2003). See text for details on phenotypes.

Sugar measurements indicated that Tre6P levels correlate inversely with glucose-6-phosphate (Glc6P) and fructose-6-phosphate (Fru6P). Hexose-phosphates were accumulated in TPP and TPH upon sucrose feeding, and in TPS plants they were depleted. This means a direct effect of Tre6 levels in hexose phosphate accumulation. Additionally, TPH developed well in trehalose levels that were detrimental for the wild-type growth. While wild-type plants accumulate high levels of Tre6P, TPH plants hydrolyzed the Tre6P to glucose and Glc6P. Only by sucrose feeding to trehalose grown wild-type plants, there is a rescue in growth. This implies that high Tre6P needs to be coordinated with availability of a metabolizable sugar for the plant to grow, which is in agreement with the

fact that sucrose itself leads to a progressive increase in Tre6P levels (Schluepmann et al., 2004).

Also interesting, was the finding of higher photosynthetic rates per unit of leaf area in TPS leaves, while the opposite was observed in TPP plants (Paul, 2007). This increase in photosynthesis was not directly correlated with more biomass because TPP plants were larger albeit its reduced photosynthesis. How Tre6P is modulating photosynthesis is still not known, but the modification of trehalose metabolism proved to be a good strategy to enhance a difficult trait such as photosynthetic capacity.

3.3.2 Trehalose as an external carbon source

Trehalose is taken up by *Arabidopsis* plants, but at high concentrations it can be toxic for the plant (Müller et al., 1995). Root elongation is inhibited in a trehalose concentration dependent manner, and addition of Validamycin A, a potent inhibitor of trehalase activity, renders plants with even shorter roots, impaired leaf expansion and growth arrest (Bae et al., 2005). However, when trehalose medium is supplemented with sucrose or glucose, plants restore their growth. The inhibitory effect of trehalose on root growth was also reversible since plants grown on trehalose and moved to sucrose supplemented medium resumed growth (Wingler et al., 2000). The later suggests that the effect of trehalose on roots is due to reduced carbon supply. Starch accumulation in the cotyledons was also evident in plants grown on trehalose. Interestingly, starch accumulation was blocked in trehalose grown plants in the dark, but not in plants grown on sucrose-trehalose media. This means that trehalose is not used as a carbon source, because starch was synthesized from carbon fixed during photosynthesis, and that the inhibitory role of trehalose in root growth is a different phenotype that the one of starch accumulation. However the strong accumulation of starch in the shoots could lead to a reduced supply of carbon to the roots (Wingler et al., 2000).

If trehalose increases starch accumulation, the activity of the first enzyme in starch synthesis, ADP-Glucose Pyrophosphorylase (AGPase), should increase. Indeed, trehalose but not sucrose, induced the activity of AGPase and of the *ApL3* gene that encodes a large subunit of AGPase. To investigate whether the effect of trehalose was in carbon allocation by induction of AGPase, Fritzius and coworkers (2001), used three mutants deficient in starch production. The *adg2-1* mutant lacks the ApL1 large subunit of the AGPase. When this mutant was grown on trehalose, it restored starch synthesis by induction of ApL3 which could replace ApL1 in the AGPase complex. However, in the *pgm1-1* mutant, lacking the plastidic phosphoglucomutase, and *adg1-1*, deficient in the small AGPase subunit (ApS), there was no restoration of starch synthesis. Interestingly, these three mutants were partially insensitive to the inhibition of root elongation by trehalose, meaning that the accumulation of starch in the shoot is to some extend responsible for the root phenotype.

AGPase post-translational redox activation and starch accumulation were used to explore the role of trehalose in starch synthesis (Kolbe et al., 2005). Posttranslational redox activation of AGPase allows the rate of starch synthesis to be increased in response to external inputs, and independently of any increase in the levels of glycolytic intermediates. With three different strategies including *Arabidopsis* leaves and potato tubers fed with trehalose, Tre6P modulated levels plants (see Schluepmann et al., 2003) and chloroplasts feed with Tre6P, the authors conclude that Tre6P increases as sucrose levels rise. This increase in Tre6P reports the cytosolic sucrose status to plastids by AGPase posttranslational redox activation which in turn promotes starch synthesis (Kolbe et al., 2005) (Figure 4). Additionally, an interplay of Tre6P with Sucrose-Non-Fermenting-Related Kinases 1 (SnRK1), which are central regulators of the transcriptome in response to stresses (Baena-Gonzalez et al., 2007), is suggested because the activation of AGPase in potato tubers is dependent on SnRK1 (Tiessen et al., 2003; Kolbe et al., 2005). However, the signaling pathway that links changes in sucrose levels to Tre6P contents and AGPase redox activation was not resolved, until a sensitive system involving liquid chromatography coupled to a triple quadrupole MS (LC/MS-Q3) to measure Tre6P was developed (Lunn et al., 2006).

Another study suggested that the accumulation of high starch levels on trehalose grown plants is a consequence of its reduced breakdown mediated by transcriptional repression of genes downstream of ABI4 (Ramon et al., 2007). Different mutants in sugar sensing and signaling, such as *abi1*, *abi2* and *abi4*, were grown on trehalose medium. The *abi4* mutant was the only one showing less sensitivity to root growth inhibition by trehalose. ABI4 is a key component in the hexokinase1 (HXK1)-dependent glucose signaling, but other mutants in this pathway such as *gin2*/*hxk1* and a HXK1 overexpressor, were as sensitive as wild-type to trehalose. Interestingly, lugol-staining of starch in *abi4* mutants grown on trehalose showed less starch accumulation than wild-type, indicating that starch breakdown was going on in this mutant. Starch mobilization and breakdown are facilitated by *STARCH EXCESS1* (*SEX1*) and *β-amylase* (BAM3) and these genes are downregulated in wild-type seedlings grown on trehalose, but not in *abi4* mutants or wild-type plants grown on trehalose and sucrose. This suggested that reduced starch degradation and export of glucose, partially controlled by ABI4, are to some extend responsible for the root phenotype of plants grown on exogenous trehalose.

4. SUGAR SENSING AND SIGNALING IN PLANTS

Sugars act as primary carriers of captured energy from the sun. Photosynthates, mainly sucrose, are transported from the leaves (source) to developing organs (sink) such as meristems, roots, flowers, seeds. In these demanding organs, sucrose is converted to hexoses by invertases and sucrose synthases or stored as starch in amyloplasts. Starch can be stored during the day and remobilized and exported as maltose or glucose to

sustain growth during the night (Rolland et al., 2006; Smith and Stitt, 2007). All these products are the result of an organized sugar metabolism in location and time. In this way, growth is regulated by sugars in a complex network of signals that involves light, stress, and hormones interactions (review by Rolland et al., 2002; Rolland et al., 2006; Ramon et al., 2008), as well as carbon and nitrogen metabolism (Coruzzi and Zhou, 2001).

Sucrose is the most important transport sugar in the plant. It is transported symplastically from the source tissues via plasmodesmata before loading the phloem. Sucrose has to be uploaded into the apoplast via H^+/-sucrose cotransporters (SUT) and sucrose transporters (SUC). There, it can be cleaved into monosaccharides that will be transported into the cell by sugar transport protein (STP), or it can be uploaded again in the symplast as sucrose by the same transporters (Lalonde et al., 2004). The cleaving enzymes of sucrose, sucrose synthase (SUS) and invertase (INV), are pivotal for maintaining the balance between both sugar signals and metabolic paths (Koch, 2004). However, as sucrose is readily hydrolyzed in glucose and fructose, most of the regulatory effects of sucrose are directed to glucose sensing and signaling. Importantly, independent signaling pathways for sucrose (Chiou and Bush, 1998; Vaughn et al., 2002), glucose (review by Rolland et al., 2006), trehalose-6-phosphate (Paul et al., 2008) and possibly fructose (Pego and Smeekens, 2000) have been reported.

4.1 Hexokinase1: the sensor of glucose

HXK1 was the first plant sugar sensor identified. It senses glucose and is also involved in the first step in glycolysis. In conditions of increased energy supply with high light, *glucose insensitive2* (*gin2/hxk1*) mutants remained small and dark green, whereas wild-type plants grew more vigorously. In anti-sense and sense lines of HXK1 and HXK2 Jang and coworkers (1997) described the hyposensitivity of the anti-sense lines whereas overexpressing HXK plants were hypersensitive. Moreover, two catalytically inactive HXK1 alleles, in the *gin2* mutant background, can restore developmental arrest on high glucose conditions and, glucose repression of chlorophyll accumulation and photosynthetic gene expression. Thus the signaling effects of HXK1 can be uncoupled from its effects on glucose metabolism (Moore et al., 2003). Arabidopsis has two HXKs and four HXK-like (HXKL) proteins, three of them have detectable kinase activity. They vary in their localization. HXKL have a predicted chloroplast transit peptide and HXK have a membrane anchor, however HXK1 is predominantly associated with the mitochondria and can translocate to the nucleus as well. Thus, protein localization is expected to play an important role coordinating substrate availability with glucose metabolism and cellular energy demand (Rolland et al., 2006).

Glucose signaling can be dependent or independent of HXK1. In the dependent, the major effect is the repression of photosynthetic gene expression, and additional target genes can be identified using the *gin2* mutant and the catalytically inactive alleles (Moore et al.,

2003). In the independent one, transported hexose is possibly sensed at the plasma membrane or, the signal is glycolysis-dependent and generated from a metabolic intermediate (Xiao et al., 2000).

In the case of sucrose, different target genes have been discovered that are specifically regulated by this sugar. Furthermore, non-metabolizable sucrose analogs, such as palatinose and turanose, can affect carbohydrate metabolism and gene expression. This suggests that a sensing system for disaccharides exists in the plasma membrane. Even though these analogs could not activate sucrose-signaling, they could be perceived as stress-related signal rather than a sucrose substitute (Ramon et al., 2008).

4.2 Sugar signaling interplays with hormones and nutrient signaling

The extensive characterization of different sugar-signaling mutants isolated in different screenings revealed diverse interactions with hormone biosynthesis and signaling pathways. An extensive list of mutants and their responses to sugar availability is presented by Ramon et al. (2008). Many of the mutants isolated were found to be allelic to ABA related genes. The *glucose insensitive1* (*gin1/sis4*) mutants are allelic to *ABA synthesis2* (*aba2*), whereas *gin5* is allelic to *aba3*, and *gin6*/*sucrose uncoupling6* (*sun6*)/*sugar insensitive5* (*sis5*) is allelic to *abi4*. These mutants have lower ABA levels and reduced seed dormancy (Arenas-Huertero et al., 2000). But not all of the *abi* mutants are insensitive to glucose, thus different signal pathways must be involved. Additionally, transcript levels of a number of ABA biosynthesis genes are increased by glucose, as well as ABA levels, indicating a mechanistic link at the molecular level (Cheng et al., 2002). Defects in *gin2* mutant growth in conditions of high light revealed glucose interactions with other hormones. Application of auxin (Indole Acetic Acid/IAA) to *gin2* mutants failed to induce cell proliferation and root formation, but mutants were hypersensitive to shoot induction by cytokinin (Moore et al., 2003). Some auxin signaling mutants, such as *auxin resistant1* (*axr1*), *axr2* and *transport inhibitor response1* (*tir1*), are resistant to growth inhibition by high glucose, similarly to plants with a constitutive cytokinin response or with cytokinin addition.

Some mutants insensitive to ethylene such as, *ethylene resistant1* (*etr1*), *ethylene insensitive2* (*ein2*) and *ein3*, are also glucose hypersensitive (Zhou et al., 1998; Yanagisawa et al., 2003). In contrast, the ethylene overproducer (*eto1*) mutant is glucose resistant (Zhou et al., 1998). One of the discovered mechanisms was the differential regulation of the EIN3 transcription factor in by glucose and ethylene. Glucose increases the proteasome-dependent degradation of EIN3 via HXK1 in the nucleus while ethylene stabilizes the protein (Yanagisawa et al., 2003). Hormones such as gibberellin (GA) are also connected to glucose signaling. REPRESSOR OF GA-LIKE2 (RGL2) and SPINDLY (SPY) are two negative regulators of GA signaling and their mutants *rgl2* and *spy* germinate normally in inhibiting glucose concentrations (Yuan and Wysocka-Diller, 2006). Also

sucrose activation of anthocyanin biosynthesis genes is repressed by GA, while ABA and jasmonate promote the effect of sucrose (Loreti et al., 2008). *brassinosteroid*, *light* and *sugar1* (*bls1*) is hypersensitive to sugars, but this can be reverted by brassinosteroid (BR) application.

Other nutrients are also part of the complex network of interactions with glucose. Microarray analyses have found that nitrogen as well as glucose and sucrose regulate specific subsets of genes through their own responsive pathways in the roots (Gutierrez et al., 2007). However, nutrients like phosphate (Pi) and nitrogen seem to modify sugar responses (Palenchar et al., 2004; Müller et al., 2007). Conversely, sugars up-regulate various nitrogen transporters and nutrient up-take genes (Lejay et al., 2008) and regulate changes in root architecture and expression of inducible genes upon Pi starvation (Karthikeyan et al., 2007).

4.3 Energy signals to cope with sugar starvation and stress

As plants are in a constantly changing environment, sensitive mechanisms are important to perceive and respond adequately to the environmental cues and continue with metabolic activity, growth and development. Energy sources may be sensed in a cell-autonomous manner to respond with changes in gene expression in the cell and the whole plant according to the stress signal (Baena-Gonzalez et al., 2007; Baena-Gonzalez, 2010). Most of the early signaling events and responses largely determine the survival of a plant under stress. These signals involve calcium fluxes, reactive oxygen species, and energy deprivation which will have an impact on light absorption, carbon fixation or oxygen availability. Thus, photosynthesis and respiration are highly affected and the effect is a low energy status in the cell.

Transcriptional changes are one of the first events happening in response to stress. Several studies to identify regulatory components of particular stresses have been conducted, however they have failed to find a common regulator triggering those responses. Only recently a central regulator of transcriptional changes in response to darkness, sugar and stress conditions has been identified (Baena-Gonzalez et al., 2007). The protein kinases KIN10 and KIN11, collectively designated as SnRK1 (Sucrose non-fermenting1 related protein kinase), are important regulators of metabolism and energy homeostasis by repressing anabolism and activating catabolism under energy deprivation conditions. These overlapping transcriptional cascades of responses assure the restoration of homeostasis, promotion of cell survival and elaboration of long-term responses for adaptation, growth and development (Baena-Gonzalez and Sheen, 2008). Accordingly, *kin10kin11* double mutant knock-out plants senesce before the onset of flowering, whereas overexpression plants have a delayed senescence and flowering and, an altered flower architecture under long-day conditions, besides an enhanced starvation tolerance.

The SnRK1 signaling cascade is far from completed. Among its components are the S-group bZIP transcription factors (TF), bZIP1, bZIP2/GBF5, bZIP11, bZIP44, and bZIP53 which regulate transcription of a subset of KIN10 target genes. This group of TFs forms heterodimers with C-group bZIP TF and are regulated in different ways by stress. However, translation of this group of TFs is repressed by sucrose, giving the framework for antagonistic regulation of the system by energy availability or deficiency. ATAF1, a member of the plant specific NAC TF family, was identified as an interacting partner of SnRK1 (Kleinow et al., 2009). It is induced by a variety of stresses and its overexpression leads to drought tolerant plants (Wu et al., 2009), but also to developmental defects due to co-suppression of the other NAC members (Kleinow et al., 2009). MYBS1 and MYB-GA TFs activation of α-*amylase3* downstream of SnRK1 has been shown to integrate glucose repression, gibberellic acid and ABA signaling, with the hypoxia response in rice (Lu et al., 2007; Lee et al., 2009).

Apart from its role in transcriptional regulation, SnRK1 also modulates enzymes by direct phosphorylation. Additionally, its activity is modulated by sugar phosphates such as Glc6P and Tre6P. Glc6P is the product of glucose phosphorylation by HXK and it represses SnRK1 activity in spinach leaf extracts (Toroser et al., 2000). Recently, it was reported in *in vitro* experiments that Tre6P could inhibit SnRK1 activity at physiological levels in growing tissues depending on an unknown intermediary factor not present in mature leaves (Zhang et al., 2010). Gene expression of seedlings with high or low Tre6P content (*otsA* and *otsB* overexpressors) revealed that SnRK1 marker genes in *otsA* plants were affected as predicted by Tre6P inhibition of SnRK1. Genes involved in photosynthesis and degradation processes which are normally upregulated by SnRK1 were downregulated in plants with high Tre6P, whereas genes involved in biosynthetic reactions behave oppositely (Zhang et al., 2010).

Among the modulated genes found in high Tre6P content plants, genes involved in cell wall biosynthesis, light and auxin signaling and circadian clock regulation were detected (Paul et al., 2010). UDPG-dehydrogenases, enzymes that use UDPG to produce UDP-glucoronic acid, are important for matrix polysaccharides in the cell wall, and were upregulated by Tre6P. It is known that the UDPG-dehydrogenases isoforms have a role in carbon partitioning between cell wall formation and sucrose synthesis, the most demanding UDPG process in cells (Klinghammer and Tenhaken, 2007). Additionally, some pectin methylesterase/invertase inhibitor genes were repressed by Tre6P. These enzymes are important in the regulation of cell wall solidity, a relevant feature when remodeling of cell wall architecture is required (Hothorn et al., 2004). Consistently, in the *tps1* mutant, cell wall biosynthesis genes were found to be downregulated (Gómez et al., 2006). *Phytochrome Interacting Factor4* (*PIF4*), a growth promoting factor, was downregulated in *otsA* mutant plants. It is regulated by the circadian clock and degraded by the 26S proteasome upon light. Together with PIF4, other genes involved in circadian clock regulation such as *Constants Like2 (COL2)*, *Early Phytochrome Responsive1* (*EPR1)* and

Constitutive Induce Resistance1/Reveille2 (CIR1/RVE2) were downregulated. Genes belonging to the 26S proteasome were induced, as opposed to some Aux/IAA TF required for auxin signaling and the auxin receptor *TIR1* that were downregulated (Paul et al., 2010). This map of genes responding to Tre6P levels complements the already known positive influence of Tre6P in plant growth and development and significantly gives direct indications to auxin and light signaling as important interactors of Tre6P metabolism.

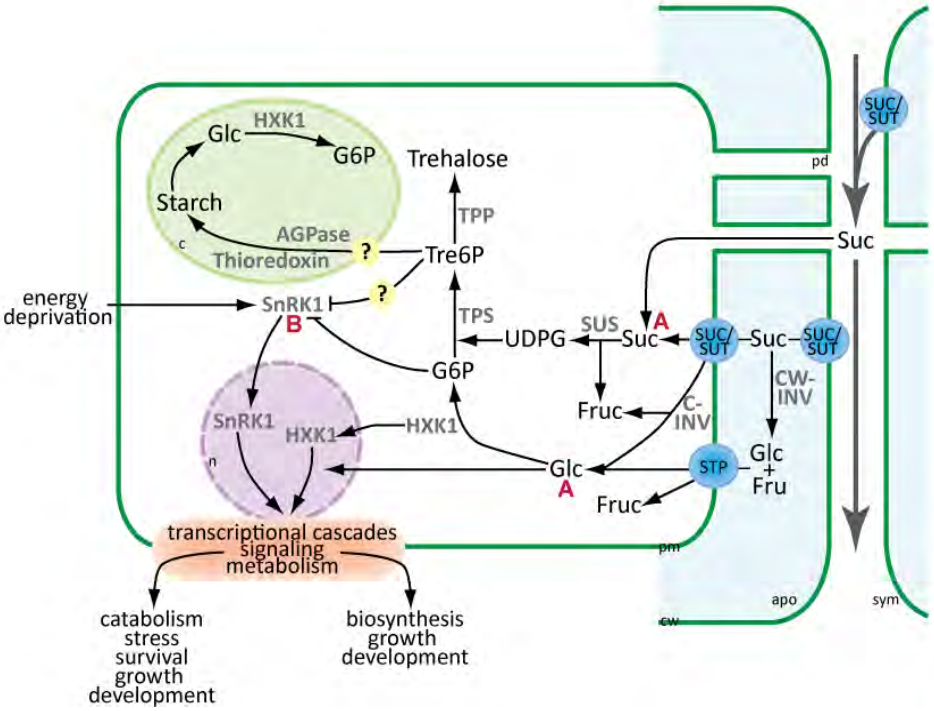


Fig. 4 Trehalose metabolism in the context of sugar signaling and stress signaling. Based on Rolland et al. (2006) , Ramon et al. (2008) and Baena-Gonzalez (2010). Plant cells have an influx of sugars that are sensed, metabolized and used in growth (A), however they are also able to sense stresses that lead to energy deprivation conditions (B). The key molecular components regulating these processes are HXK and SnRK1, respectively. They work in a complex network to keep energy balance and homeostasis in the cell. Additionally, Tre6P functions as a sucrose signal communicating the availability of energy sources. It is also an activator of starch metabolism and, importantly, inhibits SnRK1 responses via an unknown factor in young cells. This suggest that Tre6P is in a privileged position to link the energy balance by promoting growth and reducing catabolic processes in conditions of high energy. See text for details in the molecular components. cw=cell wall, pm=plasma membrane, pd=plasmodesmata, c=chloroplast, n=nuclei, apo=apoplast, sym=symplast.

Part 2. Lateral Root Development

Arabidopsis has a primary root that is initiated during embryogenesis and is visible just after germination. It grows straight downward and branches to generate several orders of lateral roots (LR). The architecture of the root is modulated by external cues such as nutrients, water availability, and stresses which in turn will trigger endogenous responses to keep the homeostasis in the plant. Stem cells in the root apical meristem (RAM) are responsible for the radial organization of the root. The center of the root is formed by vasculature files with two xylem (XP) and two phloem poles (PP). Surrounding the central vasculature are pericycle cells from which lateral roots are formed. And then endodermal, cortical and epidermal cells are radially distributed towards the outside of the root. The epidermal cells are organized in longitudinal files with different cell fates, root hairs (trichoblast cells) or non-root hairs (atrichoblast cells) (Dolan et al., 1993) (Figure 5). The organized distribution of the root has been used to unravel new components in specific developmental moments and places in the root by the generation of a spatio-temporal transcript map (Birnbaum et al., 2003; Brady et al., 2007).

1. FORMING A LATERAL ROOT

Auxin triggers responses at the molecular and cellular level regulating the majority of the events leading to the formation of a new LR (reviewed by Overvoorde et al., 2010) (Figure 6). A well characterized auxin signaling pathway involves the receptor TRANSPORT INHIBITOR RESPONSE1 (TIR1), which is the F-box component that provides substrate specificity for the E3 ubiquitin-ligase complex Skp1-Cul1-F-box (SFC). The stabilized interaction of auxin with the SCF^{TIR1} leads to ubiquitination of Aux/IAA proteins and the degradation of these TFs by the 26S proteasome. Such degradation releases the repression of the Auxin Responsive Factors (ARFs) that regulate, by activation or repression, early auxin responsive genes (reviewed by Chapman and Estelle, 2009). Defined combinations of 29 Aux/IAAs and 23 ARFs transcription factors integrate specific responses to diverse signals. Such responses are reflected in the diverse array of phenotypes found in Aux/IAA and ARF mutants. Some of the mutants that have been characterized to be involved in known molecular or cellular processes during lateral root formation, are presented by Péret et al. (2009b).

1.1 Giving a cell fate to specific xylem pole pericycle cells

Lateral roots originate in the pericycle cells next to the XP (XPP cells). Some XPP cells are designated as pericycle founder cells (PFC). These cells pass through different processes

such as initiation, patterning and emergence to finally form a functional LR (reviewed by Péret et al., 2009b). XPP cells get the PFC fate in a zone just above the RAM, called basal meristem, where temporal fluctuations of the hormone auxin will prime XPP cells to divide further on via auxin signaling in the maturation zone of the root and, guarantee the spatial distribution of LRs along the main root (De Smet et al., 2007). Recently, one of the molecular components of this process has been identified (De Rybel et al., 2010). The GATA23 TF was identified by analysis of lateral root-related transcriptomic data (Parizot et al., 2010), its expression in the basal meristem correlates with the oscillating auxin signaling maxima and it is controlled by an Aux/IAA28 dependent auxin signaling mechanism. After leaving the RAM, the pericycle cells next to the phloem pole stay in G1-phase of the cell cycle, while those XPP cells advance to G2-phase preparing themselves to start cell divisions later on in the maturation zone (Beeckman et al., 2001). In this process, the AFL4 nuclear protein is needed to keep the XPP cells in a mitosis-competent state since the mitotic marker *CyclinB1;1::GUS* induction is blocked in the *afl4* mutant, while the cell cycle marker *Cyclin Dependent Kinase B;1* (*CDKB;1::GUS*) is upregulated (DiDonato et al., 2004).

1.2 Spatial distribution of lateral roots

New lateral roots are formed in a regulated spatial pattern in the main root. Auxin is the main player in this even distribution of LRs. Its basipetal transport from the shoot through the central cylinder of the main root toward the root tip , from where it will be redistributed acropetally through the LR cap and epidermis (Luschnig et al., 1998; Müller et al., 1998; Friml et al., 2003), creates a reflux loop important for priming of XPP cells and also for gravity responses. The gravity responses produce the rhythmic waving of the root promoting the left-right positional pattern of LRs (De Smet et al., 2007) from the PFCs that are primed in the basal meristem. However, tropic and mechanical stimuli can trigger lateral root formation (LRF), allowing the primary root to adapt its architecture when needed. For example, auxin application above the basal meristem can induce LRF as well as natural gravitropic curvature or mechanical bending of the root (Lucas et al., 2008). This is due to an accumulation of auxin that triggers cell divisions in the pericycle cells. The changes in the size of the outer and inner side cells of the bend modulate the auxin fluxes and generate an auxin maximum precisely in the outer side cells of the bend (Ditengou et al., 2008; Laskowski et al., 2008). Moreover, Ca^{2+} concentrations in pericycle and adjacent tissue correlates with the mechanical stretching, and define the bending response as well (Richter et al., 2009). Seemingly the auxin oscillatory mechanism is not the only one inducing positioning of LRP and an additional pathway, dependent on the mechanical bending, is expected as well. Using these mechanisms, the plant can modulate its root architecture in response to endogenous and external stimuli (Péret et al., 2009b).

1.3 Lateral root initiation

Several millimeters distal to the RAM the first pericycle cell divisions are detected as earliest steps to forming a lateral root primordium (LRP) (Casimiro et al., 2003). One or two PFC undergo continuous rounds of anticlinal cell divisions to form a single layer primordium (Malamy and Benfey, 1997; Dubrovsky et al., 2001; Casimiro et al., 2003) that can have up to ten small cells of equal length (stage I). In stage II, periclinal divisions start forming a two layered primordium. Rounds of divisions keep on going, shaping a dome primordium (stages III-VII) that has to pass through the endodermis, cortex and epidermis cell layers to finally emerge (stage VIII) (Dubrovsky et al., 2001; Casimiro et al., 2003) (Figure 5).

1.3.1 Asymmetric cell divisions as a starting point for lateral root initiation

In stage I, the first asymmetric cell divisions of pairs of PFC lead to shorter cells in the center of the primordium and longer cells flanking it. These asymmetric cell divisions are known to give different cell fates to the daughter cells (Scheres and Benfey, 1999; reviewed by De Smet and Beeckman, 2011). The central cells that will form the LRP express marker genes associated with asymmetric cell divisions (De Smet et al., 2008), while the flanking cells do not. One of these markers is a receptor-like kinase *ARABIDOPSIS CRINKLY4* (*ACR4*), which seems to influence the specification of cell fate during LR initiation. In mutants lacking *ACR4*, LR primordia develop one next to the other or at the wrong place in opposite XP. This indicates that this kinase is important to restrict cell divisions in the adjacent cells of the primordia (De Smet et al., 2008). Weak alleles of the *ADP RIBOSYLATION FACTOR-GUANIDINE EXCHANGE FACTOR* (*ARF-GEF*) or *GNOM*, a protein that regulates the trafficking of PIN auxin efflux carrier proteins (Steinmann et al., 1999), are not able to form normal LRP upon auxin induction or fail to express asymmetric markers like ACR4. Thus GNOM seems to be required for the first asymmetrical divisions. In other mutants that are impaired to initiate LR such as the dominant auxin signaling mutant *solitary root1* (*slr1/iaa14*) transcript profiling indicates repression of a group of cell cycle genes. The overexpression of the key cell cycle gene in the transition from G1-to-S phase, D-type cyclin (*CYCD3;1*), in the *slr1* mutant activates divisions in the pericycle but fails to produce lateral roots (Vanneste et al., 2005) and induces ACR4 (De Smet et al., 2008). The authors suggest that cell cycle activation together with cell specification are needed to initiate lateral roots. Additionally, in diving pericycle cells *KIP-RELATED PROTEIN1* (*KRP1*) and *KRP2* are downregulated by auxin, whereas *Cyclin-Dependent Kinases* (*CDKs*) are upregulated. *KRPs* are repressors of *CDKs* inhibiting the G1-to-S transition which indicates that the checkpoint in G1-to-S is one of the targets for auxin-mediated LR initiation (Himanen et al., 2002; Vanneste et al., 2005). In the double mutant *auxin responsive factor 7, 19* (*arf7arf19*) that also fails to start LR, the inducible overexpression of two of their target genes, *LATERAL ORGAN BOUNDARIES-DOMAIN16/ASYMMETRIC LEAVES2-LIKE18* (*LBD16*/*ASL18*) and *LBD29*/*ASL16* complement

the mutant phenotype (Okushima et al., 2007). These two TFs seem to have a role in LR patterning acting directly downstream of ARF7 and ARF19 via auxin regulation.

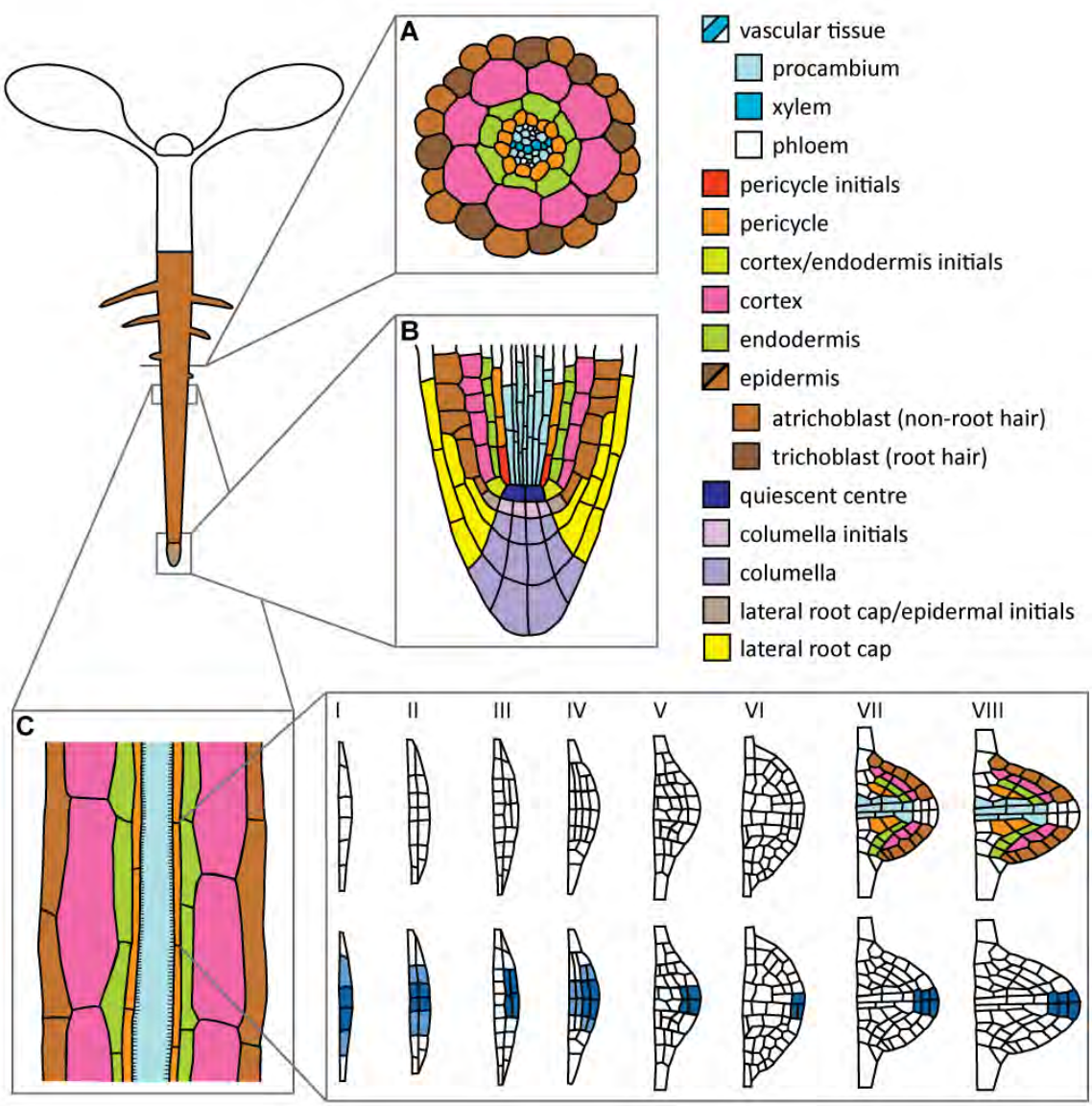


Figure 5. Map of root cell types and lateral root initiation in *Arabidopsis*. Adapted from Jansen et al. (2010) and Péret et al. (2009b). **A.** Radial distribution of cellular types in mature roots. **B.** Longitudinal view of the root apical meristem (RAM) tissue distribution. **C.** Close up to the zone where pericycle founder cells (PFC) start diving to form a new lateral. In the inset are shown all the stages for the development of a lateral root (LR) (top). This process is coordinated by the establishment of an auxin maximum (blue cells) that will pattern the primordium (bottom).

1.4 The emergence of a new meristem

Formation of a LR strongly impacts the primary root structure as endodermal, cortical and epidermal cells have to be detached and remodeled to allow the emergence of the LR. Epidermal cells are strongly attached before a new LR starts to form, however, at the time that the primordium is forming it pushes the overlaying cells, which in turn will separate

to allow contact of the new LR with the rhizosphere (reviewed by Péret et al., 2009b). This process is coordinated by auxin derived from the shoot via phloem or polar auxin transport (Bhalerao et al., 2002; Ljung et al., 2005), whereas LR initiation is regulated by auxin originated in the root tip (Casimiro et al., 2001). A number of genes working in cell wall remodeling have been reported to be expressed in front of the emerging LR primordium. They include polygalacturonase (PG) and pectin lyase (PLA2) that can cleave pectin polymers in the cell wall, xyloglucan:xyloglucosyl transferase (XTR6) that induce cell wall loosening, expansin (EXP17) and subtilisin-like protease (Cosgrove, 2000; Marin-Rodriguez et al., 2002; Vissenberg et al., 2005). Auxin influx lead by AUX1-Like 3 (LAX3) has a lot of implications in LR emergence since in its mutant, *lax3*, LR initiation is not impaired while LR emergence is. LAX3 is expressed in the cortical and epidermal cells in front of the primordium, but not in the primordium itself, suggesting that the expression of cell wall remodeling enzymes (CWR) regulated by auxin, depends on the local auxin signal from the primordium (Swarup et al., 2008a). Importantly, LAX3 generates a feedback loop for accumulation of auxin in the overlying cells while restricting auxin to go to adjacent tissues (Péret et al., 2009a). However in the endodermal cells the mechanism is different. There, the Aux/IAA3 (SHY2) repressor modulates a set of cell wall remodeling genes that differ from the LAX3 induced genes, because of the differences in cell wall composition between different root layers (Péret et al., 2009a). A strict coordination of auxin signals and fluxes and CWR enzyme responses is needed to remodel the root tissues and allow the emergence of a new LR conserving the integrity of the adjacent tissues. At the end, a new meristem is born that will take the control of the mature LR (Dubrovsky et al., 2008; Swarup et al., 2008b).

2. BUT AUXIN DOES NOT DO ALL THE WORK FORMING A LATERAL ROOT

The mechanisms of auxin-mediated LR formation have been extensively studied, but this does not mean that other endogenous and exogenous cues do not govern the process. Hormones are an important part of the signaling guiding this organogenic process (reviewed by Fukaki and Tasaka, 2009).

2.1 Cytokinin as antagonist of auxin inhibits LR formation

Cytokinin (CK) is known to play an antagonistic role to auxin in plant growth and development. Mutants in CK biosynthesis and signaling components with reduced CK levels or sensitivity, such as an overexpressors of cytokinin oxidase (*CKX*) (Werner et al., 2003; Lohar et al., 2004) and cytokinin receptors *histidine kinase*s *AHK2*, *AHK3* and *CRE1/AHK4*, display a faster growth and increased number of LR (Riefler et al., 2006), a phenotype that was also reported for mutants in *Type-B Arabidopsis Response Regulators* (*ARRs*) (Mason et al., 2005). Exogenous cytokinin inhibits LR formation by blocking PFC cycling during G2-to-M phase, and promotes LR elongation by activating the G1-to-S

transition (Li et al., 2006). However, this effect was not displayed in auxin signaling and transport deficient mutants, in which auxin application could not revert the blocking effect in LR initiation but, still was able to activate cell divisions. This suggests that accumulation of CK in the FPC arrests the LR developmental program without affecting the auxin-mediated activation of cell divisions (Li et al., 2006; Laplaze et al., 2007). This is in fact due to the inhibitory effect of CK in the expression of *PIN* genes and in the localization of these proteins in the LR primordium that disrupts the establishment of the auxin gradient needed for LR initiation (Laplaze et al., 2007).

2.2 ABA inhibits LR emergence, but is also regulated by and regulates auxin in LR formation

Another negative regulator of LR formation is the hormone abscisic acid (ABA). Studies with ABA signaling mutants demonstrate that this hormone mediates LR emergence and also has distinct roles during different moments of LR formation (reviewed by De Smet et al., 2006). Exogenously applied ABA arrests the emergence of LR primordium before the LR meristem is activated, and auxin could not rescue this phenotype. This indicates that there is a ABA check-point independent from auxin regulating emergence of LRs (De Smet et al., 2003). However, overlapping signals of auxin and ABA exist as well. ABI3 is auxin-inducible in LR primordia and is required for normal responsiveness of the LR primordium to applied auxin and auxin transport inhibitor. Moreover, a mutant of an ABI3 upstream transcription factor *ENHANCED RESPONSE TO ABA1* (*era1*), has more LRs (Brady et al., 2003). On the other hand, mutants involved in auxin signaling such as *axr2/iaa7*, *slr1/iaa14* and *indole-3-butyric acid response5* (*ibr5*) (Wilson et al., 1990; Fukaki et al., 2002; Strader et al., 2008) have different responses to ABA, this suggests that auxin as well as ABA signaling disruption have implications in the other hormone LR-mediated responses. ABA has also been reported to have a role in nitrate-mediated LR formation (Signora et al., 2001), and auxin is also involved in this response (Beeckman and Friml, 2010; Krouk et al., 2010).

2.3 Other hormones affect LR formation by modulating auxin responses

Brasinosteroids (BR) and auxin promote LR formation synergistically (Bao et al., 2004) as they do with other developmental processes in plants (Clouse, 2011). A mutant in a BR receptor (*bri1)* displays less LRs than wild-type, it is insensitive to BRs and has a reduced expression of the auxin-responsive marker *DR5::GUS*. The mechanism appears to be associated with the promotion of acropetal auxin transport in the root by BRs, and concomitant increase in LR initiation (Bao et al., 2004). Another hormone, ethylene, alters auxin transport as well but acts negatively in LR formation (Negi et al., 2008). Application of the ethylene precursor, ACC, inhibits initiation of LR primordia but activates the emergence of existing LR primordia. The inhibition of LR initiation is overruled by auxin

application only in the primordia that were already dividing. The same phenotype was observed in the ethylene overproducer *eto1* (Ivanchenko et al., 2008).

It is clear that LR formation is governed by a complex hormonal network with auxin as a central player, however a lot of research needs to be done to connect perception, synthesis and transport of these hormones with their effects in LR formation.

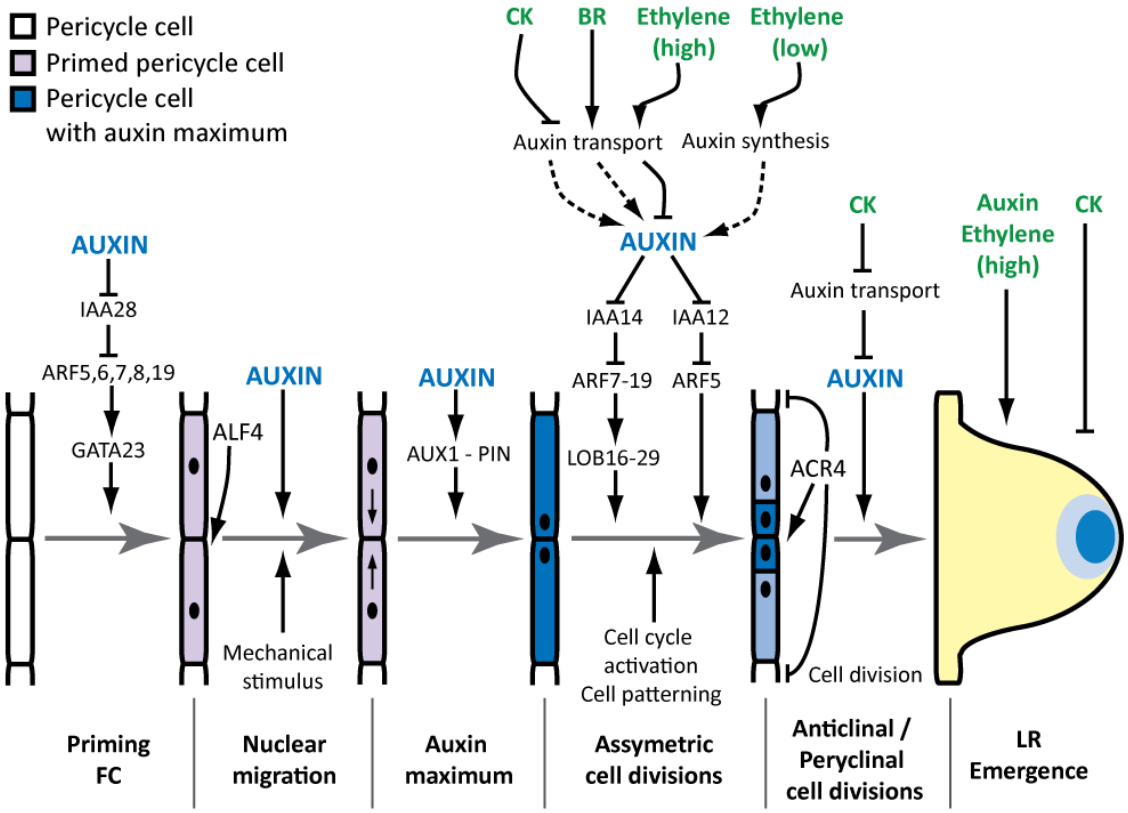


Figure 7. Molecular and hormonal map of lateral root initiation, formation and development. Based on De Rybel et al.(2010) , Péret at al.(2009a) , Fukaki and Tasaka (2009). Auxin (in blue) is the main player controlling LR initiation, its transport is necessary for priming of the FC in the basal meristem of the root. Before the first asymmetric divisions, nuclei of PFC migrate to the common cell wall, and an auxin maximum is generated to trigger cell division that will lead to a fully formed lateral root. Hormones (in green) are also important components in LR formation but mainly through regulation of auxin synthesis and transport. The molecular components are explained in detail in the text.

3. THE NEED FOR SUGARS FOR LR DEVELOPMENT

3.1 Glucose regulates several genes involved in auxin signaling

Sugars have important impacts during the whole plant life cycle by regulating metabolism, development and growth in response to environmental and endogenous cues. Increasing concentrations of sugars such as glucose increase root length and number of lateral roots until certain concentration but, passing that level, the effect is the opposite (Mishra et al.,

2009). In the glucose insensitive mutant *gin2*, the effect of high glucose in the root is the same as in wild-type but to a lesser extend. Importantly, this mutant is resistant to auxin because its reduced LR root formation upon exogenous auxin application, as well as *HXK-Like* (*HXKL1*) overexpressor mutant that lacks glucose catalytic activity (Moore et al., 2003; Karve and Moore, 2009). To study further the glucose and auxin interaction, Mishra and coworkers (2009) analyzed the effect on gene expression in plants treated with different concentrations of glucose with or without auxin. A large overlap in glucose and IAA regulated genes was found, but most importantly, glucose was found to affect several important genes for auxin biosynthesis, perception, signaling and transport. The auxin receptor *TIR1* was downregulated by glucose, while *YUCCA2*, involved in auxin biosynthesis was upregulated. *PIN2* was upregulated by glucose as well leading to an increase in basipetal auxin transport. Moreover, when mutants on auxin perception such as *tir1* and signaling including *slr1/iaa14*, *axr3/iaa17* and *axr2/iaa7*, where grown on glucose they were less responsive to glucose induction of root length and LR emergence.

3.2 Auxin mediated LR formation shows a bimodular effect under glucose

Another study revealed that auxin and glucose applied in the absence of the other increased the number of LR and LR primordia, but the effect of auxin alone was greater, indicating that auxin is the primary signal for LR formation. However, when auxin and glucose were applied together the interaction does not create an additive effect in LR formation. Adding glucose increases the number of auxin-induced lateral roots up to 0.03µM of auxin. At high levels of both components there is a plateau effect in the number of LR and LR primordia, and at high auxin levels increasing glucose beyond 0.3% reduced the number of LR and LR primordia (Booker et al., 2010). Since auxin alone has a linear effect on LR formation but addition of glucose to auxin does not have a linear effect in this response, auxin displays a bimodular effect in LR formation upon exogenous glucose. To further test these effects, mutants in the G protein complex where subjected to auxin treatments. The heterotrimeric G-protein has a role in sugar-related responses, it has one Gα subunit (GPA1), one Gβ subunit (AGB1) and two Gγ subunits (AGG1, AGG2). In the *agb1-2 gpa1-4* double mutant, or single mutants, in which the heterotrimer or the different subunits are removed, the auxin bimodal effect is enhanced. However when the receptor of D-glucose at the plasma membrane, *REGULATOR OF G-protein SIGNALING1* (*RGS1*), is removed, the glucose effect is gone. This indicates that the perception of glucose is required to have the bimodal auxin response in LR formation.

3.3 Heterotrimeric G protein regulate LR formation by modulating auxin transport

Interestingly, null mutations in the AGB1 and AGG subunits produce more lateral roots than wild-type and are hypersensitive to auxin induction of LR formation (Trusov et al., 2007), whereas the opposite was observed in *gpa1* mutants (Ullah et al., 2003). The specific expression of the AGG1 in the central stele and of AGG2 in the cortex and

epidermis, correlates with the basipetal and acropetal transport of auxin. Mutant analysis blocking each auxin flux revealed that the G-protein dimer Gβγ1 represses LR root development by reducing the acropetal auxin flux, whereas the Gβγ2 inhibits LR formation through the cortex and epidermis by impairing basipetal auxin transport (Trusov et al., 2007). An interactor with the Gβγ dimmers, N-MYC DOWNREGULATED-LIKE1 (NDL1) was described to have a positive role in LR formation. NDL1 affects auxin transport in an AGB1-dependent manner by favoring basipetal and reducing acropetal auxin transport, probably by regulating the PIN2 and AUX1 auxin transporters. A feedback mechanism regulates NDL1 levels. They increase with D-glucose and AGB1, but decrease in an auxin-concentration manner creating a negative-feedback loop. However, as AGB1 is positively regulated by auxins, it generates a positive-feedback loop (Mudgil et al., 2009).

3.4 Osmotic-stress inhibition of LR formation requires the neutral invertase CYT-INV1

A mutant encoding a neutral invertase, *Cytoplasmic Invertase 1* (*cyt-inv1*), was characterized to be insensitive to the repression on LR development under high concentrations of KNO_3, KCl or mannitol (Qi et al., 2007). This unique neutral invertase cleaves sucrose into glucose and fructose and is very specific for this sugar (Qi et al., 2007). High concentrations of KNO_3 or KCl can produce osmotic potentials as mannitol does, thus inhibiting LR formation (Signora et al., 2001; Deak and Malamy, 2005). *cyt-inv1* was insensitive to the inhibition of LR development under these osmotic conditions. This mutant did not respond with reduced mitosis as wild-type under osmotic conditions, but the LR inhibitory effect of osmotic stress was restored by ABA and glucose. The accumulation of sucrose and lack of glucose in the mutant may explain its role during osmotic stress. First, as glucose induces ABA biosynthesis and activates its signaling pathway, in the *cyt-inv1* stress responses are diminished because of low ABA biosynthesis. And secondly, CYT-INV1 cleaves sucrose into hexoses under osmotic-stress conditions elevating the osmotic potential of the cells locally (Koch, 2004) and promoting the sugar signaling pathway (Qi et al., 2007).

3.5 Sucrose uptake in the shoot is needed for LR formation

Osmotic stress not only slows down the development of the root system but highly affects its architecture. The repression of LR primordia emergence is ABA-dependent, but auxin can surpass this program with or without osmotic stress (Deak and Malamy, 2005). *lateral root development2* (*lrd2*), was found in a screen for mutants with highly branched root system under mild osmotic stress conditions (Macgregor et al., 2008). *LRD2* is an allele of *Long-Chain Fatty Acid Synthase 2* (*LACS2*) playing a role in cutin formation and aerial tissue permeability. Mutants in cutin formation resembled the LR phenotype of *lrd2* under mild osmotic stress conditions by attenuating the permeability reduction in the shoot. Interestingly, preventing the contact of the shoot with the medium arrested LR

emergence in wild-type, a phenotype that was rescued by adding sucrose to the isolated shoot grown on non-stress media but not on mild osmotic stress media. This means that a long distance communication between the shoot and root is required for sucrose to induce LR formation in plants grown *in vitro*, and this mechanism is inhibited under stress conditions. Exogenous sucrose could enter the shoot through the apoplast and join the sucrose pool coming from photosynthesis (Lalonde et al., 2004), be transported to the root and there, supply energy for LR formation. This is plausible as it is known that stimuli increasing photosynthesis and photosynthates, such as light or CO_2, have a positive effect in LR development (Crookshanks et al., 1998; Reed et al., 1998). ABA is known to regulate stress responses, and as such it was observed that roots grown on ABA media reduced permeability in the isolated shoot, thus blocking the permeability to exogenous sucrose with concomitant restriction on LR development (Macgregor et al., 2008).

Tre6P and trehalose have evolved from an obscure function to coordinators of carbohydrate metabolism with growth and development, mainly through Tre6P being a signal of sugar status. However, still little is known about the exact function of this molecule and the role of the trehalose metabolic enzymes in plants. Plant growth and development are governed by sugar utilization and hormone signals, but still there is a lot to uncover about crosstalk of these elements. The possibility of placing Tre6P and trehalose metabolism as an important part of this complex network of interactions is emerging, but the road to depict it is still in question.

4. REFERENCES

Arenas-Huertero, F., Arroyo, A., Zhou, L., Sheen, J., and Leon, P. (2000). Analysis of Arabidopsis glucose insensitive mutants, gin5 and gin6, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. Genes Dev **14,** 2085-2096.

Arguelles, J.C. (2000). Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. Arch Microbiol **174,** 217-224.

Avonce, N., Mendoza-Vargas, A., Morett, E., and Iturriaga, G. (2006). Insights on the evolution of trehalose biosynthesis. BMC Evol Biol **6,** 109.

Avonce, N., Wuyts, J., Verschooten, K., Vandesteene, L., and Van Dijck, P. (2010). The Cytophaga hutchinsonii ChTPSP: First Characterized Bifunctional TPS–TPP Protein as Putative Ancestor of All Eukaryotic Trehalose Biosynthesis Proteins. Mol Biol Evol **27,** 359-369.

Avonce, N., Leyman, B., Mascorro-Gallardo, J.O., Van Dijck, P., Thevelein, J.M., and Iturriaga, G. (2004). The Arabidopsis Trehalose-6-P Synthase AtTPS1 Gene Is a Regulator of Glucose, Abscisic Acid, and Stress Signaling. Plant Physiol **136,** 3649-3659.

Bae, H., Herman, E., Bailey, B., Bae, H.J., and Sicher, R. (2005). Exogenous trehalose alters Arabidopsis transcripts involved in cell wall modification, abiotic stress, nitrogen metabolism, and plant defense. Physiologia Plantarum **125,** 114-126.

Baena-Gonzalez, E. (2010). Energy signaling in the regulation of gene expression during stress. Mol Plant **3,** 300-313.

