Transcriptional networks of TCP transcription factors in Arabidopsis development

Selahattin Danisman

Thesis committee

Thesis supervisor

Prof. dr. ir. G.C. Angenent

Personal chair at the Laboratory of Molecular Biology

Wageningen University

Thesis co-supervisor

Dr. ir. R.G.H. Immink

Senior Researcher at Plant Research International

Wageningen

Other members

Prof. dr. B. Scheres, Utrecht University

Prof. dr. ir. H.J. Bouwmeester, Wageningen University

Dr. L. De Veylder, Ghent University, Belgium

Dr. H.J.M. Linthorst, Leiden University

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Selahattin Danisman

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Selahattin Danisman

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Table of Contents

Chapter 1

The roles of TCPs in Arabidopsis development- an overview

TCPs in the plant kingdom

When European explorers sailed to the Americas in the 15th and 16th century they came back with a cereal plant thitherto unknown to Europe: maize (*Zea mays* L.). Today, domesticated maize is one of world's major crops with a production of over 800 million tons worldwide in the year 2008 (FAO, 2008). Maize was domesticated over 8700 years ago in Mesoamerica (Piperno et al., 2009) out of its wild ancestor, which has been identified as teosinte (Beadle, 1939). One of the main differences between teosinte and domesticated maize is the branching pattern of the two species. Whereas teosinte exhibits multiple shoots and is bushy as a result, modern maize has one shoot with one male flower at the top and few female flowers in the leaf axes. Only in 1995 the morphological difference between teosinte and maize was mapped to the *teosinte branched 1* (*tb1*) locus (Doebley et al., 1995; Doebley et al.,1997). Further research showed that the *Tb1* gene belongs to a plant specific transcription factor family, later named the TCP transcription factor family.

The name TCP is an acronym of the three founding genes identified for this group: *Tb1* in maize, *CYCLOIDEA* (*CYC*) in snapdragon, and *PCF1* in rice (Cubas et al., 1999). Molecular and mutant analyses for these three genes represent the diversity of morphologic traits under the control of TCPs. Whereas the *Tb1* gene affects amongst others the outgrowth of axillary meristems of maize, the *CYC* gene is involved in the control of flower symmetry in snapdragon (Luo et al., 1996). Snapdragon usually displays a zygomorphic flower like most members of the Scrophulariaceae family. However, snapdragon flowers with a double mutation of *cyc* and its homologue *dichotoma* display radial symmetry (Luo et al., 1996). The third representative of the TCP family, *PCF1* has been found to bind the promoter of the *proliferating cell nuclear antigen* gene (*PCNA*) in rice (Kosugi and Ohashi, 1997). PCNA is a cofactor of DNA polymerase δ and is involved in DNA synthesis and repair, and cell cycle control in meristems (Bravo et al., 1987). Besides these three examples of *TCPs*, further studies proved that *TCP* genes are widely distributed in the plant kingdom. *TCP* genes are conserved in all higher plants and even in the moss *Physcomitrella patens* (Navaud et al., 2007). Most plant species have multiple *TCP* genes encoded in their genome; however, the number of *TCP* genes varies significantly between species. *Arabidopsis thaliana*, the model plant we work with in this study, has 24 TCP transcription factors encoded in its genome (Figure 1) (Aguilar-Martinez et al., 2007).

Molecular function of TCPs

Before the TCP transcription factor family was defined by Pilar Cubas and colleagues, there was little data on the molecular functions of TCP proteins (Cubas et al., 1999). Cubas and colleagues analyzed the predicted secondary structures of the TCP domain and compared them to known protein structures. The TCP domain was suggested to form a non-canonical basic helix-loop-helix structure (Cubas et al., 1999). As this domain is known to be involved in DNA binding, and additionally its importance for dimerization was shown for rice TCPs (Kosugi and Ohashi, 1997), TCP proteins were defined as a new family of transcription factors in plants, acting in a combinatorial manner (Cubas et al., 1999). They are expected to regulate growth in plants, since

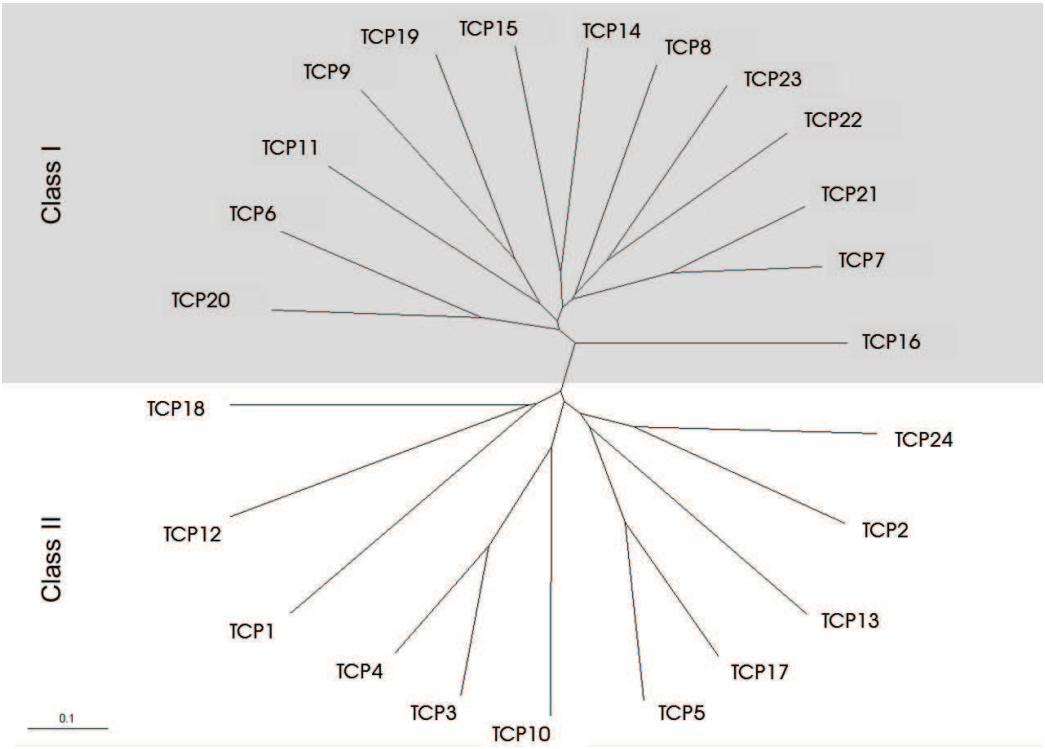


Figure 1. *Graphical representation of the 24 Arabidopsis TCPs. Full length protein sequences were compared using ClustalX. The resulting relationships are visualized using TreeView. TCPs are divided into two classes, the class I (grey background) and class II TCPs (white background).*

the first three TCPs identified are all involved in the regulation of meristem outgrowth or cell cycle-regulated organ development. Further phylogenetic and sequence analyses identified two different classes of *TCPs,* based on conservation of particular amino acid residues in the TCP domain (Kosugi and Ohashi, 2002). In the same work, yeast two-hybrid analyses revealed that TCP proteins are able to dimerize preferentially with TCP proteins of the same class. Furthermore, electrophoretic mobility shift assays (EMSA) were conducted to identify which DNA sequences can be bound by TCPs. The consensus binding sequences were identified to be GGNCCCAC for class I, and GTGGNCCC for class II TCPs (Kosugi and Ohashi, 2002). These putative binding sites overlap in sequence to such an extent that class II TCPs might bind class I TCP binding sites, and vice versa. This lack of exclusiveness raised the theory that class I and class II TCPs have common target genes that they regulate antagonistically (Li et al., 2005). According to this theory TCPs of the two classes compete for the binding sites in the promoters of their target genes. Inhibition or activation of the target genes depends on which TCP class is more abundant or which TCP class has higher affinity for the binding sites. This supposed mode of action, with competition between class I and class II TCP proteins, provides a mechanism for the tight regulation of growth related processes. Nevertheless, at the start of this thesis project evidence for common targets between class I and class II TCPs was missing.