Baena-Gonzalez, E., and Sheen, J. (2008). Convergent energy and stress signaling. Trends Plant Sci **13,** 474-482.

Baena-Gonzalez, E., Rolland, F., Thevelein, J.M., and Sheen, J. (2007). A central integrator of transcription networks in plant stress and energy signalling. Nature **448,** 938-942.

Bao, F., Shen, J., Brady, S.R., Muday, G.K., Asami, T., and Yang, Z. (2004). Brassinosteroids interact with auxin to promote lateral root development in Arabidopsis. Plant Physiol **134,** 1624-1631.

Beeckman, T., and Friml, J. (2010). Nitrate contra auxin: nutrient sensing by roots. Developmental Cell **18,** 877-878.

Beeckman, T., Burssens, S., and Inzé, D. (2001). The peri-cell-cycle in *Arabidopsis*. J Exp Bot **52,** 403-411.

Bell, W., Sun, W., Hohmann, S., Wera, S., Reinders, A., De Virgilio, C., Wiemken, A., and Thevelein, J.M. (1998). Composition and functional analysis of the Saccharomyces cerevisiae trehalose synthase complex. J Biol Chem **273,** 33311-33319.

Bhalerao, R.P., Eklof, J., Ljung, K., Marchant, A., Bennett, M., and Sandberg, G. (2002). Shoot-derived auxin is essential for early lateral root emergence in Arabidopsis seedlings. Plant J **29,** 325-332.

Bianchi, G., Gamba, A., Limiroli, R., Pozzi, N., Elster, R., Salamini, F., and Bartels, D. (1993). The unusual sugar composition in leaves of the resurrection plant Myrothamnus flabellifolia. Physiologia Plantarum **87,** 223-226.

Birnbaum, K., Shasha, D.E., Wang, J.Y., Jung, J.W., Lambert, G.M., Galbraith, D.W., and Benfey, P.N. (2003). A gene expression map of the Arabidopsis root. Science **302,** 1956-1960.

Blazquez, M.A., and Gancedo, C. (1995). Mode of action of the qcr9 and cat3 mutations in restoring the ability of Saccharomyces cerevisiae tps1 mutants to grow on glucose. Mol Gen Genet **249,** 655-664.

Blazquez, M.A., Lagunas, R., Gancedo, C., and Gancedo, J.M. (1993). Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases. FEBS Letters **329,** 51-54.

Blázquez, M.A., Santos, E., Flores, C.-l., Martínez-Zapater, J.M., Salinas, J., and Gancedo, C. (1998). Isolation and molecular characterization of the Arabidopsis TPS1 gene, encoding trehalose-6-phosphate synthase. Plant J **13,** 685-689.

Bonini, B.M., Van Vaeck, C., Larsson, C., Gustafsson, L., Ma, P., Winderickx, J., Van Dijck, P., and Thevelein, J.M. (2000). Expression of Escherichia coli otsA in a Saccharomyces cerevisiae tps1 mutant restores trehalose 6-phosphate levels and partly restores growth and fermentation with glucose and control of glucose influx into glycolysis. Biochem J **350 Pt 1,** 261-268.

Booker, K.S., Schwarz, J., Garrett, M.B., and Jones, A.M. (2010). Glucose attenuation of auxin-mediated bimodality in lateral root formation is partly coupled by the heterotrimeric G protein complex. PLoS One **5,** e12833.

Brady, S.M., Sarkar, S.F., Bonetta, D., and McCourt, P. (2003). The ABSCISIC ACID INSENSITIVE 3 (ABI3) gene is modulated by farnesylation and is involved in auxin signaling and lateral root development in Arabidopsis. Plant J **34,** 67-75.

Brady, S.M., Orlando, D.A., Lee, J.Y., Wang, J.Y., Koch, J., Dinneny, J.R., Mace, D., Ohler, U., and Benfey, P.N. (2007). A high-resolution root spatiotemporal map reveals dominant expression patterns. Science **318,** 801-806.

Casimiro, I., Beeckman, T., Graham, N., Bhalerao, R., Zhang, H., Casero, P., Sandberg, G., and Bennett, M.J. (2003). Dissecting Arabidopsis lateral root development. Trends Plant Sci **8,** 165-171.

Casimiro, I., Marchant, A., Bhalerao, R.P., Beeckman, T., Dhooge, S., Swarup, R., Graham, N., Inzé, D., Sandberg, G., Casero, P.J., and Bennett, M. (2001). Auxin transport promotes *Arabidopsis* lateral root initiation. Plant Cell **13,** 843-852.

Chapman, E.J., and Estelle, M. (2009). Mechanism of Auxin-Regulated Gene Expression in Plants. Annu Rev Genet **43,** 265-285.

Chary, S.N., Hicks, G.R., Choi, Y.G., Carter, D., and Raikhel, N.V. (2008). Trehalose-6-phosphate synthase/phosphatase regulates cell shape and plant architecture in Arabidopsis. Plant Physiol **146,** 97-107.

Cheng, W.H., Endo, A., Zhou, L., Penney, J., Chen, H.C., Arroyo, A., Leon, P., Nambara, E., Asami, T., Seo, M., Koshiba, T., and Sheen, J. (2002). A unique short-chain dehydrogenase/reductase in Arabidopsis glucose signaling and abscisic acid biosynthesis and functions. Plant Cell **14,** 2723-2743.

Chiou, T.J., and Bush, D.R. (1998). Sucrose is a signal molecule in assimilate partitioning. Proc Natl Acad Sci U S A **95,** 4784-4788.

Clouse, S.D. (2011). Brassinosteroid Signal Transduction: From Receptor Kinase Activation to Transcriptional Networks Regulating Plant Development. Plant Cell.

Cortina, C., and Culianez-Macia, F.A. (2005). Tomato abiotic stress enhanced tolerance by trehalose biosynthesis. Plant Science **169,** 75-82.

Coruzzi, G.M., and Zhou, L. (2001). Carbon and nitrogen sensing and signaling in plants: emerging 'matrix effects'. Curr Opi Plant Biol **4,** 247-253.

Cosgrove, D.J. (2000). Loosening of plant cell walls by expansins. Nature **407,** 321-326.

Crookshanks, M., Taylor, G., and Dolan, L. (1998). A model system to study the effects of elevated CO2 on the developmental physiology of roots: the use of Arabidopsis thaliana. J Exp Bot **49,** 593-597.

De Rybel, B., Vassileva, V., Parizot, B., Demeulenaere, M., Grunewald, W., Audenaert, D., Van Campenhout, J., Overvoorde, P., Jansen, L., Vanneste, S., Moller, B., Wilson, M., Holman, T., Van Isterdael, G., Brunoud, G., Vuylsteke, M., Vernoux, T., De Veylder, L., Inze, D., Weijers, D., Bennett, M.J., and Beeckman, T. (2010). A novel aux/IAA28 signaling cascade activates GATA23-dependent specification of lateral root founder cell identity. Curr Biol **20,** 1697-1706.

De Smet, I., and Beeckman, T. (2011). Asymmetric cell division in land plants and algae: the driving force for differentiation. Nature reviews. Molecular cell biology **12,** 177-188.

De Smet, I., Zhang, H., Inze, D., and Beeckman, T. (2006). A novel role for abscisic acid emerges from underground. Trends Plant Sci **11,** 434-439.

De Smet, I., Signora, L., Beeckman, T., Inze, D., Foyer, C.H., and Zhang, H. (2003). An abscisic acid-sensitive checkpoint in lateral root development of Arabidopsis. Plant J **33,** 543-555.

De Smet, I., Tetsumura, T., De Rybel, B., Frey, N.F., Laplaze, L., Casimiro, I., Swarup, R., Naudts, M., Vanneste, S., Audenaert, D., Inze, D., Bennett, M.J., and Beeckman, T. (2007). Auxin-dependent regulation of lateral root positioning in the basal meristem of Arabidopsis. Development **134,** 681-690.

De Smet, I., Vassileva, V., De Rybel, B., Levesque, M.P., Grunewald, W., Van Damme, D., Van Noorden, G., Naudts, M., Van Isterdael, G., De Clercq, R., Wang, J.Y., Meuli, N., Vanneste, S., Friml, J., Hilson, P., Jurgens, G., Ingram, G.C., Inze, D., Benfey, P.N., and Beeckman, T. (2008). Receptor-like kinase ACR4 restricts formative cell divisions in the Arabidopsis root. Science **322,** 594-597.

Deak, K.I., and Malamy, J. (2005). Osmotic regulation of root system architecture. Plant J **43,** 17-28.

DiDonato, R.J., Arbuckle, E., Buker, S., Sheets, J., Tobar, J., Totong, R., Grisafi, P., Fink, G.R., and Celenza, J.L. (2004). Arabidopsis ALF4 encodes a nuclear-localized protein required for lateral root formation. Plant J **37,** 340-353.

Ditengou, F.A., Teale, W.D., Kochersperger, P., Flittner, K.A., Kneuper, I., van der Graaff, E., Nziengui, H., Pinosa, F., Li, X., Nitschke, R., Laux, T., and Palme, K. (2008). Mechanical induction of lateral root initiation in Arabidopsis thaliana. Proc Natl Acad Sci U S A **105,** 18818-18823.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., and Scheres, B. (1993). Cellular-Organization of the Arabidopsis-Thaliana Root. Development **119,** 71-84.

Dubrovsky, J.G., Rost, T.L., Colon-Carmona, A., and Doerner, P. (2001). Early primordium morphogenesis during lateral root initiation in Arabidopsis thaliana. Planta **214,** 30-36.

Dubrovsky, J.G., Sauer, M., Napsucialy-Mendivil, S., Ivanchenko, M.G., Friml, J., Shishkova, S., Celenza, J., and Benkova, E. (2008). Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. Proc Natl Acad Sci U S A **105,** 8790-8794.

Eastmond, P.J., Van Dijken, A.J.H., Spielman, M., Kerr, A., Tissier, A.F., Dickinson, H.G., Jones, J.D.G., Smeekens, S.C., and Graham, I.A. (2002). Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for Arabidopsis embryo maturation. Plant J **29,** 225-235.

El-Bashiti, T., Hamamci, H., Oktem, H.A., and Yucel, M. (2005). Biochemical analysis of trehalose and its metabolizing enzymes in wheat under abiotic stress conditions. Plant Science **169,** 47-54.

Elbein, A.D., Pan, Y.T., Pastuszak, I., and Carroll, D. (2003). New insights on trehalose: a multifunctional molecule. Glycobiology **13,** 17R-27R.

Elliott, B., Haltiwanger, R.S., and Futcher, B. (1996). Synergy between trehalose and Hsp104 for thermotolerance in Saccharomyces cerevisiae. Genetics **144,** 923.

Ernandes, J.R., De Meirsman, C., Rolland, F., Winderickx, J., de Winde, J., Brandao, R.L., and Thevelein, J.M. (1998). During the initiation of fermentation overexpression of hexokinase PII in yeast transiently causes a similar deregulation of glycolysis as deletion of Tps1. Yeast **14,** 255-269.

Fougere, F., Le Rudulier, D., and Streeter, J.G. (1991). Effects of Salt Stress on Amino Acid, Organic Acid, and Carbohydrate Composition of Roots, Bacteroids, and Cytosol of Alfalfa (Medicago sativa L.). Plant Physiol **96,** 1228-1236.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jürgens, G. (2003). Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. Nature **426,** 147-153.

Fritzius, T., Aeschbacher, R., Wiemken, A., and Wingler, A. (2001). Induction of ApL3 expression by trehalose complements the starch-deficient Arabidopsis mutant adg2-1 lacking ApL1, the large subunit of ADP-glucose pyrophosphorylase. Plant Physiol **126,** 883-889.

Fukaki, H., and Tasaka, M. (2009). Hormone interactions during lateral root formation. Plant Mol Biol **69,** 437-449.

Fukaki, H., Tameda, S., Masuda, H., and Tasaka, M. (2002). Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of Arabidopsis. Plant J **29,** 153-168.

Gancedo, C., and Flores, C.L. (2004). The importance of a functional trehalose biosynthetic pathway for the life of yeasts and fungi. FEMS Yeast Res **4,** 351-359.

Garg, A.K., Kim, J.K., Owens, T.G., Ranwala, A.P., Choi, Y.D., Kochian, L.V., and Wu, R.J. (2002). Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci U S A **99,** 15898-15903.

Geelen, D., Royackers, K., Vanstraelen, M., De Bus, M., Inzé, D., Van Dijck, P., Thevelein, J.M., and Leyman, B. (2007). Trehalose-6-P synthase AtTPS1 high molecular weight complexes in yeast and Arabidopsis. Plant Science **173,** 426-437.

Goddijn, O.J., Verwoerd, T.C., Voogd, E., Krutwagen, R.W., de Graaf, P.T., van Dun, K., Poels, J., Ponstein, A.S., Damm, B., and Pen, J. (1997). Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. Plant Physiol **113,** 181-190.

Gómez, L.D., Baud, S., Gilday, A., Li, Y., and Graham, I.A. (2006). Delayed embryo development in the ARABIDOPSIS TREHALOSE-6-PHOSPHATE SYNTHASE 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. Plant J **46,** 69-84.

Gómez, L.D., Gilday, A., Feil, R., Lunn, J.E., and Graham, I.A. (2010). AtTPS1-mediated trehalose 6-phosphate synthesis is essential for embryogenic and vegetative growth and responsiveness to ABA in germinating seeds and stomatal guard cells. Plant J **64,** 1-13.

Gutierrez, R.A., Lejay, L.V., Dean, A., Chiaromonte, F., Shasha, D.E., and Coruzzi, G.M. (2007). Qualitative network models and genome-wide expression data define carbon/nitrogen-responsive molecular machines in Arabidopsis. Genome Biol **8,** R7.

Himanen, K., Boucheron, E., Vanneste, S., de Almeida Engler, J., Inzé, D., and Beeckman, T. (2002). Auxin-mediated cell cycle activation during early lateral root initiation. Plant Cell **14,** 2339-2351.

Hohmann, S., Neves, M.J., de Koning, W., Alijo, R., Ramos, J., and Thevelein, J.M. (1993). The growth and signalling defects of the ggs1 (fdp1/byp1) deletion mutant on glucose are suppressed by a deletion of the gene encoding hexokinase PII. Curr Genet **23,** 281-289.

Holmström, K.-O., Mantyla, E., Welin, B., Mandal, A., Palva, E.T., Tunnela, O.E., and Londesborough, J. (1996). Drought tolerance in tobacco. Nature **379,** 683-684.

Horlacher, R., Uhland, K., Klein, W., Ehrmann, M., and Boos, W. (1996). Characterization of a cytoplasmic trehalase of Escherichia coli. J Bacteriol **178,** 6250-6257.

Hothorn, M., Wolf, S., Aloy, P., Greiner, S., and Scheffzek, K. (2004). Structural insights into the target specificity of plant invertase and pectin methylesterase inhibitory proteins. Plant Cell **16,** 3437-3447.

Ivanchenko, M.G., Muday, G.K., and Dubrovsky, J.G. (2008). Ethylene-auxin interactions regulate lateral root initiation and emergence in Arabidopsis thaliana. Plant J **55,** 335-347.

Jang, I.C., Oh, S.J., Seo, J.S., Choi, W.B., Song, S.I., Kim, C.H., Kim, Y.S., Seo, H.S., Choi, Y.D., and Nahm, B.H. (2003). Expression of a bifunctional fusion of the Escherichia coli genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. Plant Physiol **131,** 516.

Jang, J.C., Leon, P., Zhou, L., and Sheen, J. (1997). Hexokinase as a sugar sensor in higher plants. Plant Cell **9,** 5-19.

Jansen, L., Rybel, B., Vassileva, V., and Beeckman, T. (2010). Root Development. In Plant Developmental Biology - Biotechnological Perspectives, E.C. Pua and M.R. Davey, eds (Springer Berlin Heidelberg), pp. 71-90.

Jules, M., Guillou, V., Francois, J., and Parrou, J.L. (2004). Two distinct pathways for trehalose assimilation in the yeast Saccharomyces cerevisiae. Appl Environ Microbiol **70,** 2771-2778.

Jules, M., Beltran, G., Francois, J., and Parrou, J.L. (2008). New insights into trehalose metabolism by Saccharomyces cerevisiae: NTH2 encodes a functional cytosolic trehalase, and deletion of TPS1 reveals Ath1p-dependent trehalose mobilization. Appl Environ Microbiol **74,** 605-614.

Kaasen, I., Falkenberg, P., Styrvold, O.B., and Strom, A.R. (1992). Molecular cloning and physical mapping of the otsBA genes, which encode the osmoregulatory trehalose pathway of Escherichia coli: evidence that transcription is activated by katF (AppR). J Bacteriol **174,** 889-898.

Karthikeyan, A.S., Varadarajan, D.K., Jain, A., Held, M.A., Carpita, N.C., and Raghothama, K.G. (2007). Phosphate starvation responses are mediated by sugar signaling in Arabidopsis. Planta **225,** 907-918.

Karve, A., and Moore, B.D. (2009). Function of Arabidopsis hexokinase-like1 as a negative regulator of plant growth. J Exp Bot **60,** 4137-4149.

Kleinow, T., Himbert, S., Krenz, B., Jeske, H., and Koncz, C. (2009). NAC domain transcription factor ATAF1 interacts with SNF1-related kinases and silencing of its subfamily causes severe developmental defects in Arabidopsis. Plant Science **177,** 360-370.

Klinghammer, M., and Tenhaken, R. (2007). Genome-wide analysis of the UDP-glucose dehydrogenase gene family in Arabidopsis, a key enzyme for matrix polysaccharides in cell walls. J Exp Bot **58,** 3609-3621.

Koch, K. (2004). Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Curr Opi Plant Biol **7,** 235-246.

Kolbe, A., Tiessen, A., Schluepmann, H., Paul, M., Ulrich, S., and Geigenberger, P. (2005). Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. Proc Natl Acad Sci U S A **102,** 11118-11123.

Krouk, G., Lacombe, B., Bielach, A., Perrine-Walker, F., Malinska, K., Mounier, E., Hoyerova, K., Tillard, P., Leon, S., Ljung, K., Zazimalova, E., Benkova, E., Nacry, P., and Gojon, A. (2010). Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. Developmental Cell **18,** 927-937.

Lalonde, S., Wipf, D., and Frommer, W.B. (2004). Transport mechanisms for organic forms of carbon and nitrogen between source and sink. Annu Rev Plant Biol **55,** 341-372.

Laplaze, L., Benkova, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, R., Weijers, D., Calvo, V., Parizot, B., Herrera-Rodriguez, M.B., Offringa, R., Graham, N., Doumas, P., Friml, J., Bogusz, D., Beeckman, T., and Bennett, M. (2007). Cytokinins act directly on lateral root founder cells to inhibit root initiation. Plant Cell **19,** 3889-3900.

Laskowski, M., Grieneisen, V.A., Hofhuis, H., Hove, C.A., Hogeweg, P., Maree, A.F., and Scheres, B. (2008). Root system architecture from coupling cell shape to auxin transport. PLoS Biol **6,** e307.

Lee, K.W., Chen, P.W., Lu, C.A., Chen, S., Ho, T.H., and Yu, S.M. (2009). Coordinated responses to oxygen and sugar deficiency allow rice seedlings to tolerate flooding. Sci Signal **2,** ra61.

Lejay, L., Wirth, J., Pervent, M., Cross, J.M., Tillard, P., and Gojon, A. (2008). Oxidative pentose phosphate pathway-dependent sugar sensing as a mechanism for regulation of root ion transporters by photosynthesis. Plant Physiol **146,** 2036-2053.

Leyman, B., Van Dijck, P., and Thevelein, J.M. (2001). An unexpected plethora of trehalose biosynthesis genes in Arabidopsis thaliana. Trends Plant Sci **6,** 510-513.

Li, X., Mo, X., Shou, H., and Wu, P. (2006). Cytokinin-mediated cell cycling arrest of pericycle founder cells in lateral root initiation of Arabidopsis. Plant Cell Physiol **47,** 1112-1123.

Ljung, K., Hull, A.K., Celenza, J., Yamada, M., Estelle, M., Normanly, J., and Sandberg, G. (2005). Sites and regulation of auxin biosynthesis in Arabidopsis roots. Plant Cell **17,** 1090-1104.

Lohar, D.P., Schaff, J.E., Laskey, J.G., Kieber, J.J., Bilyeu, K.D., and Bird, D.M. (2004). Cytokinins play opposite roles in lateral root formation, and nematode and Rhizobial symbioses. Plant J **38,** 203-214.

Londesborough, J., and Vuorio, O. (1991). Trehalose-6-phosphate synthase/phosphatase complex from bakers' yeast: purification of a proteolytically activated form. J Gen Microbiol **137,** 323-330.

Loreti, E., Povero, G., Novi, G., Solfanelli, C., Alpi, A., and Perata, P. (2008). Gibberellins, jasmonate and abscisic acid modulate the sucrose-induced expression of anthocyanin biosynthetic genes in Arabidopsis. New Phytol **179,** 1004-1016.

Lu, C.A., Lin, C.C., Lee, K.W., Chen, J.L., Huang, L.F., Ho, S.L., Liu, H.J., Hsing, Y.I., and Yu, S.M. (2007). The SnRK1A protein kinase plays a key role in sugar signaling during germination and seedling growth of rice. Plant Cell **19,** 2484-2499.

Lucas, M., Godin, C., Jay-Allemand, C., and Laplaze, L. (2008). Auxin fluxes in the root apex co-regulate gravitropism and lateral root initiation. J Exp Bot **59,** 55-66.

Lunn, J.E. (2007). Gene families and evolution of trehalose metabolism in plants. Functional Plant Biology **34,** 550-563.

Lunn, J.E., Feil, R., Hendriks, J.H., Gibon, Y., Morcuende, R., Osuna, D., Scheible, W.R., Carillo, P., Hajirezaei, M.R., and Stitt, M. (2006). Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in Arabidopsis thaliana. Biochem J **397,** 139-148.

Luschnig, C., Gaxiola, R.A., Grisafi, P., and Fink, G.R. (1998). EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. Genes Dev **12,** 2175-2187.

Macgregor, D.R., Deak, K.I., Ingram, P.A., and Malamy, J.E. (2008). Root system architecture in Arabidopsis grown in culture is regulated by sucrose uptake in the aerial tissues. Plant Cell **20,** 2643-2660.

Malamy, J.E., and Benfey, P.N. (1997). Organization and cell differentiation in lateral roots of Arabidopsis thaliana. Development **124,** 33-44.

Marin-Rodriguez, M.C., Orchard, J., and Seymour, G.B. (2002). Pectate lyases, cell wall degradation and fruit softening. J Exp Bot **53,** 2115-2119.

Mason, M.G., Mathews, D.E., Argyros, D.A., Maxwell, B.B., Kieber, J.J., Alonso, J.M., Ecker, J.R., and Schaller, G.E. (2005). Multiple type-B response regulators mediate cytokinin signal transduction in Arabidopsis. Plant Cell **17,** 3007-3018.

Miranda, J.A., Avonce, N., Suarez, R., Thevelein, J.M., Van Dijck, P., and Iturriaga, G. (2007). A bifunctional TPS-TPP enzyme from yeast confers tolerance to multiple and extreme abiotic-stress conditions in transgenic Arabidopsis. Planta **226,** 1411-1421.

Mishra, B.S., Singh, M., Aggrawal, P., and Laxmi, A. (2009). Glucose and auxin signaling interaction in controlling Arabidopsis thaliana seedlings root growth and development. PLoS One **4,** e4502.

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W.H., Liu, Y.X., Hwang, I., Jones, T., and Sheen, J. (2003). Role of the Arabidopsis glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science **300,** 332-336.

Mudgil, Y., Uhrig, J.F., Zhou, J., Temple, B., Jiang, K., and Jones, A.M. (2009). Arabidopsis N-MYC DOWNREGULATED-LIKE1, a positive regulator of auxin transport in a G protein-mediated pathway. Plant Cell **21,** 3591-3609.

Müller, A., Guan, C., Galweiler, L., Tanzler, P., Huijser, P., Marchant, A., Parry, G., Bennett, M., Wisman, E., and Palme, K. (1998). AtPIN2 defines a locus of *Arabidopsis* for root gravitropism control. Embo J **17,** 6903-6911.

Müller, J., Boller, T., and Wiemken, A. (1995). Effects of validamycin A, a potent trehalase inhibitor, and phytohormones on trehalose metabolism in roots and root nodules of soybean and cowpea. Planta **197,** 362-368.

Müller, R., Morant, M., Jarmer, H., Nilsson, L., and Nielsen, T.H. (2007). Genome-wide analysis of the Arabidopsis leaf transcriptome reveals interaction of phosphate and sugar metabolism. Plant Physiol **143,** 156-171.

Negi, S., Ivanchenko, M.G., and Muday, G.K. (2008). Ethylene regulates lateral root formation and auxin transport in Arabidopsis thaliana. Plant J **55,** 175-187.

Nwaka, S., Mechler, B., and Holzer, H. (1996). Deletion of the ATH1 gene in Saccharomyces cerevisiae prevents growth on trehalose. FEBS Letters **386,** 235-238.

Okushima, Y., Fukaki, H., Onoda, M., Theologis, A., and Tasaka, M. (2007). ARF7 and ARF19 regulate lateral root formation via direct activation of LBD/ASL genes in Arabidopsis. Plant Cell **19,** 118-130.

Overvoorde, P., Fukaki, H., and Beeckman, T. (2010). Auxin control of root development. Cold Spring Harbor Perspectives in Biology **2,** a001537.

Palenchar, P.M., Kouranov, A., Lejay, L.V., and Coruzzi, G.M. (2004). Genome-wide patterns of carbon and nitrogen regulation of gene expression validate the combined carbon and nitrogen (CN)-signaling hypothesis in plants. Genome Biol **5,** R91.

Parizot, B., De Rybel, B., and Beeckman, T. (2010). VisuaLRTC: a new view on lateral root initiation by combining specific transcriptome data sets. Plant Physiol **153,** 34-40.

Parrou, J.L., Jules, M., Beltran, G., and Francois, J. (2005). Acid trehalase in yeasts and filamentous fungi: localization, regulation and physiological function. FEMS Yeast Res **5,** 503-511.

Paul, M. (2007). Trehalose 6-phosphate. Curr Opi Plant Biol **10,** 303-309.

Paul, M.J., Primavesi, L.F., Jhurreea, D., and Zhang, Y. (2008). Trehalose metabolism and signaling. Annu Rev Plant Biol **59,** 417-441.

Paul, M.J., Jhurreea, D., Zhang, Y., Primavesi, L.F., Delatte, T., Schluepmann, H., and Wingler, A. (2010). Upregulation of biosynthetic processes associated with growth by trehalose 6-phosphate. Plant Signal Behav **5,** 386-392.

Pego, J.V., and Smeekens, S.C. (2000). Plant fructokinases: a sweet family get-together. Trends Plant Sci **5,** 531-536.

Péret, B., Larrieu, A., and Bennett, M.J. (2009a). Lateral root emergence: a difficult birth. J Exp Bot **60,** 3637-3643.

Péret, B., De Rybel, B., Casimiro, I., Benkova, E., Swarup, R., Laplaze, L., Beeckman, T., and Bennett, M.J. (2009b). Arabidopsis lateral root development: an emerging story. Trends Plant Sci **14,** 399-408.

Pilon-Smits, E.A.H., Terry, N., Sears, T., Hyeong, K.I.M., Zayed, A., Seongbin, H., and Van, D.U.N. (1998). Trehalose-producing transgenic tobacco plants show improved growth performance under drought stress. J Plant Physiol **152,** 525-532.

Piper, P.W., and Lockheart, A. (1988). A temperature-sensitive mutant of Sacchromyces cerevisiae defective in the specific phosphatase of trehalose biosynthesis. FEMS Microbiol Lett **49,** 245-250.

Pramanik, M.H., and Imai, R. (2005). Functional identification of a trehalose 6-phosphate phosphatase gene that is involved in transient induction of trehalose biosynthesis during chilling stress in rice. Plant Mol Biol **58,** 751-762.

Qi, X., Wu, Z., Li, J., Mo, X., Wu, S., Chu, J., and Wu, P. (2007). AtCYT-INV1, a neutral invertase, is involved in osmotic stress-induced inhibition on lateral root growth in Arabidopsis. Plant Mol Biol **64,** 575-587.

Ramon, M., Rolland, F., and Sheen, J. (2008). Sugar sensing and signaling. The Arabidopsis Book **6,** 1-22.

Ramon, M., Rolland, F., Thevelein, J.M., Van Dijck, P., and Leyman, B. (2007). ABI4 mediates the effects of exogenous trehalose on Arabidopsis growth and starch breakdown. Plant Mol Biol **63,** 195-206.

Ramon, M., De Smet, I., Vandesteene, L., Naudts, M., Leyman, B., Van Dijck, P., Rolland, F., Beeckman, T., and Thevelein, J.M. (2009). Extensive expression regulation and lack of heterologous enzymatic activity of the Class II trehalose metabolism proteins from Arabidopsis thaliana. Plant Cell Environ **32,** 1015-1032.

Reed, R.C., Brady, S.R., and Muday, G.K. (1998). Inhibition of auxin movement from the shoot into the root inhibits lateral root development in Arabidopsis. Plant Physiol **118,** 1369-1378.

Richter, G.L., Monshausen, G.B., Krol, A., and Gilroy, S. (2009). Mechanical stimuli modulate lateral root organogenesis. Plant Physiol **151,** 1855-1866.

Riefler, M., Novak, O., Strnad, M., and Schmulling, T. (2006). Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell **18,** 40-54.

Rolland, F., Moore, B., and Sheen, J. (2002). Sugar sensing and signaling in plants. Plant Cell **14 Suppl,** S185-205.

Rolland, F., Baena-Gonzalez, E., and Sheen, J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu Rev Plant Biol **57,** 675-709.

Romero, C., Belles, J.M., Vaya, J.L., Serrano, R., and Culianez-Macia, F.A. (1997). Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. Planta **201,** 293-297.

Satoh-Nagasawa, N., Nagasawa, N., Malcomber, S., Sakai, H., and Jackson, D. (2006). A trehalose metabolic enzyme controls inflorescence architecture in maize. Nature **441,** 227-230.

Scheres, B., and Benfey, P.N. (1999). Asymmetric Cell Division in Plants. Annu Rev Plant Physiol Plant Mol Biol **50,** 505-537.

Schluepmann, H., Pellny, T., van Dijken, A., Smeekens, S., and Paul, M. (2003). Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. Proc Natl Acad Sci U S A **100,** 6849-6854.

Schluepmann, H., van Dijken, A., Aghdasi, M., Wobbes, B., Paul, M., and Smeekens, S. (2004). Trehalose Mediated Growth Inhibition of Arabidopsis Seedlings Is Due to Trehalose-6-Phosphate Accumulation. Plant Physiol **135,** 879-890.

Shima, S., Matsui, H., Tahara, S., and Imai, R. (2007). Biochemical characterization of rice trehalose-6-phosphate phosphatases supports distinctive functions of these plant enzymes. Febs J **274,** 1192-1201.

Signora, L., De Smet, I., Foyer, C.H., and Zhang, H. (2001). ABA plays a central role in mediating the regulatory effects of nitrate on root branching in Arabidopsis. Plant J **28,** 655-662.

Smith, A.M., and Stitt, M. (2007). Coordination of carbon supply and plant growth. Plant Cell Environ **30,** 1126-1149.

Smith, L.G., and Oppenheimer, D.G. (2005). Spatial control of cell expansion by the plant cytoskeleton. Annu Rev Cell Dev Biol **21,** 271-295.

Steinmann, T., Geldner, N., Grebe, M., Mangold, S., Jackson, C.L., Paris, S., Galweiler, L., Palme, K., and Jürgens, G. (1999). Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. Science **286,** 316-318.

Strader, L.C., Monroe-Augustus, M., Rogers, K.C., Lin, G.L., and Bartel, B. (2008). Arabidopsis iba response5 suppressors separate responses to various hormones. Genetics **180,** 2019-2031.

Suzuki, N., Bajad, S., Shuman, J., Shulaev, V., and Mittler, R. (2008). The transcriptional co-activator MBF1c is a key regulator of thermotolerance in Arabidopsis thaliana. J Biol Chem **283,** 9269-9275.

Swarup, K., Benkova, E., Swarup, R., Casimiro, I., Peret, B., Yang, Y., Parry, G., Nielsen, E., De Smet, I., Vanneste, S., Levesque, M.P., Carrier, D., James, N., Calvo, V., Ljung, K., Kramer, E., Roberts, R., Graham, N., Marillonnet, S., Patel, K., Jones, J.D., Taylor, C.G., Schachtman, D.P., May, S., Sandberg, G., Benfey, P., Friml, J., Kerr, I., Beeckman, T., Laplaze, L., and Bennett, M.J. (2008a). The auxin influx carrier LAX3 promotes lateral root emergence. Nat Cell Biol **10,** 946-954.

Swarup, K., Benkova, E., Swarup, R., Casimiro, I., Peret, B., Yang, Y., Parry, G., Nielsen, E., De Smet, I., Vanneste, S., Levesque, M.P., Carrier, D., James, N., Calvo, V., Ljung, K., Kramer, E., Roberts, R., Graham, N., Marillonnet, S., Patel, K., Jones, J.D., Taylor, C.G., Schachtman, D.P., May, S., Sandberg, G., Benfey, P., Friml, J., Kerr, I., Beeckman, T., Laplaze, L., and Bennett, M.J. (2008b). The auxin influx carrier LAX3 promotes lateral root emergence. Nature cell biology.

Thaller, M.C., Schippa, S., and Rossolini, G.M. (1998). Conserved sequence motifs among bacterial, eukaryotic, and archaeal phosphatases that define a new phosphohydrolase superfamily. Protein Sci **7,** 1647-1652.

Tiessen, A., Prescha, K., Branscheid, A., Palacios, N., McKibbin, R., Halford, N.G., and Geigenberger, P. (2003). Evidence that SNF1-related kinase and hexokinase are involved in separate sugar-signalling pathways modulating post-translational redox activation of ADP-glucose pyrophosphorylase in potato tubers. Plant J **35,** 490-500.

Toroser, D., Plaut, Z., and Huber, S.C. (2000). Regulation of a plant SNF1-related protein kinase by glucose-6-phosphate. Plant Physiol **123,** 403-412.

Trusov, Y., Rookes, J.E., Tilbrook, K., Chakravorty, D., Mason, M.G., Anderson, D., Chen, J.G., Jones, A.M., and Botella, J.R. (2007). Heterotrimeric G protein gamma subunits provide functional selectivity in Gbetagamma dimer signaling in Arabidopsis. Plant Cell **19,** 1235-1250.

Ullah, H., Chen, J.G., Temple, B., Boyes, D.C., Alonso, J.M., Davis, K.R., Ecker, J.R., and Jones, A.M. (2003). The beta-subunit of the Arabidopsis G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. Plant Cell **15,** 393-409.

Van Dijck, P., Mascorro-Gallardo, J.O., De Bus, M., Royackers, K., Iturriaga, G., and Thevelein, J.M. (2002). Truncation of Arabidopsis thaliana and Selaginella lepidophylla trehalose-6-phosphate synthase unlocks high catalytic activity and supports high trehalose levels on expression in yeast. Biochem J **366,** 63-71.

van Dijken, A.J.H., Schluepmann, H., and Smeekens, S.C.M. (2004). Arabidopsis Trehalose-6-Phosphate Synthase 1 Is Essential for Normal Vegetative Growth and Transition to Flowering. Plant Physiol **135,** 969-977.

Vandesteene, L. (2009). Functional analysis of trehalose metabolism gene family in *Arabidopsis thaliana* (PhD thesis-Katholieke Universiteit Leuven).

Vandesteene, L., Ramon, M., Le Roy, K., Van Dijck, P., and Rolland, F. (2010). A Single Active Trehalose-6-P Synthase (TPS) and a Family of Putative Regulatory TPS-Like Proteins in Arabidopsis. Mol Plant **3,** 406-419.

Vanneste, S., De Rybel, B., Beemster, G.T., Ljung, K., De Smet, I., Van Isterdael, G., Naudts, M., Iida, R., Gruissem, W., Tasaka, M., Inze, D., Fukaki, H., and Beeckman, T. (2005). Cell cycle progression in the pericycle is not sufficient for SOLITARY ROOT/IAA14-mediated lateral root initiation in Arabidopsis thaliana. Plant Cell **17,** 3035-3050.

Vaughn, M.W., Harrington, G.N., and Bush, D.R. (2002). Sucrose-mediated transcriptional regulation of sucrose symporter activity in the phloem. Proc Natl Acad Sci U S A **99,** 10876-10880.

Vissenberg, K., Fry, S.C., Pauly, M., Höfte, H., and Verbelen, J.P. (2005). XTH acts at the microfibril-matrix interface during cell elongation. J Exp Bot **56,** 673-683.

Vogel, G., Aeschbacher, R.A., Müller, J., Boller, T., and Wiemken, A. (1998). Trehalose-6-phosphate phosphatases from Arabidopsis thaliana: identification by functional complementation of the yeast tps2 mutant. Plant J **13,** 673-683.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmulling, T. (2003). Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell **15,** 2532-2550.

Wiemken, A. (1990). Trehalose in yeast, stress protectant rather than reserve carbohydrate. Antonie Van Leeuwenhoek **58,** 209-217.

Wilson, A.K., Pickett, F.B., Turner, J.C., and Estelle, M. (1990). A dominant mutation in Arabidopsis confers resistance to auxin, ethylene and abscisic acid. Mol Gen Genet **222,** 377-383.

Wingler, A., Fritzius, T., Wiemken, A., Boller, T., and Aeschbacher, R.A. (2000). Trehalose Induces the ADP-Glucose Pyrophosphorylase Gene, ApL3, and Starch Synthesis in Arabidopsis. Plant Physiol **124,** 105-114.

Wu, Y., Deng, Z., Lai, J., Zhang, Y., Yang, C., Yin, B., Zhao, Q., Zhang, L., Li, Y., and Xie, Q. (2009). Dual function of Arabidopsis ATAF1 in abiotic and biotic stress responses. Cell Res **19,** 1279-1290.

Xiao, W., Sheen, J., and Jang, J.C. (2000). The role of hexokinase in plant sugar signal transduction and growth and development. Plant Mol Biol **44,** 451-461.

Yanagisawa, S., Yoo, S.D., and Sheen, J. (2003). Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature **425,** 521-525.

Yuan, K., and Wysocka-Diller, J. (2006). Phytohormone signalling pathways interact with sugars during seed germination and seedling development. J Exp Bot **57,** 3359-3367.

Zentella, R., Mascorro-Gallardo, J.O., Van Dijck, P., Folch-Mallol, J., Bonini, B., Van Vaeck, C., Gaxiola, R., Covarrubias, A.A., Nieto-Sotelo, J., Thevelein, J.M., and Iturriaga, G. (1999). A Selaginella lepidophylla trehalose-6-phosphate synthase complements growth and stress-tolerance defects in a yeast tps1 mutant. Plant Physiol **119,** 1473-1482.

Zhang, H., Mao, X., Wang, C., and Jing, R. (2010). Overexpression of a common wheat gene TaSnRK2.8 enhances tolerance to drought, salt and low temperature in Arabidopsis. PLoS One **5,** e16041.

Zhou, L., Jang, J.C., Jones, T.L., and Sheen, J. (1998). Glucose and ethylene signal transduction crosstalk revealed by an Arabidopsis glucose-insensitive mutant. Proc Natl Acad Sci U S A **95,** 10294-10299.

Chapter II

Expansive evolution of the Trehalose-6-Phosphate gene family in Arabidopsis thaliana

Adapted from Vandesteene, L.*, López-Galvis, L.*, Vanneste, K., Maere, S., Lammens, W., Rolland, F., Avonce, N., Beeckman, T. and Van Dijck, P. Manuscript under revision in Plant Physiology.
* shared first author

Expansive evolution of the Trehalose-6-phosphate phosphatase gene family in *Arabidopsis thaliana*

1. ABSTRACT

Trehalose biosynthesis in plants involves a two-step reaction in which trehalose-6-phosphate (Tre6P) is synthesized from UDP-glc and Glc6P (catalysed by Tre6P synthase, TPS), and subsequently dephosphorylated to produce the disaccharide trehalose (catalysed by Tre6P phosphatase, TPP). While higher plants typically do not accumulate high levels of trehalose, they encode large families of putative trehalose biosynthesis genes. In *Arabidopsis thaliana*, eleven genes encode proteins with both TPS- and TPP-like domains but only one of these (AtTPS1) appears to be an active (TPS) enzyme. In addition, plants contain a large family of smaller proteins with a conserved TPP domain. Here, we present an in-depth analysis of the 10 *TPP* genes and gene products in *Arabidopsis (TPPA-TPPJ)*. Phylogenetic and collinearity analysis revealed that all of these genes originate from whole genome duplication events. Heterologous expression in yeast showed that all encode active TPP enzymes with an essential role for some conserved residues in the catalytic domain. These results suggest that the *TPP* genes function in the regulation of Tre6P levels, emerging as a novel key regulator of growth and development in higher plants. Extensive gene expression analyses using a complete set of promoter-beta-glucuronidase/green fluorescent protein (GUS/GFP) reporter lines further uncovered cell- and tissue-specific expression patterns, conferring spatio-temporal control of trehalose metabolism. Consistently, phenotypic characterization of knock-down and overexpression lines of a single *TPP, AtTPPG*, points to unique properties of individual TPPs in Arabidopsis, and underlines the intimate connection between trehalose metabolism and abscisic acid (ABA)-signaling.

2. INTRODUCTION

The presence of trehalose in a wide variety of organisms and the existence of different biosynthesis pathways suggests a pivotal and ancient role for trehalose metabolism in nature. The most widely distributed metabolic pathway consists of two consecutive enzymatic reactions, with trehalose-6-phosphate (Tre6P) synthase (TPS) catalyzing the transfer of a glucosyl moiety from uridine diphosphate-glucose (UDP-glc) to glucose-6-phosphate (Glc6P) to produce Tre6P and UDP, and Tre6P phosphatase (TPP) catalyzing the dephosphorylation of Tre6P to trehalose (Cabib and Leloir, 1958). Apart from operating as a (reserve) carbon source and structural component in bacteria, fungi, and

invertebrates, trehalose also functions as a major stress protectant of proteins and membranes during adverse conditions such as dehydration, high salinity, hypoxia and nutrient starvation (Elbein et al., 2003). Trehalose accumulation is also observed in drought-tolerant non-vascular plants like green algae and liverworts and in a few lower vascular resurrection plants. Until about a decade ago, higher vascular plants were believed to have lost the ability to produce trehalose, but with the emergence of more sensitive assays, genome sequencing, and the use of yeast mutant complementation, minute amounts of trehalose and Tre6P and functional plant enzyme orthologs were found (Goddijn et al., 1997; Vogel et al., 1998; Lunn et al., 2006). In addition, heterologous expression and disruption of trehalose metabolism in plants conferred pleiotropic effects on plant growth, ranging from altered stress tolerance, leaf morphology and developmental timing to embryo-lethality (Holmström et al., 1996; Goddijn et al., 1997; Romero et al., 1997; Eastmond et al., 2002; Schluepmann et al., 2003; Avonce et al., 2004; Gómez et al., 2006; Satoh-Nagasawa et al., 2006; Miranda et al., 2007; Chary et al., 2008), pointing to an important regulatory function. The intermediate Tre6P has been highlighted as a novel signal for carbohydrate status (Paul, 2008), positively correlating with sucrose levels, redox-regulated ADP-glucose pyrophosphorylase activity, and starch biosynthesis (Lunn et al., 2006). Recently, it was reported that Tre6P inhibits the activity of the SnRK1 protein kinase to activate energy-consuming biosynthetic processes (Zhang et al., 2009).

In most bacterial and eukaryotic species, the TPS and TPP activities are found on separate proteins. Recent phylogenetic and biochemical analyses showed that some archaea and bacteria, such as *Cytophaga hutchinsonii,* express proteins that have both active TPS and TPP domains resulting from gene fusion, suggesting that such prokaryotic bifunctional proteins are the evolutionary ancestors of the large eukaryotic trehalose biosynthesis enzymes in which one or both domains have subsequently lost their catalytic activity (Avonce et al., 2010). The yeast TPP enzyme Tps2, for example, harbours an inactive N-terminal TPS domain and an active C-terminal TPP domain. In contrast to the single *TPS* and *TPP* genes in most micro-organisms, the genomes of higher plants encode a remarkably large family of putative trehalose biosynthesis enzyme homologues. These are commonly classified in three distinct subgroups, according to their similarity to the microbial TPS and TPP proteins and/or presence of specific motifs (e.g. conserved phosphatase boxes) (Thaller et al., 1998; Leyman et al., 2001; Eastmond and Graham, 2003). Even primitive plants such as the alga *Ostreococcus tauri* and the moss *Physcomitrella patens* already contain members of each of these gene families, pointing to the early establishment and conservation of these proteins in plant evolution (Lunn, 2007; Avonce et al., 2010). In Arabidopsis, the class I TPS proteins (AtTPS1-4) show most similarity to the yeast TPS Tps1, but also have a C-terminal domain with limited similarity to TPPs. However, only one of these, AtTPS1, appears to have heterologous enzymatic TPS activity in yeast (Blázquez et al., 1998; Vandesteene et al., 2010). The class II TPS proteins (AtTPS5-11) are similar bipartite proteins with a TPS-like domain but a more

conserved TPP domain. They appear to lack both heterologous TPS and TPP activity (Ramon et al., 2009). The high level of conservation of putative substrate binding residues in class I and class II proteins, however, suggests that substrates might still bind (Avonce et al., 2006; Lunn, 2007; Ramon et al., 2009; Vandesteene et al., 2010). Together with the specific expression patterns of the class I genes (van Dijken et al., 2004; Geelen et al., 2007; Vandesteene et al., 2010) and the extensive expression regulation of all class II members by plant carbon status (Baena-Gonzalez et al., 2007; Usadel et al., 2008; Ramon et al., 2009), this suggests tissue-specific regulatory functions for these proteins in metabolic regulation of plant growth and development. Finally, Arabidopsis also harbours a family of 10 smaller proteins (AtTPPA-J; 320-385aa) with limited similarity to the class I and class II proteins (795-942aa). Like class II proteins, they contain the phosphatase box consensus sequences, characteristic of the L-2-haloacid dehalogenase (HAD) super family of enzymes, which comprises a wide range of phosphatases and hydrolases (Thaller et al., 1998). It has been suggested that the origin of these plant *TPP* genes is different from the origin of the class I and II genes (Avonce et al., 2010) and that plants recruited the *TPP* genes after their divergence from fungi, most probably from proteobacteria or actinobacteria. Consistently, orthologous TPP proteins are present in proteobacteria such as *Rhodopherax ferrireducens* (Avonce et al., 2010). To date, only few of these single-domain plant TPP proteins have been subject to biochemical characterization, e.g. TPPA and TPPB from Arabidopsis (Vogel et al., 1998), OsTPP1 and OsTPP2 from rice (Pramanik and Imai, 2005; Shima et al., 2007) and RAMOSA3 (RA3) from maize (Satoh-Nagasawa et al., 2006).

The phenotypic alterations observed in plants fed with trehalose or genetically modified in trehalose biosynthesis, suggest a pivotal role for trehalose metabolism in integrating the metabolic status with growth and development. Disruption of the only known active TPS enzyme in Arabidopsis (AtTPS1) results in embryo-lethality (Eastmond et al., 2002) and, when rescued to survive embryogenesis, causes a strong disruption of vegetative and generative development and ABA-hypersensitivity (van Dijken et al., 2004; Gómez et al., 2010). Overexpressing *AtTPS1*, on the other hand, renders seedlings sugar- and ABA-insensitive (Avonce et al., 2004). These observations strongly link trehalose metabolism with ABA signaling. Interestingly, a mutation of a *TPP* gene in maize, *RA3*, results in a distinct phenotype, with incorrect axillary meristem identity and determinacy in both male and female inflorescences (Satoh-Nagasawa et al., 2006). Arabidopsis plants with overall increased Tre6P levels, such as *OtsA* (*E. coli* TPS) overexpression plants, similarly show increased inflorescence branching (Schluepmann et al., 2003; van Dijken et al., 2004).

3. RESULTS

3.1 Collinearity analysis shows that the *TPP* gene family in eudicots mainly expanded through genome duplication

It has been established that most, if not all vascular plants are ancient paleoploids (Masterson, 1994; Cui et al., 2006; Soltis et al., 2009; Van de Peer et al., 2009). Current evidence indicates that a hexaploidization event (referred to as γ) occurred around the base of the eudicots. Many eudicot lineages underwent additional genome duplication events, many of them around the Cretaceous-Tertiary boundary, 65 mya (Fawcett et al., 2009; Van de Peer et al., 2009). For instance, two additional genome duplications have occurred within the *Brassicales* lineage, referred to as β and α in Arabidopsis (Jaillon et al., 2007; Tang et al., 2008). Remnants of ancient genome duplications have also been discovered in monocot genomes, e.g. an event shared by Sorghum and Oryza probably dating back to the base of the Poaceae clade, and in the moss *Physcomitrella patens*.

Genome duplications have had a profound impact on the expansion of certain gene families. We investigated their impact on the *TPP* gene family by searching for collinearity of genomic segments containing *TPP* genes, using the PLAZA v1.0 platform (Proost et al., 2009). Strikingly, all *TPP* genes in eudicots appear to be linked through collinearity relationships, indicating that the *TPP* gene family in eudicots exclusively expanded through genome duplication. In Arabidopsis, 9 out of 10 *AtTPP* genes, all except *TPPD,* were found in duplicated blocks remaining from the most recent genome duplication (α, Ks around 0.7-0.8) (Figure 1A). Moreover, all TPPs were retained in duplicate after the α event, except for *TPPA* and *TPPD.* Whereas *TPPA* still lies in a duplicated block remaining from α, for *TPPD,* we could not find back any syntenic relationships dating back to the α event, indicating that the whole duplicated stretch, including the duplicate of *TPPD,* was lost or has degraded beyond recognition. Deeper collinearity relationships caused by previous genome duplication events were also evident. *TPPD,* for instance, has a syntenic relationship with *TPPB*, but this duplicated block is much older (Ks=1.6) and originates from an earlier genome duplication (data not shown). Extending our analysis to other species, we found clear collinearity relationships linking all *TPPs* across the eudicots (see for example Figure 1B) indicating that all eudicot *TPP* duplicates created after the monocot-dicot split have been derived from genome duplications. Several collinearities among monocot *TPP* genes were also apparent but their phylogenetic relationship is less clear.

3.2 Collinearity constraints improve the phylogenetic reconstruction of the *TPP* gene family

We used the TREEFINDER package (Jobb et al., 2004) to reconstruct a naive phylogeny of the *TPP* gene family in the green plant lineage (see Material and Methods). The resulting

tree is depicted in Figure S1A. It is in good agreement with the tree calculated with different methods by Avonce et al. (2010). The tree multifurcates in 4 branches, the Physcomitrella *TPP* genes and three clades of angiosperm *TPP* genes, all having average to high bootstrap support (78.6 – 98.3) except for the branch containing the Arabidopsis *TPPF*, *TPPG*, and *TPPA* genes which had a much lower bootstrap support value of 52.5.