Roles of TCPs in Arabidopsis development

The three founder TCP proteins are known to be involved in the regulation of different growth

processes in plants. The completion of the Arabidopsis genome in the year 2000 not only made it possible to identify 24 *TCPs* encoded in this genome, it also paved the road towards the identification of targets and biological processes downstream of these transcription factors. However, soon it was discovered that single mutations in *TCP* genes usually do not lead to obvious phenotypes. Additionally, *TCPs* of the same class appeared to have similar expression patterns. These two observations led to the conclusion that particular groups of *TCP* genes have redundant functions (Koyama et al., 2007). Redundancy means that the lack of one TCP is compensated by the action of its homologues. Functional redundancy is a common phenomenon for plant transcription factors and provides robustness against genetic disturbances.
Generating gene mutants and screening for an aberrant phenotype is a classical approach in biology; however this approach is hindered by functional redundancy. One way to overcome redundancy is to suppress the expression of a whole group of genes by ectopic expression of antisense RNAs or microRNAs (Kennerdell and Carthew, 1998). In a genetic screen Palatnik and colleagues identified the *JAGGED AND WAVY* (*jaw*-D) phenotype, which is the result of an overexpressed endogenous microRNA (*miR319a*) (Palatnik et al., 2003). The name *JAGGED AND WAVY* describes the excessive leaf curvature of *jaw*-D plants, whose serrated leaves are the most obvious phenotype they display (Figure 2).

***Figure 2**. Leaf serration phenotype of jaw-D plants (right) compared with wild type Arabidopsis leaf (left), plants are of the same age. Note that due to the prolonged cell proliferation stage in the mutant line, ultimately the jaw-D leaves will be larger than wild type (Palatnik et al., 2003).*

Proliferation of leaf cells during development is prolonged in these plants. Leaf cells usually undergo several rounds of cell division first and then enter a phase of cell expansion. In *jaw*-D plants the first phase is prolonged, leading to leaves with smaller but more cells than wild type leaves (Efroni et al., 2008). Apart from their name-giving leaf phenotype, *jaw*-D plants exhibit other phenotypes, such as late flowering, altered petal outgrowth and delayed senescence (Palatnik et al., 2003; Schommer et al., 2008; Nag et al., 2009). This plethora of effects in *jaw*-D plants is the result of microRNA-mediated downregulation of a whole clade of class II TCP genes; these being *TCP2*, *TCP3*, *TCP4*, *TCP10* and *TCP24* (Palatnik et al., 2003). Because of their sensitivity to *miR319a* they are called *jaw-TCPs*. The individual *jaw-TCP*s are downregulated at different strengths in *jaw*-D plants; *TCP4* being the strongest affected gene.

Single knockout of *TCP4* leads to a weak leaf serration phenotype, and only parallel knockout of several other *jaw-TCPs* results in enhanced phenotypes increasingly resembling the *jaw*-D phenotype (Schommer et al., 2008). Ectopic expression of a microRNA-resistant *TCP4* (called *TCP4m*) leads to a stop of meristematic proliferation in early development and is therefore lethal.

Other examples of Arabidopsis class II TCPs that exhibit growth phenotypes when downregulated simultaneously, include *TCP12* and *TCP18.* Double-knockouts of these leads to branching phenotypes similar to *teosinte branched 1* plants in maize (Aguilar-Martinez et al., 2007). Based on this finding *TCP12* and *TCP18* where renamed *BRANCHED1 (BRC1*) and *BRC2*, respectively. Downregulation of another clade of class II TCP genes (*TCP5, TCP13* and *TCP17*) results in bigger leaves and bigger flowers consisting of smaller cells (Efroni et al., 2008). Combining this mutation with the *jaw*-D mutation strongly enhances the leaf growth effects.
In contrast to class II *TCPs*, far less is known about the functions of class I *TCPs*, due to the absence of a mutant phenotype in class I *TCP* knockout plants. This will probably change once multiple knockouts of class I TCPs have been generated. A first successful study doing this was published recently. Here, Kieffer and colleagues showed that double mutants of *TCP14* and *TCP15* display, amongst others, shorter internodes (Kieffer et al., 2011). The only examples of single knockout/knockdown lines with described phenotypical effects are *tcp14* and *tcp16,* respectively (Takeda et al. 2006; Tatematsu et al., 2008). Alternative ways to unravel class I TCP functions followed two strategies.The first strategy involved identifying bound target genes and hypothesizing the function of the TCPs based on the common functions of its target genes. While this approach is valid, it is limited, especially when binding events are not linked with either activation or repression of target genes and when the search for target genes was not comprehensive but restricted to a small set of putative candidates. An example of such an approach is the work published for the *TCP20* gene. *TCP20* is a class I TCP gene closely related to maize *PCF1.* Maize *PCF1* has been shown to bind the *PCNA* promoter (Kosugi and Ohashi, 1997). Arabidopsis *TCP20* was analyzed for bound targets (Li et al., 2005) in a targeted chromatin immunoprecipitation experiment. In this experiment the promoters of *Cyclin B1*, *PCNA* and genes encoding ribosomal proteins were bound by TCP20 (Li et al., 2005). This observation led to the conclusion that TCP20 links cell cycle regulation with growth, since ribosomal proteins are directly linked to growth. However, a phenotype for *tcp20* knockout plants has not been described, probably because TCP20 functions redundantly with other class I TCPs.

The second strategy to study class I TCP functions was to express a TCP tagged with a dominant suppressor domain, and analyze plants expressing this modified TCP for transcriptional and phenotypic changes. This is what Hervé and colleagues did to study TCP20 function (Herve et al., 2009). They tagged TCP20 with either an activator or a suppressor domain (Herve et al., 2009). Ectopic expression of these tagged TCP20 proteins led to pleiotropic growth phenotypes in plants. The set of identified differentially expressed genes in these plants did not include the putative target genes found by Li and colleagues (Li et al., 2005). This indicates that binding events, transactivation or repression of target genes and eventual steady-state transcript levels are not necessarily comparable.

Much of today's transcriptional network analysis is focused on the identification of transcription factor target genes, either by straightforward genetic, or via target gene identification approaches. Target gene approaches make use of inducible promoters (Roslan et al., 2001) or post-translational activation in glucocorticoid response assays (Aoyama and Chua, 1997) in combination with transcriptome analysis. Alternatively, they look for binding of transcription factors to specific sequences *in vitro* (Singer et al., 1997) or *in vivo* (Weinmann and Farnham, 2002). Transcriptional network studies can also start from selected target genes of interest, followed by the identification of upstream regulators. Especially yeast one-hybrid analyses have been successfully applied for this purpose (Uno et al., 2000; Miao et al., 2004; Tran et al., 2004). Also TCPs have been identified using this method. The class I TCP transcription factor TCP21 has been identified to play a role in the regulation of the circadian clock (Pruneda-Paz et al., 2009), as it has been identified to bind as a dimer with TIMING OF CAB EXPRESSION (TOC1) to the promoter of *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) (Pruneda-Paz et al., 2009). It is likely that comprehensive analysis of class I TCP regulated networks and detailed analyses of multiple *tcp knockout* or *knockdown* lines will reveal some of class I TCP's functions in the near future.