Next, we sought to impose topological constraints on the tree by taking into account the genome duplication relationships among the *TPPs*. Based on the collinearity relationships

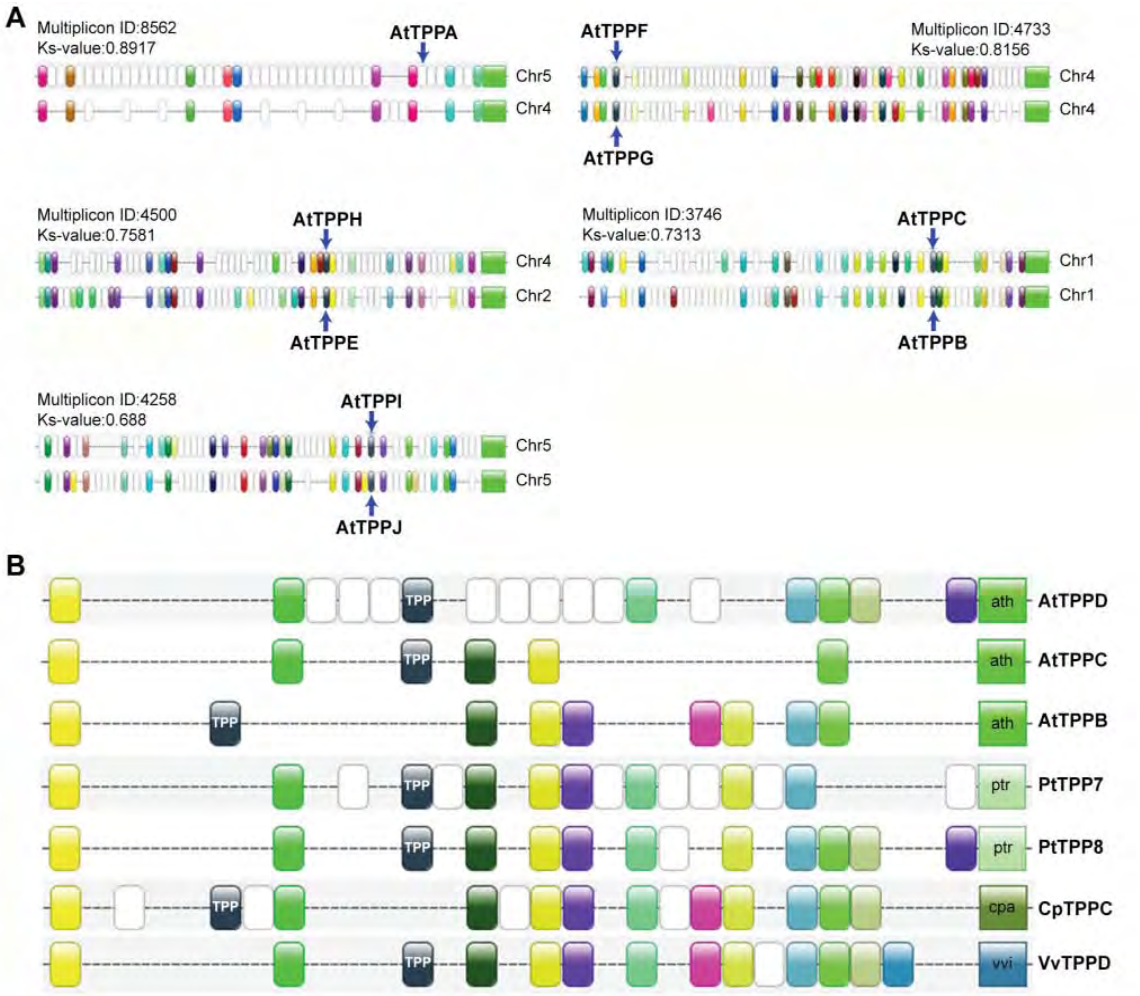


Figure 1. Collinearity of the Arabidopsis *TPP* genes. A. Information was extracted from the PLAZA v1.0 platform. For each multiplicon, the PLAZA v1.0 multiplicon ID and corresponding K_S-value are depicted. The K_S-values for all multiplicons shown here are typical of duplicated blocks remaining from the most recent genome duplication in Arabidopsis (Fawcett et al., 2009). **B.** Example of collinearity relationships among *TPPs* across the eudicots. The eudicot part of multiplicon 894 was found after a search with *AtTPPC* in the Skyline Plot utility of the PLAZA v1.0 platform. Genes with the same colour represent members of the same gene family that are present in several of the homologous blocks (also known as anchor points). *TPP* genes are coloured black. The ratio of collinear regions expected for Vitis:Poplar:Carica:Arabidopsis on the basis of their genome duplication history is 1:2:1:4 (Fawcett et al., 2009), very close to the observed ratio of 1:2:1:3, indicating that very little loss has occurred in this clade. Only the collinear region of *TPPD* created by the most recent whole genome duplication in Arabidopsis appears to have been lost.

found with PLAZA v1.0 (Proost et al., 2009) and the available knowledge on the timing and phylogenetic position of genome duplications and speciations in angiosperm plants (Fawcett et al., 2009), we were able to severely constrain the topology of the TPP tree (Figure S1B). Because the phylogenetic interpretation of the collinearities among monocot *TPP* genes was less clear, we did not use them to constrain the tree topology. The constrained tree was again fed into the TREEFINDER package to resolve the remaining unconstrained branches, using a bootstrap ML analysis with 1000 iterations. The resulting phylogenetic tree of the *TPP* gene family in the land plants is presented in Figure S1B. The constrained phylogenetic relationships fit relatively well with the unconstrained phylogeny (Figure S1A). Three major subclades of eudicot TPPs are apparent in both trees, and the within-species phylogenetic relationships among *TPP* genes are identical. Within the subclades, the between-species phylogenetic relationship of the *TPPs* is more variable. Carica *TPPs* are for instance often put outside Arabidopsis-Populus clades in the unconstrained topology, possibly an artifact of slower evolution of these genes in Carica. On two occasions, Vitis TPPs are similarly put erroneously close together in the unconstrained topology. The fact that all nodes in the unconstrained tree that do not match the constrained tree have relatively low bootstrap support is also an indication that constraining the topology on the basis of collinearity relationships significantly improves the tree.

3.3 All 10 putative Arabidopsis TPP enzymes have heterologous TPP activity in yeast

The TPP gene family in higher plants appears to have expanded mainly by whole genome duplication, but it is not clear whether all have maintained enzymatic activity. To assess TPP activity, we have used a *Sacharomyces cerevisiae* TPP mutant complementation assay. Yeast *tps2Δ* mutants exhibit thermosensitive growth due to accumulation of high levels of Tre6P at increased temperature (De Virgilio et al., 1993). Previously, complementation of this thermosensitive growth phenotype has been used to isolate the *TPPA* and *TPPB* genes from an Arabidopsis cDNA library (Vogel et al., 1998). We have now analyzed all putative Arabidopsis TPP enzymes in the yeast *tps2Δ* mutant strain with efficient expression of the 10 proteins confirmed by western blot analysis. All 10 TPP members were able to complement growth deficiency of the yeast *tps2Δ* mutant strain at elevated temperature (Figure 2A-C). Based on sequence alignment and the crystal structure of the TPP-related Tre6P Phosphatase protein from the archaean *Thermoplasma acidophylum* (TaTre6PP) (Rao et al., 2006), a strikingly conserved active site is found in all 10 Arabidopsis TPP proteins (Avonce et al., 2006; Rao et al., 2006; Lunn, 2007). We made an overall representation model with Tre6P modelled in the active site of Arabidopsis TPPG (Figure 2C). TaTre6PP uses the common catalytic reaction mechanism typical for the L-2-HaloAcid Dehalogenase (HAD) superfamily, with the active site located within the cavity at the interface of two major domains (Rao et al., 2006). Three sequence motifs make up the active site, with the N-terminal DXDX(T/V) motif (motif I, aa7-11 in TaTre6PP) being the most conserved. A nucleophilic attack of Asp7 (D7, D113 in TPPG) on the phosphorus

atom of Tre6P is thought to result in a phospho-aspartic acid intermediate, followed by hydrolysis of an activated water-molecule, generated by Asp9 (D9, D115 in TPPG). Asp9 might also assist as a general acid catalyst, protonating the oxygen atom of the leaving group (Rao et al., 2006). Here, we provide experimental evidence for the importance of these two conserved aspartate residues for TPP catalytic activity. Mutation of these aspartate residues in TPPG (D113 and D115, TPPG-DYD) to asparagine (similar spatial structure as aspartate but harbouring an uncharged polar R group) (TPPG-NYN) abolished yeast *tps2Δ* mutant growth complementation (Figure 2A-C).

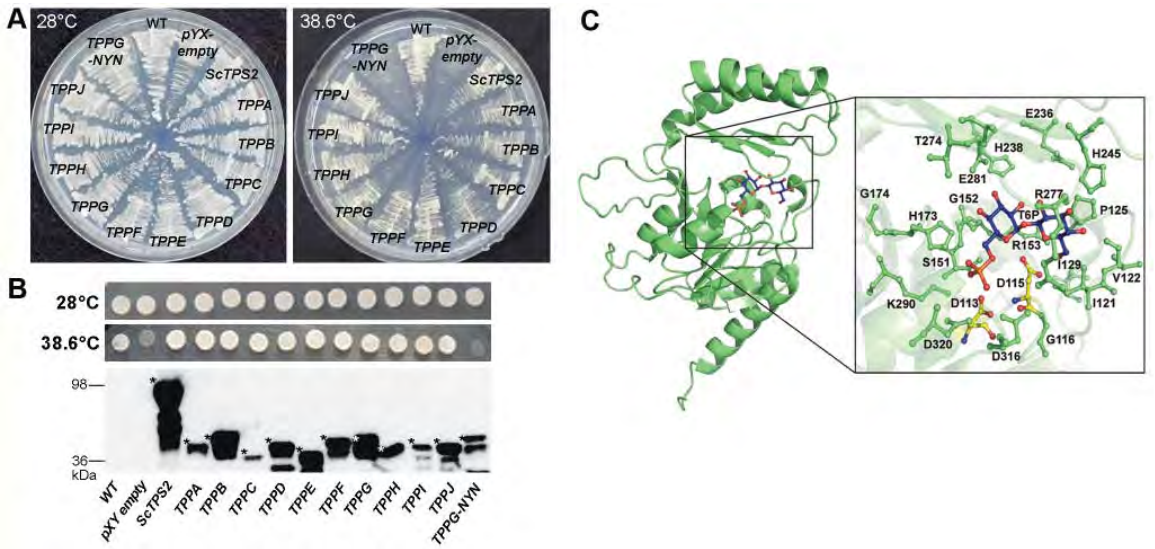


Figure 2. The 10 Arabidopsis TPP candidates display heterologous TPP activity. A. Yeast *tps2Δ* growth complementation assay of TPP Arabidopsis proteins at heat temperature of 38.6°C with strains grown at 28°C as control treatment. *tps2Δ* yeast strains expressing the *TPPG-NYN* mutant (Asp113 and Asp115 of wild type TPPG were mutated to Asn) showed heat sensitive growth phenotype, indicating the double mutation causes loss of TPPG catalytic activity. **B.** Confirmation of growth and transformation of the strains used in A. For the spot assay, cells of all samples were diluted to OD_{600} 1 and incubated at 28°C and 38.6°C. The western blot analysis confirms the expression of the HA-tagged proteins. Asterisks indicate the full-length tagged protein, based on the predicted mass (TAIR) and the pre-stained protein standard. **C.** Overall representation model of the TPPG protein (green), and detail of Tre6P (blue) modeled in the active site of TPPG. Catalytic important residues near the active site are reported in green ball and stick figures. The two Asp of Motif I, which are predicted to play a crucial role in the substrate binding and the catalytic mechanism, are indicated in yellow. Asp113 and Asp115 of TPPG (TPPG wild type) were mutated to Asn (TPPG-NYN). Figure was prepared with PYMOL (DeLano, 2002).

3.4 Differential expression profiles suggest functional diversity among the Arabidopsis TPP family

In multicellular organisms, protein function is often tightly linked to tissue- and cell type-specific expression. As all Arabidopsis TPP proteins seem to have Tre6P-phosphatase activity, it is important to determine their spatio-temporal expression *in planta* to get an initial clue about their functional specificity. We therefore fused a 2kb promoter sequence of each Arabidopsis *TPP* gene to a GUS/GFP reporter, and introduced these

constructs into *A. thaliana* ecotype *Columbia (Col-0)*. For each construct, at least three independent homozygous lines (with single insertion) were selected in the T3 generation, and results of homozygous lines with representative expression patterns are shown. A global overview of the expression patterns of the 10 *TPP* genes in various organs at different developmental stages was obtained with GUS staining and microscopic analyses (Figure 3, S3). *TPP* gene expression occurred in various and distinct organs, from root tips

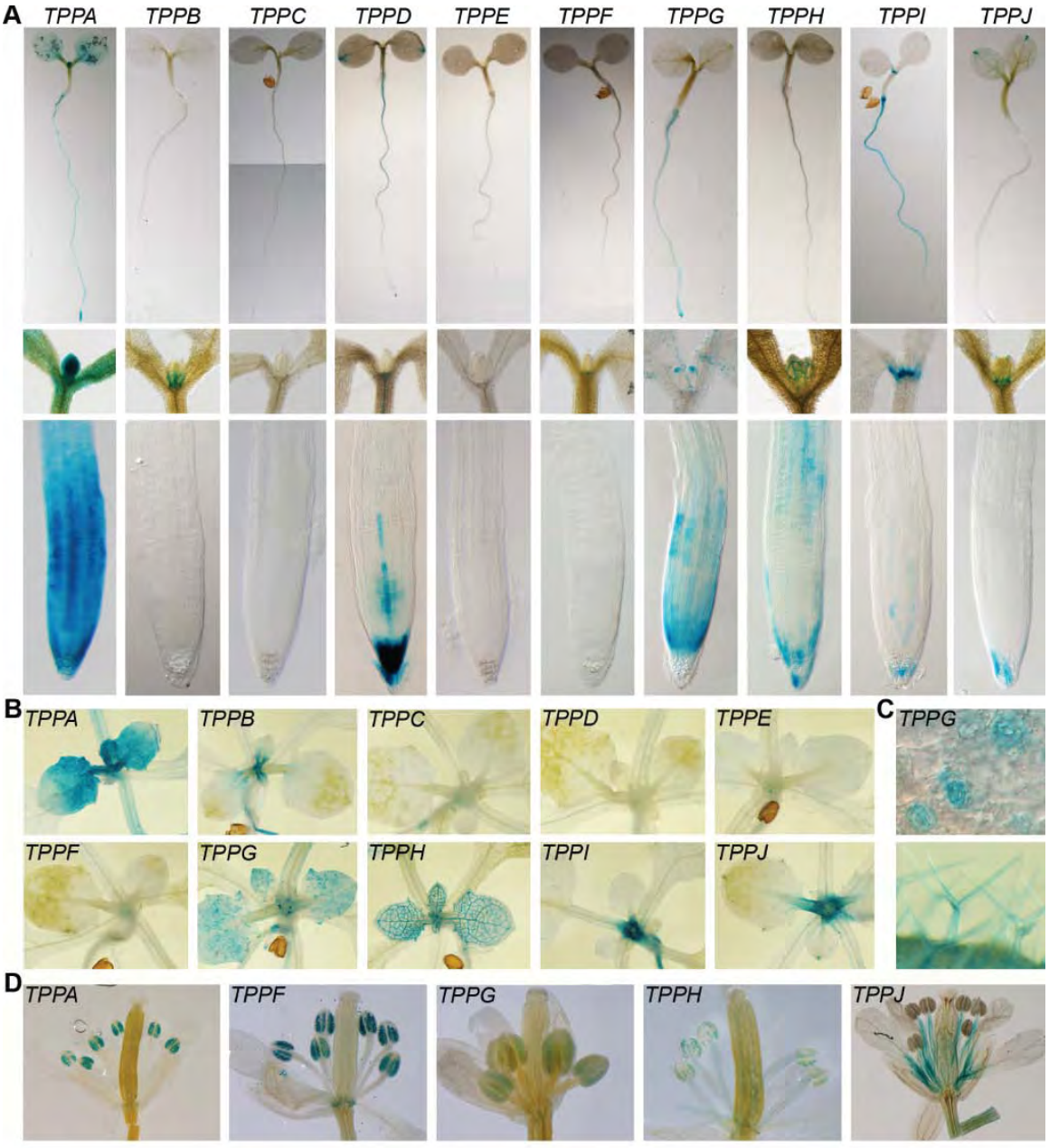


Figure 3. Differential expression profiles of Arabidopsis *TPP* genes. A. Overview of *TPP* expression patterns in young seedlings with detail view at whole seedlings, the shoot meristemic zones, and the root tips. Specific expression profiles of each *TPP* in seedlings can be noticed. **B.** Expression patterns of the *TPP* genes in shoots. Some *TPP* genes are expressed in the shoot apical meristem (SAM) as well as in the leaves, e.g. *TPPA* and *TPPG* have an epidermal pattern, *TPPH* is present in vasculature, *TPPJ* in hydathodes. **C.** Detailed *TPPG* expression profile in leaves. *TPPG* is expressed in trichomes and stomata. **D.** Overview of the *TPP* genes in flowers. Only *TPP* promoter-GUS lines with detectable GUS-expression in flowers are shown.

to leaves. At the seedling level, already soon after germination clear differences in *TPP* gene expression could be observed, with 6 *TPPs* expressed in the shoot apical meristem (SAM) and 4 *TPPs* found expressed in the root apical meristem (RAM) (Figure 3A, S3). At the tissue level, we can see *TPPD* expression in the root cap, whereas *TPPA* and *TPPG* are expressed in the protoderm (Figure 3A, S4A) . In older plants, some *TPPs* are more highly expressed in young leaves than in mature ones as seen for *TPPA*, *TPPB* and *TPPG* promoter-GUS lines (Figure 3B, S3). At the cellular level, some *TPPs* have particular expression profiles in specific cell types, e. g. *TPPG* in the stomata and trichomes of actively growing leaves young leaves, *TPPH* in the vasculature of all leaves and in stomata, and *TPPJ* in hydathodes (Figure 3B, 3C). During the reproductive stage, 5 *TPPs* are found in flowers, especially in the stamens (Figure 3D, S3). Specific expression in the anthers is found for *TPPA*, *TPPF*, *TPPG* and *TPPH*, while *TPPJ* is expressed in the filament.

Publicly available expression data (Genevestigator, Zimmermann et al., 2004; Hrutz et al., 2008; and eFP browser, Winter et al., 2007), largely fit with the GUS expression profiles. For example, in accordance with Genevestigator, we detected *TPPA, TPPF, TPPG* and *TPPH* expression in pollen grains (Figure S4B). Also consistent with Genevestigator expression levels below background, we did not detect *TPPC* and *TPPE* GUS-expression.

Additional to the different expression profiles displayed by each TPP, it was interesting to go a step further and to look into more detail at their expression during organogenic processes such as lateral root initiation (Figure S5). As it was seen in the SAM and in young leaves, TPPs are active during early events of development of new organs, *TPPA*, *TPPB*, *TPPI* and *TPPH* are present during the whole process of forming a new lateral root.

3.5 Mutation of a single *TPP* in Arabidopsis, *AtTPPG*, causes ABA-related phenotypes

To demonstrate possible functional specificity, we analyzed the phenotypes of Arabidopsis plants overexpressing or disrupted in a single *TPP*. *TPPG* shows an epidermal expression profile with remarkable cell type-specific expression in trichomes and stomata (Figure 3C). The *TPPG* overexpressor (*TPPG-1)* and T-DNA knock-down (*tppg-1*) Arabidopsis lines grow as wild type plants in normal growth conditions. However, consistent with earlier reports linking trehalose metabolism and ABA-signaling, *tppg-1* and *TPPG-1* seeds showed resistance and hypersensitivity, respectively, to ABA-inhibition of germination (Figure 4A). Consistent with *TPPG* expression in stomata, ABA addition strongly decreased stomatal aperture size in wild type and in the *TPPG-1* Arabidopsis lines but not in *tppg-1* mutants (Figure 4B), indicating that TPPG is required for normal ABA responsiveness in these cell types.

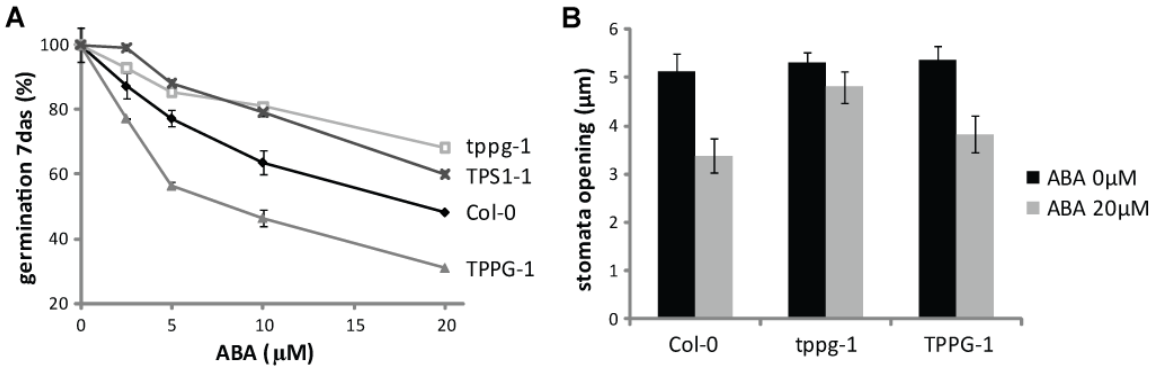


Figure 4. Response of *TPPG* knock-down (*tppg-1*) and overexpression (*TPPG-1*) Arabidopsis plants to ABA. A. Germination inhibition assay with ABA. Seeds of *tppg-1* knock-down lines, *TPPG-1, TPS1-1* overexpressors and Col-0 wild type plants were sown in different ABA concentrations; the percentage of seed germination was calculated 7 days after sowing (das). *tppg-1* seedlings were able to germinate at high levels of ABA, showing resistance to the inhibition of germination by ABA as seen with the positive control line *TPS1-1*. The opposite is observed for the *TPPG-1* overexpressor line. **B.** Stomatal aperture size in epidermal strips isolated from *tppg*-1 knock-down and *TPPG-1* overexpression plants in the absence or presence of 20μM of ABA. *tppg*-1 lines show resistance to ABA promotion of closure.

4. DISCUSSION

TPP proteins seem to be ideally positioned for the tight control of Tre6P levels and with the emerging significance of Tre6P as a reporter of carbon status in higher plants, there is an urgent need to understand the occurrence of the large number of plant TPP proteins. Arabidopsis harbours 10 putative TPP candidates, opposed to only one active TPS enzyme, AtTPS1.

Phylogenetic reconstruction of the Arabidopsis *TPP* multigene family strongly indicates that all eudicot and many monocot *TPP* genes originate from whole genome duplications. Maere et al. (2005) and Freeling (2009) found that only particular classes of genes preferentially expand through genome duplications in plants, mainly regulatory genes (transcription factors, signal transducers) and genes encoding complex-forming proteins. Similar observations have been made in mammals (Blomme et al., 2006) and other organisms (Maere and Van de Peer, 2010 and references therein). A likely explanation for this phenomenon is offered by the dosage balance hypothesis, which postulates that the stoichiometric quantities of proteins functioning within protein complexes and regulatory/signaling cascades must be preserved in order to ensure proper function (Papp et al., 2003; Birchler et al., 2005; Veitia et al., 2008; Maere and Van de Peer, 2010). This requirement is violated by small-scale gene duplications but fulfilled by whole genome duplications, where all components of such complexes and/or cascades are simultaneously duplicated. Moreover, after genome duplication, balance-sensitive gene duplicates cannot easily be lost, as this would again give rise to dosage balance effects.

The particularly strong genome duplication bias in the history of the *TPP* gene family therefore hints at a potential regulatory role for the TPP proteins.

The next step to further elucidate the functionality of these proteins was to analyze if these 10 *TPP* genes in Arabidopsis still encode Tre6P-phosphatase active enzymes. Heterologous expression in yeast showed that all 10 candidate *TPP* genes encode heterologously active TPP enzymes; apparently all 10 proteins have conserved their enzymatic function during evolution. We also confirmed the essential role of some conserved residues in the catalytic domain as predicted by an archaean crystal structures, by specific site directed mutagenesis.

Analyzing promoter GUS/GFP lines, we found very diverse spatio-temporal expression patterns for the 10 Arabidopsis *TPP* genes, indicating possible functional diversification. Our data correlate well with publicly available gene expression datasets, however, using spatio-temporal root maps, Brady and coworkers (2007) did not find detectable levels of expression for e.g. *TPPD* in the root cap, or *TPPA/TPPG* in the epidermis.

Since trehalose metabolism and especially the intermediate Tre6P seem to have profound effects on global plant performance, and interfere with plant growth and development, differential spatio-temporal regulation of trehalose metabolom is plausible. Its connection to sugar sensing/signaling suggests that cellular monitoring of, and responses to, subtle fluctuations of Tre6P levels are essential for carbon homeostasis and the integration of the nutritional status with plant growth and development. The phylogenetic analysis and the differential expression patterns indicate distinct functionality among the different TPPs; a hypothesis supported by available microarray datasets showing *TPP* expression regulation by various environmental signals (Kreps et al., 2002; Wang et al., 2003; Contento et al., 2004; Scheible et al., 2004; Thimm et al., 2004; Bläsing et al., 2005; Brenner et al., 2005; Liu et al., 2005; Roth et al., 2006), and by published co-expression analyses that cluster every individual *TPP* in separate networks associated with genes that identify different functional categories (Ma et al., 2007; Li et al., 2008). Since partial overlap is present in the promoter-GUS expression patterns, some redundancy cannot be excluded. Further research is needed to clarify if the TPPs operate by spatio-temporal Tre6P-regulation, and/or have a regulatory function uncoupled from their enzymatic TPP activity. Catalytically dead TPP versions are a nice tool to further elucidate this issue. While the Arabidopsis class II proteins still contain TPP-like domain structures, none of these proteins confers heterologous TPP activity (Ramon et al., 2009). The recruitment of the 10 *TPP* genes encoding active TPP enzymes may have enabled the evolution of the class II proteins of as yet unknown but apparently beneficial functions, different from enzymatic functions in trehalose biosynthesis.

The phenotype of Arabidopsis plants deficient in a single member of the large *TPP* family, *TPPG,* links the expression in guard cells with a regulatory function in stomatal closure.

Although *TPPH* is also expressed in leaf stomata in some growth conditions, the ABA-related stomatal closure phenotype of the *tppg-1* mutant indicates a unique role for TPPG in this response. Whether the stomatal kinetics of the *tppg-1* plants are caused by the tissue-specific accumulation of Tre6P levels in the guard cells (through the loss of the active enzyme), or by the loss of the TPPG protein itself (which might have a regulatory function, uncoupled from its catalytic activity) will be a subject for further research. Interestingly, former reports with transgenic plants overexpressing yeast *TPS1* link stomatal regulation during drought stress with trehalose metabolism (Gaff, 1996; Stiller et al., 2008). A co-expression analysis linked *AtTPPG* to the *DREB2A* and *DREB2B* expression (involved in drought response) (Li et al., 2008). The *AtTPPG* promoter also contains an ABRE binding site and DRE core motifs (ATHENA, O'Connor et al., 2005; AGRIS, Palaniswamy et al., 2006).

The alteration in ABA-sensitivity in germination in *TPPG* mutants in Arabidopsis further corroborates the strong crosstalk between ABA and trehalose metabolism. ABA is known to inhibit the induction of cell wall hydrolases that weaken and break the micropylar endosperm, allowing the radicle to grow out of the seed during the germination process (Müller et al., 2006; Gimeno-Gilles et al., 2009). Consistent with the observed phenotype, *TPPG* expression is localized in the micropylar endosperm of the developing seed (http://www.seedgenenetwork.net). The differences in ABA sensitivity of the *TPPG* mutants also fit with former observations of *TPS1* Arabidopsis mutant lines, with contrasting ABA sensitivity correlating with the differences in Tre6P content. Three TILLING alleles of *tps1* with low Tre6P levels are hypersensitive to ABA in germination assays and are affected in ABA-stomatal pore aperture regulation (Gómez et al., 2006), whereas *p35S::AtTPS1* overexpressing Arabidopsis seedlings exhibit ABA-insensitivity in germination assays and abolish glucose-induced ABA accumulation on 6% glucose (Avonce et al., 2004). These observations all indicate that there is a strong link between Tre6P levels and sensitivity to ABA.

In conclusion, our analyses point to the functional divergence of the large multigene family of *TPPs* in the model plant *A. thaliana.* All 10 genes originate from whole genome duplications, rather than from small-scale gene duplications, suggesting that this family exhibits important regulatory functions. Detailed gene expression profiles indicate that tissue-specific regulation of Tre6P levels might be the key function of this class of 10 catalytically active proteins. The ABA-related stomatal phenotype of a single *TPP* mutant in Arabidopsis, finally, illustrates the functional divergence and specificity of a single TPP protein.

5. MATERIALS AND METHODS

Phylogenetic and collinearity analysis

The genes used for the collinearity analysis and for the phylogenetic reconstruction of the TPP gene family in the green plant lineage were collected using the PLAZA v1.0 platform (Proost et al., 2009). Sequences were available for the following land plants: *Arabidopsis thaliana*, *Carica papaya*, *Populus trichocarpa*, *Vitis vinifera*, *Oryza sativa*, *Sorghum bicolor*, and the moss *Physcomitrella patens*. The sequence of an *Ostreococcus lucimarinus TPP* gene belonging to the Chlorophyta was added as an outgroup. Amino-acid level multiple sequence alignment was performed with MUSCLE (Edgar, 2004). For phylogenetic reconstruction, we used the TREEFINDER program (Jobb et al., 2004), a phylogenetic reconstruction program that infers phylogeny based on maximum likelihood under a variety of models of sequence evolution with the possibility to specify topological constraints and perform tree calibration. We used the JTT protein substitution model (Jones et al., 1992) in combination with the discrete gamma heterogeneity model (Yang, 1994) with 5 rate categories, as suggested by the TREEFINDER Model Proposer. Bootstrap analyses were performed with 1000 maximum likelihood iterations.

Modeling of T6P in the active site of AtTPPG

We modelled binding of the ligand trehalose-6-phosphate (T6P) to the active site of Arabidopsis TPPG. A model of TPPG was constructed with MODELLER 9v8 (http://salilab.org/modeller/) using the crystal structure of the TPP-related protein from the archaean *Thermoplasma acidophylum* (TaT6PP, PDB ID: 1U02), a Mg^{2+}-dependent phosphatase belonging to the L-2-HaloAcid Dehalogenase (HAD) superfamily (Rao et al., 2006) as a template. T6P (taken from PDB ID: 1BYK) (Hars et al., 1998) was manually placed into the active site of TPPG with COOT (Emsley et al., 2010) according to Rao et al. (2006) and afterwards energy-minimized using CNS (Brünger et al., 1998).

Yeast growth complementation assay

For the yeast growth complementation assay, the yeast (*Saccharomyces cerevisiae*) W303-1A wild-type strain (*Matα leu2-3, 112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC2*), the *tps1Δ* deletion strain YSH290 (W303-1A, *tps1Δ*::*TRP1*) and the *tps2Δ* deletion strain YSH448 (W303-1A, *tps2Δ*::*HIS3*) (Hohmann et al., 1993) were used (see also Ramon et al. 2009; Vandesteene et al. 2010). The different *TPP* CDS (confirmed by, and if necessary corrected to, the sequence alignment to the TAIR Arabidopsis Genome Initiative Coding Sequence dataset), were amplified from *A. thaliana* cDNA and cloned in a modified yeast multicopy pYX212 plasmid with an *HXT7* promoter and *URA3* marker, without stop codon and in frame with C-terminal double HA tag (primers, see supplementary table S2). Primers used for AtTPPG-NYN mutagenesis were 5'ATAGCTGTGTTTCTAAATTATAATGGAACACTTTCTC3' and 5'GAGAAAGTGTTCCATTATAATTTAG AAACACAGCTAT3'. As a control for the complementation assay, the *tps2Δ* strain was also transformed with the empty vector and the yeast *TPP TPS2* (Vogel et al., 1998; Zentella et al., 1999; Ramon et al., 2009). Yeast transformation was performed using the one-step method as described by Chen et al. (1992). Cultures of the transformed *tps2Δ* and WT control strains were grown overnight at 28°C on SD-ura containing 2% glucose and drop-assays were performed on SD-ura containing 2% glucose and galactose. Transformants were spotted at an OD_{600} of 1 and incubated at 28°C (control) or 38.6°C and analyzed after two days. For growth curves, pre-cultures were grown in SD-ura with 2% glucose at 28°C. Samples were then diluted to OD600 0.100 in

200µl SD-ura in 100-well Honeycomb2 plates (Thermo Electron Corporation) in three replicates. Blanks and appropriate growth controls were also included. The transformed *tps2Δ* strains and wild type strain were grown in SD-ura with 2% glucose at 28°C (control) and 38.6°C. Growth was followed by OD measurements every 15 min with continuous shaking in the Automated Microbiology Growth analysis System (Bioscreen C) (Oy Growth Curves AB Ltd).

Western blot analysis of heterologous protein expression

After overnight growth, 50ml yeast cell cultures were harvested and resuspended in ice-cold lysis buffer (500 µl 1XPBS, 0.1% Triton X-100, 10% glycerol, 2.5 mM MgCl2, 1 mM EDTA, pH8) containing protease inhibitors (Complete EDTA-free, Roche). After vortexing (0.5mm glass beads) and centrifugation at 4°C for 10 min at maximum speed, the supernatant fractions (of which protein amounts were determined with Bradford, 1974) were subjected to immunoprecipitation using anti-HA rat antibodies 3F10 (Roche) and protein G-agarose beads (Roche). Bead-bound proteins were collected by centrifugation at 4°C (30s, 2000 rpm), and washed several times with lysis buffer. Amounts of immuno-precipitated protein did not vary by more than a factor 3 between samples; SDS-PAGE sample buffer was added, followed by boiling for 5 min. After centrifugation, equal amounts of proteins were loaded on a Mini-protean precast gel (Bio-rad) together with the protein standard (Invitrogen, SeeBlue Pre-stained protein standard) in a Tris/glycine/SDS running buffer and analyzed using immunoblotting. For detection of HA-tagged proteins, we used HRP-coupled anti-HA High Affinity rat antibodies (1/1000; Roche). Proteins indicated as full-length have about the predicted size of full-length tagged proteins.

Promoter GUS/GFP constructs

To study the expression patterns of all *TPPs in vivo*, we amplified and fused the promoter/5'UTR sequences of around 2kb of all *TPPs* to GUS/GFP reporter genes in the pHGWFS7 vector (Karimi et al., 2007) using the GatewayTM technology (Invitrogen) according to manufacturer's instructions. Vectors were obtained from the Plant Systems Biology department (VIB, UGhent) (http://www.psb.ugent.be/gateway). Primers used are listed in supplementary table S2. Wild type *A. thaliana* ecotype Columbia plants were transformed by floral dip with *Agrobacterium tumefaciens* (C58C1) containing the promoter constructs (Clough and Bent, 1998). Homozygous plants for at least three (independent and single-insertion) transformed Arabidopsis lines were selected for each *TPP* promoter construct. Therefore, vapor-sterilized seeds were sown in petri dishes on full strength Murashige and Skoog Salt Mixture including vitamins (MS) medium (4.4g/l) with MES (0.5g/l) and solidified with Phytagar (8g/l) (Duchefa) with HygromycinB following a modified protocol from Harrison et al. (2006).

Histochemical and histological analysis

For detailed analysis of seedlings and roots, seedlings were grown for 2 days after germination (DAG) on vertical plates, 0.5xMS, 1% suc, continuous light. For shoot analysis, plants were grown for 15DAG, on horizontal plates, 0.5xMS, 1% suc, 16h-8h day/night cycle. For flower analysis, plants were grown on soil for 5-6 weeks, 16h-8h day/night cycle. For lateral root development analysis, seedling were grown 4DAG vertical plates, 0.5xMS, 1% suc, continuous light. The beta-glucuronidase (GUS)-staining assays were performed according to Beeckman and Engler (1994). Seedlings, shoots and flowers were afterwards cleared by mounting them in 90% lactic acid (Acros Organics) on glass microscope slides. Detailed staging of lateral roots was done as described by Malami and Benfey (1997). Samples were analyzed by differential interference contrast

microscopy (Olympus BX51). Qualitative GUS expression levels of the 10 *TPP* genes in different tissues, were clustered by the Genesis program (Sturn et al., 2002).

Physiological characterization of the AtTPPG mutants

The SALK_078443 (ecotype *Col-0*) line was available from the SALK Institute and was ordered from NASC stock center. Plants were checked for homozygosity (and single T-DNA insertion) by selection on the kanamycin marker and with PCR with a specific primer for the T-DNA insertion, Lba1 (5' TGGTTCACGTAGTGGGCCATCG3') and specific primers for the gene: LP1-RP1 primers (5' TTGGAGAGTTCGATGGATTTG3' and 5'CTGTTCCCTTCCCTCAGTACC3'). The exact insertion site of the T-DNA was determined by sequencing (384bp after starting codon; Figure S6A). *TPPG* expression was tested with real time qRT-PCR with specific primers for *TPPG* binding (5'TGGATCATCCTTCCCTTCAG3' 5'CCTTGACAAGCCTTTTACGC3') and normalized to *CDKA1;1* expression (5'ATTGCGTATTGCCACTCTCATAGG 3' 5'TCCTGACAGGGATACCGAATGC3') and *EEF1α4* (5'CTGGAGGTTTTGAGGCTGGTAT3' 5'CCAAGGGTGAAAGCAAGAAGA3'). The qRT-PCR results showed dramatic downregulation of *TPPG* in this SALK line (therefore named hereafter as *tppg-1* knock-down mutants) (Figure S5B). *AtTPPG* overexpressing plants (*TPPG-1* overexpressors) were obtained by cloning the *AtTPPG* CDS into a pDONR221 donor vector, followed by subcloning in a pK7WG2 expression vector with 35S promoter (Karimi et al., 2007) using the GatewayTM technology (Invitrogen) according to manufacturer's instructions. *Col-0* ecotype plants were transformed with the expression vector and selected on kanamycin. Homozygous, independent-single insertion lines were generated. Overexpression was tested and confirmed with qRT-PCR with the same primer sets used to test the *tppg-1* knock-down mutants (Fig. S6B).

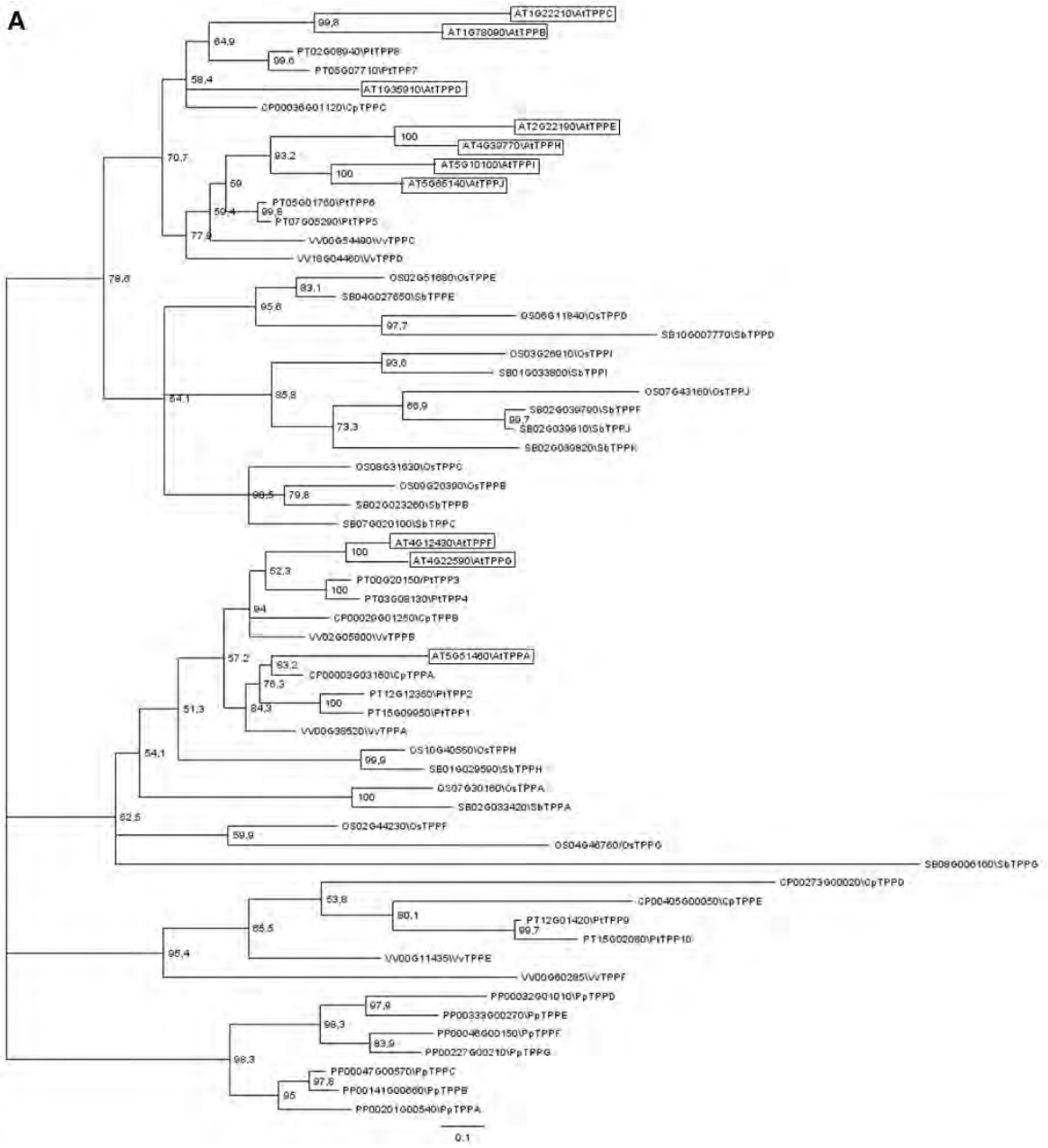
ABA germination assay

tppg-1 knock-down and *TPPG-1* overexpressor, *TPS1-1* and *Col-0* wild type seeds were sown in 0.5x MS supplemented with 1% sucrose and different concentrations (0.0, 2.5, 5.0 and 10.0 µM) of ABA (Sigma). The % of germinated seeds was daily scored for 7 days.

Measurements of stomatal aperture

The first pair of fully expanded leaves of 3 weeks old plants, grown in soil 16h-8h day/night cycle, was detached and incubated in a stomata opening buffer (10mM MES-KOH pH6.15, 10mM KCl) for 2 hours under continuous light. 20µM of ABA (0µM in controls) was added to the buffer and leaves were incubated 2 hours more. Peels of the abaxial side of the leaf were taken by pasting the abaxial side of the leaf to a microscope slide with double-sided sticky tape, the mesophyll cells were removed by gently scraping the leaf with a blade and the abaxial epidermis could be visualized in a microscope (Olympus BX51). Pictures of stomata were taken and pore aperture was measured using ImageJ software.

6. SUPPLEMENTAL INFORMATION



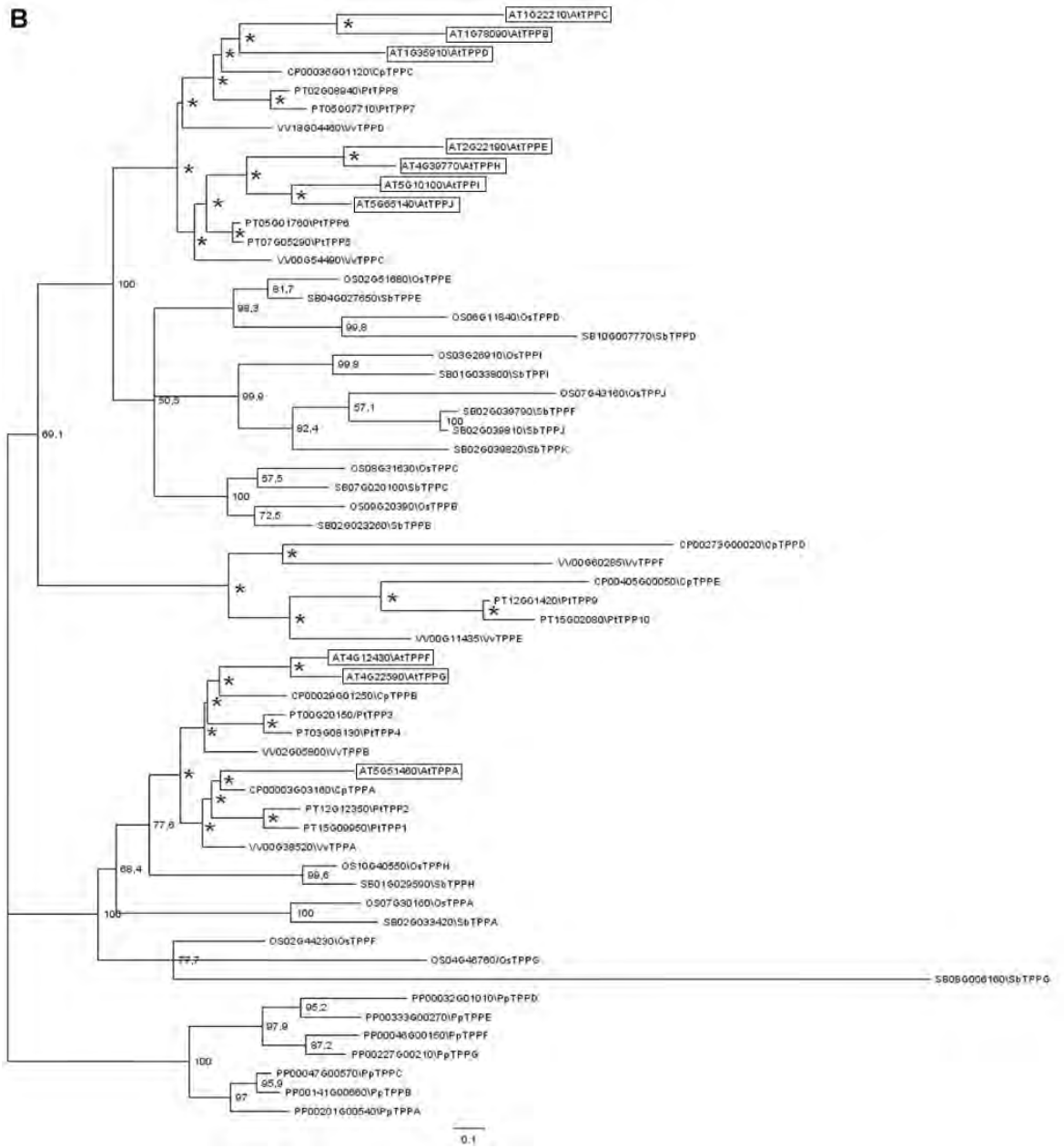


Figure S1. A. Phylogenetic tree of land plant TPP genes without any imposed topological constraints. Inner values denote bootstrap support. The branch length scale is denoted underneath the figure. **B.** Phylogenetic tree of land plant *TPP* genes with topological constraints. Inner values denote bootstrap support. The branch length scale is denoted underneath the tree. Stars represent topological constraints and the 10 members of the TPP gene family of Arabidopsis are highlighted.

Table S2. Cloning primers of the 10 *AtTPP* genes. TPPA (At5g51460.1); TPPB (At1g78090); TPPC (At1g22210) TPPD (At1g35910); TPPE (At2g22190); TPPF (At4g12430); TPPG (At4g22590); TPPH (At4g39770); TPPI (At5g10100); and TPPJ (At5g65140.1).

Primer	Sequence (5'→3')
CDS cloning	
AtTPPA-fw	ggaagatctATGGACATGAAATCTGGTCACTC
AtTPPA-rev	aaggcctACCCATTGATCTCTTCCATGTCA
AtTPPB-fw	ggaattcATGACTAACCAGAATGTCATCGTT
AtTPPB-rev	aaggcctCTCTTCTCCCACTGTCTTCCTC
AtTPPC-fw	cgggatccATGAAGATTACGGATATTTCCGG
AtTPPC-rev	aaggcctTTCTCCAAGTGTTTGTTTCTTCC
AtTPPD-fw	cgggatccATGACAAACCATAATGCCTTAATC
AtTPPD-rev	aaggcctTCTTCCTCTTAGTGACATTTGTTTC
AtTPPE-fw	cgggatccATGGTTAGATTCATCGAAGAAAA
AtTPPE-rev	aaggcctTGCCCCACACCTTGACTGTTTC
AtTPPF-fw	catgccATGGATTTAAACTCAAACCACAAATC
AtTPPF-rev	tcccccgggAAAACCAGTAGAATTCTTCTCCAAC
AtTPPG-fw	catgccATGGATTTGAATATAAACAAGACGAC
AtTPPG-rev	aaggcctAAAACTTGTTTTTGAACTTTCCATCTTC
AtTPPH-fw	cgggatccATGGTTAGATTCATAGAAGAAAACAC
AtTPPH-rev	aaggcctTGCTCCAGATCTCAATTGTTTCC
AtTPPI-fw	cgggatccATGTCAGCTAGTCAAAACATTGTC
AtTPPI-rev	aaggcctCATTCTTGGCTGCATTTGTTTCC
AtTPPJ-fw	cgggatccATGGTGAGCCAAAACGTCGTCG
AtTPPJ-rev	aaggcctTTGCTGCATCTGTTTCCACTCC
Promoter cloning	
pTPPA-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTATCGATGATCCCACATGGAG
pTPPA-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTAACAACTATATCAGCAAACTATA
pTPPB-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAATACCAAAGTGGTTGTCC
pTPPB-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTATTCTCGTTGAGACAGAGAG
pTPPC-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTAAATTAACTGAATCAACAC
pTPPC-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAGTGATCTGAGAGAGG
pTPPD-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTACTTGTAGTGCCCTTCATGG
pTPPD-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTAGGGGCTTAGGAAAAAAAAG
pTPPE-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCATCAGAAAAAATGGCATATC
pTPPE-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTCAAAGCAGATGGATGTTC
pTPPF-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGATTCTCGAGAACTTACG
pTPPF-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTAATCAAAACTCTAAACAATGCT
pTPPG-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGACCACGTCATTCTGTCATC
pTPPG-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTCGAACTCTCCAAACAATAAAATG
pTPPH-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCCTCGTAGGGTTCCAC
pTPPH-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCTGTCTGTAAATGAAAAGAG
pTPPI-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGATCAAACTAGGTTAAGAAG
pTPPI-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTATCTGTGGTTTTTCCACGAC
pTPPJ-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGTAGCACTAGTACAAGCTTAC
pTPPJ-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTATATAAAACTATAAAAACAGGG

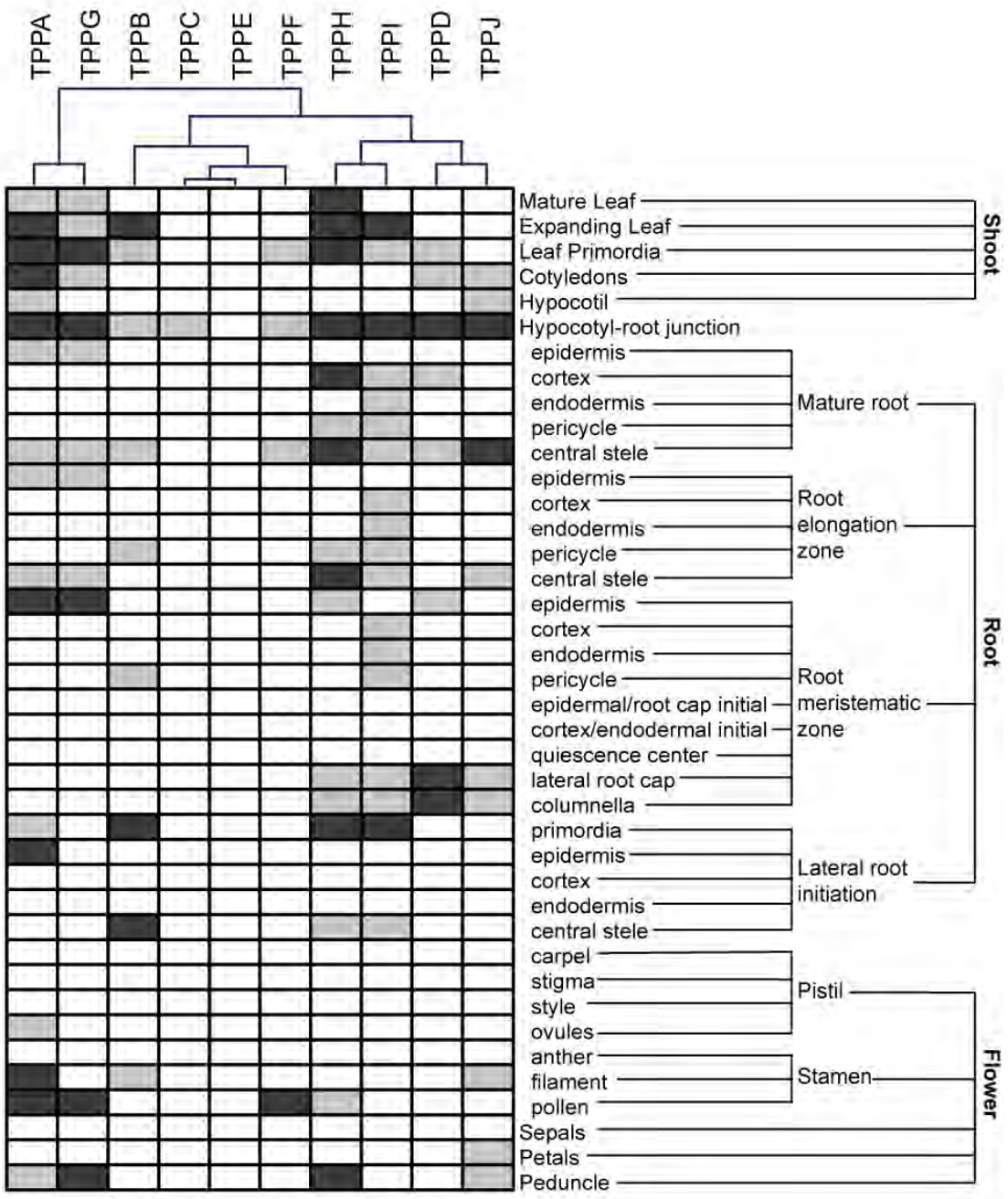


Figure S3. Clustering of the qualitative expression levels of the *TPP* genes in several tissues. White= no expression, light gray = weak expression, dark gray = strong expression. Clustering was performed with the Genesis program (Sturn et al. 2002).