Outline of this work

TCPs are an ancient plant transcription factor family. However, research on *TCP* genes is relatively young and was focusing predominantly on cell cycle and growth regulation. Although *in vitro* binding studies revealed direct interactions between TCP proteins and cell cycle promoter elements, direct effects of TCPs on the expression of core cell cycle genes were shown yet. Another problem of the current research is that it is fragmented and no efforts have been undertaken to study the complete network of genes controlled by particular TCP proteins.
We tried to address these issues in this work. This work was part of the EU-AGRONomics program, in which 14 laboratories joined forces to unravel leaf growth regulation. As part of this program, we focused on the role of TCP transcription factors in leaf development. We assumed that a comprehensive view on TCP-controlled transcriptomes will help us to address the following questions:

1. What are the target genes of selected TCPs and do they show common set of target genes?
2. Do TCPs control growth phenomena by regulating the transcription of core cell cycle genes directly?
3. Which upstream regulators control the cell cycle during leaf growth?

and

4. What are the molecular mechanisms underlying transcriptional control of target genes by TCPs?

In Chapter 2 we addressed the issue of redundancies between TCPs and how to get around this phenomenon in the search for functions of individual TCP transcription factors. We present a way of comprehensive bioinformatics analyses, making use of available data, and show that these can help identifying gene pairs that are possibly strongly redundant with each other. In this work, protein sequence, expression data and TCP-TCP protein interaction behavior was used to identify the two *jaw*-regulated *TCPs TCP10* and *TCP4* as a highly redundant pair. Subsequent glucocorticoid response assays identifying the target genes of TCP10 show that TCP10 and TCP4 share many common target genes, and control similar processes during leaf development. To our surprise we did not identify cell cycle genes as direct targets of these TCP proteins. Nevertheless, *jaw*-D plants display aberrant cell proliferation during leaf development. Our target gene analyses suggests that there must be at least one other layer of regulation linking TCPs to the core cell cycle genes.

In Chapter 3 and 4 we report about the function of TCP20, a class I TCP protein. In Chapter 3 we determined the genome-wide target gene map of the class I TCP20 protein. The goal of the analysis was to identify TCP20 targets during leaf development. TCP20-GR induction assays revealed that TCP20 is involved in regulating jasmonic acid biosynthesis and shares target genes related to this process with class II *jaw*-TCPs (Schommer et al., 2008). In depth analysis elucidated the interplay between class I and class II TCPs in the regulation of common target genes, as hypothesized by Li and colleagues (Li et al., 2005). Furthermore, we identified the class I *TCP9* gene as a target of TCP20 and showed that this gene plays a role in determination of leaf cell sizes during early developmental stages. In Chapter 4 we continued investigating the function of some other TCP20 target genes belonging to the bHLH transcription factor family that are known to be involved in iron homeostasis. These genes have been identified in root iron homeostasis, but our work shows that they are also important for iron homeostasis during photomorphogenesis, a process connected to leaf development.

In Chapter 5 we determined upstream regulators of *WEE1, E2Fa* and *DEL1,* three genes that are known to be involved in the cell cycle, in a large-scale yeast-one hybrid approach. Although many transcriptional regulators were identified, there was only one TCP transcription factor represented. In depth analyses of the candidate effector genes will help to further elucidate cell cycle control as a main issue of leaf development. In the final concluding chapter, we discuss how the results from the experimental chapters have changed our view about the way TCP transcription factors act, with a special focus on their involvement in leaf growth.

Chapter 2

Cling together, swing together: Analysis of functional redundancies within the Arabidopsis TCP transcription factor family and validation by target gene comparison for TCP4 and TCP10

Selahattin Danisman, Aalt D.J. van Dijk, Lars Hennig, Stefan de Folter, Gerco C. Angenent and Richard G.H. Immink

Abstract

Functional redundancy is a common feature in the *Arabidopsis thaliana* TCP transcription factor family, but identification of functionally redundant genes is impeded by the relative low overall sequence similarity for *TCP* genes and concomitant small differences in the encoded proteins. Furthermore, partial and unequal redundancies complicate the analysis of closely related TCP proteins. We performed pairwise protein-protein interaction studies and expression analyses during different stages of leaf development for all members of the Arabidopsis TCP family. The results were combined with publicly available expression data to predict functionally redundant TCP transcription factors. All known cases were identified, such as e.g. the five members belonging to the *CINCINNATA (CIN)/JAGGED AND WAVY (JAW)* clade. Importantly, within this clade we could differentiate between pairs with a higher versus a lower redundancy potential. One of the couples with a predicted high functional overlap are the two TCPs TCP4 and TCP10, and to further elucidate their function, we performed a genome-wide target gene analysis for TCP10. The obtained data was compared to available information about genes that act downstream of TCP4, revealing a common function for these two proteins in the regulation of jasmonate biosynthesis and response genes.

Introduction

TEOSINTE-LIKE1, CYCLOIDEA and PCF1 (TCP) transcription factors constitute a small family of plant-specific transcription factors whose members are believed to share functions in plant development (for a review, see Martín-Trillo and Cubas, 2010). The *Arabidopsis thaliana* genome encodes for 24 TCP transcription factors divided into class I and class II TCPs according to sequence similarities (Cubas et al., 1999; Kosugi and Ohashi, 2002). All TCP transcription factors share the TCP domain, a 59 amino-acids long non-canonical basic helix-loop-helix domain responsible for nuclear targeting, DNA binding and mediating protein-protein interactions (Cubas et al., 1999; Kosugi and Ohashi, 2002). Apart from this domain, TCP protein sequences are in general highly variable. Analysis of single *tcp* knockout mutants in Arabidopsis resulted in only a few distinct mutant phenotypes (Takeda et al., 2006; Schommer et al., 2008; Tatematsu et al., 2008) and most known Arabidopsis *tcp* mutant phenotypes are the result of double or multiple knockouts. For example, in the *JAGGED AND WAVY (JAW-D)* mutant, overexpression of the microRNA *miR319a* leads to the knockdown of five class II *TCPs* (below referred to as *jaw-TCPs*); *TCP2, TCP3, TCP4, TCP10* and *TCP24. Jaw*-D plants exhibit several phenotypic defects, including highly serrated leaves, altered petal development, and delayed leaf senescence (Palatnik et al., 2003; Schommer et al., 2008; Nag et al., 2009). Part of these phenotypes could be explained by a closer examination of genes that act downstream of TCP4 (Schommer et al. 2008), although a list of direct target genes is missing for this regulatory protein. The *tcp4* single knockout phenotype shows only a mild leaf serration phenotype, which can be enhanced by introducing knockouts of the other *jaw*-TCPs (Schommer et al., 2008). The degree of phenotype alterations varies and depends on which *tcp* mutant is being crossed with *tcp4* plants, suggesting that the five *jaw*-TCPs share unequal and only partially redundant functions.

Many Arabidopsis single knockout mutants show no obvious phenotypic alterations, as is the case for numerous single *tcp* mutants (Bouché and Bouchez, 2001). Apart from technical difficulties to detect inconspicuous phenotypes, single knockouts are often compensated by functionally redundant paralogs. Full genetic redundancy is likely to be genetically instable (Thomas, 1993), as the duplication of a gene lowers the selective pressure on either the new copy or the original gene (Ohno, 1970; Hughes, 1994). Hence, most duplication events either lead to an abortion of the duplicate gene or to neo- and sub-functionalization (Rastogi and Liberles, 2005). Sub-functionalization results in partially redundant genes that share common functions, but have distinct roles or expression patterns (Briggs et al., 2006). Genes that are most similar to each other at the sequence level are not always those with the closest redundant function, especially in cases where a family is defined by a single or a few conserved motifs, and where the remaining of the amino acid sequence is quite variable. In such cases prediction of redundant gene pairs by sequence similarities is impeded, and additional functional information is essential for the identification of redundant gene pairs. This can be achieved by integrating sequence information with expression and further functional data.

In this work we determine the potential degrees of functional redundancy between Arabidopsis

TCP transcription factors, integrating sequence, expression, and protein-protein interaction data. In a second step we assess the functional redundancy between the two related *jaw*-TCPs TCP4 and TCP10 by determining the genome-wide direct target genes of TCP10, and subsequently comparing them to known genes downstream of TCP4.

Materials and Methods

Plant growth and media. Wild type *Arabidopsis thaliana* (accession Columbia-0) plants were grown on rockwool for analyses of above-ground material. They received 1g/L Hyponex™ Plant food solution two- to three times a week. For Dexamethasone (DEX) induction experiments, seeds were gas-sterilized and sown out on MS-agarose media (1/2 MS + 6 g agar). Per plate, 30 to 50 seeds were sown on top of a 200 µm nylon mesh that was placed onto the agar. The plants grew at 25 degrees under long-day conditions .

Constructs. For the glucocorticoid induction experiments, we created a microRNA insensitive version of *TCP10 (TCP10m)* by site-directed mutagenesis and cloned it into a GR destination vector (Kaufmann et al., 2009). For this, the miRNA target site was mutated in the same way as it was done previously with *TCP4* (Palatnik et al., 2003). Primers used are given in Table 1. These primers allow mutation of the *miR319a* binding site without changing the expressed protein's amino acid sequence. *TCP10m* was cloned into the GATEWAY®-compatible pCR8/GW/TOPO vector (Invitrogen). It was then introduced behind the CaMV35S promoter in the GR destination vector via an LR reaction.

Transformation of Arabidopsis. Wild type Arabidopsis was grown on soil till the primary inflorescences emerged, which were cut to promote growth of secondary inflorescences and to increase the number of flowers. The binary *TCP10m-GR* construct was transformed into *Agrobacterium tumefaciens* strain C58C1-PMP90. Transformation of plants was conducted by floral dip (Clough and Bent, 1998). After transformation, plants were kept in a growth chamber till seed-set. The T1 seeds were then selected on ½MS + Agar (8 gr/L) plates containing 30 µg/mL Kanamycine for two weeks, after which rooting green T1 seedlings were transferred to soil and grown till seed-set. The following T2 generation was checked for expression of the transgene by RT-PCR.

Induction experiments. We conducted Glucocorticoid induction experiments three weeks after plant germination. Because the plants were grown on nylon meshes and on ½ MS medium with low concentrated (6g/L) Agar, we could transfer them into induction media quickly and without severely damaging the roots. The induction medium consisted of 2.3 g/L MS, 1% (w/v) sugar, 10 µM DEX, and 10 µM Cycloheximide (CYC). Samples for RNA isolation were harvested immediately before and two and four hours after start of the treatment.

Table 1: Primers used for generating TCP10m and for qRT-PCR analyses

TCP	Forward primer	Reverse primer
TCP10m-GR	TACCTTGCAAAGTAGCTTATTCCCTCATTCGTTTCG	CCCTTATTCGATGAAACGTTCCATGGGGAAACAACAAC
TCP1	GATAATCCAGAGACTGCCTCTGATA	CAAACTCCTCGATTGGTTCCTTGTA
TCP2	CATCATCATCCTCATCATCAGCATCA	AGATTGAATGCTCCGCCGTTGGA
TCP3	ACCTATGATTCGTGCTTGGTTTGAT	GAGGGATATGGTAGGGATGATGATG
TCP4	TTCGGAAGGATTCAGAGACTAGTGG	AGGAGTAGGAGGAGCGAACAGAAAC
TCP5	GGGTTTAACACCAATCATCAACAA	CGACAGTAACGTTATTACCAGATT
TCP6	GGGTTTGATCTGAATTACGGAATT	CCTTAATTCAAGTCCAGGCGT T
TCP7	AACAACATCATCATCAGCAACCATT	ACCCGGAAGATAATTCCCAACTCTA
TCP8	CAAATGGAGAGTAGTAGCAACAATA	GTACTGATCCATCGGAAAACTAAA
TCP9	TGCCGTCGTTTTCAATGTCTTTAGC	TTCTCCCTCTTCCTTCAACCTTCG
TCP10	AAGCTTCTTCGATGTTTGCTTCATC	GCTTCTTCTCCGTGGAGTAGTCTTG
TCP11	GCCACAATCGCCGAGTTGTAGGTTGGAT	CATCGGCTCTAAAAGCATCGCTGT
TCP12	GTGACTCTAGCTCCTCTTCCATTTTT	CCGTTGCTATTGAAGTTGTGTTGTGT
TCP13	CTGGTTCAGGGACTATGGAGACATT	AAATGTTTTGGGAAGACGAAGATGA
TCP14	TCGGAGAAGAAAAGAAGAATCCAAA	TGGCTGTAGATCCACTGTTGCTAGA
TCP15	TTACCTACGAGTCAGAGTCCTGCAA	ACCACTGTTGGGGTTGTAAACATCT
TCP16	CGTCAGATGGTGTTTCCGGTTTCT	CGGTAGCATTACCATTAAAGGTGGAA
TCP17	GGTAACGTCACTGTCGCATTTTCTAA	GAAACGAAGGGTACCTTGTTGGGA
TCP18	GAAAGTTGTGGAAGAAGACGCTCAT	CGCAGTGTGTCATCTCAAAAGAACT
TCP19	CTGTCTGACCCGAATCCGAAAACCAA	GATCCTCCGACCTCGTCCTTCTACT
TCP20	TTAGGCTTGTCTCAAGAAGGGAATG	GCATATGGTGAAGAACCCTACCTTG
TCP21	GTTTCTCCACTGCTTCTCTCTCCAC	ATTAACGAAGTCCCCATTGTGTGTC
TCP22	GTAGTAACTGCACCAATGGGGTCA	CCAAACCATCGCCTCTACTGCC
TCP23	GGGAGGTCAACAGTTAGGGTTAGGT	CACTCACTTGATGTTGAGGCTTTTG
TCP24	CCTTCAGTCCAATTCACAATCTCTCT	GCTTTGACTATCCATTGGAGAAGAG
LOX2	GATGCCCCAGTTCTCATTAACAGGG	CGGGTCTAGTTTGCTTATTAACGGC

RNA isolation and qRT-PCR. RNA was isolated by lithium chloride-phenol-chloroform extraction (Verwoerd et al., 1989). After DNase (Invitrogen) treatment was stopped with 1µL of a 20 mM EDTA solution and 10 minutes incubation at 65 degrees Celsius, RNA concentration was measured, and 500 ng RNA was used to perform cDNA synthesis. The cDNA made was diluted 10 times and used for quantitative Realtime PCR (qRT-PCR) using the SYBR green mix from BioRad. The SAND family gene *AT2G28390* was determined as "superior reference gene" for developmental studies (Czechowski et al., 2005) and used as reference gene for the analyses. The primers used in the transcript analyses are given in Table 1.

Yeast two-hybrid analysis. Protein-protein interactions between TCP proteins were analyzed in a matrix-based yeast two-hybrid (Y2H) GAL4 assay (de Folter et al., 2005). Bait vectors were transformed into yeast strain PJ69-4α; prey vectors were transformed into yeast strain PJ69-4a (James et al., 1996). The individual transformants were grown in liquid SD medium lacking Leu and Trp, respectively. These overnight cultures were mated by spotting 5 µL liquid culture of the individual yeast cultures on top of each other on SD complete plates. After overnight incubation, yeast was transferred by a 96-pins replicator to freshly prepared SD plates lacking Leu and Trp, selecting for diploid yeast containing both plasmids. In a last step, the mated yeast strains were

transferred on SD – Leu – Trp –Ade or SD – Leu – Trp – His medium, supplemented with 5 and 10 mM 3-Amino-1,2,4-triazole (3AT), respectively. Growth of yeast, and hence protein-protein interaction events, was scored after 5 days incubation at 30 degrees Celsius. Because of high auto-activation capacity of several TCPs, not all combinations could be analyzed reciprocally. Auto-activation capacity was determined beforehand for the baits by testing for growth of the single transformants on selective SD medium for the His and Ade protein-protein interaction markers. TCP1, 2, 4, 10, 12, 18, 20 and 24 were exhibiting auto-activation when expressed from the GAL4 BD vector and matings with these particular TCP-BD constructs were not included in the Y2H analysis. Every combination was analyzed 18 times (six replicates and three different selection markers). In the end, only pairs which scored for at least two different selection markers reproducibly positive, were taken as true protein-protein interactions.