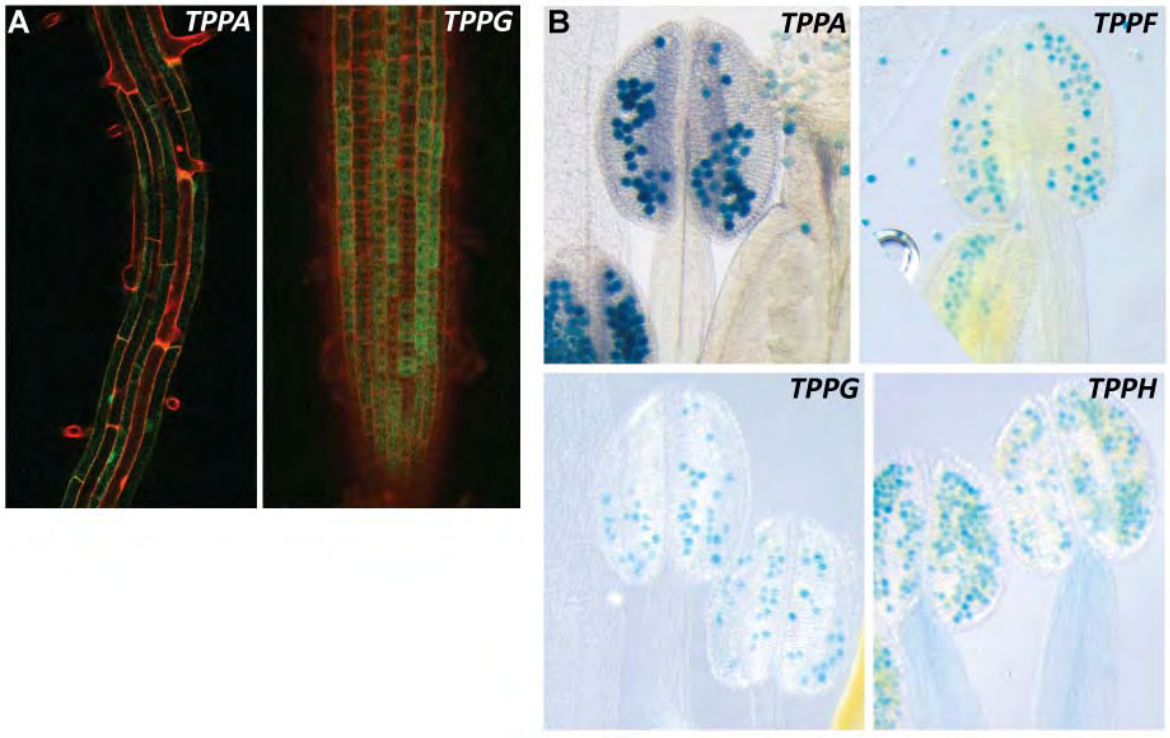


Figure S4. A. Expression of *TPPA* and *TPPG* mainly in atrichoblast cells in elongation and meristematic root zones, respectively. **B.** *TPPA, TPPF, TPPG* and *TPPH* expression in pollen grains. Only *TPP* promoter-GUS lines with detectable GUS-expression in pollen grains are shown.

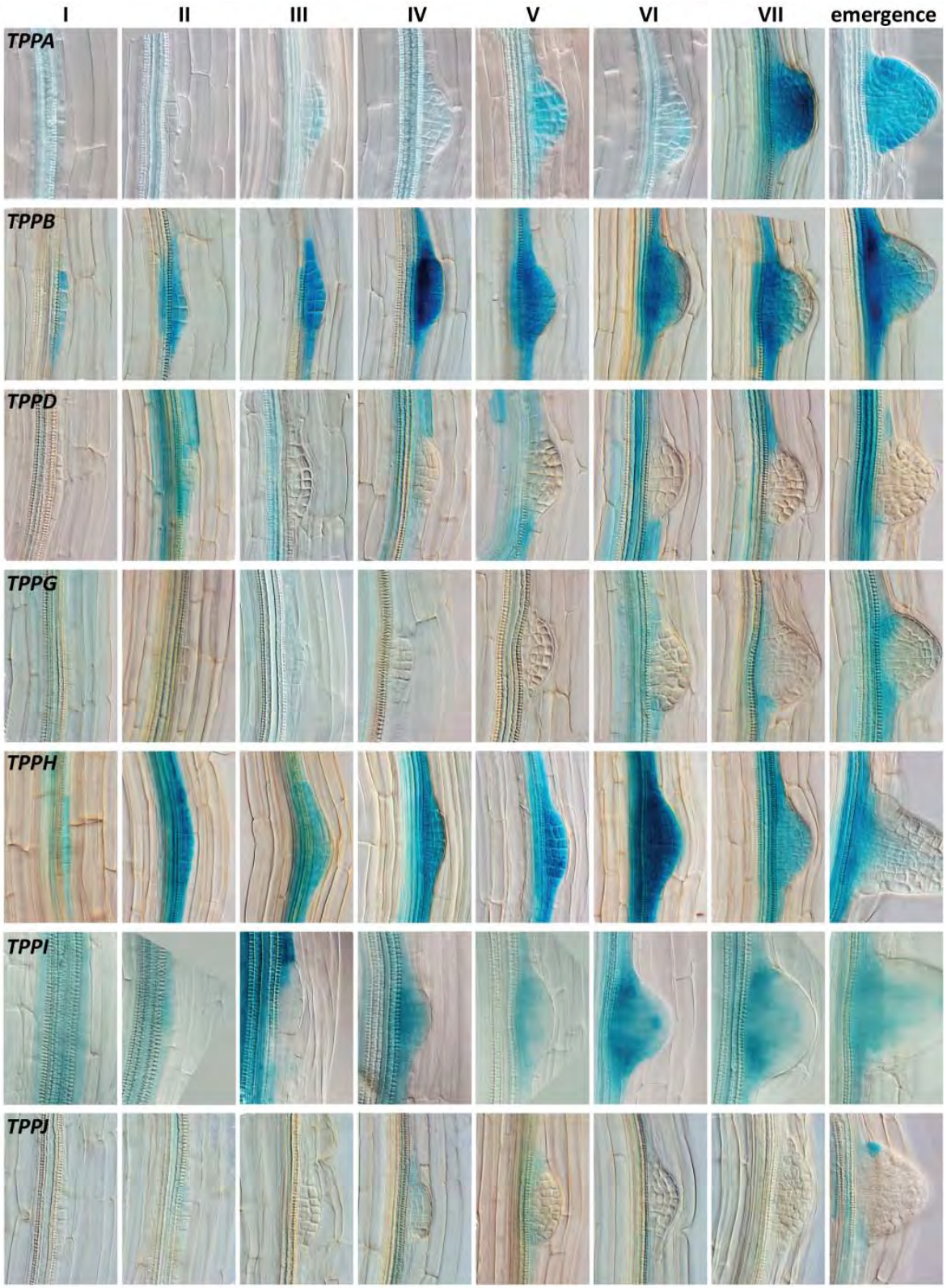


Figure S5. TPPs expression during lateral root development in 4DAG seedlings, stages were defined as described by Malamy and Benfey (1996). Only TPP lines with detectable GUS-expression in lateral root primordia are shown.

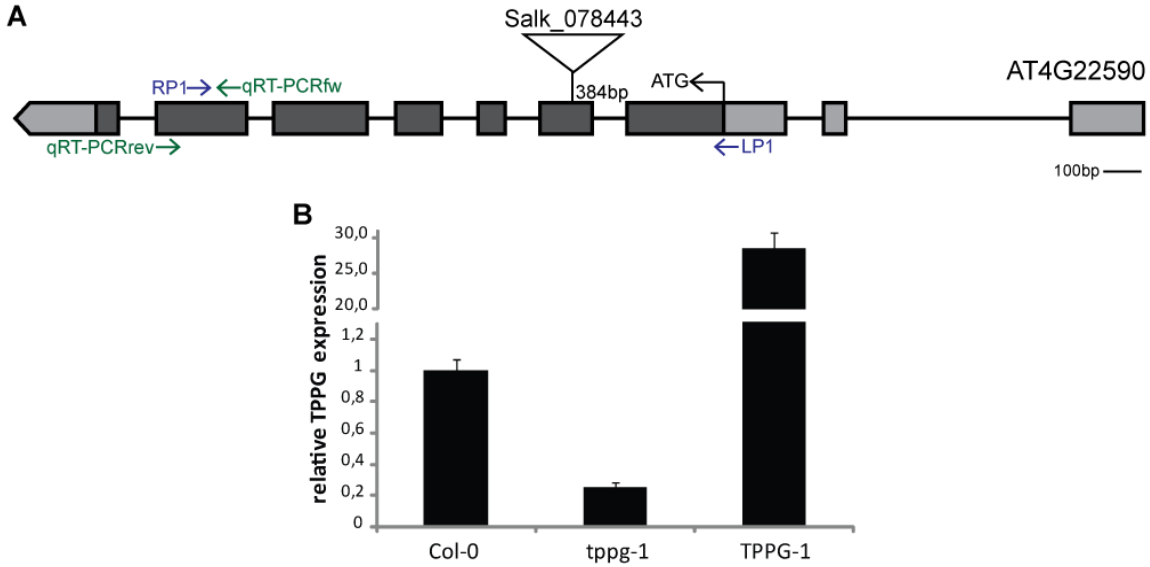


Figure S6. A. Schematic representation of the *TPPG* gene. The position of the T-DNA insertion line and the primers used for genotyping and qRT-PCR are indicated. **B.** Relative *TPPG* gene expression in *ttpg-1* knock-down and *TPPG-1* overexpressing Arabidopsis mutants.

7. REFERENCES

Avonce, N., Mendoza-Vargas, A., Morett, E., and Iturriaga, G. (2006). Insights on the evolution of trehalose biosynthesis. BMC Evol Biol **6,** 109.

Avonce, N., Wuyts, J., Verschooten, K., Vandesteene, L., and Van Dijck, P. (2010). The Cytophaga hutchinsonii ChTPSP: First Characterized Bifunctional TPS–TPP Protein as Putative Ancestor of All Eukaryotic Trehalose Biosynthesis Proteins. Mol Biol Evol **27,** 359-369.

Avonce, N., Leyman, B., Mascorro-Gallardo, J.O., Van Dijck, P., Thevelein, J.M., and Iturriaga, G. (2004). The Arabidopsis Trehalose-6-P Synthase AtTPS1 Gene Is a Regulator of Glucose, Abscisic Acid, and Stress Signaling. Plant Physiol **136,** 3649-3659.

Baena-Gonzalez, E., Rolland, F., Thevelein, J.M., and Sheen, J. (2007). A central integrator of transcription networks in plant stress and energy signalling. Nature **448,** 938-942.

Beeckman, T., and Engler, G. (1994). An easy technique for the clearing of histochemically stained plant tissue. Plant Molecular Biology Reporter **12,** 37-42.

Birchler, J.A., Riddle, N.C., Auger, D.L., and Veitia, R.A. (2005). Dosage balance in gene regulation: biological implications. Trends Genet **21,** 219-226.

Bläsing, O.E., Gibon, Y., Gunther, M., Hohne, M., Morcuende, R., Osuna, D., Thimm, O., Usadel, B., Scheible, W.R., and Stitt, M. (2005). Sugars and circadian regulation make major contributions to the global regulation of diurnal gene expression in Arabidopsis. Plant Cell **17,** 3257-3281.

Blázquez, M.A., Santos, E., Flores, C.-l., Martínez-Zapater, J.M., Salinas, J., and Gancedo, C. (1998). Isolation and molecular characterization of the Arabidopsis TPS1 gene, encoding trehalose-6-phosphate synthase. Plant J **13,** 685-689.

Blomme, T., Vandepoele, K., De Bodt, S., Simillion, C., Maere, S., and Van de Peer, Y. (2006). The gain and loss of genes during 600 million years of vertebrate evolution. Genome Biol **7,** R43.

Brady, S.M., Orlando, D.A., Lee, J.Y., Wang, J.Y., Koch, J., Dinneny, J.R., Mace, D., Ohler, U., and Benfey, P.N. (2007). A high-resolution root spatiotemporal map reveals dominant expression patterns. Science **318,** 801-806.

Brenner, W.G., Romanov, G.A., Kollmer, I., Burkle, L., and Schmulling, T. (2005). Immediate-early and delayed cytokinin response genes of Arabidopsis thaliana identified by genome-wide

expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. Plant J **44,** 314-333.

Brünger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S., Read, R.J., Rice, L.M., Simonson, T., and Warren, G.L. (1998). Crystallography & NMR system: A new software suite for macromolecular structure determination. Acta Crystallogr D Biol Crystallogr **54,** 905-921.

Cabib, E., and Leloir, L.F. (1958). The biosynthesis of trehalose phosphate. J Biol Chem **231,** 259-275.

Chary, S.N., Hicks, G.R., Choi, Y.G., Carter, D., and Raikhel, N.V. (2008). Trehalose-6-phosphate synthase/phosphatase regulates cell shape and plant architecture in Arabidopsis. Plant Physiol **146,** 97-107.

Chen, D.C., Yang, B.C., and Kuo, T.T. (1992). One-step transformation of yeast in stationary phase. Curr Genet **21,** 83-84.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16,** 735-743.

Contento, A.L., Kim, S.J., and Bassham, D.C. (2004). Transcriptome profiling of the response of Arabidopsis suspension culture cells to Suc starvation. Plant Physiol **135,** 2330-2347.

Cui, L., Wall, P.K., Leebens-Mack, J.H., Lindsay, B.G., Soltis, D.E., Doyle, J.J., Soltis, P.S., Carlson, J.E., Arumuganathan, K., Barakat, A., Albert, V.A., Ma, H., and dePamphilis, C.W. (2006). Widespread genome duplications throughout the history of flowering plants. Genome Res **16,** 738-749.

De Virgilio, C., Burckert, N., Bell, W., Jeno, P., Boller, T., and Wiemken, A. (1993). Disruption of TPS2, the gene encoding the 100-kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex in Saccharomyces cerevisiae, causes accumulation of trehalose-6-phosphate and loss of trehalose-6-phosphate phosphatase activity. Eur J Biochem **212,** 315-323.

DeLano, W.L. (2002). The PyMOL molecular graphics system. San Carlos, USA: DeLano Scientific.

Di Laurenzio, L., Wysocka-Diller, J., Malamy, J.E., Pysh, L., Helariutta, Y., Freshour, G., Hahn, M.G., Feldmann, K.A., and Benfey, P.N. (1996). The SCARECROW gene regulates an asymmetric cell division that is essential for generating the radial organization of the Arabidopsis root. Cell **86,** 423-433.

Eastmond, P.J., and Graham, I.A. (2003). Trehalose metabolism: a regulatory role for trehalose-6-phosphate? Curr Opi Plant Biol **6,** 231-235.

Eastmond, P.J., Van Dijken, A.J.H., Spielman, M., Kerr, A., Tissier, A.F., Dickinson, H.G., Jones, J.D.G., Smeekens, S.C., and Graham, I.A. (2002). Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for Arabidopsis embryo maturation. Plant J **29,** 225-235.

Edgar, R.C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics **5,** 113.

Elbein, A.D., Pan, Y.T., Pastuszak, I., and Carroll, D. (2003). New insights on trehalose: a multifunctional molecule. Glycobiology **13,** 17R-27R.

Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and development of Coot. Acta Crystallogr D Biol Crystallogr **66,** 486-501.

Fawcett, J.A., Maere, S., and Van de Peer, Y. (2009). Plants with double genomes might have had a better chance to survive the Cretaceous-Tertiary extinction event. Proc Natl Acad Sci U S A **106,** 5737-5742.

Freeling, M. (2009). Bias in plant gene content following different sorts of duplication: tandem, whole-genome, segmental, or by transposition. Annu Rev Plant Biol **60,** 433-453.

Gaff, D. (1996). Tobacco-plant desiccation tolerance. Nature **382,** 502-502.

Geelen, D., Royackers, K., Vanstraelen, M., De Bus, M., Inzé, D., Van Dijck, P., Thevelein, J.M., and Leyman, B. (2007). Trehalose-6-P synthase AtTPS1 high molecular weight complexes in yeast and Arabidopsis. Plant Science **173,** 426-437.

Gimeno-Gilles, C., Lelievre, E., Viau, L., Malik-Ghulam, M., Ricoult, C., Niebel, A., Leduc, N., and Limami, A.M. (2009). ABA-mediated inhibition of germination is related to the inhibition of genes encoding cell-wall biosynthetic and architecture: modifying enzymes and structural proteins in Medicago truncatula embryo axis. Mol Plant **2,** 108-119.

Goddijn, O.J., Verwoerd, T.C., Voogd, E., Krutwagen, R.W., de Graaf, P.T., van Dun, K., Poels, J., Ponstein, A.S., Damm, B., and Pen, J. (1997). Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. Plant Physiol **113,** 181-190.

Gómez, L.D., Baud, S., Gilday, A., Li, Y., and Graham, I.A. (2006). Delayed embryo development in the ARABIDOPSIS TREHALOSE-6-PHOSPHATE SYNTHASE 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. Plant J **46,** 69-84.

Gómez, L.D., Gilday, A., Feil, R., Lunn, J.E., and Graham, I.A. (2010). AtTPS1-mediated trehalose 6-phosphate synthesis is essential for embryogenic and vegetative growth and responsiveness to ABA in germinating seeds and stomatal guard cells. Plant J **64,** 1-13.

Harrison, S.J., Mott, E.K., Parsley, K., Aspinall, S., Gray, J.C., and Cottage, A. (2006). A rapid and robust method of identifying transformed Arabidopsis thaliana seedlings following floral dip transformation. Plant Methods **2,** 19.

Hars, U., Horlacher, R., Boos, W., Welte, W., and Diederichs, K. (1998). Crystal structure of the effector-binding domain of the trehalose-repressor of Escherichia coli, a member of the LacI family, in its complexes with inducer trehalose-6-phosphate and noninducer trehalose. Protein Sci **7,** 2511-2521.

Hohmann, S., Neves, M.J., de Koning, W., Alijo, R., Ramos, J., and Thevelein, J.M. (1993). The growth and signalling defects of the ggs1 (fdp1/byp1) deletion mutant on glucose are suppressed by a deletion of the gene encoding hexokinase PII. Curr Genet **23,** 281-289.

Holmström, K.-O., Mantyla, E., Welin, B., Mandal, A., Palva, E.T., Tunnela, O.E., and Londesborough, J. (1996). Drought tolerance in tobacco. Nature **379,** 683-684.

Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., Widmayer, P., Gruissem, W., and Zimmermann, P. (2008). Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. Adv Bioinformatics **2008,** 420747.

Jaillon, O., Aury, J.M., Noel, B., Policriti, A., Clepet, C., Casagrande, A., Choisne, N., Aubourg, S., Vitulo, N., Jubin, C., Vezzi, A., Legeai, F., Hugueney, P., Dasilva, C., Horner, D., Mica, E., Jublot, D., Poulain, J., Bruyere, C., Billault, A., Segurens, B., Gouyvenoux, M., Ugarte, E., Cattonaro, F., Anthouard, V., Vico, V., Del Fabbro, C., Alaux, M., Di Gaspero, G., Dumas, V., Felice, N., Paillard, S., Juman, I., Moroldo, M., Scalabrin, S., Canaguier, A., Le Clainche, I., Malacrida, G., Durand, E., Pesole, G., Laucou, V., Chatelet, P., Merdinoglu, D., Delledonne, M., Pezzotti, M., Lecharny, A., Scarpelli, C., Artiguenave, F., Pe, M.E., Valle, G., Morgante, M., Caboche, M., Adam-Blondon, A.F., Weissenbach, J., Quetier, F., and Wincker, P. (2007). The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature **449,** 463-467.

Jobb, G., von Haeseler, A., and Strimmer, K. (2004). TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol Biol **4,** 18.

Jones, D.T., Taylor, W.R., and Thornton, J.M. (1992). The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci **8,** 275-282.

Karimi, M., Depicker, A., and Hilson, P. (2007). Recombinational cloning with plant gateway vectors. Plant Physiol **145,** 1144-1154.

Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X., and Harper, J.F. (2002). Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiol **130,** 2129-2141.

Leyman, B., Van Dijck, P., and Thevelein, J.M. (2001). An unexpected plethora of trehalose biosynthesis genes in Arabidopsis thaliana. Trends Plant Sci **6,** 510-513.

Li, P., Ma, S., and Bohnert, H.J. (2008). Coexpression characteristics of trehalose-6-phosphate phosphatase subfamily genes reveal different functions in a network context. Physiologia Plantarum **133,** 544-556.

Liu, F., Vantoai, T., Moy, L.P., Bock, G., Linford, L.D., and Quackenbush, J. (2005). Global transcription profiling reveals comprehensive insights into hypoxic response in Arabidopsis. Plant Physiol **137,** 1115-1129.

Lunn, J.E. (2007). Gene families and evolution of trehalose metabolism in plants. Functional Plant Biology **34,** 550-563.

Lunn, J.E., Feil, R., Hendriks, J.H., Gibon, Y., Morcuende, R., Osuna, D., Scheible, W.R., Carillo, P., Hajirezaei, M.R., and Stitt, M. (2006). Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in Arabidopsis thaliana. Biochem J **397,** 139-148.

Ma, S., Gong, Q., and Bohnert, H.J. (2007). An Arabidopsis gene network based on the graphical Gaussian model. Genome Res **17,** 1614-1625.

Maere, S., and Van de Peer, Y. (2010). Duplicate retention after small- and large-scale duplications. In Evolution after gene duplication, D.A. Liberles and K. Dittmar, eds (Wiley Press).

Maere, S., De Bodt, S., Raes, J., Casneuf, T., Van Montagu, M., Kuiper, M., and Van de Peer, Y. (2005). Modeling gene and genome duplications in eukaryotes. Proc Natl Acad Sci U S A **102,** 5454-5459.

Malamy, J.E., and Benfey, P.N. (1997). Organization and cell differentiation in lateral roots of Arabidopsis thaliana. Development **124,** 33-44.

Masterson, J. (1994). Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. Science **264,** 421-424.

Miranda, J.A., Avonce, N., Suarez, R., Thevelein, J.M., Van Dijck, P., and Iturriaga, G. (2007). A bifunctional TPS-TPP enzyme from yeast confers tolerance to multiple and extreme abiotic-stress conditions in transgenic Arabidopsis. Planta **226,** 1411-1421.

Müller, K., Tintelnot, S., and Leubner-Metzger, G. (2006). Endosperm-limited Brassicaceae seed germination: abscisic acid inhibits embryo-induced endosperm weakening of Lepidium sativum (cress) and endosperm rupture of cress and Arabidopsis thaliana. Plant Cell Physiol **47,** 864-877.

O'Connor, T.R., Dyreson, C., and Wyrick, J.J. (2005). Athena: a resource for rapid visualization and systematic analysis of Arabidopsis promoter sequences. Bioinformatics **21,** 4411-4413.

Palaniswamy, S.K., James, S., Sun, H., Lamb, R.S., Davuluri, R.V., and Grotewold, E. (2006). AGRIS and AtRegNet. a platform to link cis-regulatory elements and transcription factors into regulatory networks. Plant Physiol **140,** 818-829.

Papp, B., Pal, C., and Hurst, L.D. (2003). Dosage sensitivity and the evolution of gene families in yeast. Nature **424,** 194-197.

Paul, M.J. (2008). Trehalose 6-phosphate: a signal of sucrose status. Biochem J **412,** e1-e2.

Pramanik, M.H., and Imai, R. (2005). Functional identification of a trehalose 6-phosphate phosphatase gene that is involved in transient induction of trehalose biosynthesis during chilling stress in rice. Plant Mol Biol **58,** 751-762.

Proost, S., Van Bel, M., Sterck, L., Billiau, K., Van Parys, T., Van de Peer, Y., and Vandepoele, K. (2009). PLAZA: a comparative genomics resource to study gene and genome evolution in plants. Plant Cell **21,** 3718-3731.

Ramon, M., De Smet, I., Vandesteene, L., Naudts, M., Leyman, B., Van Dijck, P., Rolland, F., Beeckman, T., and Thevelein, J.M. (2009). Extensive expression regulation and lack of heterologous enzymatic activity of the Class II trehalose metabolism proteins from Arabidopsis thaliana. Plant Cell Environ **32,** 1015-1032.

Rao, K.N., Kumaran, D., Seetharaman, J., Bonanno, J.B., Burley, S.K., and Swaminathan, S. (2006). Crystal structure of trehalose-6-phosphate phosphatase-related protein: biochemical and biological implications. Protein Sci **15,** 1735-1744.

Romero, C., Belles, J.M., Vaya, J.L., Serrano, R., and Culianez-Macia, F.A. (1997). Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. Planta **201,** 293-297.

Roth, U., von Roepenack-Lahaye, E., and Clemens, S. (2006). Proteome changes in Arabidopsis thaliana roots upon exposure to Cd2+. J Exp Bot **57,** 4003-4013.

Satoh-Nagasawa, N., Nagasawa, N., Malcomber, S., Sakai, H., and Jackson, D. (2006). A trehalose metabolic enzyme controls inflorescence architecture in maize. Nature **441,** 227-230.

Scheible, W.R., Morcuende, R., Czechowski, T., Fritz, C., Osuna, D., Palacios-Rojas, N., Schindelasch, D., Thimm, O., Udvardi, M.K., and Stitt, M. (2004). Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen. Plant Physiol **136,** 2483-2499.

Schluepmann, H., Pellny, T., van Dijken, A., Smeekens, S., and Paul, M. (2003). Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. Proc Natl Acad Sci U S A **100,** 6849-6854.

Shima, S., Matsui, H., Tahara, S., and Imai, R. (2007). Biochemical characterization of rice trehalose-6-phosphate phosphatases supports distinctive functions of these plant enzymes. Febs J **274,** 1192-1201.

Soltis, D.E., Albert, V.A., Leebens-Mack, J., Bell, C.D., Paterson, A.H., Zheng, C., Sankoff, D., dePamphilis, C.W., Wall, P.K., and Soltis, P.S. (2009). Polyploidy and angiosperm diversification. Am J Bot **96,** 336.

Stiller, I., Dulai, S., Kondrak, M., Tarnai, R., Szabo, L., Toldi, O., and Banfalvi, Z. (2008). Effects of drought on water content and photosynthetic parameters in potato plants expressing the trehalose-6-phosphate synthase gene of Saccharomyces cerevisiae. Planta **227,** 299-308.

Sturn, A., Quackenbush, J., and Trajanoski, Z. (2002). Genesis: cluster analysis of microarray data. Bioinformatics **18,** 207-208.

Tang, H., Bowers, J.E., Wang, X., Ming, R., Alam, M., and Paterson, A.H. (2008). Synteny and Collinearity in Plant Genomes. Science **320,** 486-488.

Thaller, M.C., Schippa, S., and Rossolini, G.M. (1998). Conserved sequence motifs among bacterial, eukaryotic, and archaeal phosphatases that define a new phosphohydrolase superfamily. Protein Sci **7,** 1647-1652.

Thimm, O., Blasing, O., Gibon, Y., Nagel, A., Meyer, S., Kruger, P., Selbig, J., Muller, L.A., Rhee, S.Y., and Stitt, M. (2004). MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J **37,** 914-939.

Usadel, B., Blasing, O.E., Gibon, Y., Retzlaff, K., Hohne, M., Gunther, M., and Stitt, M. (2008). Global transcript levels respond to small changes of the carbon status during progressive exhaustion of carbohydrates in Arabidopsis rosettes. Plant Physiol **146,** 1834-1861.

Van de Peer, Y., Maere, S., and Meyer, A. (2009). The evolutionary significance of ancient genome duplications. Nat Rev Genet **10,** 725-732.

van Dijken, A.J., Schluepmann, H., and Smeekens, S.C. (2004). Arabidopsis trehalose-6-phosphate synthase 1 is essential for normal vegetative growth and transition to flowering. Plant Physiol **135,** 969-977.

Vandesteene, L., Ramon, M., Le Roy, K., Van Dijck, P., and Rolland, F. (2010). A Single Active Trehalose-6-P Synthase (TPS) and a Family of Putative Regulatory TPS-Like Proteins in Arabidopsis. Mol Plant **3,** 406-419.

Veitia, R.A., Bottani, S., and Birchler, J.A. (2008). Cellular reactions to gene dosage imbalance: genomic, transcriptomic and proteomic effects. Trends Genet **24,** 390-397.

Vogel, G., Aeschbacher, R.A., Müller, J., Boller, T., and Wiemken, A. (1998). Trehalose-6-phosphate phosphatases from Arabidopsis thaliana: identification by functional complementation of the yeast tps2 mutant. Plant J **13,** 673-683.

Wang, R., Okamoto, M., Xing, X., and Crawford, N.M. (2003). Microarray Analysis of the Nitrate Response in Arabidopsis Roots and Shoots Reveals over 1,000 Rapidly Responding Genes and New Linkages to Glucose, Trehalose-6-Phosphate, Iron, and Sulfate Metabolism. Plant Physiol **132,** 556-567.

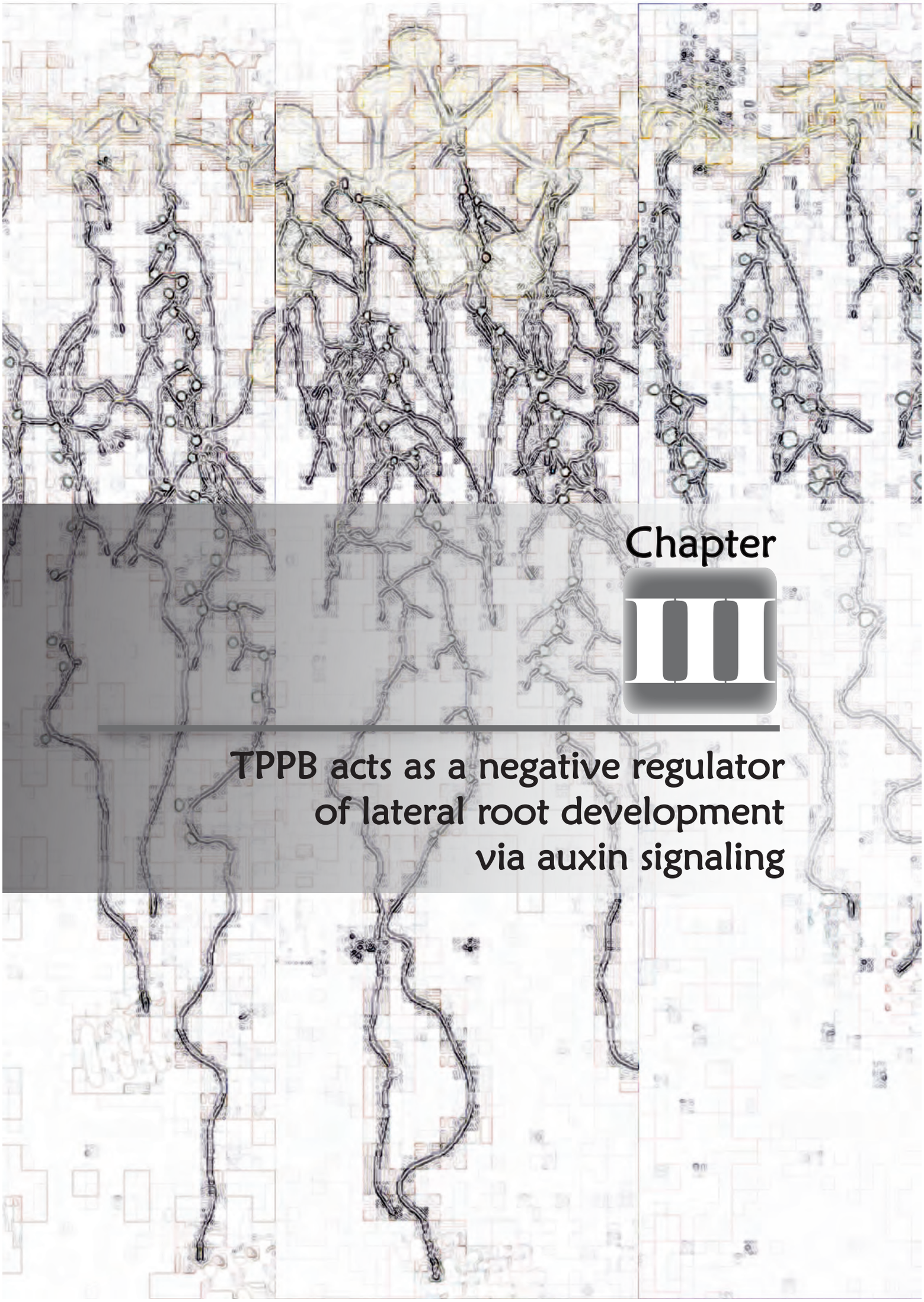
Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V., and Provart, N.J. (2007). An "electronic fluorescent pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS One **2,** e718.

Yang, Z. (1994). Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. J Mol Evol **39,** 306-314.

Zentella, R., Mascorro-Gallardo, J.O., Van Dijck, P., Folch-Mallol, J., Bonini, B., Van Vaeck, C., Gaxiola, R., Covarrubias, A.A., Nieto-Sotelo, J., Thevelein, J.M., and Iturriaga, G. (1999). A Selaginella lepidophylla trehalose-6-phosphate synthase complements growth and stress-tolerance defects in a yeast tps1 mutant. Plant Physiol **119,** 1473-1482.

Zhang, Y., Primavesi, L.F., Jhurreea, D., Andralojc, P.J., Mitchell, R.A.C., Powers, S.J., Schluepmann, H., Delatte, T., Wingler, A., and Paul, M.J. (2009). Inhibition of SNF1-Related Protein Kinase1 Activity and Regulation of Metabolic Pathways by Trehalose-6-Phosphate. Plant Physiol **149,** 1860-1871.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004). GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol **136,** 2621-2632.



Chapter

III

TPPB acts as a negative regulator of lateral root development via auxin signaling

TPPB acts as a negative regulator of lateral root development via auxin signaling

1. ABSTRACT

Trehalose metabolism has been related to stress resistance, ABA and sugar signaling and embryo development, among other processes in plants. The intermediate of trehalose biosynthesis, Tre6P, has been postulated as a sucrose signal in the cell and therefore it can be an important connector between energy sources and processes such as growth. Here we describe for the first time the role of a TPP in the initiation and development of a new organ such as a lateral root. *TPPB* is expressed in pericycle cells during lateral root initiation and emergence. This, together with the *TPPB* mutant root phenotype of a higher lateral root density in *TPPB-KO* lines, revealed the inhibitory effect of *TPPB* in lateral root development. Auxin is an important player in the development of a new lateral root and *TPPB* was found to be negatively regulated by auxin, possibly through a repressing ARF.

2. INTRODUCTION

Lateral root development is a well studied event. These roots are formed from pericycle founder cells (PFC) which are located next to the xylem pole (XPP). These PFC initiate a cycle of divisions to pattern and form a lateral root primordium which later on will emerge from the main root (Casimiro et al., 2003; reviewed by Péret et al., 2009). Genetic and hormonal players in this process have been extensively described as well, auxin being one of the most important ones. Many auxin signaling mutants are affected in lateral root formation (Casimiro et al., 2003). One of the first mutants described by its impaired lateral root phenotype was SOLITARY ROOT (*slr-1*), which happened to be a gain-of-function mutation in the IAA14 gene, one of the members of the Auxin/Indole 3-Acetic Acid (Aux/IAA) protein family (Fukaki et al., 2002). Later, a double mutant of two members of the Auxin-Response Factor family (ARF), *arf7arf19* loss-of-function mutant was found to have the same phenotype as *slr-1* (Okushima et al., 2005). The auxin signaling cascade controlling lateral root development and other processes, starts from the perception of auxin by the SCF^{TIR1} (Skp1-Cul1-F-box-TIR1) complex. Upon binding of auxin to the TIR1 receptor, Aux/IAA proteins are targeted to degradation in an ubiquitin (Ub)-mediated way. Aux/IAA proteins regulate auxin-dependent transcription by forming dimers with ARF transcription factors, and in this way Aux/IAAs inhibit ARFs from inducing transcription of their target genes. Therefore, ARFs function in the transcriptional regulation of early auxin responsive genes (Fukaki et al., 2002).

The Aux/IAA and ARF protein families are encoded by 29 and 23 genes, respectively. Extensive structural analysis on ARF proteins define them as having an N-terminal DNA binding domain (DBD), sufficient to recruit them to the target genes; a middle region (MR) that determines its function as repressor or as activator of transcription; and a C-terminal domain containing motifs III and IV important for interaction with Aux/IAA and other ARFs (Ulmasov et al., 1999; Tiwari et al., 2003). ARFs bind to Auxin-response Elements (AuxREs, TGTCTC) sequences in promoters of early-response auxin genes, but the regulation of transcription depends on the MR in the ARF. Five ARFs are transcriptional activators by having a glutamine-rich MR, 15 ARFs may function as repressors and 3 remain unknown (Ulmasov et al., 1999; Tiwari et al., 2003). However recent studies have suggested that other transcription factors act with ARF proteins, thus their activity can vary depending on the developmental context (Shin et al., 2007; Varaud et al., 2011).

In the quest to find new molecular players of lateral root initiation, a lateral root-induction system (LRIS) was developed. At the onset of lateral root initiation, auxin activates different processes to induce XPP cells for asymmetric cell divisions. The LRIS uses an auxin transport inhibitor (N-1-naphthylphthalamic acid, NPA) followed by auxin (1-naphthalene acetic acid, NAA) application to synchronously activate divisions in the XPP cells. The first asymmetric divisions occurred 6h after transfer from NPA to NAA, indicating that this is a suitable system to systematically follow transcriptional events that occur prior lateral root initiation (LRI) (Himanen et al., 2002). Using the LRIS, mutants such as *slr-1* and GFP sorted protoplasts from XPP-GFP marker line (J0121, Laplaze et al., 2005) had been subjected to transcriptomic analysis (Vanneste et al., 2005; De Smet et al., 2008). Additional to these microarray experiments, auxin treated *arf19* and *arf7*, single and double mutants have been transcriptionally analyzed to elucidate transductional components mediating auxin regulated lateral root initiation (Okushima et al., 2007). Recently, a Visual Lateral Root Transcriptome Compendium (VisuaLRTC) including these microarray datasets and others more was published (Parizot et al., 2010). This compendium is a helpful tool to obtain an initial idea of the involvement of a gene of interest with lateral root initiation via auxin regulation.

In this study, *TPPB* was identified as a novel regulator of lateral root initiation and emergence. Its particular expression in pericycle cells and its activation from early stages of lateral root initiation, together with the lateral root phenotypes of *TPPB* mutants, indicate a clear role of this TPP enzyme in an organogenesis process. Additionally TPPB seems to be a direct target of auxin signaling, and it is transcriptionally repressed probably through a repressing ARF.

3. RESULTS

3.1 TPPB is specifically expressed in pericycle cells and lateral root primordia

In general, by gus staining in seedlings 2 days after germination (DAG) of *promTPPB::GUS-GFP* lines, we could see that *TPPB* is expressed in the SAM and in the hypocotyl-root junction (Figure 1A). Even though 2 DAG *TPPB* is absent in the RAM, by 10 DAG its expression there (Figure 1A), as well as in the RAM of lateral roots is evident (Figure 1C). Along the root *TPPB* is expressed in mature pericycle cells that are forming new lateral roots Figure 1B). During lateral root development *TPPB* is highly expressed from stage I till emergence of the lateral root primordia. Expression is clearly visible in the base and actively dividing cells from stage I until V. After stage V it is more expressed in the base of the primordia and in the central stele while, after emergence it fades away in the stele, and stays mainly at the root tip (Figure 1C).

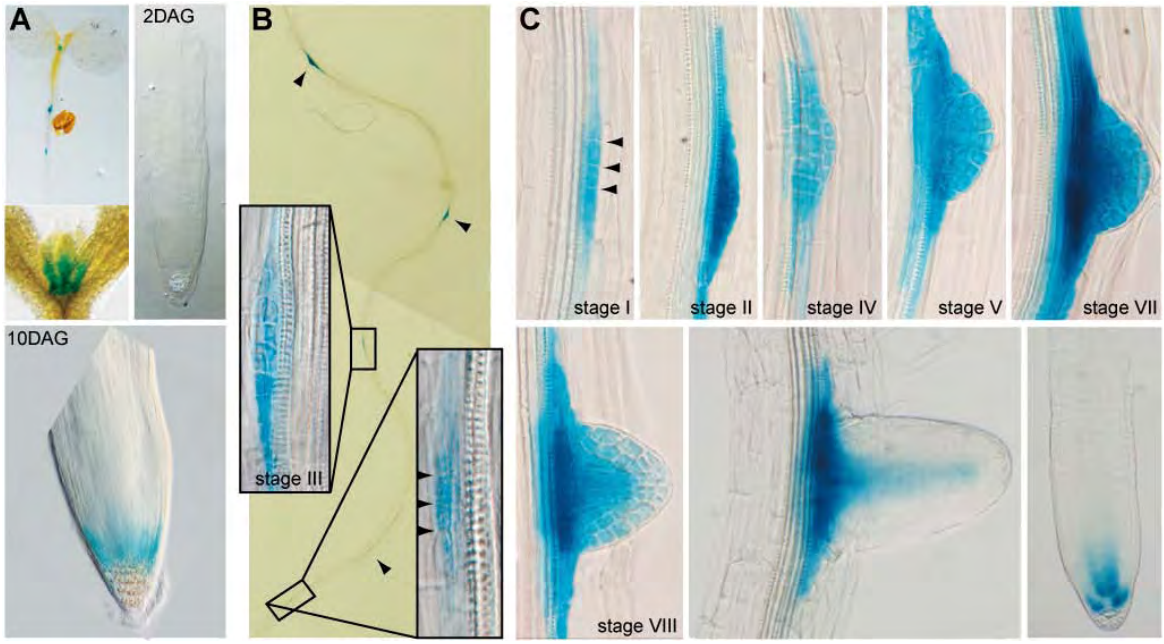


Figure 1. TPPB expression in seedlings. A. Expression in 2 DAG seedlings, and in 10 DAG root tip. **B.** Expression in 10 DAG roots, insets correspond to expression during early stages of lateral root initiation. **C.** Expression during different stages of lateral root initiation until emergence.

3.2 TPPB mutants have lateral root and shoot phenotypes

Based on the specific expression pattern during lateral root initiation, T-DNA insertion lines were selected and OE lines were generated in order to check for possible phenotypes related to lateral root development. Two different insertion lines were selected, SALK_037324 and SAIL_191F08 which harbor insertions in exon 3 and 9 of the gene, respectively (Figure 2A). After getting homozygous lines (see material and methods), relative expression of TPPB was checked by qPCR detecting a full knock-out (KO) in the salk line, hereafter *tppb-1*, and a knock-down (KD) of 80% in the sail line,

hereafter *tppb-2* (Figure 2B). In parallel, overexpression (OE) lines were generated by fusing the coding sequence of TPPB with a 35S promoter. T3 -single insertion- homozygous lines were checked by qPCR and confirmed upregulation of *TPPB* by more than 15-fold in *TPPB-1* and 8-fold in *TPPB-2* (Figure 2C).

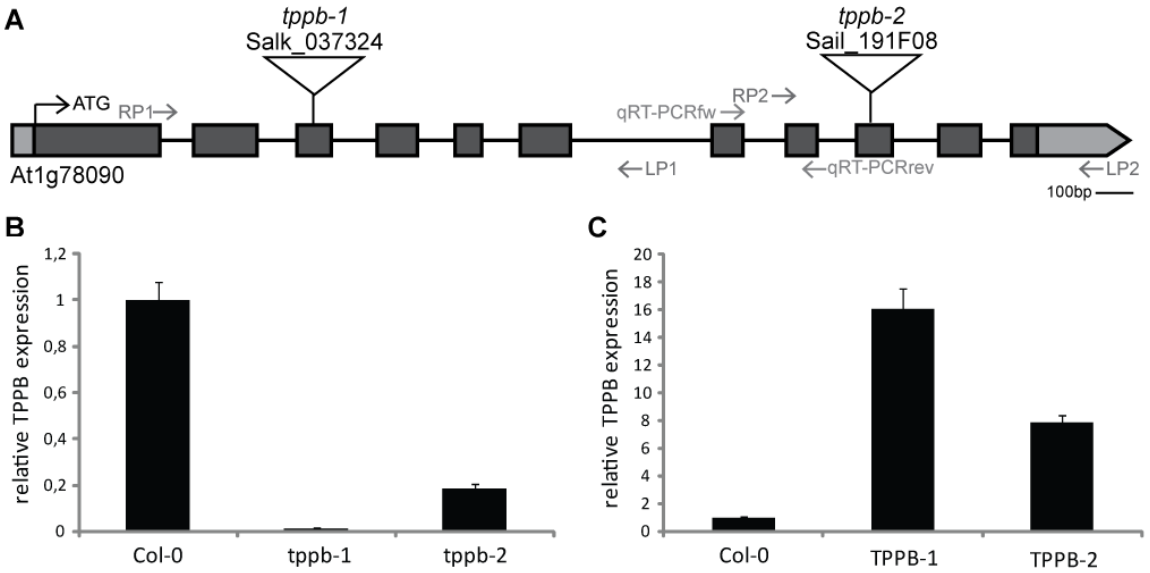


Figure 2. TPPB T-DNA insertion and OE mutants. **A.** Genomic TPPB scheme showing UTRs in light gray boxes, exons in dark gray boxes and introns as black line. The two T-DNA lines that were isolated are indicated in the map. **B.** Relative *TPPB* expression levels in T-DNA lines showing downregulation of TPPB. **C.** Relative *TPPB* expression levels in OE lines showing upregulation of TPPB.

The lateral root phenotype of *TPPB* mutant lines was checked in vertically grown seedlings 12 DAG. *TPPB-KO* seedlings presented a larger root system than wt and *TPPB-OE* lines (Figure 3A). No differences in the main root length were found between wt and *TPPB-KO* plants, although for *TPPB-OE* plants the main root was shorter (Figure 3B). *TPPB-KO* plants had slightly more lateral roots than wt whereas for *TPPB-OE* few lateral roots were found (Figure 3C). In the case of lateral root density, *TPPB-KO* plants had more lateral roots per cm than wt and *TPPB-OE* plants showed the opposite phenotype. When these mutants were subjected to a detailed analysis of lateral root initiation, i.e. staging of lateral root primordia, *TPPB-KO* lines were noticed for their higher density of primordia at early stages and in general for a higher density of emerged and non-emerged lateral roots, whereas for *TPPB-OE* densities were comparable to wt, and as described above the main differences were found in emerged lateral root density (Figure 3E). This suggests that pericycle cells in *TPPB-KO* are more active to start cell divisions and form a new lateral root. These results indicate that *TPPB* functions as a negative regulator of lateral root initiation and development.

TPPB mutant root phenotypes were consistent in all conditions tested, such as low sucrose, short days, or nitrogen starvation conditions (data not shown). This is in part in

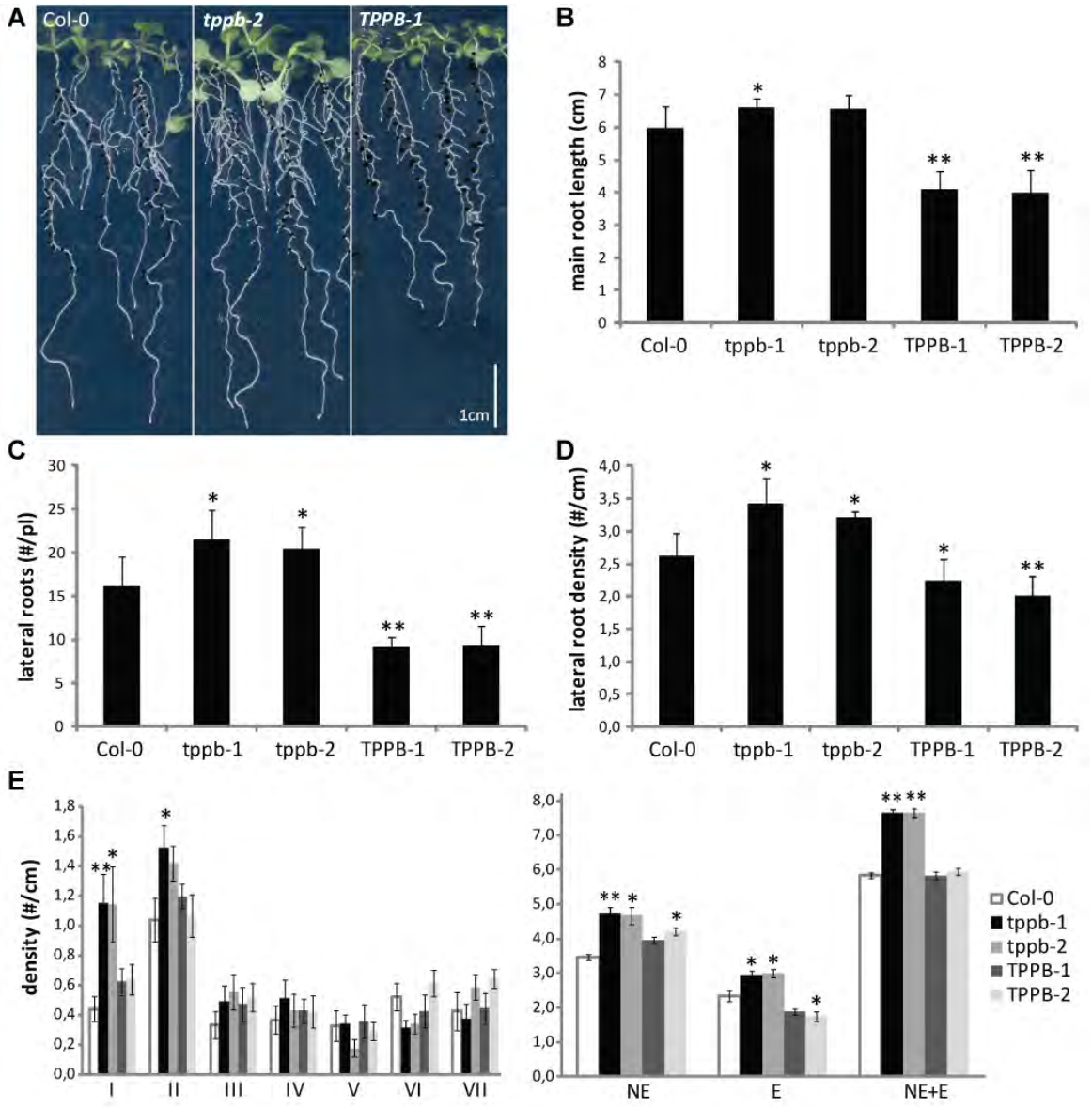


Figure 3. Phenotype of *TPPB-K0* and *OE* mutants. A. 12 DAG plants grown on vertical plates, a larger root system is evident for *tppb-2* line. **B.** 12 DAG *TPPB* mutants main root length, *TPPB-OE* lines have a shorter main root that wt. **C.** 12 DAG *TPPB* mutants lateral root number, *TPPB-OE* lines have less lateral roots per plant than wt. **D.** 12 DAG *TPPB* mutants lateral root density, *TPP-KO* plants show a higher density than wt and the opposite is true for *TPPB-OE* plants. **E.** Staging of lateral root primordia in 6 DAG plants expressed as number of primordia/cm of main root, stages=I-VII, NE=total of non emerged primordia, E=emerged lateral roots, NE+E=total of primordia and lateral roots, higher primordia densities at early stages and more emerged lateral roots is the phenotype of *TPPB-KO* plants.*P<0.05, **P<0.01.

disagreement with the root growth phenotype of mutants with modified trehalose and Tre6P content grown under high levels of sugars (Schluepmann et al., 2003; Avonce et al., 2004). Therefore, we measured trehalose and Tre6P, among other sugars, in *TPPB* mutants to validate, in some way, the function of TPPB as trehalose biosynthesis enzyme (Table S1). *TPPB-KO* and *OE* mutants had, respectively, lower and higher trehalose levels than wt, in the root as well as in the shoot. However differences in Tre6P content were subtle. As the *TPPB-OE* lines were constructed with a 35Spromoter, we expected to find

more differences in sugar contents in this line than in the *KO* plants. As such glucose, Suc6P, Gluc6P and Fruc6P contents were high in *TPPB-OE* plants, whilst for *KO* plants, only glucose content was found to be lower than in wt. Additionally starch content was measured showing that *TPPB-OE* plants have higher starch than wt.

Unexpectedly, *TPPB* mutants shoot phenotype was also noticeable. In essence, *TPPB-KO* plants were bigger than wt when grown in vitro (Figure S2A) or in-soil (data not shown) and the opposite occurred with OE plants. Detailed analysis of this phenotype was done in 21 DAG *in vitro* plants by dissecting the rosette in the different leaves. The individual and total leaf area was measured and showed that *TPPB-KO* plants had bigger leaves than wt, whereas OE plants were smaller. Moreover, *TPPB-KO* plants have on average 2 more leaves than wt (Figure S2B), while *TPPB-OE* plants seemed to be delayed in growth. These results led us to investigate in more detail whether the phenotype in leaf size is due to more cell divisions or larger cells (De Veylder et al., 2002). The results of the analysis on the abaxial epidermis of the first pair of true leaves in three-week old mutants are shown in Table S3. *TPPB-KO* leaves were 17% larger than wt, while *TPPB-OE* were smaller. No difference was found for the cell size or in the stomatal index. This suggests that the enlargement in leaf size is due to higher cell division rates in the *TPPB-KO* plants.

3.3 TPPB is strongly downregulated by auxin repression that depends on SLR and ARF7

By looking at the publicly available data sets involved in lateral root development summarized in the spreadsheet tool Visual Lateral Root Transcriptome Compendium (VisuaLRTC, Parizot et al., 2010) the regulation of *TPPB* was checked. In the lateral root initiation set (Vanneste et al., 2005), plants in the lateral root-inducible system (LRIS) (Himanen et al., 2002) showed that *TPPB* is strongly downregulated after a short auxin treatment with NAA on pretreated NPA plants. However TPPB repression by auxin is partially dependent on *SLR/IAA14,* as in the *slr-1* mutant this repression is to some extent reduced. For the asymmetric division set (De Smet et al., 2008), XPP protoplasts from plants under the LRIS showed strong *TPPB* downregulation. For the auxin response (Okushima et al., 2005), *TPPB* is repressed by auxin, but its repression depends on *ARF7*, since in the *arf7* and *arf7arf19* loss-of-function mutants there was no effect of the auxin treatment on *TPPB* expression. The pericycle differential expression set showed that *TPPB* is enriched in the phloem pole pericycle (PPP) cells, data that correlates also with the cell type dataset (Brady et al., 2007). Finally, *TBB* is not related to a specific cell cycle phase (Menges et al., 2003). In summary, according to the VisuaLRTC we can define that *TPPB* is repressed by auxin and this response depends on some important players in LRI.

Some of the results extracted from the VisuaLRTC were confirmed by qPCR. As reported before, *TPPB* is highly downregulated by auxin in wt plants. 30 min after NAA (10µM) treatment the relative *TPPB* expression drops to 50%, and it almost disappears after 6h treatment (Figure 4A). When *promTPPB::GUS* plants were tested in the LRIS, we see that

under NPA (10µM) *TPPB* is upregulated in the basal meristem, a place where in normal conditions it is not expressed. After transfer to NAA (10µM) for 2h or 4h *TPPB* expression is absent in most of the plants. However, when NAA is present for 6h, more than half of the plants show expression in the basal meristem and the meristem itself (Figure 4B). However, this upregulation of *TPPB* is not seen in the newly formed primordia after NAA treatment (data not shown). This result confirmed indirectly the repressive action of auxin in *TPPB*. The upregulation of *TPPB* by auxin indicated in the later time points of the LRIS does not correlate with the qPCR results, it could be that the upregulation in the root tip is not detected at the RNA level when qPCR is done in entire roots, or the treatment stabilizes the protein.

Relative *TPPB* expression was also checked in the *slr-1* and *arf7arf19* mutants (Figure 4C). Upon auxin treatment *TPPB* is 80% expressed in *slr-1* and no changes are detected in *arf7arf19* compared to untreated plants, this means that the repression of *TPPB* by auxin is dependent on this known Aux/IAA-ARF module.

3.4 TPPB is a direct target of auxin signaling and could be repressed by ARF4

Auxin regulates SCF^{TIR1}-dependent degradation of Aux/IAA proteins by targeting them for proteasome degradation (Gray et al., 2001), de-repressing ARFs responsible of positive or negative responses in early auxin responsive genes. TPPB could be a direct or indirect target of auxin, meaning that it is directly regulated by an ARF, or an ARF is targeting an early auxin responsive gene that later on will regulate TPPB expression. Since ARFs are binding to identified Auxin responsive elements (Aux-REs) in the promoter of primary auxin responsive genes (Ulmasov et al., 1997), we found that TPPB has an Aux-REs (TGTCTC) motif 15 bp upstream of the start codon, which can indicate that it is an early auxin response gene. Also, we checked *TPPB* expression in wt plants treated with or without auxin, cycloheximide (CHX) and MG132. With CHX the new protein synthesis is blocked causing a rapid depletion of Aux/IAA repressors and thus activating ARFs. These ARFs will repress/activate early auxin responsive genes that are direct targets of auxin and do not need novel protein synthesis to be regulated. *TPPB* is still downregulated by auxin after CHX pretreatment (Figure 4D) meaning that is a direct target of auxin signaling. However, *TPPB* is also downregulated by CHX, response that could be in agreement with the effect of this inhibitor in the transcriptional regulation of primary auxin response genes. It is known that Aux/IAA are specifically induced by CHX (Hagen and Guilfoyle, 2002), as well as some ARF (Lau et al., 2011) and some early auxin response genes (Frigerio et al., 2006; Okushima et al., 2007), in which CHX has the same transcriptional effect as auxin. In the case of impairment of Aux/IAA degradation by inhibition of the 26S proteasome with MG132, which should imply a stabilization of ARF repression, still some TPPB downregulation upon auxin treatment could be reported (Figure 4D).

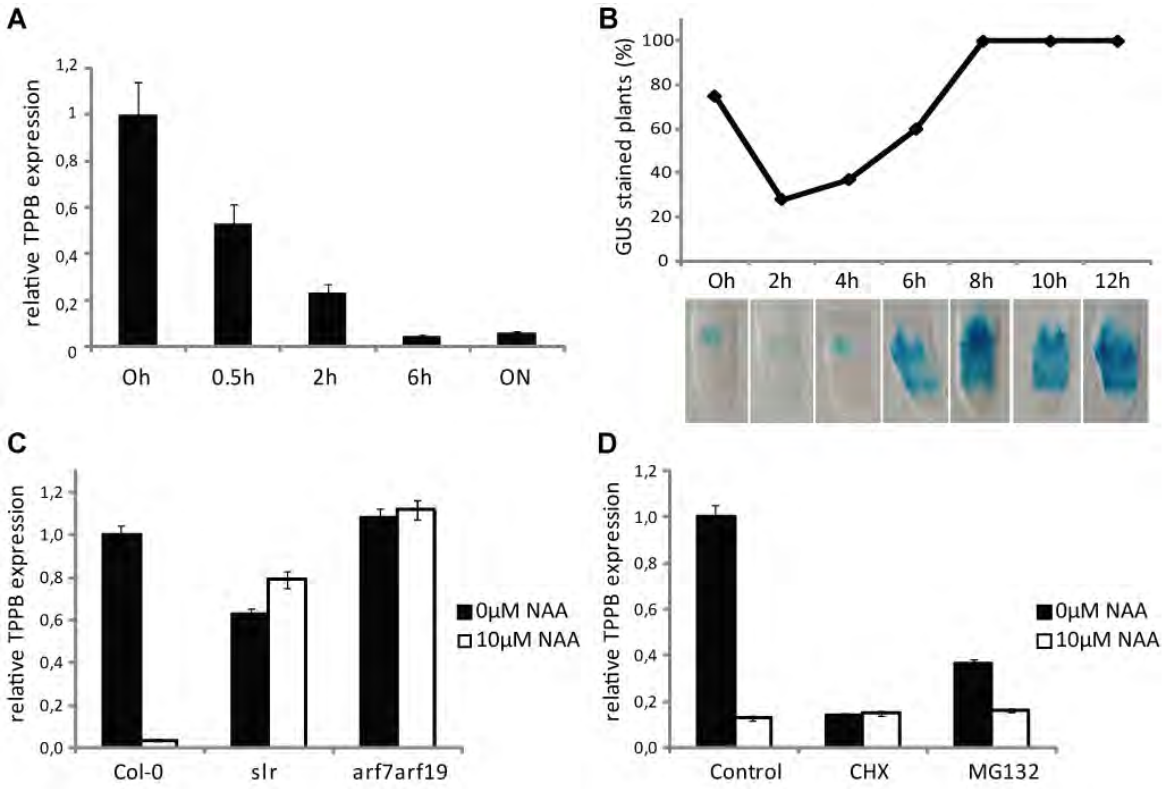


Figure 4. *TPPB* responses to auxin. A. Relative *TPPB* expression on Col-0 roots treated with 10µM NAA following different lengths of auxin treatment. **B.** LRIS, 72HAG *promTPPB::GUS/GFP* plants grown on 10µM NPA supplemented media were transferred to 10µM NAA media for the times indicated, graph corresponds to the percentage of plants showing GUS expression as seen in the pictures. **C.** Relative *TPPB* expression on *slr-1* and *arf7arf19* 5 DAG old roots treated with 10 µM NAA for 6 hours compared to mock treatment (0µM NAA, DMSO). **D.** Relative *TPPB* expression on Col-0 roots treated for 2 hours with 10 µM NAA or mock (0µM NAA, DMSO) and pretreated 30 minutes with 50µM CHX or 50µM MG132.

Since TPPB is a direct target of auxin response, the next question was which is the Aux/IAA – ARF module controlling TPPB expression? Measuring TPPB expression in some aux/iaa gain-of-function mutants could give us an indication of its upstream regulators (Figure 5A). *TPPB* was downregulated in *iaa7*/*axr2* and *iaa17*/*axr3* mutants and overexpressed in *iaa3*/*shy2*, whereas for *iaa14*/*slr*, *iaa1*, *iaa12*/*bdl*, *iaa19*/*msg2*-1 and *iaa28-1* mutants its expression remain unchanged. If the *Aux*/*IAA* is stabilized there could not be repression of *TPPB*, meaning that the last group could be involved in the *TPPB* transcriptional regulation.

Although in *iaa14/slr-1*, *arf7* and *arf7arf19* mutants the repression of *TPPB* by auxin is partially to completely lost, and *TPPB* expression seems to be upregulated in the basal meristem in these mutants (Figure 5B), this module might not be the one triggering the direct response to auxin. This is because ARF7 as well as ARF19 are known to be activators of downstream genes (Tiwari et al., 2003). However we can not discard the possibility that ARF7 could be positively acting on a repressor of TPPB. Therefore, as *TPPB* is downregulated by auxin we should focus on a negative ARF. According to the literature

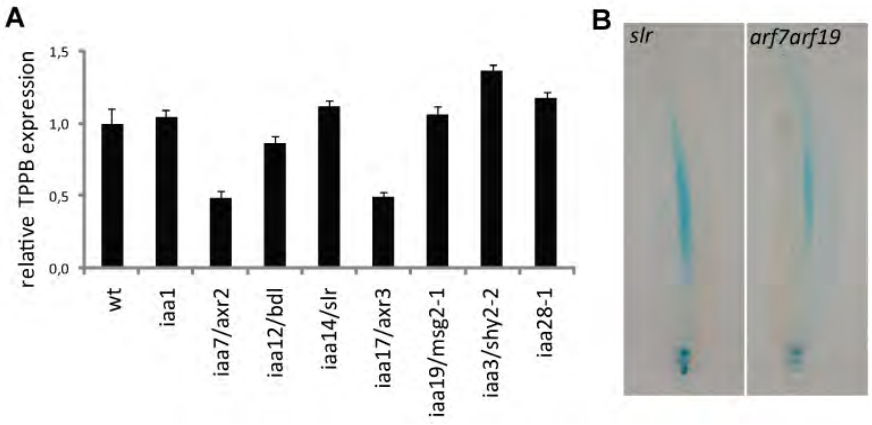


Figure 5. Expression of *TPPB* in auxin signaling mutants A. *TPPB* expression in 5DAG auxin signaling gain-of-function mutants relative to their wt. **B.** *pTPPB::GUS* expression in 7DAG *slr-1* and *arf7arf19* mutants background shows *TPPB* upregulation in the basal meristem.

among the 23 ARFs, 5 are considered as activators and the rest act as repressors (Tiwari et al., 2003). Based on the expression pattern of these transcription factors (Root lab ARF analysis, PSB-UGent, unpublished results) we could think of ARF4 as a possible regulator

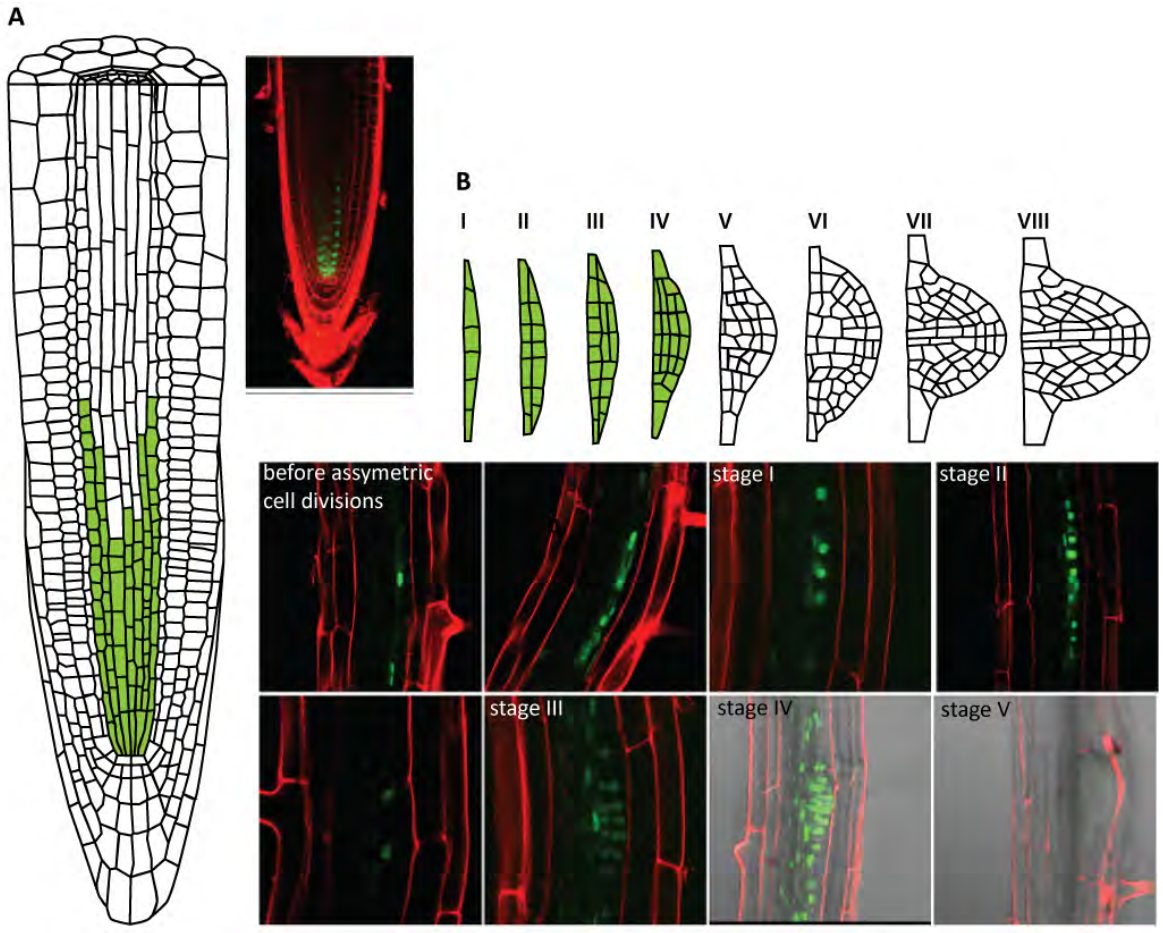


Figure 6. Expression pattern of ARF4 in roots. *pARF4::3x-GFP* plants were analyzed by confocal microscopy. **A.** *ARF4* expression in 5DAG RAM, expression is confined to the central stele. **B.** Expression pattern of *ARF4* during lateral root initiation, expression is noticed before stage I until stage IV.

of *TPPB* because its expression in the central stele in the root tip and in pericycle cells before the first asymmetric divisions until stage IV of lateral root initiation (Figure 6), overlaps in part with *TPPB* expression.

If ARF4 is controlling *TPPB* expression an Aux/IAA should be also involved, therefore we looked at the Y2H interaction Aux/IAA-ARF dataset (Cell cycle lab, PSB-UGent, unpublished results) to unravel a possible module to work further with, surprisingly we found that there is not a single positive interaction of an ARF or Aux/IAA with ARF4. This highlights the possibility that repressor *ARF4* is not regulated by Aux/IAAs during auxin signaling, which have been already proposed by Tiwari et al. (2003).

4. DISCUSSION

Trehalose metabolism, and especially its intermediate Tre6P, is emerging as an interesting bridge linking sucrose status in the cell with growth (Schluepmann et al., 2003; Schluepmann et al., 2004; Paul, 2008). However little is known about the specific function of the 10 TPP genes putatively involved in the de-phosphorylation of Tre6P to produce trehalose. Here we describe for the first time, the involvement of a TPP in an organogenesis process such as lateral root development. *TPPB* has a particular expression in the pericycle cells and in the early stages of lateral root initiation, an expression that is correlated with the phenotype of *TPPB* mutants. Loss-of-function mutants of *TPPB* display a larger root system with a higher lateral root density and higher early lateral root primordia densities than the wt, whereas OE mutants show the opposite. These results identify *TPPB* as a negative regulator of lateral root initiation. Moreover the bigger shoot in KO mutants caused by a higher cell division in epidermal cells, could be a consequence of a better root system in these plants, since a larger root system extracts more nutrients and water from the media. However experiments such as grafting could help us to corroborate this hypothesis by playing with the root and shoot of *TPPB-KO* and wt plants.

Lateral root development is a well studied process and key components of its molecular basis have been elucidated (Fukaki et al., 2002; Okushima et al., 2007; De Smet et al., 2008; Fukaki and Tasaka, 2009; Péret et al., 2009; De Rybel et al., 2010). These components are regulated by auxin, via upregulation of the downstream genes of the Aux/IAA –ARF module, resulting in the priming, initiation and development of the lateral roots. *TPPB* has an AuxRE box in its promoter which is important for ARF binding. Moreover, by CHX treatments, we show *TPPB* may be a direct target of the auxin signaling because *TPPB* is still repressed by auxin without de-novo protein synthesis. Analysis of TPPB in the VisuaLRTC (Parizot et al., 2010) indicates that *TPPB* is regulated by the known IAA14/ARF7-ARF19 module because its repression by auxin is not evident in the mutants *slr-1* and *arf7arf19*. However as *TPPB* is dowregulated by auxin, the ARFs involved in its regulation should be acting as repressors, not as activators which is the case for *ARF7* and

ARF19 (Tiwari et al., 2003). That leads us to think about a repressing ARF. Among the 23 ARFs, ARF1 to ARF4 and ARF9 are reported to act as repressors of a synthetic marker of auxin, however by sequence analysis the repressors list can be extended by a further 10 ARFs (Tiwari et al., 2003). By identifying the expression pattern of these transcription factors, we could spatially place *TPPB* and its regulator *ARF*. With this condition, *ARF4* looks like a putative candidate regulating *TPPB.* They share similar expression patterns in the central stele and at the initial stages of lateral root initiation, while other ARFs are restricted to epidermis, root cap or absent in the root. However if ARF4 is repressing *TPPB,* the expression of *TPPB* should be absent where or when *ARF4* is expressed, so we could expect that there is a threshold in the level of auxin that regulates *ARF4* expression and in consequence regulates *TPPB*.

ARF4 is a target of the *TAS3 TasiRNAs* (*trans-acting small interference RNA*), and together with *miR390*, *ARF2* and *ARF3* conform an auxin-responsive regulatory network controlling lateral root growth (Marin et al., 2010). *ARF4* is repressed by *TAS3 TasiRNAs* which are products of the cleavage of *TAS3* by miR390. *ARF4* together with *ARF2* and *ARF3* are triggering elongation of lateral roots in a controlled balance. ARF4 is the one restricting miRNA139 to the basal cells of the lateral root primordia, while *ARF2* and probably *ARF3* are positively regulating accumulation of miR390 (Marin et al., 2010). As for root phenotypes, *arf4-2* plants had less emerged lateral roots than wt, and the opposite phenotype occurred in *ARF4-OE* plants (Yoon et al., 2010). However, Marin and coworkers (2010) reported *arf4-2* as having longer roots, but no differences in other root traits. Interestingly, in the two publications the growth conditions were the same, so it makes sense to repeat these experiments in house to corroborate their results. The smaller lateral root density phenotype of *TPPB-OE* plants resembles the *arf4-2* phenotype reported by Yoon et al. (2010), which is another suggestion of the direct control of *TPPB* by ARF4. However, as *TPPB* response to auxin is gone in *arf7arf19* mutants, which are known to be activating ARFs, it is possible that TPPB is regulated by different ARFs (activators and repressors) that depend on SLR/IAA14 or other Aux/IAAs. As there is a highly redundant regulation of auxin responsive genes by ARFs and Aux/IAAs, it will not be surprising that *TPPB* is regulated in place and time by more than one *ARF* depending on auxin signals.

5. MATERIALS AND METHODS

Plant material

pTPPB:GUS-GFP were generated as described in Chapter II. T-DNA insertion lines Salk_037324 and Sail_191F08 were obtained from NASC (Alonso et al., 2003) and genotyped with specific primers according to T-DNA express : Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress) (Table S2). *TPPB-OE* lines were generated by cloning the *TPPB* CDS with a 2xHA-tagged sequence into a pDONR221 donor vector, followed by subcloning in a pK7WG2 expression vector with 35S promoter (Karimi et al., 2007); using the GatewayTM technology (Invitrogen)

according to manufacturer's instructions. *Col-0* ecotype plants were transformed with the expression vector and selected on kanamycin. Homozygous, independent-single insertion lines were generated.

Plant growing conditions

For GUS analysis seedlings were grown for 3 or 10 DAG, for root phenotypic analysis, seedlings were grown for 12 DAG, in vertical plates on standard half strength MS supplemented with 1% sucrose under continuous light (110 $\mu E.m^{-2}.s^{-1}$ photosynthetically active radiation) at $22^{O}C$. For shoot phenotypic analysis plants were grown 21 DAG in the media described above under 16h/8h day/night regime. Media was supplemented with NPA, NAA, CHX and MG132 in the concentrations and times indicated in the text.

Histochemical and histological analysis

The beta-glucuronidase (GUS)-staining assays were performed according to Beeckman and Engler (1994). Samples were cleared by mounting them in 90% lactic acid (Acros Organics) on glass microscope slides and analyzed by differential interference contrast microscopy (Olympus BX51). For root staging, 4 DAG seedlings were cleared as described by Malamy and Benfey (1997) mounted on microscopy slides and analyzed as described above. For shoot analysis, the first pair of true leaves were cleared in 100% acetone for 1h and then ON, transferred to 9:1 ethanol:acetic acid mix for 6h and kept in 90% lactic acid for microscopy analysis done as described above. Pictures of the abaxial epidermis close to the apex and the base of 5-6 leaves of an area covering around 30-40 pavement cells were taken. Analysis was done as described by De Veylder et al., (2002). Pictures were processed with Adobe Photoshop CS4.

Quantitative real-time PCR

RNA was extracted with RNeasy kit (Qiagen). cDNA was prepared from 1 µg of total RNA with iScript cDNA synthesis kit (Bio-Rad) and analyzed on a LightCycler 480 Real-Time PCR instrument (Roche Applied Science) with the SYBR Green I Master (Roche Applied Science) according to manufacturer's instructions. All reactions were done in triplicate. Relative expression levels of target genes were quantified with specific primer sets (Table S4), analyzed with qBase (Hellemans et al., 2007) and normalized to *cDKA 1;1* (At3g48750) and *EEF1α4* (At5g60390)expression levels.

Metabolite measurements

Between 10-15 plants grown for 11 DAG as indicated above were pooled and frozen immediately in liquid nitrogen. Tre6P, phosphorylated intermediates and organic acids were measured by liquid-tandem mass spectrometry as indicated in Lunn et al. (2006). Sucrose, glucose and fructose contents were measured in ethanol extracts as described by Geigenberger et al. (1996). Starch contents were determined as glucose equivalents as described by Hendricks et al. (2003). Trehalose was measured in the same plant extracts used for Tre6P measurements (Lunn et al., 2006) using trehalase (from *E.coli*, TREF) to hydrolyze the trehalose into glucose, which was measured in a fluorescence assay using glucose oxidase (GOD) and Amplex Red (Invitrogen). For each sample a calibration curve was done by adding to different sample aliquots a known concentration of trehalose. In a black 96-well microplate, 5ul of plant extract were incubated with 35µl of assay mix (4µl of buffer mix [phosphate buffer 500mM pH7,5, 10mM $MgCl_2$, 100mM NaCl, keep at $4^{o}C$], 0,2µl catalase/GOD mix [0,5µl catalase 30 KU/ml in 20µl GOD 20 KU/ml] and 30,6µl H_2O), blank and standards had 0,2µl TREF [1mg/ml] as well, at $30^{o}C$ for 60 min shaking. The plate was heated at $80^{o}C$ for 15 min, cooled and spun down. On ice, 5µl of H_2O were added to the samples and blank, and 5µl of trehalose standards (2,5 to 40 pmol) were used for the samples of the calibration curve. 55µl of determination mix were added to each sample (6µl of buffer mix, 0,2µl GOD [10 KU/ml], 0,2µl TREF [1mg/ml], 0,2µl horse radish peroxidase (HRP), 0,25µl Amplex Red). The plate was read in a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc.) in the fluorescence mode till the slope was stable.

6. SUPLEMENTAL INFORMATION

Table S1. Levels of Tre6P, sugar phosphates, sugars, trehalose and starch in TPPB mutants. Measurements were done in 12 DAG plants grown on vertical plates supplemented with 1%sucrose under continuous light. n=4 independent experiments, ±SD

Shoot	Col-0	*tppb-1*	*tppb-2*	*TPPB-1*	*TPPB-2*
Tre6P (nmol/gFW)	0,262±0,02	0,180±0,03	0,183±0,02	0,262±0,01	0,255±0,01
Suc6P (nmol/gFW)	0,575±0,09	0,516±0,03	0,489±0,03	0,894±0,04	0,699±0,08
Glc6P (nmol/gFW)	160,9±18,7	192,1±7,6	180,6±6,7	200,0±19,9	230,1±18,9
Fru6P (nmol/gFW)	68,4±1,3	73,7±2,5	70,9±3,0	79,4±7,5	80,8±2,7
UDPG (nmol/gFW)	54,1±7,4	50,7±2,4	46,9±4,1	59,2±1,2	54,0±4,9
Sucrose (µmol/gFW)	1,9±0,2	1,9±0,2	1,6±0,1	3,9±0,5	4,5±0,8
Glucose (µmol/gFW)	5,2±1,1	3,6±0,1	4,0±0,9	6,3±0,9	6,3±0,6
Fructose (µmol/gFW)	2,7±0,4	1,7±0,5	1,6±0,3	3,1±0,1	3,4±1,2
Trehalose(nmol/gFW)	22,9±2,6	14,3±3,4	18,2±7,0	99,1±13,2	50,7±5,8
Starch (µmol gluc equivalents/gFW)	36,3±3,9	29,7±4,9	36,4±3,0	58,8±6,5	67,1±4,1
Root					
Tre6P (nmol/gFW)	0,409±0,07	0,374±0,019	0,430±0,045	0,411±0,046	0,410±0,036
Suc6P (nmol/gFW)	0,368±0,05	0,358±0,035	0,378±0,061	0,819±0,115	0,685±0,109
Glc6P (nmol/gFW)	161,5±24,6	157,4±15,1	164,2±9,5	264,5±47,9	199,3±16,0
Fru6P (nmol/gFW)	60,3±5,1	62,5±2,2	64,7±2,2	90,1±3,9	86,0±8,1
UDPG (nmol/gFW)	56,6±12,8	54,7±3,9	56,4±5,6	71,0±8,5	64,9±3,0
Sucrose (µmol/gFW)	5,1±0,6	8,1±3,3	7,4±0,8	8,0±1,2	8,0±0,8
Glucose (µmol/gFW)	18,9±2,4	16,0±4,2	14,7±0,2	22,1±0,6	24,7±0,2
Fructose (µmol/gFW)	11,8±1,7	9,5±3,2	8,0±0,9	11,4±0,9	14,1±0,9
Trehalose(pmol/gFW)	34,6±4,2	19,7±2,8	26,6±5,3	253,7±58,1	164,7±53,4

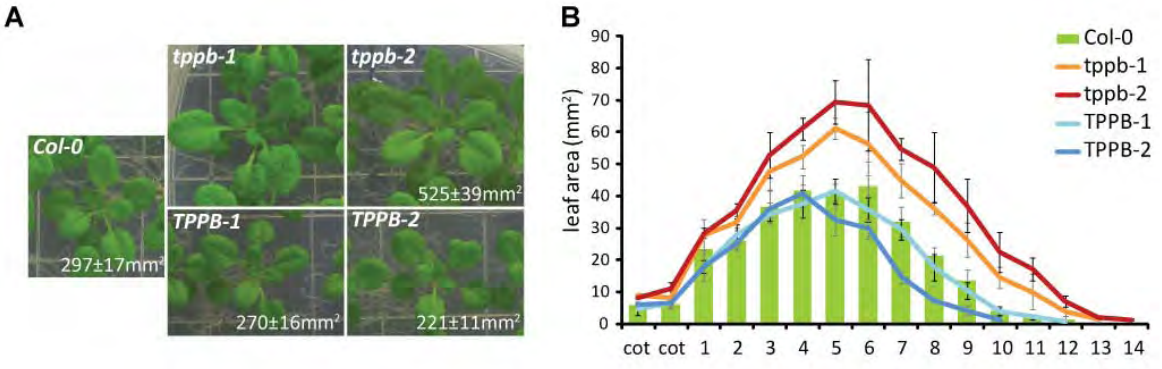


Figure S2. Shoot phenotype of TPPB mutant plants. A. Shoot of 21 DAG plants grown *in vitro*, numbers indicate the average total leaf area, *TPPB-KO* plants are bigger than wt. **B.** Area of rosette leaves of 21 DAG plants, KO and OE plants have an opposite phenotype.

Table S3. Abaxial epidermal analysis of first pair of true leaves in three-week-old *TPPB* mutants. Values are average ± stdev. n=6-10 *P<0.05 **P<0.01

Line	Leaf size (mm^2)	Epidermal cell size (μm^2)	Estimated number of cells/leaf	Stomatal index
Col-0	28,03 ± 2,52	1934 ± 191	24821 ± 3078	21,6 ± 0,8
tppb-1	36,30 ± 4,67**	1962 ± 127	33907 ± 4943**	22,6 ± 0,4
tppb-2	32,88 ± 3,62*	2111 ± 141	28035 ± 1533*	22,8 ± 2,0
TPPB-1	21,83 ± 5,91**	1819 ± 244	21709 ± 3660*	22,5 ± 2,2
TPPB-2	26,17 ± 3,85	1950 ± 136	24823 ± 4091	22,8 ± 1,2

Table S4. Specific primer sets for genotyping of T-DNA insertion lines and for qPCR analysis.

Primer	Sequence (5'→3')
Cloning	
TPPB-OE-attB1	GGAGATAGAACCATGACTAACCAGAATGTCATCGTTTCC
TPPB-OE-attB2	GGGTCACCGCCTCCGGATCAAGCGTAGTCTGGAACGTCG
Genotyping	
tppb-1_LP1	GTGCGGAAAAATGAAATATCG
tppb-1 _RP1	TTCAATCATTGGACGGATTTC
tppb-2_LP2	CAAGAAAGTGAACAAAGGAAAGG
tppb-2 _RP2	TTTGTAACAGAGATGGCCTGC
qPCR	
TPPB_fw	GATGAGAAGAGATGGCCTGC
TPPB_rev	CCCTTGTCCCATTTGATTGT
CDKA1;1_fw	ATTGCGTATTGCCACTCTCATAGG
CDKA1;1_rev	TCCTGACAGGGATACCGAATGC
EEF1α4_fw	CTGGAGGTTTTGAGGCTGGTAT
EEF1α4_rev	CCAAGGGTGAAAGCAAGAAGA

7. REFERENCES

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., and Ecker, J.R. (2003). Genome-Wide Insertional Mutagenesis of Arabidopsis thaliana. Science **301,** 653-657.

Avonce, N., Leyman, B., Mascorro-Gallardo, J.O., Van Dijck, P., Thevelein, J.M., and Iturriaga, G. (2004). The Arabidopsis Trehalose-6-P Synthase AtTPS1 Gene Is a Regulator of Glucose, Abscisic Acid, and Stress Signaling. Plant Physiol **136,** 3649-3659.

Beeckman, T., and Engler, G. (1994). An easy technique for the clearing of histochemically stained plant tissue. Plant Molecular Biology Reporter **12,** 37-42.

Brady, S.M., Orlando, D.A., Lee, J.Y., Wang, J.Y., Koch, J., Dinneny, J.R., Mace, D., Ohler, U., and Benfey, P.N. (2007). A high-resolution root spatiotemporal map reveals dominant expression patterns. Science **318,** 801-806.

Casimiro, I., Beeckman, T., Graham, N., Bhalerao, R., Zhang, H., Casero, P., Sandberg, G., and Bennett, M.J. (2003). Dissecting Arabidopsis lateral root development. Trends Plant Sci **8,** 165-171.

De Rybel, B., Vassileva, V., Parizot, B., Demeulenaere, M., Grunewald, W., Audenaert, D., Van Campenhout, J., Overvoorde, P., Jansen, L., Vanneste, S., Moller, B., Wilson, M., Holman, T., Van Isterdael, G., Brunoud, G., Vuylsteke, M., Vernoux, T., De Veylder, L., Inze, D., Weijers, D., Bennett, M.J., and Beeckman, T. (2010). A novel aux/IAA28 signaling cascade activates GATA23-dependent specification of lateral root founder cell identity. Curr Biol **20,** 1697-1706.

De Smet, I., Vassileva, V., De Rybel, B., Levesque, M.P., Grunewald, W., Van Damme, D., Van Noorden, G., Naudts, M., Van Isterdael, G., De Clercq, R., Wang, J.Y., Meuli, N., Vanneste, S., Friml, J., Hilson, P., Jurgens, G., Ingram, G.C., Inze, D., Benfey, P.N., and Beeckman, T. (2008). Receptor-like kinase ACR4 restricts formative cell divisions in the Arabidopsis root. Science **322,** 594-597.

De Veylder, L., Beeckman, T., Beemster, G.T., de Almeida Engler, J., Ormenese, S., Maes, S., Naudts, M., van der Schueren, E., Jacqmard, A., Engler, G., and Inzé, D. (2002). Control of proliferation, endoreduplication and differentiation by the *Arabidopsis* E2Fa-DPa transcription factor. Embo J **21,** 1360-1368.

Frigerio, M., Alabadi, D., Perez-Gomez, J., Garcia-Carcel, L., Phillips, A.L., Hedden, P., and Blazquez, M.A. (2006). Transcriptional regulation of gibberellin metabolism genes by auxin signaling in Arabidopsis. Plant Physiol **142,** 553-563.

Fukaki, H., and Tasaka, M. (2009). Hormone interactions during lateral root formation. Plant Mol Biol **69,** 437-449.

Fukaki, H., Tameda, S., Masuda, H., and Tasaka, M. (2002). Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of Arabidopsis. Plant J **29,** 153-168.

Geigenberger, P., Lerchi, J., Stitt, M., and Sonnewald, U. (1996). Phloem specific expression of pyrophosphatase inhibits long distance transport of carbohydrates and amino acids in tobacco plants. Plant Cell Environ **19,** 43-55.

Gray, W.M., Kepinski, S., Rouse, D., Leyser, O., and Estelle, M. (2001). Auxin regulates SCF^{TIR1}-dependent degradation of AUX/IAA proteins. Nature **414,** 271-276.

Hagen, G., and Guilfoyle, T. (2002). Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Mol Biol **49,** 373-385.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol **8,** R19.

Hendriks, J.H., Kolbe, A., Gibon, Y., Stitt, M., and Geigenberger, P. (2003). ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of Arabidopsis and other plant species. Plant Physiol **133,** 838-849.

Himanen, K., Boucheron, E., Vanneste, S., de Almeida Engler, J., Inzé, D., and Beeckman, T. (2002). Auxin-mediated cell cycle activation during early lateral root initiation. Plant Cell **14,** 2339-2351.

Karimi, M., Depicker, A., and Hilson, P. (2007). Recombinational cloning with plant gateway vectors. Plant Physiol **145,** 1144-1154.

Laplaze, L., Parizot, B., Baker, A., Ricaud, L., Martiniere, A., Auguy, F., Franche, C., Nussaume, L., Bogusz, D., and Haseloff, J. (2005). GAL4-GFP enhancer trap lines for genetic manipulation of lateral root development in Arabidopsis thaliana. J Exp Bot **56,** 2433-2442.

Lau, S., Smet, I.D., Kolb, M., Meinhardt, H., and Jurgens, G. (2011). Auxin triggers a genetic switch. Nat Cell Biol **13,** 611-615.

Lunn, J.E., Feil, R., Hendriks, J.H., Gibon, Y., Morcuende, R., Osuna, D., Scheible, W.R., Carillo, P., Hajirezaei, M.R., and Stitt, M. (2006). Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in Arabidopsis thaliana. Biochem J **397,** 139-148.

Malamy, J.E., and Benfey, P.N. (1997). Organization and cell differentiation in lateral roots of Arabidopsis thaliana. Development **124,** 33-44.

Marin, E., Jouannet, V., Herz, A., Lokerse, A.S., Weijers, D., Vaucheret, H., Nussaume, L., Crespi, M.D., and Maizel, A. (2010). miR390, Arabidopsis TAS3 tasiRNAs, and Their AUXIN RESPONSE FACTOR Targets Define an Autoregulatory Network Quantitatively Regulating Lateral Root Growth. Plant Cell **22,** 1104-1117.

Menges, M., Hennig, L., Gruissem, W., and Murray, J.A. (2003). Genome-wide gene expression in an Arabidopsis cell suspension. Plant Mol Biol **53,** 423-442.

Okushima, Y., Fukaki, H., Onoda, M., Theologis, A., and Tasaka, M. (2007). ARF7 and ARF19 regulate lateral root formation via direct activation of LBD/ASL genes in Arabidopsis. Plant Cell **19,** 118-130.

Okushima, Y., Overvoorde, P.J., Arima, K., Alonso, J.M., Chan, A., Chang, C., Ecker, J.R., Hughes, B., Lui, A., Nguyen, D., Onodera, C., Quach, H., Smith, A., Yu, G., and Theologis, A. (2005). Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in Arabidopsis thaliana: unique and overlapping functions of ARF7 and ARF19. Plant Cell **17,** 444-463.

Parizot, B., De Rybel, B., and Beeckman, T. (2010). VisuaLRTC: a new view on lateral root initiation by combining specific transcriptome data sets. Plant Physiol **153,** 34-40.

Paul, M.J. (2008). Trehalose 6-phosphate: a signal of sucrose status. Biochem J **412,** e1-e2.

Péret, B., De Rybel, B., Casimiro, I., Benkova, E., Swarup, R., Laplaze, L., Beeckman, T., and Bennett, M.J. (2009). Arabidopsis lateral root development: an emerging story. Trends Plant Sci **14,** 399-408.

Schluepmann, H., Pellny, T., van Dijken, A., Smeekens, S., and Paul, M. (2003). Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. Proc Natl Acad Sci U S A **100,** 6849-6854.

Schluepmann, H., van Dijken, A., Aghdasi, M., Wobbes, B., Paul, M., and Smeekens, S. (2004). Trehalose Mediated Growth Inhibition of Arabidopsis Seedlings Is Due to Trehalose-6-Phosphate Accumulation. Plant Physiol **135,** 879-890.

Shin, R., Burch, A.Y., Huppert, K.A., Tiwari, S.B., Murphy, A.S., Guilfoyle, T.J., and Schachtman, D.P. (2007). The Arabidopsis transcription factor MYB77 modulates auxin signal transduction. Plant Cell **19,** 2440-2453.

Tiwari, S.B., Hagen, G., and Guilfoyle, T. (2003). The Roles of Auxin Response Factor Domains in Auxin-Responsive Transcription. Plant Cell **15,** 533-543.

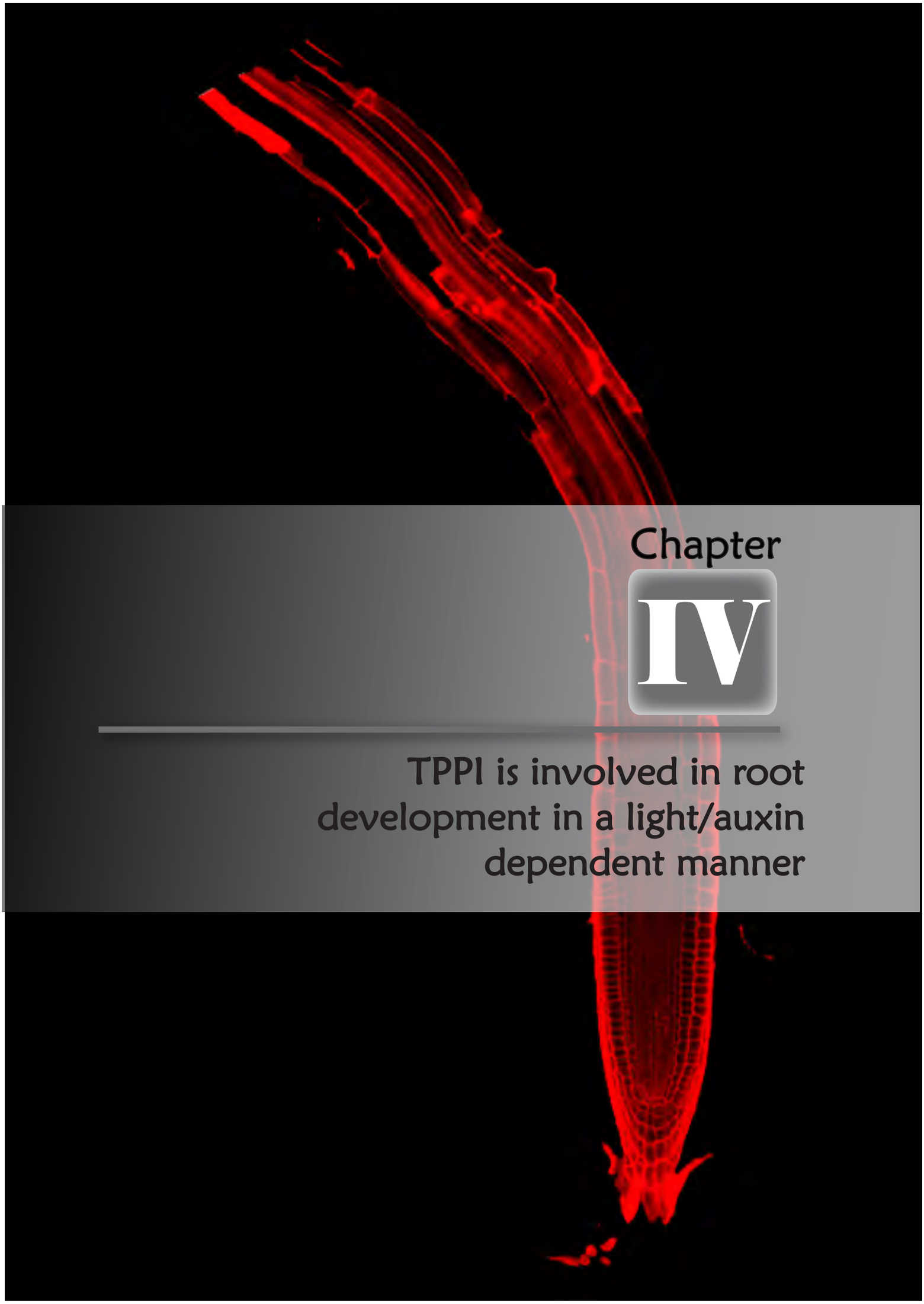
Ulmasov, T., Hagen, G., and Guilfoyle, T.J. (1997). ARF1, a transcription factor that binds to auxin response elements. Science **276,** 1865-1868.

Ulmasov, T., Hagen, G., and Guilfoyle, T.J. (1999). Activation and repression of transcription by auxin-response factors. Proc Natl Acad Sci U S A **96,** 5844-5849.

Vanneste, S., De Rybel, B., Beemster, G.T., Ljung, K., De Smet, I., Van Isterdael, G., Naudts, M., Iida, R., Gruissem, W., Tasaka, M., Inze, D., Fukaki, H., and Beeckman, T. (2005). Cell cycle progression in the pericycle is not sufficient for SOLITARY ROOT/IAA14-mediated lateral root initiation in Arabidopsis thaliana. Plant Cell **17,** 3035-3050.

Varaud, E., Brioudes, F., Szécsi, J., Leroux, J., Brown, S., Perrot-Rechenmann, C., and Bendahmane, M. (2011). AUXIN RESPONSE FACTOR8 Regulates Arabidopsis Petal Growth by Interacting with the bHLH Transcription Factor BIGPETALp. Plant Cell **23,** 973-983.

Yoon, E.K., Yang, J.H., Lim, J., Kim, S.H., Kim, S.-K., and Lee, W.S. (2010). Auxin regulation of the microRNA390-dependent transacting small interfering RNA pathway in Arabidopsis lateral root development. Nucleic Acids Res **38,** 1382-1391.



Chapter

IV

TPPI is involved in root development in a light/auxin dependent manner

TPPI is involved in root development in a light and auxin dependent manner

1. ABSTRACT

Arabidopsis thaliana harbors a TPP family of 10 members, but besides the fact that they are active Tre6P phosphatases, little is known about their function in particular cell types and organs during *Arabidopsis* development. One of the TPP genes, *TPPI*, is expressed in pericycle cells, endodermis and cortex in the root. A loss-of-function of *TPPI* showed reduced primary root growth and lateral root density compared to wild-type plants, and also a yellow and wilthy shoot after two weeks of growth in *in vitro* conditions. *tppi-1* plants grown on soil, showed a more elaborated root system and displayed a normal shoot, suggesting that the root phenotype is conditional and is that the root system is important for the development of a normal shoot. *tppi-1* plants also had a slow rate of root growth *in vitro* due to a shorter RAM and elongation zone with less cells, and probably due to the repression of mitotic cyclins in the root tip of this mutant. Additionally, *TPPI* was found to be regulated by light and auxin signaling. This points to a complex connection between environmental and hormonal stimulus with trehalose metabolism in organogenesis processes.

2. INTRODUCTION

Trehalose and its intermediate Tre6P have recently been postulated as regulators of carbohydrate metabolism and growth, mainly due to the positive correlation of Tre6P with sucrose levels in the cell and starch metabolism. Recently, it was demonstrated that Tre6P is an inhibitor of SnRK1 which is an important regulator of responses under starvation conditions (Zhang et al., 2009). Thus, phenotypes seen in plants with modified levels of Tre6P could also be related to SnRK1 regulation. Phenotypes reported in mutants overexpressing TPS and TPP are associated with abiotic stress resistance (Goddijn et al., 1997; Romero et al., 1997; Garg et al., 2002; Jang et al., 2003; Avonce et al., 2004; Karim et al., 2007; Miranda et al., 2007; Ge et al., 2008; Suarez et al., 2008) and sensitivity to external sugars and ABA (Schluepmann et al., 2003; Avonce et al., 2004), among others. Mutants in the *Arabidopsis* TPS1 are embryo lethal, have decreased cell division and accumulate starch (Gómez et al., 2006), while TPS6 mutants have changes in epidermal cell shape, branching of trichomes and plant architecture (Chary et al., 2008). However, for TPPs, only one TPP (RAMOSA3, RA3) from maize has been described. RA3 is expressed in particular domains of the axilliary meristems in inflorescences, and its mutant, *ra3*,

shows an incorrect meristem identity and determinancy in both male and female inflorescences (Satoh-Nagasawa et al., 2006).

Detailed analysis of the expression patterns of TPPs in *Arabidopsis* showed that 4 TPPs out of 10 are expressed during different stages of lateral root development (Chapter II). Lateral roots have been used to study events of cell division and growth in response to hormones, sugars, light and other environmental stimuli. In this way, some of the molecular and hormonal components of lateral root initiation, patterning and emergence have been identified (for review see Fukaki and Tasaka, 2009; Péret et al., 2009). Recently, a GATA transcription factor was described as the earliest marker in lateral root initiation. GATA23 has a particular expression in the pericycle cells close to the basal meristem that correlates with the oscillation of auxin maxima fluxes in the root tip (De Rybel et al., 2010), leading to the priming of pericycle cells that further on will form a lateral root (De Smet et al., 2007). Hormones such as auxin, are important players in the different steps forming a lateral root. Many of the components that mediate auxin regulated lateral root initiation were already described (Fukaki et al., 2007). Among these auxin signaling proteins, SOLITARY ROOT/INDOLE ACETIC ACID 14 (SLR/IAA14) is one of the most important ones. This protein belongs to the Aux/IAA family, and acts as a repressor of auxin regulated transcription by repressing Auxin Responsive Factors (ARF) such as ARF7 and ARF19 (Fukaki et al., 2005). ARFs are activators of early auxin responsive genes, upon auxin Aux/IAA are degraded and in this way de-repressed ARFs are able to transcriptionally activate genes involved in primary auxin responses (Tiwari et al., 2003; Tiwari et al., 2004; review by Guilfoyle and Hagen, 2007).

SLR/IAA14 is not only regulated by auxin, environmental stimuli such as light are also involved in its regulation. Studies have suggested that LONG HYPOCOTIL (HY5), a major component of the light signaling pathway, plays a role in suppressing auxin signaling (Cluis et al., 2004; Sibout et al., 2006). SLR/IAA14 and AUXIN RESISTANT 2 (AXR2/IAA7) are activated by HY5 (Lee et al., 2007), and the phenotype of loss-of-function *hy5* mutants seems to be the result of decreased expression of these negative regulators of auxin signaling. HY5 also promotes photomorphogenesis by direct transcriptional regulation of light-responsive genes. In darkness, CONSTITUTIVE PHOTOMORPHOGENIC (COP1), an E3 ubiquitin ligase, targets HY5 for degradation through the proteasome preventing photomorphogenic development. Upon light, COP1 is repressed by signals from the photoreceptors allowing accumulation of HY5 and activation of light signaling responses (Osterlund et al., 2000).

In this study, we describe the involvement of a TPP, TPPI, in lateral root development, and we looked further in regulators of TPPI such as light and auxin. The *tppi-1* loss-of-function mutant is impaired in the formation of lateral roots when grown in *in vitro* conditions. In addition, *tppi-1* also has a short main root that was related to a decrease rate of cell division in the RAM. This is a conditional phenotype since *tppi-1* plants grown on soil

showed a root system comparable to wt grown on similar conditions. In parallel, *TPPI* was found to be regulated by light and auxin with HY5 as common player regulating key parts of both pathways.

3. RESULTS

3.1 *TPPI* is expressed in pericycle cells and lateral root primordia

In 2 days after germination (DAG) *pTPPI::GUS-GFP* seedlings (Figure 1A) show GUS-staining in the SAM (Figure 1B), in the hypocotyl-root junction (Figure 1C), and over the whole main root (Figure 1A). In more detail, *TPPI* is expressed in the central vasculature, pericycle, endodermis and cortex in the mature root as well as in the elongation zone (Figure 1D, 1E). Interestingly, TPPI is expressed in the basal meristem, where pericycle founder cells (PFC) are primed (Figure 1F). In the root tip, *TPPI* is clearly noticed in pericycle cells, endodermis, cortex and in differentiated columella cells (Figure 1G). In older seedlings, expression is evident during the different stages of lateral root initiation. Before stage I, TPPI is expressed in the PFC, and is present in the following cell divisions, but after stage VI when vascular domains start to establish, it seems to be confined to the flanking cells of the vasculature at the base of the primordia, (Figure 1H, Chapter II-Figure S5). To confirm the pattern of expression and to visualize the subcellular localization of *TPPI*, a translational fusion line *promTPPI::TPPI::GFP* was generated. Confocal imaging of the RAM shows TPPI in nuclei of pericycle, endodermis and central stele cells in the root tip (Figure 1I).