Microarray analysis. Transcript profiling starting with 1 µg of DNA-free RNA was performed using Affymetrix Arabidopsis AGRONOMICS1 tiling microarrays (Affymetrix, Santa Clara, CA). Labeling of samples, hybridizations and measurements were performed as described (Rehrauer et al., 2010). Signal values were derived using the RMA algorithm implemented in the statistical language R (R Development Core Team, 2008) using probe sets comprising exonic probes based on the TAIR10 genome annotation. For details of probe set definition and low level data analysis see Rehrauer et al., 2010. Differentially expressed genes were selected using the RankProduct algorithm (Breitling et al., 2004). Genes were considered as differentially expressed if $p<0.05$. The microarray data is made available on ArrayExpress (accession number: E-TABM-1191).

Computational analysis. *Arabidopsis thaliana* TCP protein sequences were obtained from TAIR9 and their phylogeny was constructed using PhyML (Guindon and Gascuel, 2003) with the JTT substitution model, a distance-based tree as starting tree and maximum likelihood estimation for the gamma distribution parameter. Trees were visualized using the R-package Ape (Paradis et al., 2004).

To generate trees based on the Y2H or expression datasets, the information in those datasets was first converted to distances between pairs of TCPs. For the Y2H dataset, this distance was calculated as the number of proteins that were interacting with only one out of the pair for which the distance was calculated. This is equivalent to encoding the interaction pattern of each TCP as a binary vector with 1 indicating interaction and 0 indicating non-interaction, and then calculating the distance between two TCPs by subtracting two vectors and using the squared length of the resulting vector. The distance matrix obtained by calculating this distance for each pair of TCPs was subsequently scaled such that the maximum value was 1.0 and the minimum value 0.0 by applying a linear transformation: $dnew=(dold-dmin)/(dmax-dmin)$ where dold and dnew indicate the values of the distance before and after transformation, respectively; dmax and dmin indicate the maximum and minimum distance before scaling. The scaling does not change the relative ordering of pairs of TCPs but makes distances more comparable when comparing different

datasets. For the expression datasets, the distance was calculated by summing the absolute value of the difference between expression in each tissue or condition, followed by the same scaling as described above for the Y2H dataset. The way in which the distance calculation is performed for the expression data is completely equivalent to the calculation for the Y2H data. An alternative way to calculate the distance would be to take the square root of the sum of squares of the differences between expression in each tissue or condition; we tested this as well and found that it gives virtually indistinguishable results (Pearson correlation coefficient between the two sets of distances is ~0.99, ranking of the pairs is very similar).

For comparison of these interaction- or expression-based distances with sequence-based distances, a sequence based distance matrix was obtained using ClustalW (Thompson et al., 2002). The similarity between those sets of distances was characterized by the Pearson correlation coefficient. For the Y2H and expression datasets, trees were obtained based on the distance matrices using the NJ algorithm as implemented in the R-package Ape (Paradis et al., 2004).

Results and Discussion

Phylogenetic relationships and comparison of TCP gene expression patterns

Initially, we built a phylogenetic tree based on publicly available protein sequence data for all 24 Arabidopsis TCP transcription factors (Figure 1A). Similarly to previously described phylogenetic trees (Cubas et al., 1999; Aguilar-Martinez et al., 2007), the resulting phylogenetic tree divides the TCP family into two distinct classes. In a next step differences in *TCP* expression during Arabidopsis development were analyzed based on publicly available microarray data from AtGenExpress (Figure 1B, Table 2) (Schmid et al., 2005). Some of the *TCPs* did not show significant expression for any of the analyzed tissues: these were *TCP1, TCP6, TCP7, TCP12, TCP16, TCP18* and *TCP22.* We extended the AtGenExpress data by analyzing the expression of all 24 TCP genes by RT-PCR in a leaf development series (Figure 1C, Table 3). For this purpose we harvested above ground parts of seedlings, four and seven days after germination, and the complete first leaf at 11, 14, 16, 21 and 28 days after germination, respectively. Per time point three biological replicate samples were taken, consisting of 30 plants each. Also here, for some of the *TCP* genes no expression could be detected (*TCP1, TCP6, TCP11, TCP12, TCP16* and *TCP18*). Expression-based distances between pairs of TCPs were calculated for the AtGenExpress expression data set and our generated expression data as described in the Methods section; these distances exhibited high correlation between the two datasets (Figure 1D), irrespective of differences in detection methods and analyzed samples. Despite the high correlation between the two datasets, there are differences in the final expression trees (Figure 1B and 1C), which is the reason why the two data sets were used separately in later data integration. Some TCP genes that are closely related and cluster in the phylogenetic analysis, exhibit considerably different expression patterns, such as the pairs *TCP13-TCP17* and *TCP14-TCP15*, suggesting sub-functionalization of paralogs. Recently, TCP14 and TCP15 have been found to be redundant in the regulation of internode expansion in Arabidopsis (Kieffer et al., 2011). In this