3.2 TPPI is involved in lateral root development

The specific expression of *TPPI* in pericycle cells during lateral root development and also its expression in the basal meristem suggest that TPPI may be involved in lateral root formation. To investigate this, we searched for knock-out (KO) lines and created overexpression (OE) lines. T-DNA insertion lines were selected from the available collections, SAIL_1242A06 has an insertion in intron 4, and SAIL_354D09 has an insertion in the exon 4 (Figure 2A). After genotyping the T-DNA lines and antibiotic selection for the overexpression lines, homozygous seeds were grown to check the relative expression of *TPPI*. qPCR on OE lines shows relative *TPPI* levels increasing more than 35 fold confirming the overexpression of this gene (hereafter, *TPPI-1* and *TPPI-2*) (Figure 2B). In the T-DNA lines, only in the case of the SAIL_354D09 line *TPPI* expression level was downregulated, therefore this one was selected as *TPPI-KO* (hereafter *tppi-1*) (Figure 2B).

The lateral root phenotype of the KO and OE lines was checked in vertically grown seedlings 10 DAG (Figure 2C). Interestingly, the *TPPI-KO* seedlings had a shorter root system than wt and *TPPI-OE* lines, *TPPI-KO* main root was 2/3 shorter than wt, and had

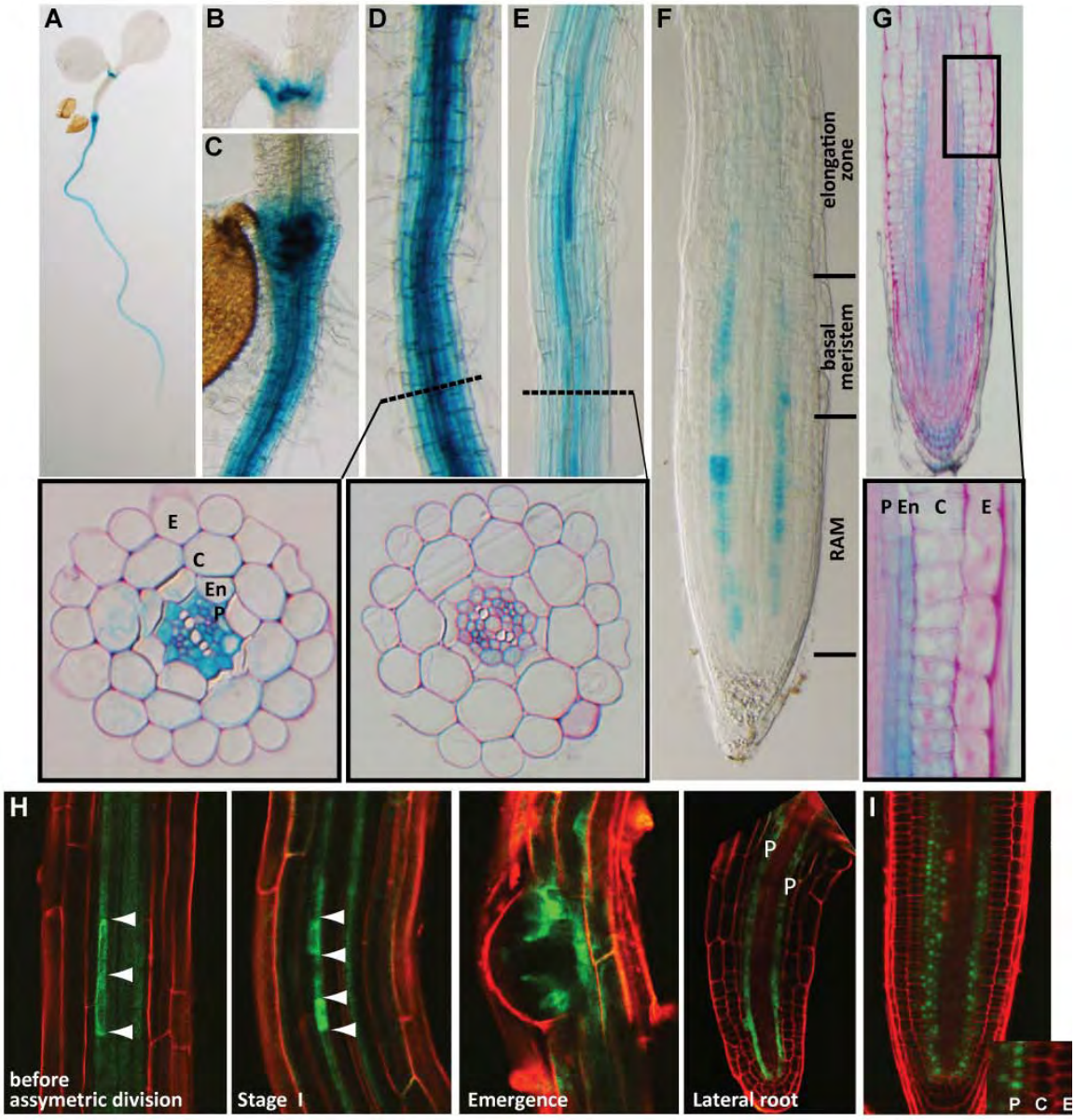


Figure 1. *TPPI* expression in seedlings and during lateral root development. 2 DAG *promTPPI::GUS-GFP* seedlings **A.** Seedlings. **B.** SAM. **C.** Hypocotyl-root junction. **D.** Root maturation zone, with a transversal section showing expression in central stele, pericycle, endodermis and cortex. **E.** Root elongation zone, zoom to a transversal section where light expression can be detected in pericycle and central stele. **F.** Root tip showing the RAM, basal meristem and elongation zone before TPPI fades away. **G.** Longitudinal section in the root tip, insset shows expression in pericycle, endodermis and cortex. **H.** GFP visualization of *TPPI* expression in PFC before the first asymmetric division until emergence of lateral roots, expression remains in the pericycle from early stages till developed lateral roots. Arrowheads point to the cells that are dividing. **I.** Protein localization of TPPI in the nuclei of endodermis and pericycle cells in the root tip. E=Epidermis, C=Cortex, En=Endodermis, P=Pericycle.

few lateral roots. In addition, the lateral root density was less than half compared to Col-0 (Figure 2D), and it was obvious that the elongation of the lateral roots was delayed. In case of *TPPI-1*, differences in main root length and number of lateral roots were found, but not in the lateral root density which was comparable to wt (Figure 2D).

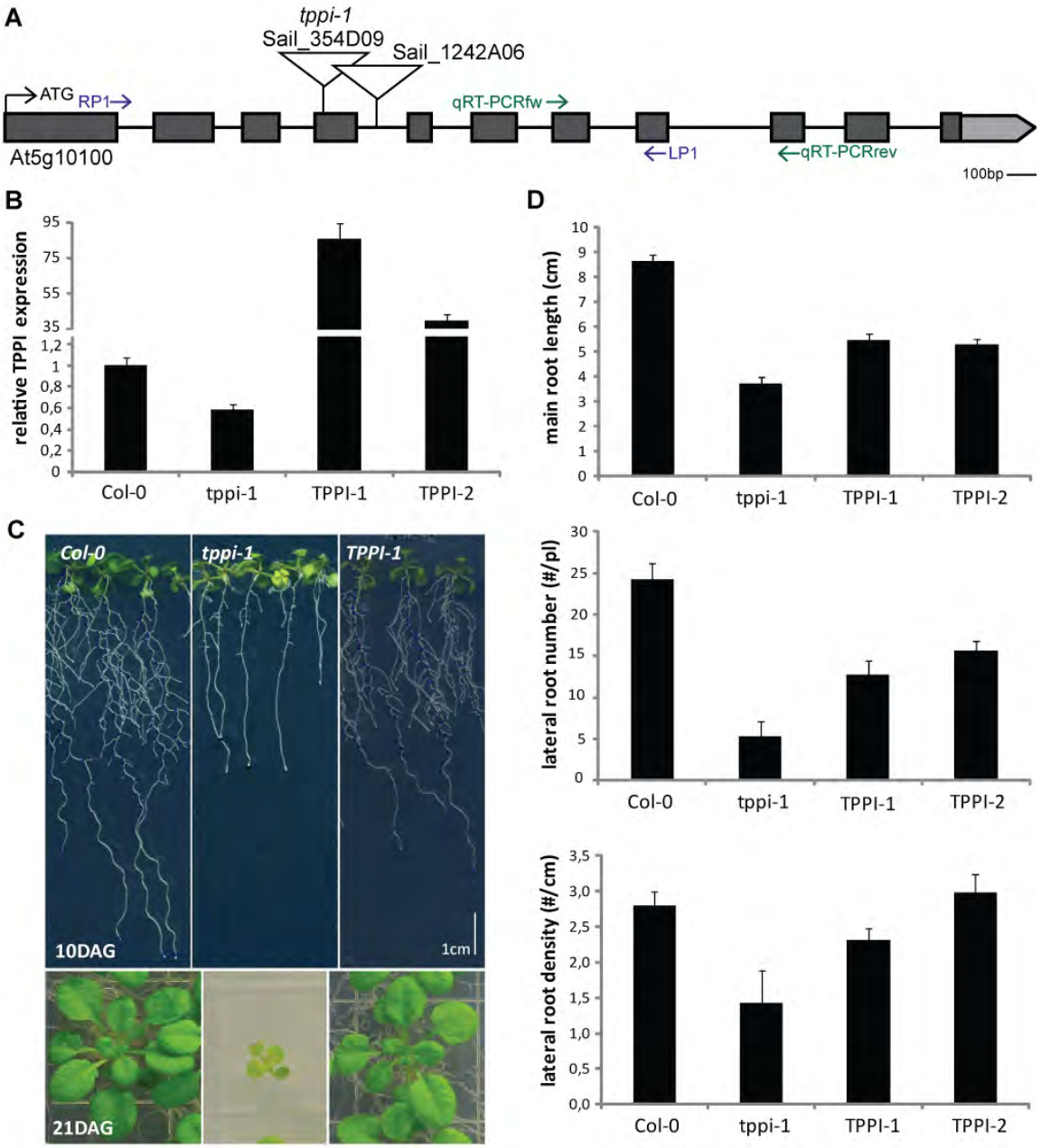


Figure 2. Characterization of *TPPI* T-DNA insertion and OE mutants. **A.** Genomic TPPI scheme showing UTRs in light grey boxes, exons in dark grey boxes and introns as a black line. T-DNA lines are indicated in the map as well as the primers used for genotyping and qPCR. **B.** Relative *TPPI* expression levels in the T-DNA line showing downregulation of *TPPI*, and in two OE lines showing upregulation of *TPPI*. **C.** Root phenotype of 10 DAG *TPPI* mutants grown on vertical plates under (upper panel) and shoot phenotype of 21 DAG plants grown on horizontal plates, notice *tppi-1* wilting phenotype under this condition. **D.** Root traits of 10 DAG *TPPI* mutants grown in vertical plates, main root length (upper graph), average number of lateral roots (middle graph), and lateral root density (lower graph).

3.3 *tppi-1* phenotype is not restored by sugars

TPPI is an active T6P phosphatase, which means that its mutants can result in plants that have different levels of metabolites, in this case Tre6P or sugars as trehalose. As the primary phenotype of *tppi-1* is found in the roots we measured sugars in roots and shoots

separately (Table 1). We could detect differences in Tre6P which is higher in *tppi-1* roots and lower in *TPPI-1* compared to wt, and these levels also correlate with sucrose levels. In the case of trehalose, the OE line shows higher levels in shoot and root as expected.

Table 1. Levels of Tre6P, sugar phosphates, sugars, trehalose and starch in TPPI mutants. Measurements were done in 12 DAG plants grown on vertical plates supplemented with 1% sucrose under continuous light. n=4 independent experiments, ±SD, nd=not determined

	Shoot			Root		
	Col-0	*tppi-1*	*TPPI-1*	Col-0	*tppi-1*	*TPPI-1*
Tre6P (nmol/gFW)	0,262±0,02	0,201±0,03	0,186±0,02	0,409±0,07	0,420±0,02	0,248±0,02
Suc6P (nmol/gFW)	0,575±0,09	0,521±0,06	0,653±0,07	0,368±0,05	0,414±0,10	0,441±0,06
Glc6P (nmol/gFW)	160,9±18,7	136,7±11,7	200,7±5,7	161,5±24,6	145,9±36,3	134,9±19,1
Fru6P (nmol/gFW)	68,4±1,3	58,6±3,3	81,1±1,6	60,3±5,1	60,1±5,1	60,5±3,7
UDPG (nmol/gFW)	54,1±7,4	43,3±3,7	58,3±5,1	56,6±12,8	46,8±9,7	51,2±12,8
Sucrose (µmol/gFW)	1,9±0,2	4,5±0,8	2,9±0,4	5,1±0,6	7,3±0,5	5,4±1,1
Glucose (µmol/gFW)	5,2±1,1	6,6±0,1	5,1±0,5	18,9±2,4	19,7±1,5	19,2±0,8
Fructose (µmol/gFW)	2,7±0,4	4,2±0,3	2,2±0,4	11,8±1,7	15,7±1,0	11,8±0,9
Trehalose (nmol/gFW)	22,9±6,1	21,2±6,1	28,0±2,8	34,6±4,2	77,1±6,4	70,4±25,6
Starch (µmol gluc equivalents/gFW)	36,3±3,9	67,0±11,6	48,0±5,2	nd	nd	nd

Metabolite measurements in whole roots do not show the real situation in those particular cell types where TPPI is expressed, especially in the KO plants in which the function of TPPI is reflected in some cells in specific moments of development. It is difficult to know whether *tppi-1* is deprived of trehalose or has higher contents of Tre6P in the cell types where it is expressed. Therefore we analyzed whether addition of trehalose to the media could rescue the root phenotype of *tppi-1*. As trehalose can be used as an external carbon source for the plant, addition of Validamycin A, and inhibitor of trehalase activity, allows us to see only the effect of trehalose itself and not of the breakdown to glucose. Additionally, other carbon sources, such as glucose and sucrose in different concentrations, where tested (Table S1). As reported before, trehalose inhibits root elongation in a concentration-dependent manner, and this reduction is higher when Validamycin A is present in the media (Wingler et al., 2000). In the *tppi-1* mutant, the relative reduction in root growth is not as much as in wt, showing less sensitivity to trehalose (Figure 3). However the root growth phenotype was not rescued by trehalose or any other sugars in the media. Interestingly, growth of *tppi-1* plants during shorter days was better compared to the relative growth of Col-0 under similar conditions (Figure S1). This may indicate that *tppi-1* is not able to link the availability of energy sources with growth, as growth is not reduced depending on day length. This suggests that *TPPI* might be needed to connect the use of energy sources, such as light, with growth (discussed later).

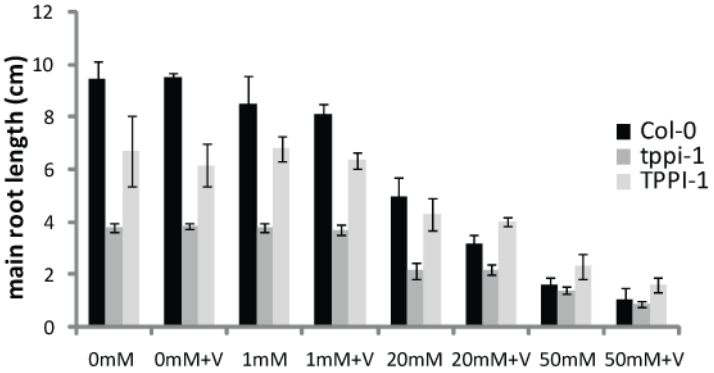


Figure 3. Root phenotype of *TPPI* mutants on medium containing trehalose as carbon source. Main root length (cm) of 10 DAG plants grown on vertical plates, 16h/8h day/night conditions, with different concentrations of trehalose without or with Validamycin A (V,10µM). n=10 plants, ±SD.

3.4 *TPPI-KO* has a conditional lateral root phenotype that is translated in a shoot phenotype

It was interesting to see that when *tppi-1* plants were kept for more than 2 weeks on vertical plates, they had a very poor root system and the shoot started to become yellow and wilted. Similar results were obtained when plants were grown in horizontal plates (Figure 2C). After germination, the plant needs the root system to extract water and nutrients from the medium until the first leaves emerge, and start to use light to produce carbohydrates that can be translated into growth. Therefore we can assume that at early stages there is a dependence of the shoot on root growth, which in the case of *tppi-1* suggest that the shoot phenotype is a consequence of its poor lateral root system.

Interestingly, when *tppi-1* plants were grown in soil they develop a normal shoot (Figure 4A), except for a phenotype in the trichomes (Figure S2). This led us to investigate which differences between *in vitro* and soil conditions could explain the different shoot phenotype observed in the two conditions. Important differences between the agar medium and the soil are porosity, availability of water and gases, and different mineral composition. We first tried the availability of water in the medium by reducing the agar content. Lower percentages of agar in the media helped *TPPI* mutant seedlings to develop a longer root system and a bigger shoot than in normal agar conditions. However, in this water-like medium leaf vitrification was evident (Figure 4A). To test whether the better root growth observed in the previous experiment was associated with the shoot growth, root traits were investigated in agar and in soil conditions. 3 and 7 DAG the complete root of *tppi-1* and Col-0 plants was extracted from the soil and, clean roots were checked under a microscope to count primordia stages of lateral root development. 3 DAG *tppi-1* plants had less primordia at early stages than control, however 7 DAG plants have comparable number of emerged lateral roots and early primordia stages (Figure 4B). When seeds were grown in vertical plates for 8 DAG it was obvious that *tppi-1* plants had

less number of primordia and emerged lateral roots than control plants (Figure 4C). These results suggest that the shoot growth phenotype is associated with the lack of lateral roots.

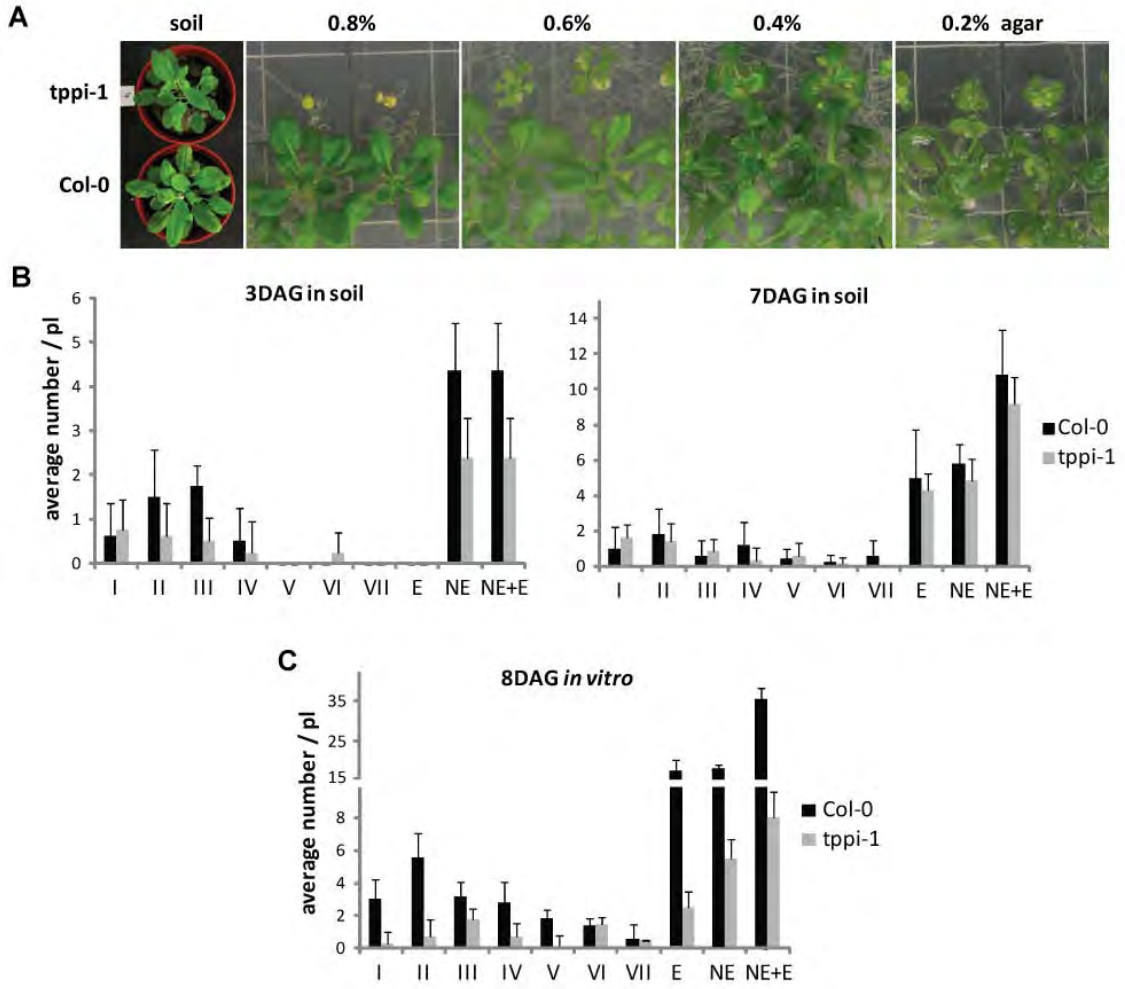


Figure 4. Conditional shoot phenotype of *tppi-1* mutant plants. **A.** 21 DAG plants grown in soil or in different concentrations of agar. Notice the normal shoot in soil conditions and the partial recovery of it in a less solid media. **B.** Detailed lateral root staging analysis, expressed as an average number of primordia per plant (average number/pl), of 3 DAG and 7 DAG seedlings grown in soil. There were less number of primordia in early stages of lateral root initiation in *tppi-1* compared to Col-0, whereas in one week old plants these differences were not noticed anymore. **C.** Detailed lateral root staging analysis, expressed as an average number of primordia per plant (average number/pl), of 8 DAG seedlings grown in vertical plates. A large difference in number of primordia and emerged lateral roots was evident between *tppi-1* and Col-0.

Furthermore, to boost lateral root initiation and emergence in *tppi-1 in vitro* grown plants, 5 DAG seedlings were transfer to different auxin (NAA) concentration media for 3 days and lateral roots and primordia were counted. There was a correlation between the amount of auxin and the number of primordia and emerged lateral roots in the control plants. However, for *tppi-1* this positive correlation was only observed with the number of lateral root primordia but not with emerged ones (Figure 5A). This may indicate that even though there are primordia being formed, the emergence is still delayed, or that the whole cell division process leading to the emergence of a lateral root is at a lower speed,

as it was seen in the RAM of this mutant (see further). However, even when these plants had more roots, the shoot was still yellow and small when plants were kept in the auxin treatment. It looks like the plant was not able to cope with the auxin addition. Therefore, in a second experiment, a low concentration of auxin was used, but then plants were moved to standard media (without auxin) afterwards. Surprisingly *tppi-1* lateral roots emerged, and this resulted in the complementation of the shoot phenotype (Figure 5B).

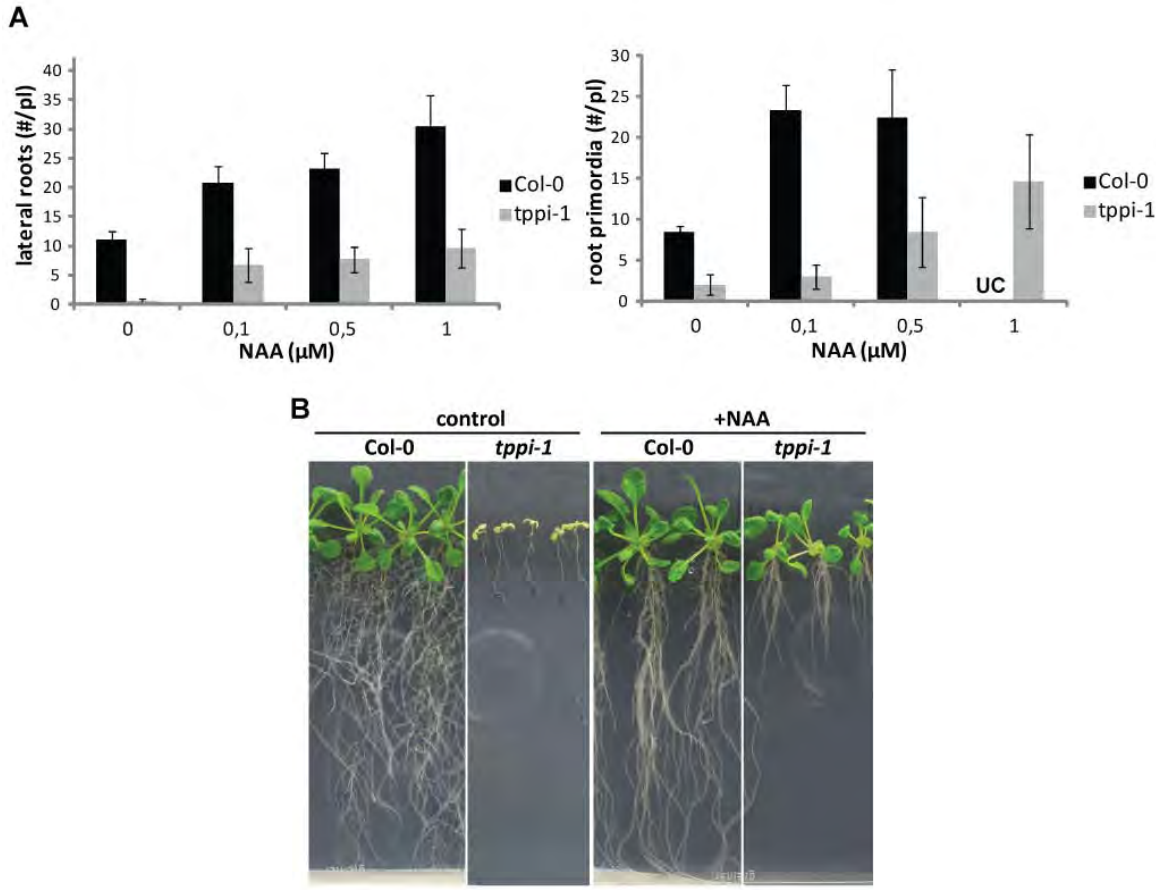


Figure 5. *tppi-1* shoot complementation with auxin. **A.** Characterization of lateral roots and root primordia in *tppi-1* seedlings grown under different concentrations of auxin, shows an increase in lateral root emergence and primordia in the mutant, but this is not comparable to its effect in wt, UC=uncountable. **B.** Shoot complementation phenotype of *tppi-1* in 5 DAG plants treated for 4 days in auxin (0,1 µM NAA) and transferred again to standard media for 6 days. The low–short auxin treatment triggers pericycle divisions and the transfer to standard media allows the primordia to develop and emerge, then the larger root system is translated in a better shoot.

3.5 *tppi-1* has shorter RAM and elongation zone

tppi-1 shows a shorter main root and in general a smaller root system than wt. We have investigated this phenotype by following the daily growth of the main root for 12 DAG, just before the shoot starts to wilt. As shown in Figure 6A, the root growth rate is strikingly reduced in *tppi-1*. Following germination, the elongation rate of the root increases over time due to an enlarged number of dividing cells in the RAM (Baskin et al., 1995). However, *tppi-1* has a slow rate of growth suggesting different cell division rates in the RAM of the mutant in comparison to wt. To elucidate this phenotype, we analyzed

the RAM size by counting and measuring the meristem cortical cells from the QC until the first elongated cell in the cortex (Beemster and Baskin, 1998; Dello Ioio et al., 2007) (Figure 6B). The RAM size in *tppi-1* is about 72% of the RAM in wild-type (Figure 6C). This RAM was composed of 28 cortical cells in comparison with 50 found in wild-type, meaning that the shorter meristem is due to lower cell division rates than to differences in cell size, which is the same in *tppi-1* and wild-type. This was extended to the cortical cells in the elongation zone, counted from the first rapidly elongated cell until the cell where the first root hairs appear (Beemster and Baskin, 1998). In this zone, we found the same cell size and half of the cell number in *tppi-1* compared to wt (Figure 6C). These observations suggest a possible cell cycle misregulation in the RAM. As a preliminary approach, we analyzed in root tips of *tppi-1* plants, the relative expression of some genes known to be key components of the cell cycle. Among the different cell cycle genes, expression of CDKA;1, CYCA3;1 and CYCB1;1 was downregulated (Figure 6D). The first two are markers for the transition of G1 to S phase, and the last one is a marker for G2 to M phase. The reduced expression of these mitotic genes correlates with the reduced number of dividing cells in the RAM of *tppi-1*, confirming a low rate of cell division or giving a hint to a slow cell cycle.

3.6 TPPI is regulated by light and auxin

By chromatin immunoprecipitation (ChIP) coupled to DNA chip hybridization (ChIP-chip) it was found that TPPI is an *in vivo* target of LONG HYPOCOTYL5 (HY5) (Lee et al., 2007). HY5 is a basic domain leucine/zipper (bZIP) transcription factor that controls light-induced gene expression downstream of photoreceptors, and plays an important role in the switch of seedling shoots from dark-adapted to light-adapted development (Sibout et al., 2006). To confirm whether TPPI is regulated by HY5 and by light, we started doing a GUS staining in seedlings grown in the light and in the dark. A clear upregulation of TPPI under light conditions in comparison with seedlings grown in the dark was observed. Furthermore, this regulation is not dependent on sugar in the medium (Figure 7A). We studied in more detail the influence of light in the regulation of TPPI by checking its daily expression in 3 DAG seedlings grown in 12h day-12h night regime. If light is a transcriptional activator of TPPI, changes in expression levels should be noticed at dawn. However there is not a clear change in level of expression when plants enter in the light cycle, there is only a slight upregulation some hours after dawn (Figure S2). On the other hand, the regulation of *TPPI* by HY5 was investigated by qPCR and expression analysis. In the *hy5-221* mutant where *HY5* expression is downregulated, *TPPI* expression was found to be reduced (Figure 7B). In addition, by crossing the *promTPPI::GUS* marker line with *hy5-221* we found that the expression of *TPPI* in the root tip totally disappears (Figure 7C). This indicates the dependence of *TPPI* on HY5 for its light regulation.

HY5 is also regulating different genes involved in hormone responses. *hy5-221* seedlings are defective in the light-induced inhibition of hypocotyl elongation, and have an elevated

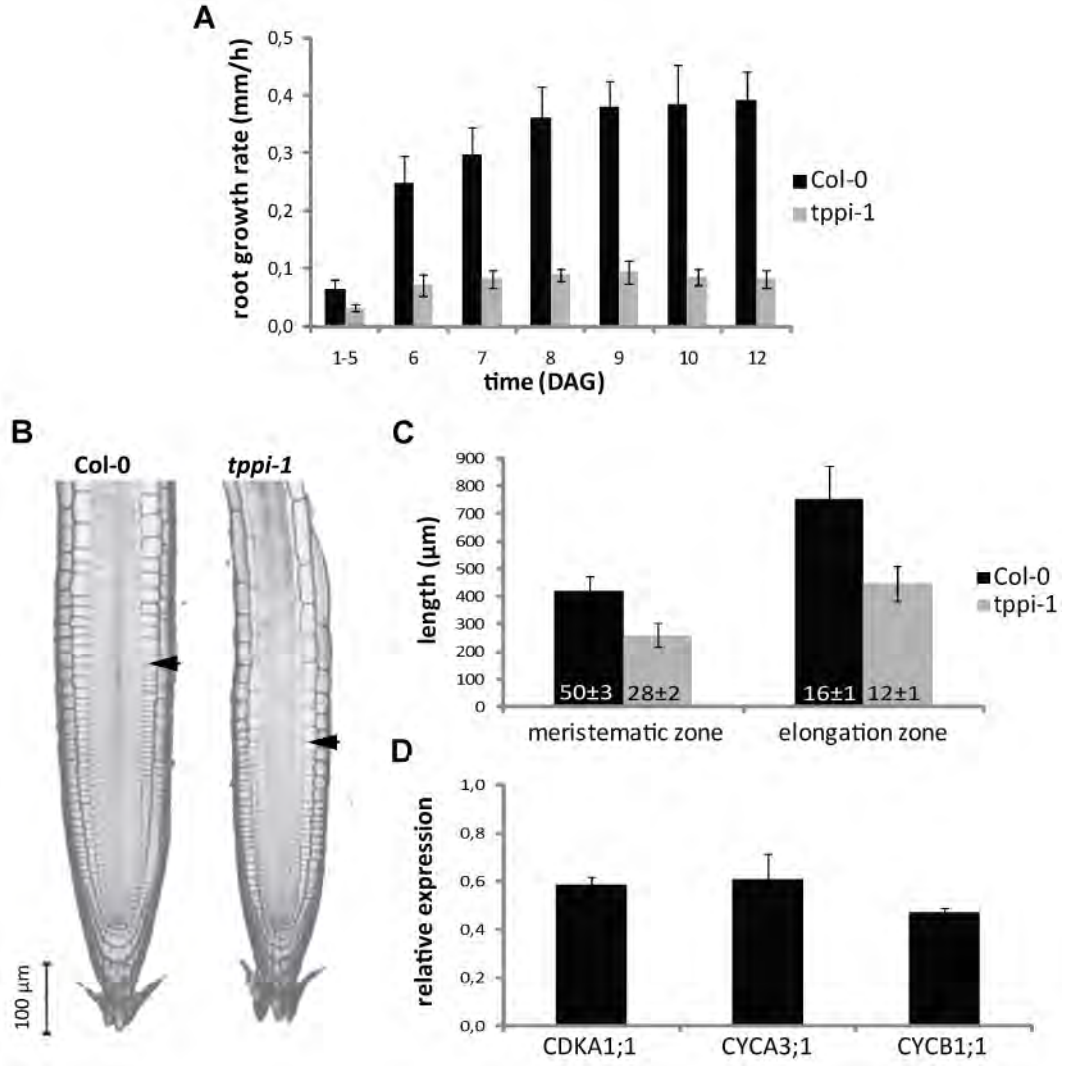


Figure 6. *tppi-1* root meristem phenotype. **A.** Root growth rate of *tppi-1* was followed for 12 DAG, notice the reduced growth rate of *tppi-1*. **B.** Confocal images comparing the root meristem of *tppi-1* with Col-0, arrowheads indicate the cortical cell where there is a switch in size indicating an arrest in cell division an a preference for cell elongation. **C.** Total length of the meristematic and elongation zones in *tppi-1* compared to Col-0, the numbers in each bar correspond to the number of cortical cells that where counted in each zone. **D.** Relative expression of some cell cycle genes in root tips of *tppi-1*.

number of lateral roots, that grow faster and are less responsive to gravitropic stimulus (Oyama et al., 1997). These phenotypes had been described partially as a result of elevated auxin signaling since factors involved in the negative regulation of auxin signaling, such as AXR2/IAA7 and SLR/IAA14, are repressed in the mutant (Cluis et al., 2004). The SLR/IAA14 is probably the central regulator of lateral root initiation, as its gain-of-function mutant, *slr-1*, lacks lateral roots (Fukaki et al., 2002). Additionally, the double mutant (*arf7arf19*) of lost-of-function alleles in ARF7 and ARF19, which are target ARFs of SLR/IAA14, lack lateral roots as well (Okushima et al., 2005). The lateral root phenotype of *tppi-1* is similar to the mutants described above that have impaired auxin signaling, therefore we investigate the regulation of *TPPI* by auxin. In Col-0 plants treated with auxin, *TPPI* is induced, this induction depends on SLR/IAA14 and ARF7ARF19 because

when these genes are mutated *TPPI* is no longer induced by auxin (Figure 7D). This indicates that in a given way, light and auxin are playing roles in the regulation of TPPI. However more analyses are needed to define the nature of the distinct and/or overlapping roles of light and auxin in the function of TPPI in lateral root development.

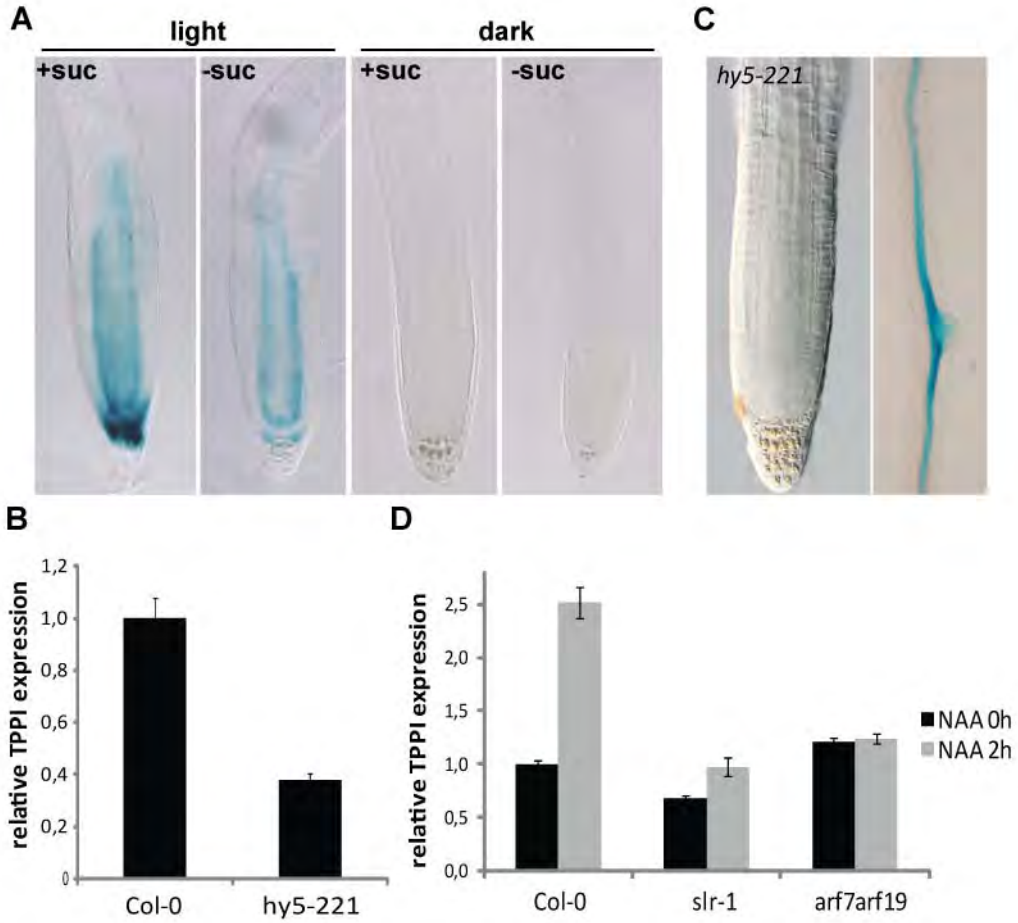


Figure 7. *TPPI* regulation by light, HY5 and auxin. **A.** Light regulates *TPPI* expression in the root tip and it is not dependent on sucrose availability, +suc (3%), -suc (0%). **B.** Relative *TPPI* expression is reduced in *hy5-221* mutant. **C.** Expression of *TPPI* in *hy5-221* seen by GUS staining is absent in the root tip, but not in other root zones. **D.** Relative *TPPI* expression on 5 DAG old roots treated with 10 µM NAA for 6 hours compared to mock treatment (DMSO) in *slr-1* and *arf7arf19* mutant plants, *TPPI* is regulated by auxin in a SLR/ARF7ARF19 manner.

4. DISCUSSION

Particular phenotypes have been discovered by looking at different mutants of TPP genes, however, the function of each TPP has not been elucidated yet. Here we describe the effect on root growth when TPPI expression is reduced in the plant, and implicate light and auxin signaling in the regulation of TPPI.

TPPI is found to be highly expressed in pericycle cells, endodermis and cortex from the root tip to the basal meristem, and reappears later close to the maturation zone. The

particular expression in pericycle cells is present also before the first divisions to form a new lateral root until its emergence. Plants with low *TPPI* expression showed a clear phenotype having lower lateral root density, a short main root and a underdeveloped shoot in *in vitro* conditions but not in soil. Detailed analysis of lateral root primordia in *tppi-1* plants grown on both conditions revealed that *tppi-1* plants in soil have a comparable root system to wt. Moreover, auxin treatments on mutant plants grown *in vitro* restored the lateral root and the shoot phenotype as well. This suggested that a better root system is translated in a well developed shoot. If we look at other mutants defective in lateral roots such as *slr-1* or *arf7arf19*, they show a normal shoot phenotype (data not shown) contrary to *tppi-1* when grown *in vitro*, suggesting that in these mutants, the main root is sufficient to extract the necessary elements from the medium to sustain growth. Additionally, the expression of TPPI in the basal meristem and the reduced lateral root density in its mutant could point to a positive role of TPPI in lateral root priming, that depends on special conditions of the *in vitro* system.

Another phenotype of *tppi-1* plants was a slower rate of root growth due to a shorter RAM. *tppi-1* plants had less cells in the RAM with no differences in cell size when compared to wild-type. This, together with the finding that there is downregulation of mitotic cyclins in the root tip could suggest that cell division in the RAM is slowed down or cells are progressing to differentiation faster. Interestingly, in the shoot we found a phenotype related to cell cycle regulation as well. Preliminary experiments showed that the extra branched trichomes in the *tppi-1* mutants are related to a higher DNA content (data not shown). It is possible that trichomes switch faster to differentiation and have more cycles of endoreduplication in comparison with wt, and this is also happening in the RAM. Additionally, the arrest in mitosis is also seen in the slow rate of lateral root growth, which needs a the addition of low auxin to trigger further growth. Mutants in cell cycle genes such as *ccs52a2,* also have a shorter root meristem. CCS52a2 is involved in the switch from mitotic cell division to endoreduplication cycles by activating the anaphase-promoting complex/cyclosome (APC/C), which is involved in the elimination of the components needed to progress through mitosis (Lammens et al., 2008). The *ccs52a2* RAM phenotype is associated with low repression of mitotic activity in the quiescent center (QC) cells and stem cells (Vanstraelen et al., 2009). It is conceivable that *TPPI* is controlled by the cell cycle machinery involved in the RAM maintenance. On the other hand, auxin also plays a role in maintaining the RAM. Some mutants of auxin transport showed shorter roots and meristems, this is the case with *pin1* and *pin2* mutants. When *pin2* is crossed with other pin mutants such as *pin3*, *pin4* and *pin7* there is a more-than-additive reduction on root and meristem size, suggesting that *pin2* plays an important role in cell division control, because it is needed to guide the basipetal flow of auxin to the meristematic cells (Blilou et al., 2005). However auxin is not working alone in maintaining the RAM. A reduced level of cytokinin in the transition zone is responsible for a proper meristem size by the regulation of cell differentiation (Dello Ioio et al., 2007), and also brassinosteroids have been found to act as regulators of the cell cycle in the

RAM (González-García et al., 2011). This suggests that may be more than one hormone is involved in the regulation of TPPI in the RAM, or TPPI could be directly regulated by cell cycle genes. In summary, there is still a lot to discover to elucidate the function of TPPI in the RAM if hormonal crosstalk and the cell cycle machinery are involved.

Interestingly, environmental clues such as light are also regulating *TPPI*. Lee and coworkers (2007) found that the transcription factor HY5, which is a regulator of light signaling, binds to the promoter of TPPI to regulate its expression. TPPI showed upregulation under light conditions and its expression in the root tip depends on the presence of HY5. HY5 regulates a large number of genes involved in light responses including auxin signaling factors such as SLR/IAA14. As *TPPI* is upregulated by auxin in a SLR/ARF7ARF19 manner, it is possible that HY5 regulates TPPI and at the same time SLR/IAA14 leading to a crosstalk between light and auxin controlling transcriptionally TPPI. Exploring the crosstalk between light and auxin pathways in the control of *TPPI* transcription is an interesting subject for further research.

Trehalose metabolism is turning out to be a key part in the control of plant growth. Here we prove that lowering the expression of TPPI, which is expressed in the RAM and pericycle cells, has important effects in meristem size and lateral root development. These phenotypes are connected to the development of a proper shoot, showing the importance of the root system in sustaining plant growth. Hormones such as auxin, seem to be important to restore the lateral root phenotype of *tppi-1* plants. This, together with a direct regulation of TPPI by light via HY5, suggests that a link between trehalose or Tre6P with root growth is regulated by environmental signals.

5. MATERIALS AND METHODS

Plant material

promTPPI:GUS-GFP lines were generated as described in Chapter II. T-DNA insertion lines Sail_354D09 and Sail_1242A06 were obtained from NASC and genotyped with specific primers (Table S4). The exact insertion place of Sail_354D09 was determined by sequencing (711bp after the starting codon, Figure 2). TPPI-OE lines were generated by cloning the *TPPI* CDS with a 2xHA-tagged sequence into a pDONR221 donor vector, followed by subcloning in a pK7WG2 expression vector with 35S promoter (Karimi et al., 2007); and *promTPPI::TPPI::GFP* lines were created by cloning TPPI promoter (used for the GUS marker lines) in a pDONRP4-P1r, TPPI-CDS in pDONR221 and GFP in a pENTRY2-3 donor vectors followed by a subcloning in the multisite vector pK7m34GW (Karimi et al., 2007), using the GatewayTM technology (Invitrogen) according to manufacturer's instructions. *Col-0* ecotype plants were transformed with the expression vector and selected on kanamycin. Homozygous, independent-single insertion lines were generated.

Plant growing conditions

For GUS analysis, metabolite and RAM size measurements, seedlings were grown for 2 DAG, 11

DAG and 4 DAG, respectively, in vertical plates on standard half strength MS supplemented with 1% sucrose under continuous light (110 µE.m^{-2}.s^{-1} photosynthetically active radiation) at 22^{O}C. For phenotypic analysis, media was supplemented with sucrose, glucose, trehalose and Validamycin A (Sigma Aldrich), auxin and agar in the concentrations and under the light regime indicated in the text. For root staging assays in plant grown on soil, seeds were sown directly in 2:1 V/V soil:sand mixture.

Histochemical and histological analysis

The beta-glucuronidase (GUS)-staining assays were performed according to Beeckman and Engler (1994). Samples were cleared by mounting them in 90% lactic acid (Acros Organics) on glass microscope slides and analyzed by differential interference contrast microscopy (Olympus BX51). For anatomical sections, GUS-stained samples were fixed overnight, dehydrated and embedded as described by De Smet et al. (2004). For GFP visualization, roots were stained with 10 µg.ml^{-1} propidium iodide (Sigma-Aldrich), mounted in water in microscope slides and analyzed in a confocal microscope with package software LSM510 version 3.2 (Zeiss, Jena , Germany). For root staging, roots from plants grown on soil were carefully cleaned and, together with the ones grown on agar plates, cleared as described by Malamy and Benfey (1997) mounted on microscopy slides and analyzed as described above. Pictures were processed with Adobe Photoshop CS4.

Quantitative real-time PCR

RNA was extracted with RNeasy kit (Qiagen). cDNA was prepared from 1 µg of total RNA with iScript cDNA synthesis kit (Bio-Rad) and analyzed on a LightCycler 480 Real-Time PCR instrument (Roche Applied Science) with the SYBR Green I Master (Roche Applied Science) according to manufacturer's instructions. All reactions were done in triplicate. Relative expression levels of target genes were quantified with specific primer sets (Table S4), analyzed with qBase (Hellemans et al., 2007) and normalized to ACTIN2 and CYTOCHROME2 expression levels.

RAM measurements

RAM measurements were done by counting and measuring the cortical cells from the quiescent center in the root tip until the first cells with a switch in cell size. The elongation zone was measured following the cortical cells from the first cell leaving the RAM until the place where root hairs appeared. 4 DAG roots were stained with propidium iodide and analyzed in a confocal microscope as described above. Cell number and sizes were determined by using Image J software.

Metabolite measurements

Between 10-15 plants grown for 11 DAG as indicated above, were pooled and froze immediately in liquid nitrogen. Tre6P, phosphorylated intermediates and organic acids were measured by liquid-tandem mass spectrometry as indicated in Lunn et al. (2006). Sucrose, glucose and fructose contents were measured in ethanol extracts as described by Geigenberger et al. (1996). Starch contents were determined as glucose equivalents as described by Hendriks et al. (2003). Trehalose was measured in the same plant extracts used for Tre6P measurements (Lunn et al., 2006) using trehalase (from *E.coli*, TREF) to hydrolyze the trehalose into glucose, which was measured in a fluorescence assay using glucose oxidase (GOD) and Amplex Red (Invitrogen). For each sample a calibration curve was done by adding to different sample aliquots a known concentration of trehalose. In a black 96-well microplate, 5ul of plant extract were incubated with

35µl of assay mix (4µl of buffer mix [phosphate buffer 500mM pH7,5, 10mM $MgCl_2$, 100mM NaCl, keep at 4°C], 0,2µl catalase/GOD mix [0,5µl catalase 30 KU/ml in 20µl GOD 20 KU/ml] and 30,6µl H_2O. Blank and standards had 0,2µl TREF [1mg/ml] as well), at 30°C for 60 min shaking. The plate was heated at 80°C for 15 min, cooled and spun down. On ice, 5µl of H_2O were added to the samples and blank, and 5µl of trehalose standards (2,5 to 40 pmol) were used for the samples of the calibration curve. 55µl of determination mix were added to each sample (6µl of buffer mix, 0,2µl GOD [10 KU/ml], 0,2µl TREF [1mg/ml], 0,2µl horse radish peroxidase (HRP), 0,25µl Amplex Red). The plate was read in a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc.) in the fluorescence mode till the slope was stable.

6. SUPPLEMENTAL INFORMATION

Table S1. Root phenotype of *tppi-1* on different sugar concentrations. Main root length (cm) of 10 DAG plants grown on vertical plates, 16h/8h day/night conditions. n=10 plants, ±SD

	sugar	Sucrose			Glucose		
	0%	1%	3%	6%	1%	3%	6%
Col	9,2±0,9	11,8±0,5	15,0±0,4	10,7±0,7	11,0±0,6	7,3±0,6	1,8±0,2
tppi-1	4,1±0,2	3,6±0,4	3,4±0,2	1,1±0,2	3,6±0,4	1,1±0,2	0±0

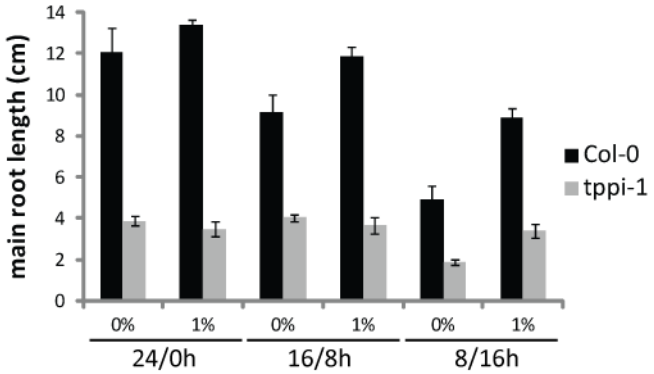


Figure S1. *tppi-1* root growth under continuous light (24h), long day (16/8h) and short day (8/16h)conditions with (1%) or without sucrose (0%).

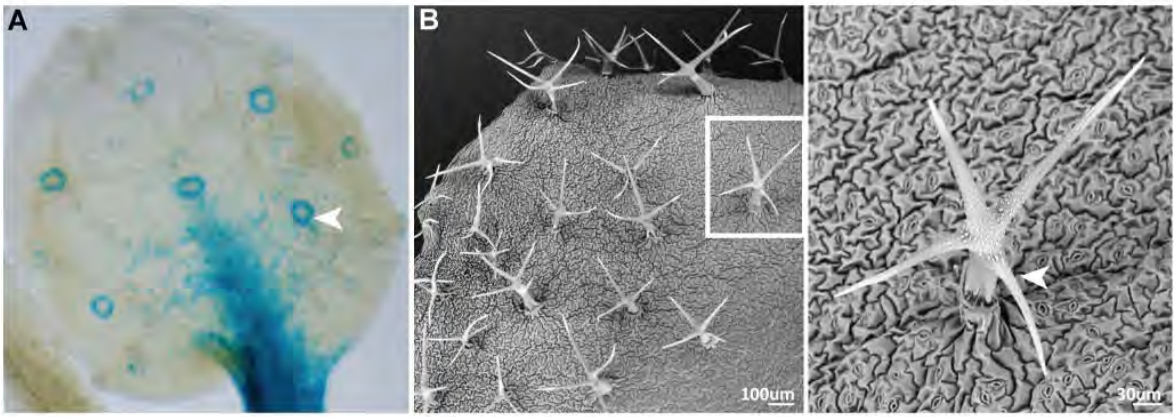


Figure S2. A. *promTPPI::GUS* expression in the subsidiary cells of trichomes. **B.** Trichomes in *tppi-1* mutant plants show one extra branch.

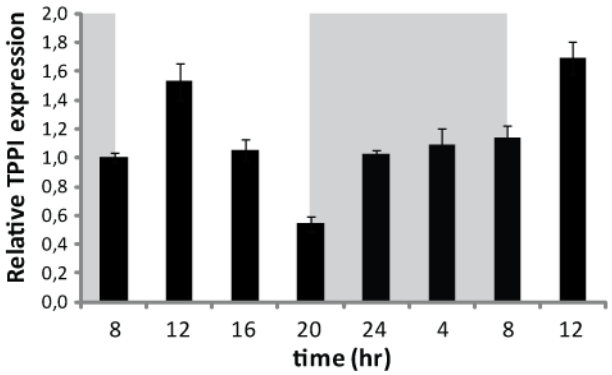


Figure S3. Relative *TPPI* expression in 4 DAG seedlings grown in a 12h/12h day/night regime. Grey boxes correspond to the dark phase of the 24h day.

Table S4. Specific primer sets for genotyping of T-DNA insertion lines and for qPCR analysis.

Primer	Sequence (5'→3')
Cloning	
TPPI-OE-attB1	GGAGATAGAACCATGTCAGCTAGTCAAAACATTGTCGTATC
TPPI-OE-attB2	GGGTCACCGCCTCCGGATCAAGCGTAGTCTGGAACGTCG
pTPPI-attB4	GGGGACAACTTTGTATAGAAAAGTTGATGATCAAACTAGGTTAAGAAG
pTPPI-attB1	GGGGACTGCTTTTTTGTACAAACTTGTATCTGTGGTTTTTCCACGAC
TPPI-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTCAGCTAGTCAAAACATTG
TPPI-atttB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCTTGGCTGCATTTGTTTCC
Genotyping	
tppi1_LP1	ACGACAGATGCAACATCCTTC
tppi1 _RP1	TTCAATCATTGGACGGATTTC
qPCR	
TPPI_fw	GAAGAAATGGAGCGAACTGG
TPPI_rev	CGTCACCAATATAAACCGGG
CDKA1;1_fw	CCTCTTGAAAGAAATGCAGCACAGC
CDKA1;1_rev	GAGAAATCAGGAGTAGAATCCATGTGC
CYCA3;1_fw	AGGAAACCCTACAAGCAACACG
CYCA3;1_rev	AGCAGCCACAGTAGACGGAAG
CYCB1;1_fw	CTCAAAATCCCACGCTTCTTGTGG
CYCB1;1_rev	CACGTCTACTACCTTTGGTTTCCC
ACT_fw	GGCTCCTCTTAACCCAAAGGC
ACT_rev	CACACCATCACCAGAATCCAGC
CK2_fw	ACCACCATTAACGTGCGTCAAC
CK2_rev	GATCTTGGCGAGAGAATCGGTATC

7. REFERENCES

Avonce, N., Leyman, B., Mascorro-Gallardo, J.O., Van Dijck, P., Thevelein, J.M., and Iturriaga, G. (2004). The Arabidopsis Trehalose-6-P Synthase AtTPS1 Gene Is a Regulator of Glucose, Abscisic Acid, and Stress Signaling. Plant Physiol **136,** 3649-3659.

Baskin, T.I., Cork, A., Williamson, R.E., and Gorst, J.R. (1995). STUNTED PLANT 1, A Gene Required for Expansion in Rapidly Elongating but Not in Dividing Cells and Mediating Root Growth Responses to Applied Cytokinin. Plant Physiol **107,** 233-243.

Beeckman, T., and Engler, G. (1994). An easy technique for the clearing of histochemically stained plant tissue. Plant Molecular Biology Reporter **12,** 37-42.

Beemster, G.T.S., and Baskin, T.I. (1998). Analysis of Cell Division and Elongation Underlying the Developmental Acceleration of Root Growth in Arabidopsis thaliana. Plant Physiol **116,** 1515-1526.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K., and Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. Nature **433,** 39-44.

Chary, S.N., Hicks, G.R., Choi, Y.G., Carter, D., and Raikhel, N.V. (2008). Trehalose-6-phosphate synthase/phosphatase regulates cell shape and plant architecture in Arabidopsis. Plant Physiol **146,** 97-107.

Cluis, C.P., Mouchel, C.F., and Hardtke, C.S. (2004). The *Arabidopsis* transcription factor HY5 integrates light and hormone signaling pathways. Plant J **38,** 332-347.

De Rybel, B., Vassileva, V., Parizot, B., Demeulenaere, M., Grunewald, W., Audenaert, D., Van Campenhout, J., Overvoorde, P., Jansen, L., Vanneste, S., Moller, B., Wilson, M., Holman, T., Van Isterdael, G., Brunoud, G., Vuylsteke, M., Vernoux, T., De Veylder, L., Inze, D., Weijers, D., Bennett, M.J., and Beeckman, T. (2010). A novel aux/IAA28 signaling cascade activates GATA23-dependent specification of lateral root founder cell identity. Curr Biol **20,** 1697-1706.

De Smet, I., Chaerle, P., Vanneste, S., De Rycke, R., Inze, D., and Beeckman, T. (2004). An easy and versatile embedding method for transverse sections. J Microsc **213,** 76-80.

De Smet, I., Tetsumura, T., De Rybel, B., Frey, N.F., Laplaze, L., Casimiro, I., Swarup, R., Naudts, M., Vanneste, S., Audenaert, D., Inze, D., Bennett, M.J., and Beeckman, T. (2007). Auxin-dependent regulation of lateral root positioning in the basal meristem of Arabidopsis. Development **134,** 681-690.

Dello Ioio, R., Linhares, F.S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., and Sabatini, S. (2007). Cytokinins Determine Arabidopsis Root-Meristem Size by Controlling Cell Differentiation. Curr Biol **17,** 678-682.

Fukaki, H., and Tasaka, M. (2009). Hormone interactions during lateral root formation. Plant Mol Biol **69,** 437-449.

Fukaki, H., Okushima, Y., and Tasaka, M. (2007). Auxin-mediated lateral root formation in higher plants. Int Rev Cytol **256,** 111-137.

Fukaki, H., Tameda, S., Masuda, H., and Tasaka, M. (2002). Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of Arabidopsis. Plant J **29,** 153-168.

Fukaki, H., Nakao, Y., Okushima, Y., Theologis, A., and Tasaka, M. (2005). Tissue-specific expression of stabilized SOLITARY-ROOT/IAA14 alters lateral root development in Arabidopsis. Plant J **44,** 382-395.

Garg, A.K., Kim, J.K., Owens, T.G., Ranwala, A.P., Choi, Y.D., Kochian, L.V., and Wu, R.J. (2002). Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci U S A **99,** 15898-15903.

Ge, L.-F., Chao, D.-Y., Shi, M., Zhu, M.-Z., Gao, J.-P., and Lin, H.-X. (2008). Overexpression of the trehalose-6-phosphate phosphatase gene *OsTPP1* confers stress tolerance in rice and results in the activation of stress responsive genes. Planta **228,** 191-201.

Geigenberger, P., Lerchi, J., Stitt, M., and Sonnewald, U. (1996). Phloem specific expression of pyrophosphatase inhibits long distance transport of carbohydrates and amino acids in tobacco plants. Plant Cell Environ **19,** 43-55.

Goddijn, O.J., Verwoerd, T.C., Voogd, E., Krutwagen, R.W., de Graaf, P.T., van Dun, K., Poels, J., Ponstein, A.S., Damm, B., and Pen, J. (1997). Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. Plant Physiol **113,** 181-190.

Gómez, L.D., Baud, S., Gilday, A., Li, Y., and Graham, I.A. (2006). Delayed embryo development in the ARABIDOPSIS TREHALOSE-6-PHOSPHATE SYNTHASE 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. Plant J **46,** 69-84.

González-García, M.-P., Vilarrasa-Blasi, J., Zhiponova, M., Divol, F., Mora-García, S., Russinova, E., and Caño-Delgado, A.I. (2011). Brassinosteroids control meristem size by promoting cell cycle progression in Arabidopsis roots. Development **138,** 849-859.

Guilfoyle, T.J., and Hagen, G. (2007). Auxin response factors. Curr Opi Plant Biol **10,** 453-460.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol **8,** R19.

Hendriks, J.H., Kolbe, A., Gibon, Y., Stitt, M., and Geigenberger, P. (2003). ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of Arabidopsis and other plant species. Plant Physiol **133,** 838-849.

Jang, I.C., Oh, S.J., Seo, J.S., Choi, W.B., Song, S.I., Kim, C.H., Kim, Y.S., Seo, H.S., Choi, Y.D., and Nahm, B.H. (2003). Expression of a bifunctional fusion of the Escherichia coli genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. Plant Physiol **131,** 516.

Karim, S., Aronsson, H., Ericson, H., Pirhonen, M., Leyman, B., Welin, B., Mantyla, E., Palva, E.T., Van Dijck, P., and Holmstrom, K.O. (2007). Improved drought tolerance without undesired side effects in transgenic plants producing trehalose. Plant Mol Biol **64,** 371-386.

Karimi, M., Depicker, A., and Hilson, P. (2007). Recombinational cloning with plant gateway vectors. Plant Physiol **145,** 1144-1154.

Lammens, T., Boudolf, V., Kheibarshekan, L., Panagiotis Zalmas, L., Gaamouche, T., Maes, S., Vanstraelen, M., Kondorosi, E., La Thangue, N.B., Govaerts, W., Inzé, D., and De Veylder, L. (2008). Atypical E2F activity restrains APC/CCCS52A2 function obligatory for endocycle onset. Proc Natl Acad Sci U S A **105,** 14721-14726.

Lee, J., He, K., Stolc, V., Lee, H., Figueroa, P., Gao, Y., Tongprasit, W., Zhao, H., Lee, I., and Deng, X.W. (2007). Analysis of Transcription Factor HY5 Genomic Binding Sites Revealed Its Hierarchical Role in Light Regulation of Development. Plant Cell **19,** 731-749.

Lunn, J.E., Feil, R., Hendriks, J.H., Gibon, Y., Morcuende, R., Osuna, D., Scheible, W.R., Carillo, P., Hajirezaei, M.R., and Stitt, M. (2006). Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in Arabidopsis thaliana. Biochem J **397,** 139-148.

Malamy, J.E., and Benfey, P.N. (1997). Organization and cell differentiation in lateral roots of Arabidopsis thaliana. Development **124,** 33-44.

Miranda, J.A., Avonce, N., Suarez, R., Thevelein, J.M., Van Dijck, P., and Iturriaga, G. (2007). A bifunctional TPS-TPP enzyme from yeast confers tolerance to multiple and extreme abiotic-stress conditions in transgenic Arabidopsis. Planta **226,** 1411-1421.

Okushima, Y., Overvoorde, P.J., Arima, K., Alonso, J.M., Chan, A., Chang, C., Ecker, J.R., Hughes, B., Lui, A., Nguyen, D., Onodera, C., Quach, H., Smith, A., Yu, G., and Theologis, A. (2005). Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in Arabidopsis thaliana: unique and overlapping functions of ARF7 and ARF19. Plant Cell **17,** 444-463.

Osterlund, M.T., Hardtke, C.S., Wei, N., and Deng, X.W. (2000). Targeted destabilization of HY5 during light-regulated development of Arabidopsis. Nature **405,** 462-466.

Oyama, T., Shimura, Y., and Okada, K. (1997). The Arabidopsis HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. Genes Dev **11,** 2983-2995.

Péret, B., De Rybel, B., Casimiro, I., Benkova, E., Swarup, R., Laplaze, L., Beeckman, T., and Bennett, M.J. (2009). Arabidopsis lateral root development: an emerging story. Trends Plant Sci **14,** 399-408.

Romero, C., Belles, J.M., Vaya, J.L., Serrano, R., and Culianez-Macia, F.A. (1997). Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. Planta **201,** 293-297.

Satoh-Nagasawa, N., Nagasawa, N., Malcomber, S., Sakai, H., and Jackson, D. (2006). A trehalose metabolic enzyme controls inflorescence architecture in maize. Nature **441,** 227-230.

Schluepmann, H., Pellny, T., van Dijken, A., Smeekens, S., and Paul, M. (2003). Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. Proc Natl Acad Sci U S A **100,** 6849-6854.

Sibout, R., Sukumar, P., Hettiarachchi, C., Holm, M., Muday, G.K., and Hardtke, C.S. (2006). Opposite Root Growth Phenotypes of *hy5* versus *hy5 hyh* Mutants Correlate with Increased Constitutive Auxin Signaling. PLoS Genet **2,** e202.

Suarez, R., Wong, A., Ramirez, M., Barraza, A., Orozco Mdel, C., Cevallos, M.A., Lara, M., Hernandez, G., and Iturriaga, G. (2008). Improvement of drought tolerance and grain yield in common bean by overexpressing trehalose-6-phosphate synthase in rhizobia. Mol Plant Microbe Interact **21,** 958-966.

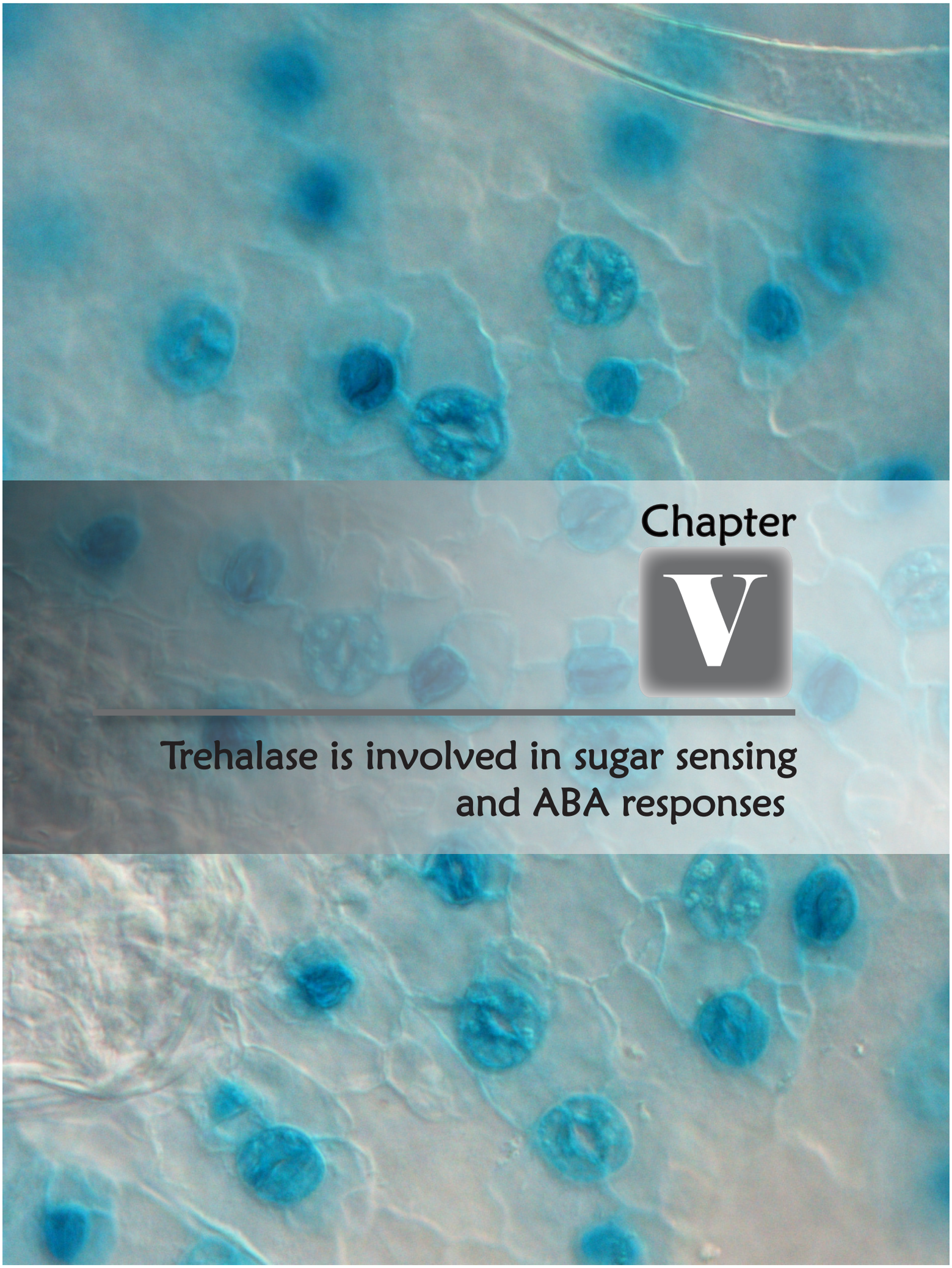
Tiwari, S.B., Hagen, G., and Guilfoyle, T. (2003). The Roles of Auxin Response Factor Domains in Auxin-Responsive Transcription. Plant Cell **15,** 533-543.

Tiwari, S.B., Hagen, G., and Guilfoyle, T.J. (2004). Aux/IAA proteins contain a potent transcriptional repression domain. Plant Cell **16,** 533-543.

Vanstraelen, M., Baloban, M., Da Ines, O., Cultrone, A., Lammens, T., Boudolf, V., Brown, S.C., De Veylder, L., Mergaert, P., and Kondorosi, E. (2009). APC/CCCS52A complexes control meristem maintenance in the Arabidopsis root. Proc Natl Acad Sci U S A **106,** 11806-11811.

Wingler, A., Fritzius, T., Wiemken, A., Boller, T., and Aeschbacher, R.A. (2000). Trehalose Induces the ADP-Glucose Pyrophosphorylase Gene, ApL3, and Starch Synthesis in Arabidopsis. Plant Physiol **124,** 105-114.

Zhang, Y., Primavesi, L.F., Jhurreea, D., Andralojc, P.J., Mitchell, R.A.C., Powers, S.J., Schluepmann, H., Delatte, T., Wingler, A., and Paul, M.J. (2009). Inhibition of SNF1-Related Protein Kinase1 Activity and Regulation of Metabolic Pathways by Trehalose-6-Phosphate. Plant Physiol **149,** 1860-1871.



Chapter

V

Trehalase is involved in sugar sensing and ABA responses

Trehalase is involved in sugar sensing and ABA responses

1. ABSTRACT

Trehalose metabolism, including the intermediate Tre6P, has been associated with different developmental processes in plants. However the role of trehalase (TRE1), the only enzyme hydrolyzing trehalose, has been more studied in plant-pathogen interactions than in plant development. Here we provide evidence that TRE1 is directly involved controlling trehalose and indirectly controls Tre6P levels in plants. *TRE1* mutants show sensitivity to sugars in the media that are correlated with Tre6P and trehalose levels. Additionally, TRE1 knock-out plants have altered responses to ABA regulation of stomatal closure. This study importantly proves the highly connected mechanism involving trehalose and Tre6P with sugar sensing and use for growth, and the close link between trehalose metabolism and ABA signaling.

2. INTRODUCTION

Trehalase is an enzyme that hydrolyzes trehalose into two glucose molecules. Trehalase enzymes have been extensively characterized in insects, fungi and yeast. Trehalose is the major sugar in the haemolymph of insects and its hydrolysis is catalyzed by trehalase and therefore trehalase has been looked at as a target for insect control (Silva et al., 2010). In *Saccharomyces cerevisiae*, three trehalases have been described. Two are neutral trehalases NTH1 and NTH2. NTH1 hydrolyze intracellular trehalose and it is localized in the cytosol (San Miguel and Arguelles, 1994). The other one is the acid trehalase (ATH1) which is vacuolar (Huang et al., 2007), and is in charge of the degradation of extracellular trehalose to be used as a carbon source for growth (Nwaka et al., 1996; Basu et al., 2006). However, as in other plant genomes, the Arabidopsis genome encodes only one trehalase, TRE1. Trehalase in plants is thought to be highly active as trehalose levels are very low, but this has not yet been defined. TRE1 is a plasma membrane-bound protein, as was demonstrated by transient expression in epidermal leaf tobacco cells (Frison et al., 2007). In the same study, it was demonstrated that TRE1 can functionally replace the extracellular active yeast Ath1p, and that its catalytic domain is apoplastic.

Only very recently, a TRE1 downregulated line was reported (Delatte et al., 2011). This line has high levels of Tre6P compared to wt, implying that TRE1 has a role in Tre6P regulation. Arabidopsis mutants in the trehalose pathway, displaying modified trehalose and Tre6P levels, had shown phenotypes related to developmental processes in embryo

and flowering transition (van Dijken et al., 2004; Gómez et al., 2006), sugar and ABA signaling pathways (Schluepmann et al., 2003; Avonce et al., 2004; Schluepmann et al., 2004), and abiotic and biotic stresses (Yeo et al., 2000; Garg et al., 2002; Jang et al., 2003; Pramanik and Imai, 2005; Miranda et al., 2007), among others. Still, trehalase function has been described more in the context of plant associated-microorganisms. Its activity is high in root legume nodules of soybean and also in clubroot (*Plasmodiophora brassicaceae*) infected organs in cabbage (reviewed by Müller et al., 1995b), and Arabidopsis (Brodmann et al., 2002), suggesting that trehalase may function in plant defenses by regulating microbial trehalose and its interference with carbon metabolism in plant cells (Müller et al., 1995b, a; Brodmann et al., 2002)

Little is known of the specific function of TRE1 in Arabidopsis plants. In this study we explore the phenotypes of TRE1 mutants under different sucrose conditions, and associate its sugar hypersensitivity with differences in trehalose and Tre6P contents. The TRE1 pattern of expression is also discussed and linked to the resistance to ABA-inhibition of stomatal aperture in TRE1 knock-down plants.

3. RESULTS

3.1 *TRE1* mutant plants are hypersensitive to exogenous sugars

As reported previously by Vandesteene (2009), *TRE1* mutant lines were found in different T-DNA collections: GT_16843 in the Ler background and Salk_151791 in the Col-0 background (Figure 1A). To check the *TRE1* level of expression in these lines, 12 DAG plants were grown and used for qPCR analysis with *TRE1* specific primers. As shown in Figure 1B the GT line showed downregulation of *TRE1*, herein after *tre1-1*, whereas in the Salk line the gene is upregulated, here after *TRE1-1*. This Salk line has a T-DNA inserted in the promoter of TRE1 just before the 5'UTR. The T-DNA contains a 35S CaMV promoter in the direction of the gene, thus it is not surprising that it leads to *TRE1* overexpression. Alternatively, T-DNA insertion could be located in an important box for transcriptional regulation, and the disruption of such a box could induce *TRE1* upregulation. However no binding motifs were found in the particular place of the *TRE1-1* promoter (ATHENA, O'Connor et al., 2005; AGRIS, Palaniswamy et al., 2006). Additionally, functional activity of these lines is reported to be in accordance with the repression and induction of *TRE1*. In *TRE1-1* lines trehalase activity is up to 25 times higher in the mutant leaves than in Col-0 leaves, while in *tre1-1* plants, very low activity is detected (Vandesteene, 2009).

Since we had the *TRE1* mutants, we also tested their root growth phenotype in seedlings. In standard growth conditions, i.e. continuous light, 1% sucrose, *tre1-1* seedlings looked the same as Ler, with only a slight increase in root length. *TRE1-1* seedlings, however, showed a reduced main root length compared to Col-0 roots (Figure 1C). Although, when

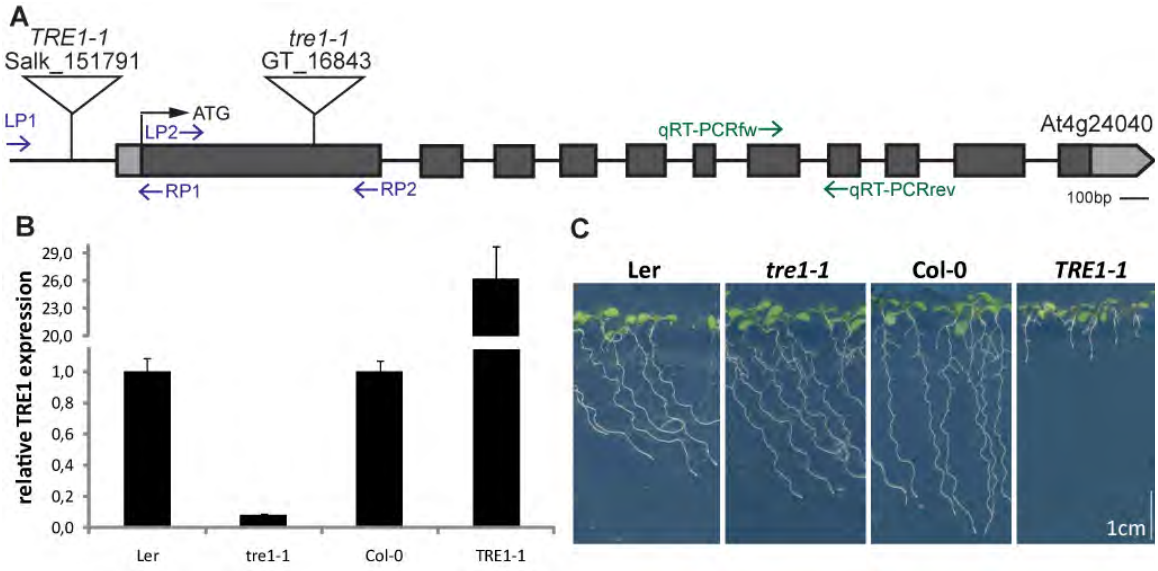


Figure 1. Characterization of *TRE1* T-DNA insertion mutants. **A.** Genomic *TRE1* scheme showing UTRs in light gray boxes, exons in dark gray boxes and introns as a black line. T-DNA lines are indicated in the map as well as the primers used for genotyping and qPCR (Table S1). **B.** Relative *TRE1* expression levels in 11 DAG T-DNA lines with downregulation and upregulation of *TRE1* in comparison with their respective wt. **C.** Root phenotype of 6 DAG *TRE1* mutants grown on vertical plates with continuous light and 1% sucrose.

media is deprived of sugar, *TRE1-1* has a restored root length (Figure 2) whilst *tre1-1* shows a slightly opposite phenotype. These preliminary results suggest that TRE1-OE is sensitive to extra carbon sources in the media, while the opposite is happening with *tre1-1* plants that grow better under high sugar conditions (Vandesteene, 2009).

Under trehalose supplemented media, plants can hydrolyze trehalose and use glucose as a carbon source. Though, when Validamicyn A, an inhibitor of trehalase activity, is added to the media, trehalose levels are high and this has an inhibitory effect in root elongation. This results in wild type seedlings arrested in growth shortly after germination, showing only a short primary root (Wingler et al., 2000). Interestingly, while for *tre1-1* plants low levels of trehalose (0,5mM) in the media are detrimental for its growth, *TRE1-1* mutants do not show inhibition of root growth even under high trehalose concentrations (25mM) confirming the high trehalase activity in these plants (Vandesteene, 2009).

3.2 Trehalose measurements reflect the enzymatic function of *TRE1*

The specific phenotypes found in *TRE1* mutants indicate that trehalase function is tightly connected with sugar sources in the plant. 12 DAG *TRE1-KO* and *OE* plants grown under continuous light in 1% sucrose supplemented media, were harvested to measure sugar levels, trehalose and Tre6P. As expected the trehalose level in the *tre1-1* mutant was higher than in the corresponding wild type line, and this level correlated with a higher Tre6P level as well. The opposite was found in the *TRE1-1* mutant with lower trehalose and Tre6P levels compared to the corresponding wild type line (Figure 3).

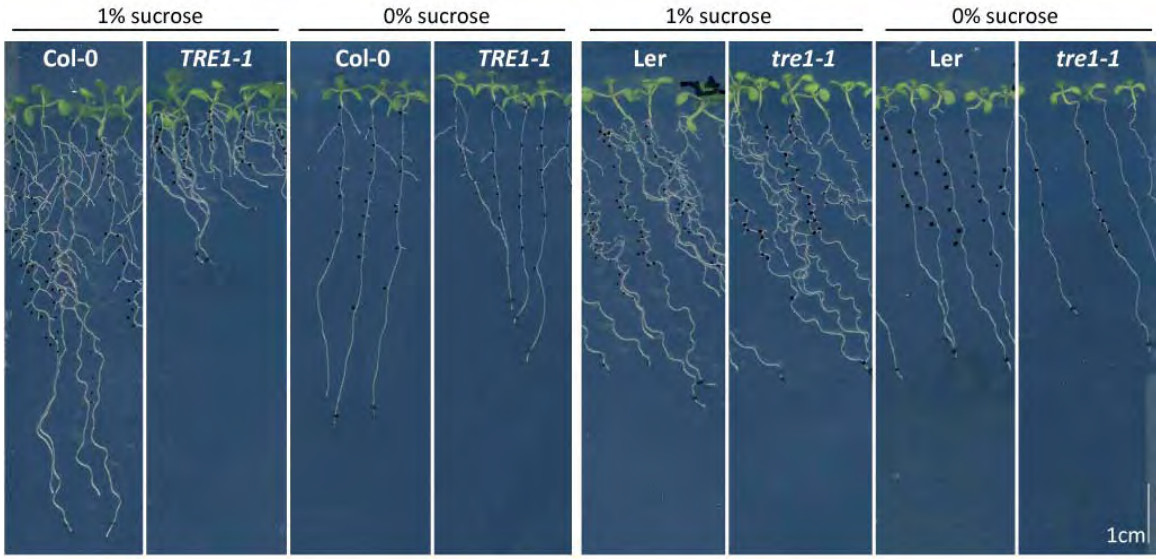


Figure 2. Phenotype of *TRE1* T-DNA insertion mutants under different sugar conditions. 9 DAG seedlings were grown on vertical plates in a 16/8h light/night cycle with or without sucrose. TRE-OE plants have a restored root in media without sucrose.

Tre6P is a signal metabolite for sucrose status in the plant as it rises with increasing sucrose levels, together with a stimulation of starch synthesis *in vivo* (Lunn et al., 2006). As *tre1-1* had an increase in Tre6P levels we could expect an increase in sucrose and starch as well. However, in *tre1-1* plants, sucrose and starch contents were not positively correlating with Tre6P levels, though we could only detect a marginal increase in hexose phosphates (Table 1). On the other hand, the lower sucrose, fructose and glucose contents of *TRE1-1* plants were correlating with its lower Tre6P level. Furthermore, the *TRE1-1* mutant showed a slightly higher starch content, and together with its reduced growth under the conditions of this experiment (Figure 1C), suggest a defect in starch breakdown and use. Therefore, we further checked genes involved in carbohydrate metabolism that are known to be responsive to changes in carbon status. We looked at the transcriptional levels, in *TRE1-1* plants, of genes such as *SUCROSE-PHOSPHATE SYNTHASE* (*SPS*) and *SUCROSE SYNTHASE1* (*SUS1*), which are involved in sucrose synthesis

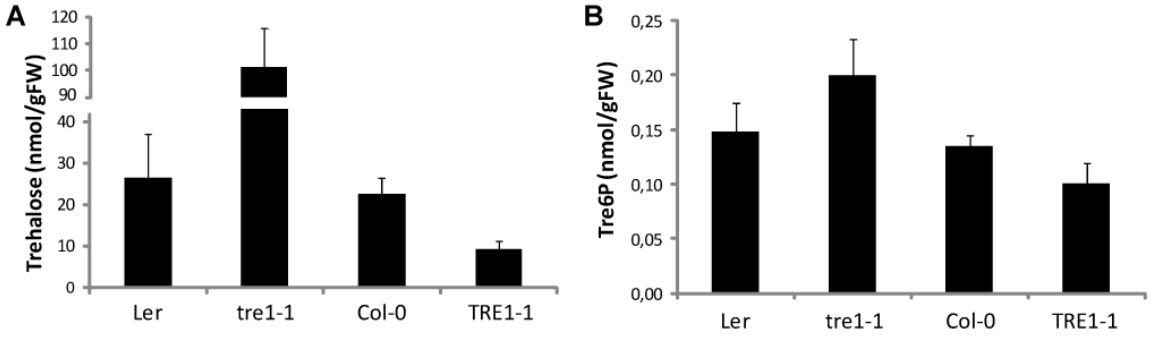


Figure 3. Trehalose and Tre6P level in *TRE1* mutants. Plants were grown on vertical plates for 11 DAG in continuous light and 1% sucrose supplemented media. **A.** Trehalose levels in *tre1-1* mutant was higher than in wt, whilst *TRE1-1* had less trehalose. **B.** Tre6P levels were higher in *tre1-1* and lower in *TRE1-1* compared to their respective controls.

and degradation respectively (Thimm et al., 2004). Additionally, we tested *APL3* which is involved in starch synthesis as it is part of the large subunit of ADP glucose phyrophosphorylase, and *STARCH EXCESS1* (*SEX1*) which regulates starch mobilization (Yu et al., 2001). qPCR analysis shows that *SPS* and *SUS1* are slightly upregulated in *TRE1-1* whereas, *APL3* transcript levels are 40% higher in *TRE1-1* plants in comparison with the wt. A strong increase in *SEX1* expression in *TRE1-1* plants was observed, which does not fit with the fact that we see higher starch levels in the mutant (Figure 4).

Table 1. Levels of sugar phosphates, sugars and starch in *TRE1* mutants. Measurements were done in 11 DAG plants grown on vertical plates supplemented with 1%sucrose under continuous light. n=4 independent experiments, ±SD

	Ler	*tre1-1*	Col-0	*TRE1-1*
Suc6P (nmol/gFW)	0,57±0,01	0,60±0,06	0,49±0,02	0,43±0,04
Glc6P (nmol/gFW)	108,4±12,6	121,3±10,9	163,2±17,2	156,0±2,4
Fru6P (nmol/gFW)	51,1±2,9	54,9±5,3	64,8±3,5	61,4±4,1
UDPG (nmol/gFW)	41,2±3,9	48,5±5,8	58,8±4,1	52,0±3,9
Sucrose (µmol/gFW)	3,0±0,6	2,6±0,3	2,5±0,0	2,0±0,1
Glucose (µmol/gFW)	4,6±0,3	4,8±0,7	3,9±0,2	2,1±0,1
Fructose (µmol/gFW)	9,4±0,8	9,2±2,4	9,5±0,8	5,2±0,9
Starch (µmol gluc equivalents/gFW)	28,9±5,7	32,7±6,3	20,8±1,8	26,0±3,8

It is important to note that the plants used to measure sugars and metabolites contents were grown under sucrose conditions, which has detrimental effects on *TRE1-1* growth (Figure 1C, 2). Therefore, we could anticipate that this preliminary experiment is useful, but more conditions, including low and high sucrose treatments, should be tested in order to relate metabolite and sugar contents with effects on growth in trehalase mutants.

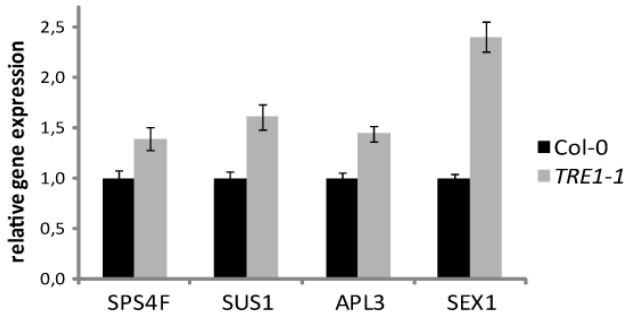


Figure 4. Expression of genes associated with carbohydrate metabolism in *TRE1-1* mutant. Plants were grown 11 DAG in continuous light and 1% sucrose supplemented media. Genes involved in sucrose and starch metabolism were upregulated in *TRE1-OE* plants.

3.3 Trehalase acts in ABA–dependent stomatal responses

Despite the association of trehalose metabolism and its intermediate Tre6P with sensing and use of sucrose, trehalose metabolism has been tightly connected with ABA responses via a crosstalk between glucose and ABA (Avonce et al., 2004; Ramon et al., 2007; Gómez et al., 2010). *TPS1-OE* seeds are able to germinate in high levels of exogenously applied ABA. On the other hand, *tps1* weak alleles, besides their sensitivity to ABA-inhibition of germination, showed sensitivity to the promotion of stomatal closure by the same hormone. These phenotypes are positively correlating with the contents of Tre6P in these mutants, meaning that more Tre6P results in higher resistance to ABA. Since *TRE1* mutants are displaying contrasting levels of Tre6P and trehalose, and as *TRE1* is highly expressed in stomata (Figure 5), among other tissues in the plant (Figure S1), we investigated further the involvement of trehalase in ABA stomatal responses. *TRE1* mutant epidermal strips were treated with ABA (20µM) after incubation in a stomatal aperture buffer (Figure 6). ABA promotes stomatal closure in *TRE1-OE* plants but not in *TRE1-KO* plants with a higher Tre6P. This response in the *TRE1-KO* plants is in agreement with the effect of ABA in other trehalose metabolism mutants such as *TPS1-OE* and *TPPG-KO* (Chapter 2) that also have higher Tre6P levels. This experiment strongly suggests that,

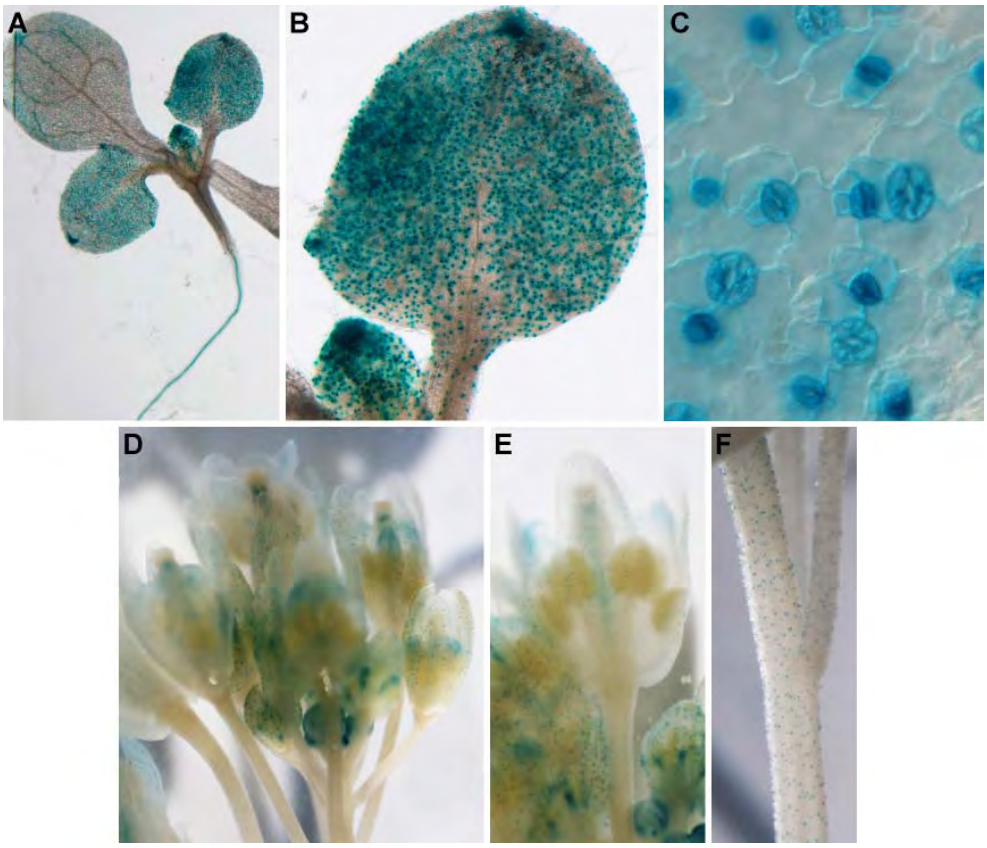


Figure 5. *TRE1* expression in stomata. A-C. 12 DAG *pTRE1::GUS-GFP* plants grown on vertical plates under continuous light, 1%suc. **A.** Shoot showing expression in first true leaves **B.** First true leaf close up showing *TRE1* expression in stomata. **C.** Expression in the central stele in maturation zone of roots. **D-F.** Five week old *pTRE1::GUS-GFP* plants grown on soil. **D.** Inflorescence showing expression in stigma of flowers. **E.** Close-up to flowers with TRE1 expression in stomata of sepals. **F.** Inflorescence peduncle with stained stomata.

Tre6P could be inhibiting ABA responses in stomatal closure and more generally that Tre6P levels correlate positively with resistance to ABA.

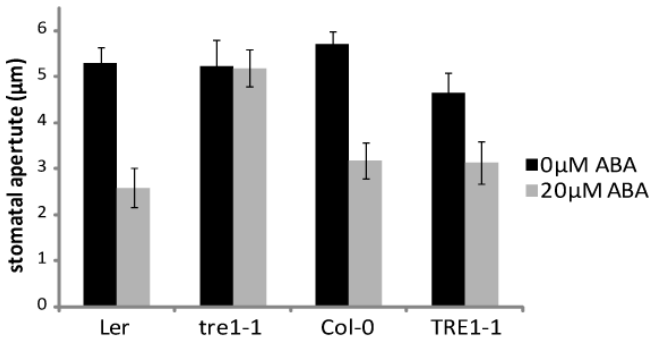


Figure 6. *tre1-1* resistance to ABA promotion of stomatal closure. Epidermal strips of *TRE1* mutants were incubated for 2h in a stomatal promotion of aperture buffer and then treated for 2h with or without ABA, *TRE1-KO* is resistant to ABA promotion of closure of stomata. n=40-50 stomata/leaf in 3 different leaves.

Additionally, we also looked at the transcriptional levels of some ABA responsive genes such as ABA-insensitive *ABI3* and *ABI4,* that have been found also in screenings for insensitivity to glucose (Arenas-Huertero et al., 2000; Finkelstein et al., 2002), and *RAB18* and *EM1* (Figure 7). For the last two genes there were no differences in transcript levels between the *TRE1* mutants and controls. However, *ABI4* levels were increased in *tre1-1* mutants whereas *ABI3* is upregulated in *TRE1-1* plants. As these two transcription factors have been associated with different ABA responses, it is plausible that they are regulated in different ways in *TRE1* mutants.

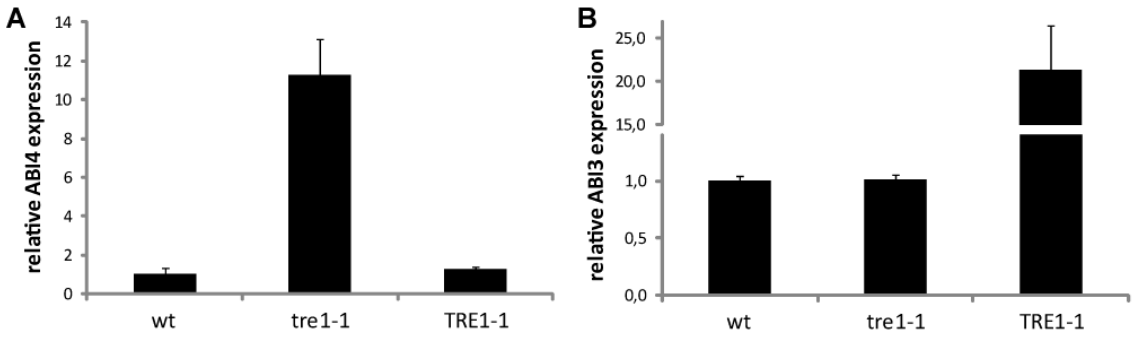


Figure 7. Expression of ABA responsive genes in *TRE1* mutants. *TRE1* mutants were grown 11 DAG in continuous light and 1% sucrose supplemented media. **A.** Relative *ABI4* expression in comparison to the respective wild type. **B.** Relative *ABI3* expression in comparison to the respective wild type.

4. DISCUSSION

Arabidopsis harbors one gene encoding trehalase, which is the only enzyme able to hydrolyze trehalose in glucose units. Here we associate the function of *TRE1* with

regulation of growth depending on endogenous sugar levels. We also connect its particular expression pattern in stomata to ABA- dependent responses in stomata.

TRE1 is expressed in all stomata in the plant, from cotyledons to sepals and peduncle in flowers. This is in line with the use of *TRE1* promoter for guard cell-specific gene expression (Kim et al., 1999). Additionally, it is found in the stigma and siliques particularly in the chalaza of developing seeds, suggesting a high activity of this enzyme in reproductive organs. Trehalase activity in Arabidopsis is the highest in flowers, siliques and developing seeds, but is very low in roots and leaves (Müller et al., 2001). Therefore, trehalase might be important for reproductive processes, or specifically in the use or allocation of sugars to the embryo due to its presence in the chalaza.

By exploring *TRE1* mutant lines, we found an interesting phenotype in roots grown on media of different sucrose contents. Plants overexpressing trehalase were hypersensitive to sucrose in the medium, had a short main root and had numerous lateral roots, a phenotype that is restored to wild type in media without sucrose. As reported already by Vandesteene (2009) this mutant is resistant to the inhibition of root elongation by high levels of exogenously added trehalose, which is in line with its enzymatic activity. The subsequent endogenous increase in glucose seems to be growth promoting in *TRE1-1* plants. But, interestingly they can not cope with high exogenous sucrose levels, because when sucrose is added to the medium, *TRE1-1* plants were unable to elongate the roots. It is conceivable that *TRE1* mutant plants are altered in carbon utilization, as it has been shown when *E. coli TPS* (*otsA*) or *TPP* (*otsB*) are overexpressed in Arabidopsis plants (Schluepmann et al., 2003). Under high sugar conditions *otsB-OE* seedlings are not able to grow, these plants have a low amount of Tre6P, but higher levels of hexose phosphates when compared to wt, and the opposite happens with *otsA-OE* seedlings. In *TRE1* mutants the level of Tre6P is low for OE seedlings and high for KO seedlings, while the hexose phosphate contents are not so altered in comparison to their respective wt. However, independently from the hexose phosphate contents, *TRE1-1* and *tre1-1* mutants, do show sensitivity and resistance, respectively, to high sucrose levels in the medium, pointing at Tre6P as key metabolite coordinating exogenous levels of sugars with root growth. As another example, weak alleles of the only active TPS enzyme in Arabidopsis, TPS1, show reduced levels of Tre6P but high contents of soluble sugars and starch. This is not in agreement with Tre6P being a signal of sucrose status in the plant, and could not be linked to the changes in transcript levels of genes associated with carbohydrate metabolism. However, the authors claimed that rather than a direct effect of Tre6P, decreased growth is affecting the strength of the sink by reducing the sugar pools and starch in *tps1* weak alleles (Gómez et al., 2010). This is not the case for the reduced growth of *TRE1-1* plants on high sucrose medium, given that Tre6P as well as sugars and starch contents in this mutant are low compared to wt, indicates that actually Tre6P, and not changes in other sugars, is the signal for the use of carbon sources into growth.

Another explanation to associate Tre6P and sugars with growth is through the inhibitory effect of Tre6P in SnRK1 activity (Zhang et al., 2009). SnRK1 is a global regulator of transcriptional networks in response to sugar and energy deprivation (Baena-Gonzalez et al., 2007). *TRE1-1* reduced Tre6P levels will activate SnRK1 to switch *TRE1-OE* plants program from anabolism to catabolism leading to a reduced growth. It would be really interesting to look in more detail whether *TRE1* mutants have differential expression of SnRK1 kinase protein complex target genes and connect, in a developmental and growth context, this energy sensor with the sucrose signaling metabolite Tre6P.

Trehalose metabolism has also been linked to ABA signaling, trehalose biosynthesis mutants with high Tre6P levels are ABA insensitive to the inhibition of germination and to the promotion of stomatal closure (Chapter II). This phenotype was also found in *tre1-1* plants which are resistant to ABA promotion of stomatal closure. However, no differences where found in *TRE1-1* mutant with low Tre6P. In fact the stomata of this mutant do not open as much as the wild type, phenotype that was also observed in *tps1-12* weak allele (Gómez et al., 2010). Interestingly *ABI4*, a transcription factor involved in ABA and sugar signaling, is highly upregulated in *tre1-1* plants. High expression of this gene was also found in *35S::TPS1* lines, indicating that *ABI4* is an important part of the mechanism leading to insensitivity to ABA in mutants with increased Tre6P level. It is possible that *ABI4* is directly mediating responses to ABA or activating other ABA related genes when Tre6P levels are changing. Although *ABI4* has been reported to be involved in different ABA process i.e. seed development and germination (Finkelstein et al., 1998), mitochondrial and chloroplast retrograde signaling (Giraud et al., 2009), and even in the reduction of polar auxin transport affecting ABA and cytokinin inhibition of lateral root formation (Shkolnik-Inbar and Bar-Zvi, 2010), there is no evidence of the role of *ABI4* in stomatal responses to ABA. On the other hand, *ABI3* is upregulated in *TRE1-1* plants, *ABI3* overexpressor plants are hypersensitive to sugars (Finkelstein et al., 2002) which is linked to the sugar phenotype of *TRE1-1* mutants.

As it has been described here with the analysis of *TRE1* mutants grown on sucrose or treated with ABA, there are still big question marks about the mechanism connecting trehalose metabolism with ABA signaling. Different players can be involved depending on the developmental stage and the environmental clues, suggesting a complex map of interconnections linking sugar, Tre6P and ABA responses, and also SnRK1 regulation through Tre6P.

5. MATERIALS AND METHODS

Plant material

pTRE1::GUS-GFP lines were generated by cloning the TRE1 promoter/5'UTR sequence of around 2kb using specific primers with attBsites and fused to GUS/GFP reporter genes in the pHGWFS7

vector (Karimi et al., 2007) by means of the GatewayTM technology (Invitrogen) according to manufacturer's instructions. Wild type *A. thaliana* ecotype Columbia plants were transformed by floral dip with *Agrobacterium tumefaciens* (C58C1) containing the promoter constructs (Clough and Bent, 1998). Homozygous plants for at least three (independent and single-insertion) transformed Arabidopsis lines were selected in standard full MS media including vitamins and supplemented with HygromycinB following a modified protocol from Harrison et al. (2006). *tre1-1* (GT_16843 in Ler background) and *TRE1-1* mutant seeds (Salk_151791 in Col-0 background) were obtained from NASC and genotyped by Vandesteene (2009) with specific primers (Table S1).

Plant growing conditions

For GUS analysis and metabolite measurements and qPCR, seedlings were grown for 12 DAG and 11 DAG, respectively, on vertical plates, 0.5xMS, 1% suc, continuous light (110 $\mu E.m^{-2}.s^{-1}$ photosynthetically activeradiation) at 22^{O}C . For GUS staining in flowers, plants were grown on soil for 5-6 weeks, 16h-8h day/night cycle. For sucrose experiments plants were grown without or with 1% suc for 9 DAG on vertical plates at 16h/8h day/night regime at 22^{O}C. For ABA treatments in stomata, plants were grown on soil for 3 weeks, 16h-8h day/night cycle, with controlled watering.

Histochemical and histological analysis

The beta-glucuronidase (GUS)-staining assays were performed according to Beeckman and Engler (1994). Samples were cleared by mounting them in 90% lactic acid (Acros Organics) on glass microscope slides, and analyzed by differential interference contrast microscopy (Olympus BX51). Flowers were visualized directly after GUS staining in a binocular Leica. Pictures were processed with Adobe Photoshop CS4.

Quantitative real-time PCR

RNA was extracted with RNeasy kit (Qiagen). cDNA was prepared from 1 µg of total RNA with iScript cDNA synthesis kit (Bio-Rad) and analyzed on a LightCycler 480 Real-Time PCR instrument (Roche Applied Science) with the SYBR Green I Master (Roche Applied Science) according to manufacturer's instructions. All individual reactions were done in triplicate. Relative expression levels of target genes were quantified with specific primer sets (Table S2), analyzed with qBase (Hellemans et al., 2007) and normalized to ACTIN2 (At3g18780) and CKA2 (At3g50000) expression levels.

Metabolite measurements

Between 10-15 plants grown for 11 DAG as indicated above, were pooled and froze immediately in liquid nitrogen. Tre6P, phosphorylated intermediates and organic acids were measured by liquid-tandem mass spectrometry as indicated in Lunn et al. (2006). Sucrose, glucose and fructose contents were measured in ethanol extracts as described by Geigenberger et al. (1996). Starch contents were determined as glucose equivalents as described by Hendricks et al. (2003). Trehalose was measured in the same plant extracts used for Tre6P measurements (Lunn et al., 2006) using trehalase (from *E.coli*, TREF) to hydrolyze the trehalose into glucose, which was measured in a fluorescence assay using glucose oxidase (GOD) and Amplex Red (Invitrogen). For each sample a calibration curve was done by adding to different sample aliquots a known concentration of trehalose. In a black 96-well microplate, 5ul of plant extract were incubated with 35µl of assay mix (4µl of buffer mix [phosphate buffer 500mM pH7,5, 10mM $MgCl_2$, 100mM NaCl, keep at 4^{o}C], 0,2µl catalase/GOD mix [0,5µl catalase 30 KU/ml in 20µl GOD 20 KU/ml] and 30,6µl H_2O. Blank and standards had 0,2µl TREF [1mg/ml] as well), at 30^{o}C for 60 min shaking. The plate was heated at 80^{o}C for 15 min, cooled and spun down. On ice, 5µl of H_2O were added to the samples and blank, and 5µl of trehalose standards (2,5 to 40 pmol) were used for the samples of the calibration curve. 55µl of determination mix were added to each sample (6µl of buffer mix, 0,2µl GOD [10 KU/ml], 0,2µl TREF [1mg/ml], 0,2µl horse radish peroxidase (HRP), 0,25µl Amplex

Red). The plate was read in a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc.) in the fluorescence mode till the slope was stable .