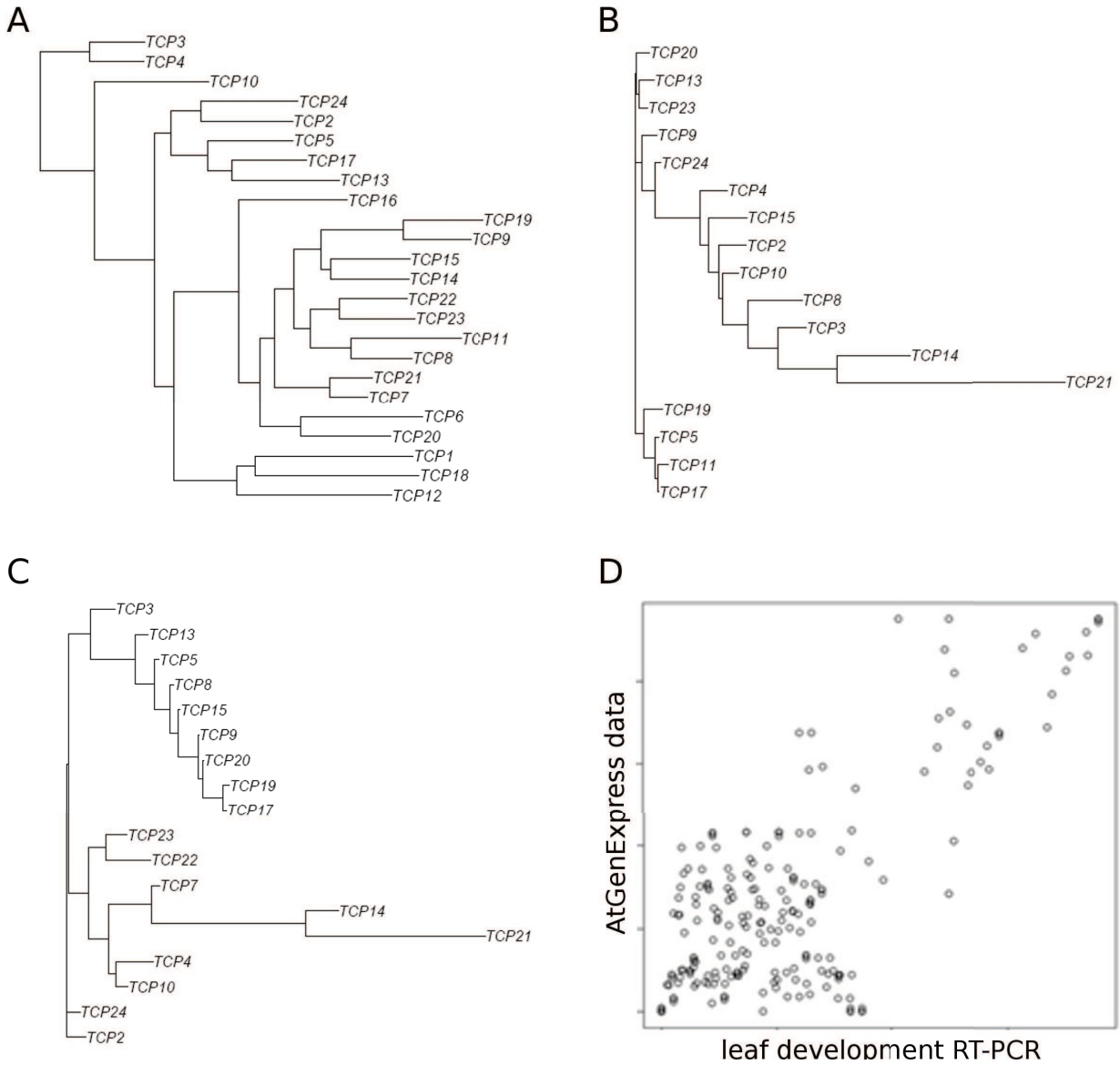


Figure 1. Relationships between TCP proteins based on (A) protein sequences, (B) global expression patterns and (C) leaf development specific expression patterns. *The phylogenetic tree for TCP protein sequences was generated using PhyML. Trees representing expression data were generated by first converting expression patterns to distances between pairs of genes and then applying the NJ-algorithm. Expression data for (B) were from the AtGenExpress microarray expression compendium by Schmid et al. 2005; expression data for (C) are from this study and where generated with qRT-PCR. (D) shows the correlation between data taken from AtGenExpress and the leaf expression data generated for this study. The correlation is high (R=0.74) despite the different samples and methods used to generate the two expression data sets.*

Table 2: AtGenExpress data for all TCP transcription factors. *Data from micro array experiments taken from the AtGenExpress Tool (Goda et al. 2008). The first five columns explain the sample on which the microarray analysis were performed, including tissues, genotypes, age, and sample ID of the sample. Each following column shows the absolute values observed for the different TCPs in the microarray experiments.*

tissue cluster	tissue	genotype	age	sample id	TCP1	TCP2	TCP3	TCP4	TCP5	TCP6	TCP7	TCP8	TCP9	TCP10	TCP11	TCP13	TCP14	TCP15	TCP16	TCP17	TCP19	TCP20	TCP21	TCP22	TCP23	TCP24
root	roots	Wt	7 days	ATGE_3	N/A	149,0575	52,6571	20,433	5,6968	N/A	N/A	395,3009	78,3945	10,9807	4,9269	5,8059	174,4751	32,5542	N/A	5,0946	47,4218	57,7081	299,2225	N/A	28,5815	85,1521
root	roots	Wt	17 days	ATGE_9	N/A	141,3477	55,1097	23,419	6,0788	N/A	N/A	347,264	242,3681	9,7857	4,5393	4,2263	205,0915	51,6822	N/A	5,7386	37,1503	64,6722	440,4953	N/A	24,3392	68,8144
root	root	Wt	15 days	ATGE_93	N/A	60,4732	48,037	32,3301	5,7278	N/A	N/A	345,6568	26,7264	13,4087	4,8222	6,9991	275,7185	62,7184	N/A	5,6533	17,5174	78,0542	279,5316	N/A	29,722	26,5237
root	root	Wt	8 days	ATGE_94	N/A	11,5789	13,3612	11,6972	5,2971	N/A	N/A	435,1785	33,4274	7,4891	4,8178	5,3238	533,4361	45,2515	N/A	5,1688	27,3804	77,7947	196,0213	N/A	31,2116	6,6672
root	root	Wt	8 days	ATGE_95	N/A	13,7296	10,4947	15,3461	5,2274	N/A	N/A	349,0061	24,144	7,4143	5,0422	4,8362	347,4954	42,6098	N/A	5,7458	14,4807	69,7046	185,9613	N/A	32,7428	8,4302
root	root	Wt	21 days	ATGE_98	N/A	15,5438	17,465	12,6901	5,229	N/A	N/A	516,8483	30,5248	7,9274	4,8234	8,6442	785,5189	43,2398	N/A	5,2867	24,1056	85,2187	155,4835	N/A	37,7062	11,1221
root	root	Wt	21 days	ATGE_99	N/A	36,988	23,2391	13,5186	5,0573	N/A	N/A	299,9744	33,0819	12,4543	4,799	7,8864	498,8665	48,0878	N/A	5,2368	12,1796	94,5891	123,8388	N/A	31,433	16,1625
stem	hypocotyl	Wt	7 days	ATGE_2	N/A	9,2653	18,1119	9,391	5,4638	N/A	N/A	334,7794	44,4134	12,0992	4,6848	5,9152	718,9617	75,4386	N/A	5,9698	21,338	57,9295	272,1999	N/A	55,0456	20,1591
stem	1st node	Wt	21+ days	ATGE_28	N/A	30,6274	15,6202	9,6998	4,9195	N/A	N/A	305,0748	80,8857	13,2017	5,1941	4,7337	302,5043	215,2397	N/A	6,123	19,6026	88,1012	532,0749	N/A	50,3491	25,0449
leaf	cotyledons	Wt	7 days	ATGE_1	N/A	192,2759	381,1789	144,7966	67,952	N/A	N/A	260,113	190,4994	222,4346	5,5065	199,0141	377,9346	48,0985	N/A	17,8297	12,111	74,3402	761,1817	N/A	123,5152	121,7122
leaf	leaves 1 + 2	Wt	7 days	ATGE_5	N/A	218,1899	370,4194	221,1379	45,5544	N/A	N/A	263,6151	110,9519	249,914	5,7519	88,7611	346,7455	116,6884	N/A	19,1652	39,1895	60,7721	1667,199	N/A	75,01	104,3385
leaf	rosette leaf # 2	Wt	17 days	ATGE_12	N/A	175,1325	458,9162	104,5278	69,5932	N/A	N/A	376,4576	194,5036	391,3009	4,9555	218,2469	811,9656	59,7857	N/A	30,6614	19,0247	82,8577	909,6162	N/A	138,2444	114,5105
leaf	rosette leaf # 4	Wt	17 days	ATGE_13	N/A	163,9702	587,3754	111,5958	31,8922	N/A	N/A	292,036	174,4915	284,482	5,1476	129,2189	647,9193	102,5173	N/A	17,77	17,5349	89,0317	1255,3043	N/A	116,2959	110,8409
leaf	rosette leaf # 6	Wt	17 days	ATGE_14	N/A	155,3482	627,3584	164,1798	22,6692	N/A	N/A	296,42	163,121	269,9781	5,337	101,3657	507,4334	98,4902	N/A	16,8913	10,839	85,7315	1595,9703	N/A	68,0471	103,4386
leaf	rosette leaf # 8	Wt	17 days	ATGE_15	N/A	169,9947	678,5757	195,0773	23,1486	N/A	N/A	300,0747	186,3502	247,9348	5,2852	75,0169	496,2571	142,1771	N/A	15,2742	24,1865	73,7621	1553,4095	N/A	62,7247	125,7522
leaf	rosette leaf # 10	Wt	17 days	ATGE_16	N/A	199,5306	583,9719	234,8585	16,6843	N/A	N/A	274,1832	134,116	225,5592	5,5216	67,2186	448,258	163,9801	N/A	18,0646	21,0156	63,86	1341,8211	N/A	43,482	114,4426
leaf	rosette leaf # 12	Wt	17 days	ATGE_17	N/A	236,7699	477,2504	308,5695	24,8361	N/A	N/A	278,4112	122,1795	229,379	5,3036	41,149	467,4574	231,1154	N/A	21,7895	56,8535	61,3503	1199,3118	N/A	37,1187	134,1778
leaf	rosette leaf # 12	gl1-T	17 days	ATGE_18	N/A	309,1807	616,1206	410,956	28,8626	N/A	N/A	218,6508	53,4859	266,3215	5,3152	43,5922	457,291	322,313	N/A	21,2363	56,3166	48,1825	935,9825	N/A	45,6361	136,8648
leaf	leaf	Wt	15 days	ATGE_91	N/A	517,749	301,3487	324,9134	78,7484	N/A	N/A	218,5337	26,9813	322,3916	5,5866	102,2234	887,5234	212,6302	N/A	36,479	58,203	64,2277	2054,3506	N/A	57,8509	175,0929
leaf	senescing leaves	Wt	35 days	ATGE_25	N/A	268,7031	254,9783	61,2762	15,6618	N/A	N/A	243,982	114,6236	275,9125	4,664	348,6608	372,078	112,1974	N/A	21,2401	10,52	130,8158	57,9257	N/A	180,5887	100,6469
leaf	cauline leaves	Wt	21+ days	ATGE_26	N/A	213,72	545,0693	168,9365	37,5441	N/A	N/A	255,8978	159,6041	332,541	5,1471	147,9126	575,639	228,1236	N/A	60,2832	25,1783	77,1753	316,7844	N/A	203,06	115,6671
whole plant	as above	Wt	22 days	ATGE_23	N/A	234,6241	505,0159	280,0987	20,9121	N/A	N/A	208,0965	40,6516	216,2921	5,0701	53,7408	487,1393	278,56	N/A	20,2102	24,4871	57,8617	879,2859	N/A	63,628	136,7993
whole plant	as above	Wt	23 days	ATGE_24	N/A	248,7002	499,7167	223,0353	20,2186	N/A	N/A	214,4493	50,9742	229,1095	5,3517	48,205	493,2334	331,1489	N/A	21,0113	19,2737	58,7244	840,7745	N/A	71,2237	124,1901
whole plant	vegetative rosette	Wt	7 days	ATGE_87	N/A	581,3752	344,0884	398,8658	91,4602	N/A	N/A	232,8577	64,0405	405,5076	6,4537	92,1267	741,9214	189,2524	N/A	38,6173	92,8041	56,2971	1314,7604	N/A	81,0169	166,9738
whole plant	vegetative rosette	Wt	14 days	ATGE_89	N/A	410,9497	330,3611	460,7202	67,8588	N/A	N/A	241,1297	46,9398	283,9376	5,7836	58,9465	595,7315	255,9098	N/A	40,4761	137,5709	48,3658	1208,4344	N/A	53,9612	163,8813
whole plant	vegetative rosette	Wt	21 days	ATGE_90	N/A	444,056	283,2013	483,5172	63,6713	N/A	N/A	262,0356	36,7766	296,4016	6,2046	49,415	704,3285	384,6062	N/A	54,4682	155,6791	47,6549	1082,1233	N/A	41,5442	158,4324
flowers	flowers stage 9	Wt	21+ days	ATGE_31	N/A	238,5787	213,616	144,1497	8,343	N/A	N/A	258,6877	44,3518	270,2164	6,2603	20,3968	358,3511	201,3774	N/A	26,2309	157,0065	53,6454	210,9652	N/A	48,5124	97,0282
flowers	flowers stage 10/11	Wt	21+ days	ATGE_32	N/A	251,9386	303,059	272,2724	8,0998	N/A	N/A	285,259	60,621	272,3072	5,0687	29,9302	349,1605	234,0329	N/A	24,3743	134,4738	48,9907	197,7862	N/A	36,9912	81,8474
flowers	flowers stage 12	Wt	21+ days	ATGE_33	N/A	172,3649	171,6548	176,2142	6,2893	N/A	N/A	263,5641	68,0066	126,5813	4,8208	31,9463	249,3348	215,7047	N/A	16,6718	66,2633	43	131,0322	N/A	20,3616	56,7237
flowers	flower stage 12; multi-carpel gynoece	clv3-7	21+ days	ATGE_53	N/A	186,2776	222,7242	200,6591	5,6038	N/A	N/A	262,9304	78,3904	105,6441	4,7056	28,482	269,4624	279,8724	N/A	12,5674	78,4439	40,7253	174,7164	N/A	21,7953	57,9668
flowers	flower stage 12; shoot characteristics;	lfy-12	21+ days	ATGE_54	N/A	487,4729	406,9372	281,2111	19,2104	N/A	N/A	302,2338	78,83	489,8881	6,171	39,708	488,9851	626,6187	N/A	64,6174	167,9131	53,1569	303,1974	N/A	42,99	112,2363
flowers	flower stage 12; no sepals or petals	ap2-6	21+ days	ATGE_56	N/A	251,8291	184,236	87,5021	7,72	N/A	N/A	248,6888	80,9306	181,2218	5,4812	8,5473	289,199	401,134	N/A	15,1722	149,7812	49,2445	222,5473	N/A	22,2707	75,5452
flowers	flower stage 12; filamentous organs ir	ufo-1	21+ days	ATGE_59	N/A	201,2989	171,3551	137,417	7,5678	N/A	N/A	287,5771	91,1822	174,0071	6,0428	38,1689	359,9468	321,7363	N/A	18,539	100,309	41,4703	214,8552	N/A	24,3832	74,4627
flowers	flower stage 12; no petals or stamens	ap3-6	21+ days	ATGE_57	N/A	316,7776	226,1895	127,338	7,9868	N/A	N/A	262,0997	90,1525	221,5342	5,3366	46,7035	304,1246	532,3346	N/A	27,0307	103,6828	56,8101	180,6907	N/A	17,0702	98,112
flowers	flower stage 12; no stamens or carpels	ag-12	21+ days	ATGE_58	N/A	300,6104	441,2498	556,2319	9,3028	N/A	N/A	229,1937	61,0653	233,3568	4,8975	56,0167	207,2626	259,6742	N/A	19,8419	83,7391	62,4989	191,9281	N/A	46,5532	95,9558
flowers	flowers stage 15	Wt	21+ days	ATGE_39	N/A	111,5651	151,3024	65,8852	9,3699	N/A	N/A	264,7192	68,6885	104,4656	4,4615	108,7125	268,2303	232,3521	N/A	17,2337	43,1375	57,1692	97,6197	N/A	18,4908	46,4454
flowers	flower	Wt	28 days	ATGE_92	N/A	315,3717	166,2442	166,6231	8,1926	N/A	N/A	287,2018	54,9678	254,7272	6,3691	24,5358	459,2115	459,6906	N/A	15,7676	111,1289	51,1185	347,428	N/A	31,5057	91,128
floral organs	mature pollen	Wt	6 wk	ATGE_73	N/A	39,2618	31,8281	21,1052	22,3265	N/A	N/A	38,8303	13,5303	39,9215	12,5049	7,2184	14,6482	11,6999	N/A	9,878	8,3523	48,9607	33,717	N/A	61,245	12,0897

Table 3: Realtime RT-PCR data showing TCP expression during leaf growth. *Averages of three biological replicates are depicted. All transcripts were analyzed on the same samples. The data was normalized using the reference gene AT2G28390, and expressions are depicted in comparison to the reference gene.*