Measurements of stomatal aperture

The first pair of fully expanded leaves of 3 weeks old plants, grown in soil 16h-8h day/night cycle, was detached and incubated in a stomata opening buffer (10mM MES-KOH pH6.15, 10mM KCl) for 2 hours under continuous light. 20µM of ABA (0µM in controls) was added to the buffer and leaves were incubated 2 hours more. Peels of the abaxial side of the leaf were taken by pasting the abaxial side of the leaf to a microscope slide with double-sided sticky tape, the mesophyll cells were removed by gently scraping the leaf with a blade and the abaxial epidermis could be visualized in a microscope (Olympus BX51). Pictures of stomata were taken and pore aperture was measured using ImageJ software.

6. SUPLEMENTARY INFORMATION

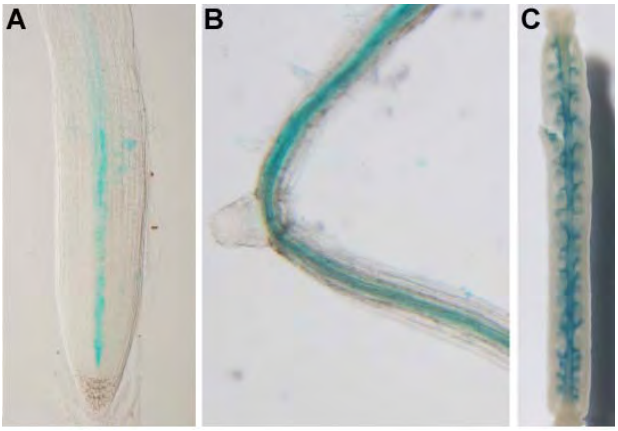


Figure S1. *TRE1* expression in roots and siliques. A-B. 12 DAG *pTRE1::GUS-GFP* plants grown on vertical plates. **A.** RAM where *TRE1* is expressed in the central bundle. **B.** Expression in the central stele in maturation zone of roots. **C.** Siliques of five weeks old *pTRE1::GUS-GFP* plants grown on soil. *TRE1* is expressed particularly in the chalaza of developing seeds.

Table S2. Specific primer sets for genotyping of T-DNA insertion lines and for qPCR analysis.

Primer	Sequence (5'→3')
Cloning	
pTRE1-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGTTTACATTGAAAAAATATCA
pTRE1-atttB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTGCCAAATGTTTGAATTCTG
Genotyping	
tre1-1 LP2	ACTCATCTCCACGCACACAC
tre1-1 RP2	AATTCTCGAACCGGGAATGAT
TRE1-1 LP1	GCCTTTTTCAAACTCAAGTTGCAC
TRE1-1 RP1	GAGTTTATTATGTGTGTGCGTGGA
qPCR	
TRE1_fw	AGCGAGAGAGAAAGCGTTTC
TRE1_rev	CCTTCCATGTCTCAGATTCC
ABI3_fw	AAGCAAAGCGACGTGGGTAA

ABI3_rev	GTGTGTCTCAGCTTCTTTTTTTGG
ABI4_fw	CGGTGGGTTCGAGTCTATCAA
ABI4_rev	ACCCATAGAACATACCGGATCAA
ApL3_fw	TCAGCACCATGTCGATAGTAAAGC
ApL3_rev	CAGTTGGTTTCTCAGAGAAATGGA
EM1_fw	CACAGCCTCGAAGCTCAAGAG
EM1_rev	CGTCTGCCCTCCCTTGCT
RAB18_fw	CGGGACTGAAGGCTTTGGA
RAB18_rev	GGAGCGGTGAAGCATTCCT
SEX1_fw	AGAACTTTTGCGGATGATAATGTCTA
SEX1_rev	GATTCGTTGCCCGACATCTC
SPS4F_fw	CCCATGACCAACAAGCCATT
SPS4F_rev	CACTCAGCCCAGAGATGTTTGTTA
SUS1_fw	CCAAGGGTGCTTTTGTCCAA
SUS1_rev	CACAAGTCATAGCCTCCACAACA
ACT_fw	GGCTCCTCTTAACCCAAAGGC
ACT_rev	CACACCATCACCAGAATCCAGC
CKA2_fw	ACCACCATTAACGTGCGTCAAC
CKA2_rev	GATCTTGGCGAGAGAATCGGTATC

7. REFERENCES

Arenas-Huertero, F., Arroyo, A., Zhou, L., Sheen, J., and Leon, P. (2000). Analysis of Arabidopsis glucose insensitive mutants, gin5 and gin6, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. Genes Dev **14,** 2085-2096.

Avonce, N., Leyman, B., Mascorro-Gallardo, J.O., Van Dijck, P., Thevelein, J.M., and Iturriaga, G. (2004). The Arabidopsis Trehalose-6-P Synthase AtTPS1 Gene Is a Regulator of Glucose, Abscisic Acid, and Stress Signaling. Plant Physiol **136,** 3649-3659.

Baena-Gonzalez, E., Rolland, F., Thevelein, J.M., and Sheen, J. (2007). A central integrator of transcription networks in plant stress and energy signalling. Nature **448,** 938-942.

Basu, A., Bhattacharyya, S., Chaudhuri, P., Sengupta, S., and Ghosh, A.K. (2006). Extracellular trehalose utilization by Saccharomyces cerevisiae. Biochim Biophys Acta **1760,** 134-140.

Beeckman, T., and Engler, G. (1994). An easy technique for the clearing of histochemically stained plant tissue. Plant Molecular Biology Reporter **12,** 37-42.

Brodmann, A., Schuller, A., Ludwig-Muller, J., Aeschbacher, R.A., Wiemken, A., Boller, T., and Wingler, A. (2002). Induction of trehalase in Arabidopsis plants infected with the trehalose-producing pathogen Plasmodiophora brassicae. Mol Plant Microbe Interact **15,** 693-700.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16,** 735-743.

Delatte, T.L., Schluepmann, H., Smeekens, S.C., de Jong, G.J., and Somsen, G.W. (2011). Capillary electrophoresis-mass spectrometry analysis of trehalose-6-phosphate in Arabidopsis thaliana seedlings. Anal Bioanal Chem **400,** 1137-1144.

Finkelstein, R.R., Gampala, S.S., and Rock, C.D. (2002). Abscisic acid signaling in seeds and seedlings. Plant Cell **14 Suppl,** S15-45.

Finkelstein, R.R., Wang, M.L., Lynch, T.J., Rao, S., and Goodman, H.M. (1998). The Arabidopsis abscisic acid response locus ABI4 encodes an APETALA 2 domain protein. Plant Cell **10,** 1043-1054.

Frison, M., Parrou, J.L., Guillaumot, D., Masquelier, D., Francois, J., Chaumont, F., and Batoko, H. (2007). The Arabidopsis thaliana trehalase is a plasma membrane-bound enzyme with extracellular activity. FEBS Letters **581,** 4010-4016.

Garg, A.K., Kim, J.K., Owens, T.G., Ranwala, A.P., Choi, Y.D., Kochian, L.V., and Wu, R.J. (2002). Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci U S A **99,** 15898-15903.

Geigenberger, P., Lerchi, J., Stitt, M., and Sonnewald, U. (1996). Phloem specific expression of pyrophosphatase inhibits long distance transport of carbohydrates and amino acids in tobacco plants. Plant Cell Environ **19,** 43-55.

Giraud, E., Van Aken, O., Ho, L.H., and Whelan, J. (2009). The transcription factor ABI4 is a regulator of mitochondrial retrograde expression of ALTERNATIVE OXIDASE1a. Plant Physiol **150,** 1286-1296.

Gómez, L.D., Baud, S., Gilday, A., Li, Y., and Graham, I.A. (2006). Delayed embryo development in the ARABIDOPSIS TREHALOSE-6-PHOSPHATE SYNTHASE 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. Plant J **46,** 69-84.

Gómez, L.D., Gilday, A., Feil, R., Lunn, J.E., and Graham, I.A. (2010). AtTPS1-mediated trehalose 6-phosphate synthesis is essential for embryogenic and vegetative growth and responsiveness to ABA in germinating seeds and stomatal guard cells. Plant J **64,** 1-13.

Harrison, S.J., Mott, E.K., Parsley, K., Aspinall, S., Gray, J.C., and Cottage, A. (2006). A rapid and robust method of identifying transformed Arabidopsis thaliana seedlings following floral dip transformation. Plant Methods **2,** 19.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol **8,** R19.

Hendriks, J.H., Kolbe, A., Gibon, Y., Stitt, M., and Geigenberger, P. (2003). ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of Arabidopsis and other plant species. Plant Physiol **133,** 838-849.

Huang, J., Reggiori, F., and Klionsky, D.J. (2007). The transmembrane domain of acid trehalase mediates ubiquitin-independent multivesicular body pathway sorting. Mol Biol Cell **18,** 2511-2524.

Jang, I.C., Oh, S.J., Seo, J.S., Choi, W.B., Song, S.I., Kim, C.H., Kim, Y.S., Seo, H.S., Choi, Y.D., and Nahm, B.H. (2003). Expression of a bifunctional fusion of the Escherichia coli genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. Plant Physiol **131,** 516.

Karimi, M., Depicker, A., and Hilson, P. (2007). Recombinational cloning with plant gateway vectors. Plant Physiol **145,** 1144-1154.

Kim, Y.C., Park, S., Cho, J., and Chung, C. (1999). Promoter for guard cell-specific gene expression in plants, EPO, ed.

Lunn, J.E., Feil, R., Hendriks, J.H., Gibon, Y., Morcuende, R., Osuna, D., Scheible, W.R., Carillo, P., Hajirezaei, M.R., and Stitt, M. (2006). Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in Arabidopsis thaliana. Biochem J **397,** 139-148.

Miranda, J.A., Avonce, N., Suarez, R., Thevelein, J.M., Van Dijck, P., and Iturriaga, G. (2007). A bifunctional TPS-TPP enzyme from yeast confers tolerance to multiple and extreme abiotic-stress conditions in transgenic Arabidopsis. Planta **226,** 1411-1421.

Müller, J., Boller, T., and Wiemken, A. (1995a). Effects of validamycin A, a potent trehalase inhibitor, and phytohormones on trehalose metabolism in roots and root nodules of soybean and cowpea. Planta **197,** 362-368.

Müller, J., Boller, T., and Wiemken, A. (1995b). Trehalose and trehalase in plants: recent developments. Plant Science **112,** 1-9.

Müller, J., Aeschbacher, R.A., Wingler, A., Boller, T., and Wiemken, A. (2001). Trehalose and Trehalase in Arabidopsis. Plant Physiol **125,** 1086-1093.

Nwaka, S., Mechler, B., and Holzer, H. (1996). Deletion of the ATH1 gene in Saccharomyces cerevisiae prevents growth on trehalose. FEBS Letters **386,** 235-238.

O'Connor, T.R., Dyreson, C., and Wyrick, J.J. (2005). Athena: a resource for rapid visualization and systematic analysis of Arabidopsis promoter sequences. Bioinformatics **21,** 4411-4413.

Palaniswamy, S.K., James, S., Sun, H., Lamb, R.S., Davuluri, R.V., and Grotewold, E. (2006). AGRIS and AtRegNet. a platform to link cis-regulatory elements and transcription factors into regulatory networks. Plant Physiol **140,** 818-829.

Pramanik, M.H., and Imai, R. (2005). Functional identification of a trehalose 6-phosphate phosphatase gene that is involved in transient induction of trehalose biosynthesis during chilling stress in rice. Plant Mol Biol **58,** 751-762.

Ramon, M., Rolland, F., Thevelein, J.M., Van Dijck, P., and Leyman, B. (2007). ABI4 mediates the effects of exogenous trehalose on Arabidopsis growth and starch breakdown. Plant Mol Biol **63,** 195-206.

San Miguel, P.F., and Arguelles, J.C. (1994). Differential changes in the activity of cytosolic and vacuolar trehalases along the growth cycle of Saccharomyces cerevisiae. Biochim Biophys Acta **1200,** 155-160.

Schluepmann, H., Pellny, T., van Dijken, A., Smeekens, S., and Paul, M. (2003). Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. Proc Natl Acad Sci U S A **100,** 6849-6854.

Schluepmann, H., van Dijken, A., Aghdasi, M., Wobbes, B., Paul, M., and Smeekens, S. (2004). Trehalose Mediated Growth Inhibition of Arabidopsis Seedlings Is Due to Trehalose-6-Phosphate Accumulation. Plant Physiol **135,** 879-890.

Shkolnik-Inbar, D., and Bar-Zvi, D. (2010). ABI4 mediates abscisic acid and cytokinin inhibition of lateral root formation by reducing polar auxin transport in Arabidopsis. Plant Cell **22,** 3560-3573.

Silva, M.C., Terra, W.R., and Ferreira, C. (2010). The catalytic and other residues essential for the activity of the midgut trehalase from Spodoptera frugiperda. Insect Biochem Mol Biol **40,** 733-741.

Thimm, O., Blasing, O., Gibon, Y., Nagel, A., Meyer, S., Kruger, P., Selbig, J., Muller, L.A., Rhee, S.Y., and Stitt, M. (2004). MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J **37,** 914-939.

van Dijken, A.J., Schluepmann, H., and Smeekens, S.C. (2004). Arabidopsis trehalose-6-phosphate synthase 1 is essential for normal vegetative growth and transition to flowering. Plant Physiol **135,** 969-977.

Vandesteene, L. (2009). Functional analysis of trehalose metabolism gene family in *Arabidopsis thaliana* (PhD thesis-Katholieke Universiteit Leuven).

Wingler, A., Fritzius, T., Wiemken, A., Boller, T., and Aeschbacher, R.A. (2000). Trehalose Induces the ADP-Glucose Pyrophosphorylase Gene, ApL3, and Starch Synthesis in Arabidopsis. Plant Physiol **124,** 105-114.

Yeo, E.T., Kwon, H.B., Han, S.E., Lee, J.T., Ryu, J.C., and Byu, M.O. (2000). Genetic engineering of drought resistant potato plants by introduction of the trehalose-6-phosphate synthase (TPS1) gene from Saccharomyces cerevisiae. Mol Cells **10,** 263.

Yu, T.S., Kofler, H., Hausler, R.E., Hille, D., Flugge, U.I., Zeeman, S.C., Smith, A.M., Kossmann, J., Lloyd, J., Ritte, G., Steup, M., Lue, W.L., Chen, J., and Weber, A. (2001). The Arabidopsis sex1 mutant is defective in the R1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. Plant Cell **13,** 1907-1918.

Zhang, Y., Primavesi, L.F., Jhurreea, D., Andralojc, P.J., Mitchell, R.A.C., Powers, S.J., Schluepmann, H., Delatte, T., Wingler, A., and Paul, M.J. (2009). Inhibition of SNF1-Related Protein Kinase1 Activity and Regulation of Metabolic Pathways by Trehalose-6-Phosphate. Plant Physiol **149,** 1860-1871.

Chapter

VI

Concluding remarks and perspectives

Concluding remarks and perspectives

Trehalose metabolism is widespread in living organisms, for some of them, it is the main energy source to sustain locomotion (insects), or in others, it is the only way they can stand dehydration and recover after water is available again (plant resurrection species). But in higher plants, the fundamental biological value of this pathway is waiting to be discovered. Trehalose-6-phosphate (Tre6P) levels are a sensor of sucrose availability in the cell, and activate starch synthesis (Lunn et al., 2006). On the other hand, Tre6P inhibits the activity of the master regulator of starvation responses, SnRK1 (Zhang et al., 2009), that promotes catabolism. Consequently, Tre6P is placed strategically in the signaling and metabolic networks responsible for sensing sugars and connecting their levels with carbohydrate metabolism and starvation responses, which ultimately will determine the energy homeostasis in the cell. However, most of the efforts have been made in deciphering the physiological context of trehalose metabolism, but not in a developmental manner. One intriguing question is why *Arabidopsis* needs such a large group of putative enzymes for trehalose metabolism? Out of the 21 proteins, only one is an active synthase, ten are active phosphatases and the rest do not display any activity (Ramon et al., 2009; Vandesteene et al., 2010). Additionally, while TPS1 is constitutively expressed in the plant, TPPs appear to be more specific in certain cell types during particular developmental processes (Chapter II). Thus, TPS1 synthesizes Tre6P, and the active TPP in each cell type will regulate Tre6P levels depending on the developmental context of cells and tissues. This suggests that each TPP must respond to endogenous and exogenous cues that control development and growth. This was exposed by coexpression analysis of each TPP where each isoform was placed in different network and functional contexts (Li et al., 2008). For particular TPPs, only two reports are available, one linking *TPPB* to nitrate responses (Wang et al., 2003) and, a second showing that *TPPI* is directly regulated by HY5 in light (Lee et al., 2007). Together, these data suggest that the functional role of each TPP has to be analyzed *in vivo* to understand specifically how the trehalose pathway is regulated.

Mutant analysis of the TPP family revealed an assortment of morphological and developmental phenotypes, which were correlated with the expression domains of each particular TPP. However, some overlapping domains exits, pointing at redundant functions for some members of the TPP family. The antagonistic roles of *TPPB* and *TPPI* in lateral root development can not be expected from their general function as phosphatases. In *TPPB-KO* and *TPPI-KO* mutants, there should be an excess of Tre6P in the dividing pericycle cells forming a lateral root, but this excess is not translated in the same phenotype in both mutants (Chapter III and IV). These two genes are direct targets

of auxin signaling, being positively (*TPPI*) and negatively (*TPPB*) regulated by auxin. This regulation leads to *TPPI* favoring lateral root formation whereas *TPPB* is inhibiting this process. Importantly, *TPPB* is regulated by the well studied auxin signaling module, SLR1/ARF7ARF19 that positively regulates early auxin responsive genes. This means that *TPPB* is target of positive ARFs and negative ones. It is possible that different ARFs are competing in the regulation of *TPPB* and depending on the developmental window they will affect *TPPB* downstream responses. Furthermore, additional stimuli are involved in the regulation of *TPPB* and *TPPI* genes. Light upregulates *TPPI* through the HY5 transcriptional regulator, while cytokinin has a negative effect in the regulation of *TPPB*. Interestingly, in a treatment with both hormones, there is a strong negative synergistic effect (data not shown), suggesting an important hormonal crosstalk that defines the function of this gene.

Another important aspect of the studies done with the *TPPB* and *TPPI* mutants was the effect of their mutations in the shoot/root balance. The larger shoot and root phenotype of *TPPB-KO* plants was the opposite to the one observed in *TPPI-KO* plants. It could be that a larger root system had a direct effect in the shoot size by allocating more water and nutrients in the leaves, which in turn will activate processes that would lead to a bigger shoot. On the other hand, an insufficient root can not deliver enough nutrients as expected to keep the shoot/root ratio. Intriguingly, this is not a general rule as in mutants without lateral roots (*arf7arf19*) the shoot looks as wild type. This suggests that this is a particular phenotype for trehalose metabolism mutants, however this suggestions need further work in order to be proven. For instance, grafting experiments by which the shoot or the root of the mutant can be coupled to the one of the wild-type, to see if the causal is the root or the shoot. Another option is to follow in time different growth traits of the shoot and root to find where and what is the primary cause to keep a balance in the shoot/root ratio.

Another phenotype of the *TPPI-KO* lines was the extra branched trichomes. Trichomes are specialized epidermal cells in leaves that have gone through sequential cycles of endoreduplication. Additional branches in trichomes have been associated with extra endoreduplication cycles. Interestingly, *TPPI-KO* roots display a shorter meristem size due to less mitotic cycles, leading to an earlier differentiation. It is possible that this is also happening in the trichomes of this mutant. Several hormones have been reported to regulate RAM size, it will be interesting to test the effect of these hormones in the *TPPI-KO* mutant.

The general idea that overexpression of TPPs will deliver larger plants with higher yields (Schluepmann et al., 2003), was reevaluated with this study since, individual lines overexpressing each TPP showed plants that were similar or even smaller to wild-type, displaying aberrant flowers as well (see summary). Controversially, different experiments changing the energy conditions in which TPP KO and OE plants were grown, by varying

the sugar content in the media under diverse light regimes, did not yield a sugar sensitive phenotype in the OE plants, as the one observed with *otsB* overexpresing plants (Schluepmann et al., 2003).

In this study additional proof of the interaction of trehalose metabolism with ABA responses was found. This observation was made in mutants of two different genes expressed in stomata, *TPPG* and *TRE1* (Chapter II and V). Here, we have two different regulators of the same process but with different functions in trehalose metabolism and degradation. The hypothesis is that high Tre6P level correlates with insensitivity to ABA stomatal responses. The molecular mechanism of this regulation is unknown, but further analysis including ABA central regulators will shed light into the dissection of the regulatory network which integrates trehalose and ABA signaling.

Although this study was focused on trehalose metabolism effects in lateral root development, additional developmental programs were disturbed by other members of the TPP family. *TPPA* and *TPPG* are expressed in the epidermal cells of the root, preferentially in the atrichoblast cells. A double knock out of *TPPA* and *TPPG* revealed defects in root hair cell specification since, continuous root hair files were formed (see summary). This suggests that these genes are potentially interacting with the regulators of the epidermal cell fate in roots. The analysis of the expression of *TPPA* and *TPPG* in the different mutants of the known genes mediating cell fate specification of epidermal cells, will help situating each gene within the cascade that controls this developmental process, work that is currently ongoing.

This study describes the developmental relevance of trehalose metabolism in Arabidopsis and, provides a functional context for some TPPs that is worth of further investigation.

REFERENCES

Lee, J., He, K., Stolc, V., Lee, H., Figueroa, P., Gao, Y., Tongprasit, W., Zhao, H., Lee, I., and Deng, X.W. (2007). Analysis of Transcription Factor HY5 Genomic Binding Sites Revealed Its Hierarchical Role in Light Regulation of Development. Plant Cell **19,** 731-749.

Li, P., Ma, S., and Bohnert, H.J. (2008). Coexpression characteristics of trehalose-6-phosphate phosphatase subfamily genes reveal different functions in a network context. Physiologia Plantarum **133,** 544-556.

Lunn, J.E., Feil, R., Hendriks, J.H., Gibon, Y., Morcuende, R., Osuna, D., Scheible, W.R., Carillo, P., Hajirezaei, M.R., and Stitt, M. (2006). Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in Arabidopsis thaliana. Biochem J **397,** 139-148.

Ramon, M., De Smet, I., Vandesteene, L., Naudts, M., Leyman, B., Van Dijck, P., Rolland, F., Beeckman, T., and Thevelein, J.M. (2009). Extensive expression regulation and lack of heterologous enzymatic activity of the Class II trehalose metabolism proteins from Arabidopsis thaliana. Plant, Cell & Environment **32,** 1015-1032.

Schluepmann, H., Pellny, T., van Dijken, A., Smeekens, S., and Paul, M. (2003). Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA **100,** 6849-6854.

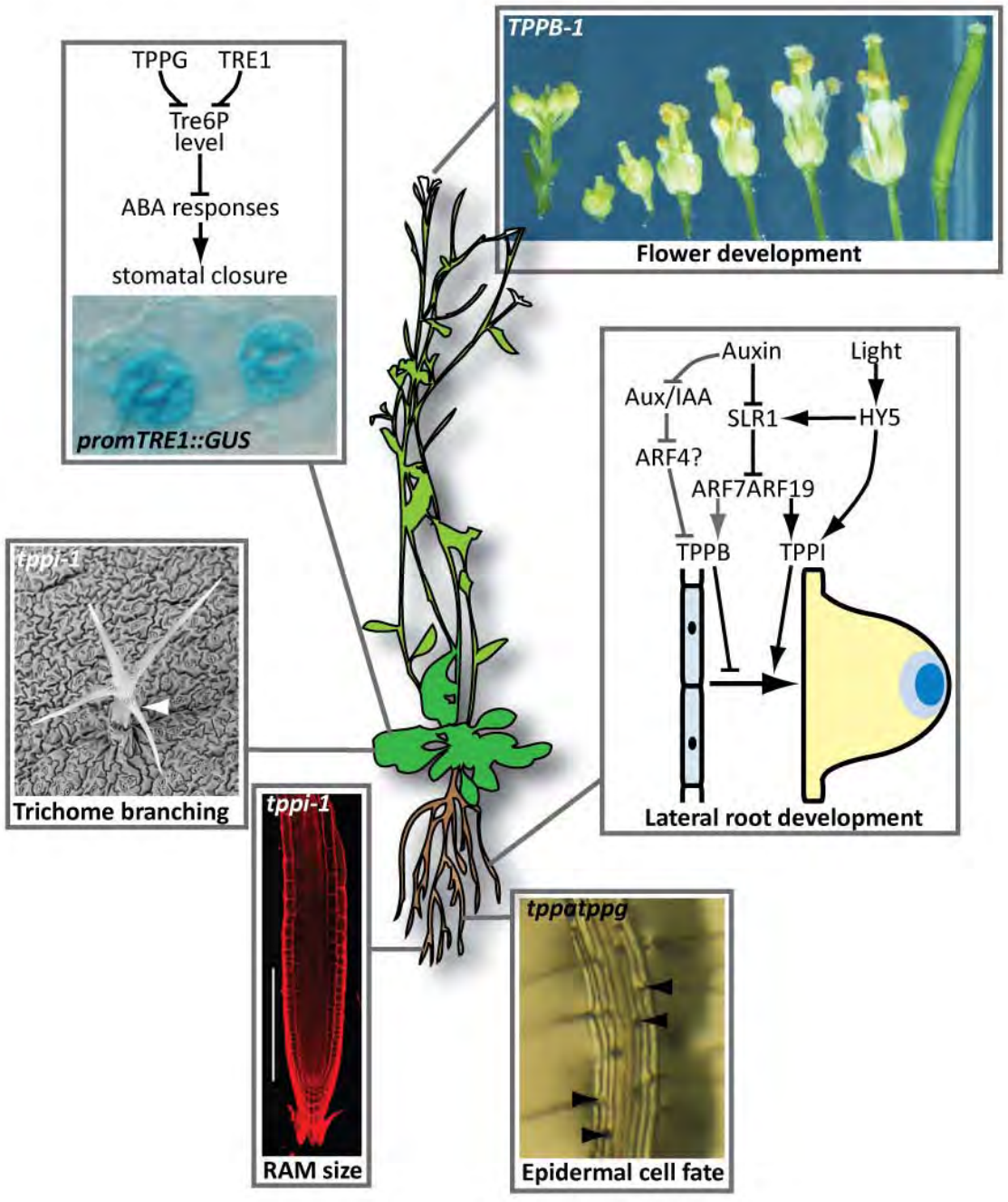
Vandesteene, L., Ramon, M., Le Roy, K., Van Dijck, P., and Rolland, F. (2010). A Single Active Trehalose-6-P Synthase (TPS) and a Family of Putative Regulatory TPS-Like Proteins in Arabidopsis. Molecular Plant **3,** 406-419.

Wang, R., Okamoto, M., Xing, X., and Crawford, N.M. (2003). Microarray Analysis of the Nitrate Response in Arabidopsis Roots and Shoots Reveals over 1,000 Rapidly Responding Genes and New Linkages to Glucose, Trehalose-6-Phosphate, Iron, and Sulfate Metabolism. Plant Physiol **132,** 556-567.

Zhang, Y., Primavesi, L.F., Jhurreea, D., Andralojc, P.J., Mitchell, R.A.C., Powers, S.J., Schluepmann, H., Delatte, T., Wingler, A., and Paul, M.J. (2009). Inhibition of SNF1-Related Protein Kinase1 Activity and Regulation of Metabolic Pathways by Trehalose-6-Phosphate. Plant Physiol **149,** 1860-1871.

Summary - Samenvatting

Summary



Summary of the different developmental processes in which TPPs are involved. Lateral root development, RAM size, ABA stomatal responses, root epidermal cell fate, trichome branching and flower development are processes in which TPP genes were found to have a role. The grey arrows in the inset represent probable links that need further study to be resolved.

Trehalose metabolism was thought to be not relevant in higher plants since they lost their ability to accumulate trehalose. Studies revealed the relevant role of this pathway in plant

carbohydrate metabolism and stress responses, as contrasting morphological and developmental phenotypes of microbial trehalose-6-phosphate synthases (TPS) and trehalose-6-phosphate phosphatases (TPP) overexpressing plants were found. Intriguingly, contrary to other phyla, *Arabidopsis* harbors a large family of 21 trehalose metabolic enzymes. There is a single active TPS and ten active TPPs (Chapter II). TPS1 was described to be involved in embryo development, sugar signaling, ABA and stress responses. However the function of each TPP is making its way to be discovered.

This study was focused on the role of the TPPs in lateral root development. The generation of GUS/GFP marker lines for each TPP revealed distinct and specific domains of expression of the TPP family (Chapter II). Four members of the family were expressed during early events of lateral root initiation, and two of them were investigated further. *TPPB* was found to repress lateral root formation (Chapter III). This gene is transcriptionally regulated by auxin, showing a rapid downregulation upon auxin treatment. This auxin effect on *TPPB* was not observed in *slr-1* and *arf7arf19* mutants, suggesting that *TPPB* depends on this canonical auxin signaling module for lateral root development. The repression of *TPPB* expression by auxin must be through the activation of a repressor *ARF*.

TPPI was also involved in lateral root development but in an opposite manner than TPPB (Chapter IV). *TPPI* acts as a positive regulator of lateral root initiation and is upregulated by auxin. TPPI is also involved in RAM size maintenance and its absence induce early differentiation of the cells in the RAM.

This study also explored the interaction of trehalose synthesis and degradation with ABA responses. *TPPG* showed to be involved in ABA mediated responses in stomatal closure and inhibition of germination (Chapter II). *TRE1* regulates the levels of Tre6P affecting ABA mediated stomatal closure (Chapter V). This gene is also important for sugar sensitivity in the plant.

In summary, several developmental programs are affected by TPP genes. Here its shown that TPP enzymes are particularly expressed in certain domains and developmental windows. TPP proteins are tightly controlled by endogenous and exogenous signals to regulate Tre6P levels in the cells and, in turn affect carbohydrate metabolism and stress responses.

Samenvatting

Aangezien hogere planten niet beschikken over de mogelijkheid om trehalose te accumuleren, een kenmerk dat wel aanwezig is in primitievere organismen, werd trehalose metabolisme niet als relevant beschouwd voor deze taxa. De identificatie van contrasterende morfologische en ontwikkelingsfenotypes gekoppeld aan overexpressie van microbiële trehalose-6-fosfaat synthase (TPS) en trehalose-6-fosfaat fosfatase (TPP) wees echter op een mogelijke betekenis van deze moleculaire processen voor het koolstofmetabolisme en stressreacties van planten. In tegenstelling tot andere taxa, vertoont Arabidopsis een grote familie met 21 trehalose metabolische enzymen waarvan de activiteit voor één TPS en voor tien TPP eiwitten werd aangetoond (zie Hoofdstuk II). Een functie in embryo ontwikkeling, suiker signalisatie, ABA en stress reacties werd toegeschreven aan TPS1. Onderzoek naar de functie van de verschillende TPP's is momenteel nog lopende.

Het hier voorgestelde project beoogde de functie van de TPP genen in zijwortel ontwikkeling te bestuderen. Via het maken van GUS/GFP merkerlijnen voor elk TPP gen konden verschillende en specifieke expressiedomeinen voor deze familie beschreven worden (Hoofdstuk II). Vier genen van deze familie komen tijdens de vroege stadia van zijwortelontwikkeling tot expressie, waarvan 2 genen verder in detail bestudeerd werden. Het TPPB gen vertoont een inhiberende rol voor zijwortelontwikkeling (Hoofdstuk III). Dit gen wordt transcriptioneel gecontroleerd door auxine waarbij na behandeling met auxine een snelle neerregulatie waargenomen werd. Dit effect blijkt echter niet op te treden in *slr-1* en *arf7arf19* mutanten wat wijst op een controle op *TPPB*-expressie via de Aux/IAA – ARF consensus module die eerder beschreven werd als essentieel voor zijwortelontwikkeling. Daarom zou de snelle inhibitie van *TPPB*-expressie dan ook het gevolg kunnen zijn van het vrijstellen van een inhiberende ARF factor.

TPPI blijkt eveneens een rol tijdens zijwortelontwikkeling te spelen maar dan wel in tegengestelde zin in vergelijking met TPPB (Hoofdstuk IV). *TPPI* werkt als een positieve factor voor zijwortelinitiatie en wordt geïnduceerd door auxine. TPPI is eveneens betrokken bij de controle op de omvang van het apicale wortelmeristeem, waarbij verlies aan TPPI leidt tot een vroegtijdig differentiëren van de cellen in het meristeem.

In deze studie werd eveneens de interactie tussen trehalose-afbraak/-synthese en ABA (abscisinezuur)-signaaloverdracht onderzocht. *TPPG* bleek namelijk betrokken te zijn tijdens het door ABA-gecontroleerde sluiten van de sluitcellen van de huidmondjes en bij de inhibitie van zaadkieming (Hoofdstuk II). Hierbij blijkt *TRE1* het niveau van Tre6P te controleren wat op zijn beurt het ABA-afhankelijk sluiten van huidmondjes beïnvloedt (Hoofdstuk V). Dit gen is tevens belangrijk voor de suiker-gevoeligheid van de plant.

Samenvattend kan gesteld worden dat diverse ontwikkelingsprogramma's in de plant door TPP genen beïnvloed worden. In deze studie wordt aangetoond dat TPP enzymen heel specifiek in bepaalde weefsels en tijdens bepaalde fasen in de ontwikkeling tot expressie komen. De TPP eiwitten worden strikt gecontroleerd door zowel endogene als exogene signalen om de trehalose-6-fosfaat niveau's van de cellen te regelen en waardoor vervolgens het koolwaterstof metabolisme en stress-reacties kunnen beïnvloed worden.

Acknowledgements

Acknowledgements

This was a surprising road for me, four years of an overwhelming learning experience, scientifically and personally. I am grateful that my steps took my to this wonderful country and that I had the opportunity to meet extraordinary people here. I have to say thanks to so many people starting from the ones who opened the door for me at VIB, Mark Veugelers and Marijke Lein, thank you for your lovely spirit and your trust in me. To my promoters Patrick Van Dijck, thank you for all your support and, Tom Beeckman, you are a wonderful teacher and a positive soul, printing your passion for science even in moments when the horizon does not look so promising. To my jury members, Johan Thevelein, Filip Rolland, Alain Goossens, John Lunn and Nelson Avonce, thank you for your feedback on my thesis work. Filip I am really glad that I meet such a nice person like you, thank you for the scientific discussions, for the life stories. Nelson it was great to share my PhD road in my own language with a person like you, thanks a lot for the help, support, and for being my friend. John thanks for guiding me in the world of metabolites and sugars, and for letting Regina and Marina to help me during my stay in your lab. To Johan and Françoise, thanks for all your efforts to maintain the lab in a privilege position.

Thanks to the girls, former and recent, of the Plant Lab-KUL, Lies, Katrien, Hilde, Ines, Suzana and Bety. Lies you introduce me to the pipettes and enzymes being my teacher when I started my PhD, thank you for sharing your knowledge and the ups and downs of the crazy trehalose world with me. To the eager to learn Hilde, I know that trehalase has a lot of surprises waiting to be discovered. To Ines, your mosses will rule the world. To Suzana, if you get a drought resistant banana we will sow it in Colombia. To Bety, thank you for your sweetness and helpful hands, for the conversations and your big smile. Also to Matthew, although we did not share to much time in the lab, you taught me that “trehalose is hot”. A mis queridas secretarias, Hilde and Leni, thanks a lot for your help solving my thousand questions, for the translations and dealing with my papers. To Nico and Jan, the lab does not work without you guys. To the technicians, Evy, Tom, Catherina, Martine, Deborah, Paul, Ilse and Willy for the unconditional help and, to Evy for the parties as well. To the PhD students in the lab, Thiago, Harish, Giovanni, Ben, Frederik, Tom DA, Mekonnen, Judi, Georg, Marlies and Jurgen keep on the hard work. To the ones that are finishing their PhD around this time, Sarah, Steve, Steven, Sona, Bram, Katrijn and Wim, we are done!. Thanks also to Marta, Beatriz, Maria and Alessandro for the nice conversations, the parties and a lot of other things that I will keep in my heart.

I am indebt to so many people in the PSB-UGent… Thanks to Dirk, all the group leaders, technicians and administrative support, for making PSB a unique and perfect place to do plant sciences. Thanks to the rooties, Gert, Maria, Giel, Boris, Leen, Dominique, Wei, Long, Marlies, Ianto, Kun, Inge, Philippe and, to the ex-rooties, Miranda, Ive, Stefan and Wim, for the nice atmosphere, discussion, conversations and all the help. Gert thanks for your designer skills, I had not made nice figures for my thesis without your help. Giel, where is the next morning talk? In Australia?. Wei, I know you will be a great daddy back home after finishing an excellent PhD here. Maria, thanks for the nice conversations and sharing so many things with me. Boris, I miss the opportunity to share more time with you to learn French and a lot of more things. Leen, the PhD road is coming to an end, I wish you the best and all the success finishing this point in your life. To Dominique and Long keep up with the chemicals. Marlies and Ianto, roots are amazing the exploration just started. Also to Bert because besides being a great teacher for me at PSB, you never complain for the noise from my talkative Colombian and friends. Also to Valya, who is like a mother to all of us. To all the brassinos, and to Camilla, Simone and the noisy Niloufer, for the good spirit and the excellent coffee. To Nathalie for all the help working with leaves, and also for the parties, barbeques, etc. To Megan for the nice conversations and your big smile anytime anywhere. A Ana, ya sabes que tu espíritu de trabajo te va a llevar lejos, solo te quedan unos pasos y serás una excelente Doctora. A Miguelito, que la moto te llevará lejos pero el PhD aún mas =P. Jayson creo que modelar es mejor en la pasarela que en las matemáticas =D.

To my friends, the ones I share lunch and a lot of nice evenings, parties and trips. Jefri, Barbara, Wilson, Nubia, Dorita, Sinta, thanks a lot for the laughs, the jokes, the discussions, the gossips and for making us feel home. Jefri you are the last of the group to finish with this mission, it will be great to see you as Doctor. Barbara long life to beers, parties, enjoy the life, that is the priority. Nubia gracias por el tiempo que has compartido conmigo y por el que compartiremos haciendo ejercicio =P. Dorita is it time to change the enter key?. A Tio Vito gracias mil por todo, por tu ayuda incondicional, por el tiempo que nos has dedicado, por abrirnos las puertas de tu casa como si fueramos tu familia, seguro no hubiéramos logrado esto sin ti al lado de nosotros =). Mis queridos amigos algún día nos veremos en Colombia!

To former PSB people, Juan Carlos gracias por la buena vibra. Gustavo gracias por enseñarme tu amor por los estomas. Mira I will never forget the amazing person you are. Vanessa de seguro dentro de poco resultas siendo mi jefa. Dianita gracias por tu amistad, ya nos veremos de nuevo al son de un café. Mandy, my official proof reader, thanks for your time reading and correcting my thesis. Tarik thanks for teaching me your cloning tips. Amandincita keep your sweetness with more and more chocolate and metal songs. Joanna, sigue así con tu español, seguro dentro de poco te veré viviendo feliz en Suramérica. Clairezinha gracias por estar pendiente de mí, por los muchos mensajes y por tener el animo arriba incluso cuando la vida es dura con nosotros.

To all the PhD students in PSB, Ricardo, Luckazs, Anas, Agnieszka, Peter M., Petra N., Maria, Petra Z., Oana, Joanna, Corina, Marta, Tomasz, Peter G., Elke, Michail, Pooneh, Sara, Azra, Sarieh, Dalong, Lieven, Sintayehu, Tessa, Michel, Amparo, Astrid N., Jacob, Astrid G., Vikram, Robin, Liana, Cezary, Inge DC, Annelies, Lorin, Nathan, Elisabeth S., Stijn, Liesbeth, Rahul, Elisabeth W., this is a crazy road, all the ups and downs sooner or later will be compensated =).

Thanks also, to people from the dark side of the building. Mara it was great meeting you and sharing so many nice moments. Gert, Alexander, Bram, Trui, thank you for helping my significant other in this tough road. Itati, gracias por tu compañia y por las lindas conversaciones. Aleksandra, larga vida al metal y a los videojuegos.

A mi familia por todo el apoyo, las mil oraciones, el amor y el sacrificio. Mom gracias por tu herencia como mujer y como persona, por el amor que le has dado a nuestra familia, por tus inmensos sacrificios por vernos felices. Gracias Dad por tu espíritu de echa'o pa'lante, porque de ti tengo el espíritu trabajador y luchador, el que busca la felicidad por encima de todo el resto de cosas. A mis hermanitos a los que adoro, que son mi orgullo y los dueños de mi corazón. Mambrú, gracias por estar allí, por cuidar de todos, por ser mi espía en casa y por ser mi amiga. Tianito, gracias por quererme tanto a pesar de que he estado lejos de ti por tanto tiempo, tu camino apenas empieza pero se que llegarás muy lejos. A mi abue Gayota por ser mi mamá también, gracias por tenernos presente todas las noches en tus oraciones, por ser fuerte y seguir con tu alma guerrera luchando por la vida. A mi abue Quequeta, que se que pidió mucho a Dios por nosotros y que se que nos cuida desde el cielo. A Rubelita, gracias por cuidar de mi familia, por ser otra mamá y querernos tanto. A Aurilla y a mi Tio Jorge, gracias por la buena energía y por estar pendientes de mi familia. A toda mi familia y amigos, Walter, Alejandro, Heinz, Deivy, Andres, Leonardo, Andreita, Paquita, Guaji, Carlitos, Christiam, José, Marce, Valerio, Joisse, Felipe y Milena, Leito, María Augusta e Iván, la doctora Mary y Keoma, que me tuvieron en su corazón y siempre desearon lo mejor para mí, mil y mil gracias. A JOP, mi ángelito de la guarda, gracias por cuidarme desde el cielo.

A los papás de Mauricio, Señora Mary y Don Alberto, gracias por el apoyo incondicional, por tenerme tan presente en sus oraciones y desear con todo su corazón lo mejor para mí, gracias por abrirme la puerta de su hogar y hacerme sentir en casa. A Maito, mi medio limón, mi profesor, mi cantante, mi payaso, mi luz, gracias por aguantarme tanto, por darme tanto amor y hacerme ver lo importante de la vida. También por tu amor al trabajo y a hacer las cosas bien, me siento muy orgullosa de ti, de lo que has logrado como científico y de todo lo que verás hacerse realidad con tu empeño y dedicación. Gracias por caminar a mi lado y no soltar mi mano.

Gracias mil,
Thank you.

Curriculum vitae

Curriculum vitae
Yenni Lorena López Galvis

Personal Information

Address	Koning Leopold II laan 159, 9000-Gent, Belgium
Tel	+32 0498567364
Birth Date	August 2, 1982. Bogotá (Cundinamarca), Colombia
Nationality	Colombian
Civil Status	Single
Languages	Spanish (Native), English (Fluent), French (Basic)
Current work	PhD student. VIB, Laboratory of Molecular Cell Biology-KULeuven Kasteelpark Arenberg 31 bus 2438 B-3001 Leuven-Heverlee, and Root Development Lab –PSB-UGent Technologiepark 927 B-9052 Gent, Belgium.
Tel:	+32 (0) 498567364
e-mail	lorena.lopez@mmbio.vib-kuleuven.be lorena.lopez@psb.vib-ugent.be lorena.lopezgalvis@gmail.com

Education

Bachelor and Master	Agronomist Engineer Universidad Nacional de Colombia, Bogotá D.C., Colombia. February 1999 - April 2005. *Thesis title:* Drought tolerance evaluation in common bean (*Phaseolus vulgaris* L.) under greenhouse conditions.
Postgraduate	PhD in Sciences Biology VIB, Laboratory of Molecular Cell Biology-KULeuven and VIB, Root Development Lab-PSB-UGent, Belgium. January 2007 - June 2011. *Thesis title:* Characterization of a family of *Arabidopsis* trehalose biosynthesis genes and their role in root development and stress tolerance.

Workshops and Trainings

Vector NTI Workshop. BITS-VIB, Belgium. February 2009.
Genevestigator Workshop. BITS-VIB, Belgium. March 2009.
Effective writing in life sciences. VIB, Belgium. June 2009.
Tech transfer course. VIB, Belgium. August 2009.
Metabolomics summer school. KULeuven, Belgium. September 2009.

Imaging course. VRTC, Belgium, December 2009.
Microarrays Workshop. VRTC, Belgium, Febreary 2010.
Science Ethics. VRTC, Belgium, February 2010.
Microscopy training. DMBR-VIB, UGent, February 2010.
Introduction to Bioinformatics. BITS-VIB, Belgium. February 2010.
Protein Structure Analysis. BITS-VIB, Belgium. November 2010.

Work and Research experience

Teacher Assistant	Universidad Nacional de Colombia Agronomy Department Irrigation and Drainage Lab practices. Bogotá D.C., Colombia August 2001 – June 2002
Crop Field Engineer internship	GRAJALES S.A. La Unión, Valle del Cauca, Colombia January 2003 – July 2003 *Internship title*: Effect of bioorganic fertilizers on post-harvesting traits of papaya (*Carica papaya*), grape (*Vitis vinifera*), melon (*Cucumis melo*) and soursop (*Annona muricata*) fruit.
Thesis Student	International Center for Tropical Agriculture (CIAT) Cali, Colombia December 2003 – January 2005 *Thesis title:* Drought tolerance evaluation on common bean (*Phaseolus vulgaris* L.) under greenhouse conditions.
Research Assistant	International Center for Tropical Agriculture (CIAT) Cali, Colombia April 2005 – November 2006 *Project:* Evaluation of drought tolerance in common bean and rice in greenhouse and field conditions.
Researcher	VIB Laboratory of Molecular Cell Biology-KULeuven and Root Development Lab-PSB-UGent. Leuven and Gent, Belgium. January 2007 – July 2011
Visiting PhD student	Max Planck Institute of Molecular Plant Physiology. Gölm, Potsdam, Germany. August 16th – September 5 th 2010. Supervised by Dr. John Lunn.

Awarded grants

VIB International PhD program scholarship. Belgium, 2007-2011

Luis C. Jota award to best student, I-2002, Faculty of Agronomy, Universidad Nacional de Colombia, Bogotá, Colombia. September 2002.

Publications

López, L., J. Polanía, R. García, S. Beebe e I. Rao. 2006. *Genotypic differences in drought adaptation among advanced lines grown in transparent plastic cylinders in the greenhouse.* Bean Project: Annual Report 2005. International Center for Tropical Agriculture (CIAT), Cali, Colombia.

López, L., J. Polanía, R. García, S. Beebe e I. Rao. 2006. *Genotypic differences in drought adaptation among advanced lines grown in small pots in the greenhouse.* Bean Project: Annual Report 2005. International Center for Tropical Agriculture (CIAT), Cali, Colombia.

López, L. 2005. *Drought tolerance evaluation on common bean (Phaseolus vulgaris, L) under greenhouse conditions*. Undergraduate thesis. Universidad Nacional de Colombia & International Center for Tropical Agriculture (CIAT), Cali, Colombia.

López, L., J. Polanía, R. García, J. Ricaurte, S. Beebe e I. Rao. 2004. *Genotypic differences in root distribution and drought tolerance of RIL´s parents*. Bean Project: Annual Report 2003. International Center for Tropical Agriculture (CIAT), Cali, Colombia.

Meeting Abstracts

López, L., Vandesteene, L., Beeckman, T. and Van Dijck, P. "*Trehalose Phosphate Phosphatase function in lateral root development*". Abstract book XVIII Congress of the Federation of the European Societies of Plant Biology (FESPB 2010), Valencia, Spain. July 4^{th} - 9^{th} 2010.

Van Houtte, H., Vandesteene, **L., Lopez**, L., Avonce, N., Van Dijck, P. (2010). "*Trehalase and its role in plant stress and sugar signalling in Arabidopsis thaliana and Physcomitrella patens*". Abstract book Gordon Conference: Salt and water stress in plants. Les Diablerets, Switzerland, June 13^{th} -18^{th} 2010.

Vandesteene, L., **Lopez, L.**, Avonce, N., Van Houtte, H., Van Dijck, P. (2009). "*The trehalase enzyme is involved in the response to drought and ABA in Arabidopsis thaliana and Physcomitrella patens"*. Plant Hormones: New insights for Biotechnology. Gembloux, Belgium. November 13^{th} 2009.

López, L., Vandesteene, L., Beeckman, T y Van Dijck, P. 2009. "*Insights into the Trehalose Phosphate Phosphatase Family in Arabidopsis thaliana"*. Abstract book 20^{th} International

Conference on Arabidopsis Research (ICAR); Edinburgh, Scotland, UK, June 30th – July 3rd, 2009.

L. Vandesteene, L., **Lopez L**., Ramon M., Rolland F. y Van Dijck, P. "*Functional analysis of the Arabidopsis thaliana trehalose-6-phopshate phosphatase family"*. Abstract book XVI Congress of the Federation of European Societies of Plant Biology (FESPB 2008); Tampere, Finland, August 17 th – 22nd, 2008.

Galindo L. M., **Lopez L**., Rao I., Tohme J. e Ishitani M. 2006. *"Are DREB gene(s)associated with the deeper rooting phenotype of common bean under drought stress?".* Poster presented in the Sackler Colloquium: From Functional Genomics of Model Organisms to Crop Plants for Global Health; Washington, USA, April 3 th – 5th, 2006.

Manuscripts in preparation

Vandesteene, L.*, **López-Galvis, L.***, Vanneste, K., Maere, S., Lammens, T., Rolland, F., Avonce, N., Beeckman, T. and Van Dijck, P. "*Expansive evolution of the Trehalose-6-phosphate phosphatase gene family in Arabidopsis thaliana".* *shared first author. Under revision in Plant Physiology.

López-Galvis, L., Vandesteene, L., Van Dijck, P. and Beeckman, T. "*The trehalose biosynthesis gene, TPPB, acts as an inhibitor of lateral root initiation via auxin signaling"*. Manuscript in preparation.

López-Galvis, L., Vandesteene, L., Van Dijck, P. and Beeckman, T. "*TPPI, a trehalose-6-phosphate phosphatase, is involved in root apical meristem maintenance under light conditions"*. Manuscript in preparation.

Attended meetings

International Symposium Growth and Development of Roots. Louvain-La-Neuve. January 27th, 2011.

2nd VIB International PhD student Symposium, VIBes in Biosciences 2010. Leuven, Belgium. October 13th -15th, 2010.

XVIII Congress of the Federation of the European Societies of Plant Biology (FESPB 2010), Valencia, Spain. July 4th – 9th 2010. Poster presented: López, L., Vandesteene, L., Beeckman, T. and Van Dijck, P. "*Trehalose Phosphate Phosphatase function in lateral root development*".

20th International Conference on Arabidopsis Research (ICAR), Edinburgh, Scotland, UK. June 30th – July 4th 2009. Poster presented: López, L., Vandesteene, L., Beeckman, T and Van Dijck, P. "*Insights into the Trehalose Phosphate Phosphatase Family in Arabidopsis thaliana*".

1st VIB International PhD student Symposium, VIBes in Biosciences 2008. Gent, Belgium. September 13th – 15th, 2010.

IX Congreso de Fitomejoramiento y Producción de Cultivos; Palmira, Colombia, April 21st – 23th, 2005. Poster presented: López, L., J. Polanía, G. Ligarreto, S. Beebe and I. Rao. "*Greenhouse methodology to evaluate drought resistance on common bean (Phaseolus vulgaris, L) based on root distribution*".

Supervised students

Celine De Maesschalk, "*Unraveling the function of trehalose bisynthesis genes in lateral root development*". Thesis for the degree of Master of Biochemistry and Biotechnology 2009-2010. Department of Plant Biotechnology and Genetics, UGent.

Jolien Duchou, "*Role of trehalose biosynthesis genes in root development*". Thesis for the degree of 1st master of Biochemistry and Biotechnology 2009-2010. Department of Plant Biotechnology and Genetics, UGent.

Additional activities

Member of BIOGENIC : Biólogos Genetistas Colombianos. http://biogenic-colombia.blogspot.com/

Organizer of the 2nd VIB International PhD student Symposium, VIBes in Biosciences 2010. Leuven, Belgium. October 13th -15th, 2010.

Arenberg Doctoral School of Science, Engineering & Technology
Faculty of Sciences
Department of Biology
Laboratory of Molecular Cell Biology
Kasteepark Arenberg 31
B-3001 Leuven