DAS	TCP1	TCP2	TCP3	TCP4	TCP5	TCP6	TCP7	TCP8	TCP9	TCP10	TCP11	TCP12
4	N/A	0,826468	2,57072	1,248478	0,799252	N/A	2,505396	0,516991	0,445567	1,445479	N/A	N/A
7	N/A	1,339935	1,279426	1,809366	1,01074	N/A	3,330027	0,597114	0,459214	1,765785	N/A	N/A
11	N/A	1,653059	1,697827	3,200526	0,944412	N/A	2,564817	0,609778	0,453707	2,871013	N/A	N/A
14	N/A	1,065978	1,074863	2,286211	0,751578	N/A	2,252675	0,524417	0,312819	2,024036	N/A	N/A
16	N/A	1,65878	1,497741	2,325731	0,784235	N/A	2,693368	0,891419	0,293677	1,977055	N/A	N/A
21	N/A	2,950978	0,97977	1,168312	0,744222	N/A	2,228676	0,633183	0,367421	2,091004	N/A	N/A
28	N/A	1,894228	1,039517	0,814745	0,378166	N/A	1,731921	0,631992	0,437641	1,651016	N/A	N/A
DAS	TCP13	TCP14	TCP15	TCP16	TCP17	TCP18	TCP19	TCP20	TCP21	TCP22	TCP23	TCP24
4	0,739719	4,389516	0,369439	N/A	0,063909	N/A	0,16063	0,363332	5,956745	2,807647	1,556484	2,08286
7	0,672442	4,102705	0,744131	N/A	0,129291	N/A	0,199704	0,369759	6,564918	1,639221	1,652093	1,403849
11	0,610782	4,346602	0,770971	N/A	0,146701	N/A	0,215824	0,249814	6,870515	1,109702	1,350056	1,76988
14	0,611064	2,754182	0,508942	N/A	0,183688	N/A	0,079582	0,356432	4,46324	1,310656	1,978315	1,099888
16	0,80107	3,869184	0,720475	N/A	0,154691	N/A	0,0814	0,324541	6,261847	1,894896	2,47545	1,662001
21	1,603162	6,266321	0,470597	N/A	0,201104	N/A	0,11665	0,389859	5,794817	3,965777	3,400275	2,99921
28	1,481726	3,550164	0,403171	N/A	0,170656	N/A	0,045735	0,430936	1,887881	3,567482	2,878158	1,459403

case, a redundant function could only be found where the expression patterns of the two *TCPs* overlapped, whereas in leaves, where *TCP14* and *TCP15* expression differ significantly, redundant effects were almost absent (Kieffer et al., 2011). The opposite case is also detected in our analysis: some pairs show quite similar expression patterns although based on sequence similarity they are relatively distinct. For example, *TCP14* and *TCP21* are both class I *TCPs* but are distinct in protein sequence. However, in both analyses the two transcripts of these genes prove to be highly co-expressed. Although an almost identical expression pattern does not mean that two genes act functionally redundant, similar expression patterns are essential for a fully redundant function. All together, these observations show that the percentage of sequence similarity only is not always sufficient to predict functional redundancy.

Yeast two-hybrid analyses show class preference in TCP-TCP interactions

We used a matrix-based yeast two-hybrid analysis to expand our knowledge of TCP function and redundancy by investigation of protein-protein interaction capacities. TCP transcription factors are known to form dimers (Kosugi and Ohashi, 2002), but a comprehensive interaction map is missing. The assay resulted in 64 detected dimer combinations, including seven homodimers and 57 heterodimers (Figure 2).

Class I TCPs prefer interactions with other class I TCPs, and the same holds for class II proteins. In addition, the number of dimerization partners per TCP protein among class I TCPs (average = 7.3) is higher than among the class II TCP clade members (average=2.5) (Student's t-test p=0.0045). Based on phylogeny (Figure 1A) class II TCPs group into three sub-clades, of which the TEOSINTE BRANCHED-LIKE sub-clade (TCP1, TCP12, TCP18) does neither show up in the yeast two-hybrid (Figure 2), nor in the leaf expression analyses. Note however that all these three TCP proteins give auto-activation in the yeast-two hybrid analysis and therefore, could not be tested for homodimerization capacity or direct reciprocal interactions. Interestingly, the other two class II TCP sub-clades that belong to the CINNCINATA (CIN) clade, being the *jaw*-TCPs and the proteins TCP5, TCP13 and TCP17 (TCP5-likes), appear to form dimers almost

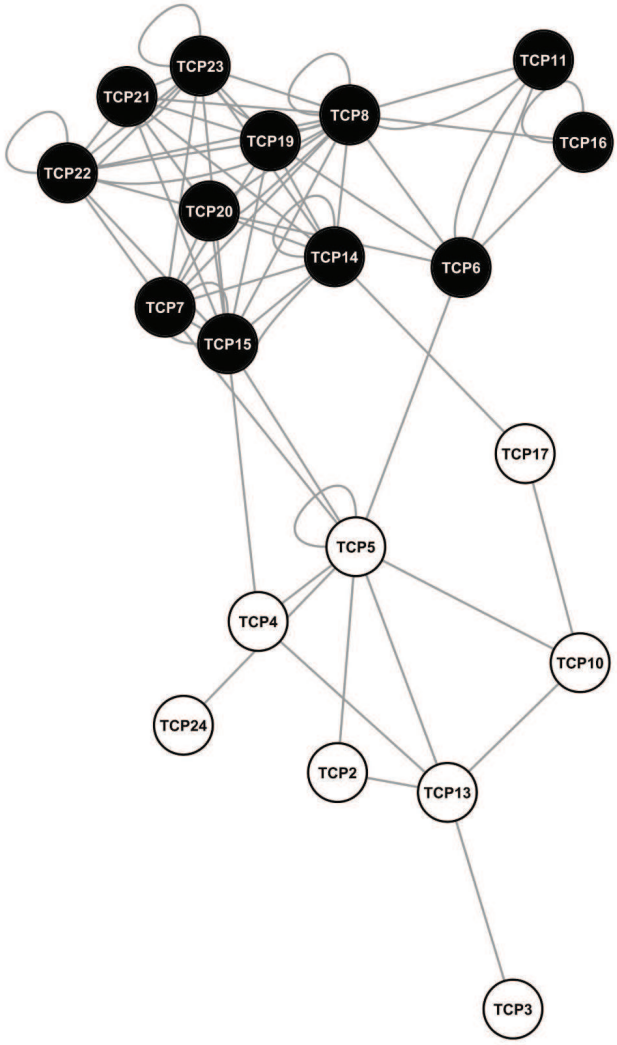


Figure 2: Results of the matrix-based yeast two-hybrid analysis of TCP-TCP interactions. *Nodes represent the TCPs, edges represent protein-protein interaction between these. White nodes are class II TCPs, black nodes are class I TCPs. As the graphical layout is spring embedded, a group of nodes is placed closer to each other equivalent to the amount of edges between them. Hence, the layout reflects the natural ordering occurring in the data. The representation reveals that interactions are preferred between TCPs of the same class. We used Cytoscape version 2.6.2 to visualize protein-protein interactions (Shannon et al., 2003).*

exclusively with each other. The fact that a cross between *jaw*-D mutants and plants expressing an artificial microRNA against all TCP5-like *TCPs* leads to an enhanced *JAW* phenotype and that members of the two sub-clades prefer to form heterodimers with each other, leads to the theory that they share common functions in leaf and flower development (Efroni et al., 2008). However, it is possible that different roles are fulfilled by different heterodimers consisting of *jaw*-TCPs and TCP5-like TCPs, or that different heterodimers are formed in different organs, as it was shown for other dimerizing plant transcription factors (Toledo-Ortiz et al., 2003; de Folter et al., 2005).

Integrative analysis strengthens the prediction of redundancy within gene families

In a next step, we determined pairs of proteins with a high potential for functional redundancy by ranking the distances in protein-sequence, expression and protein-protein interaction patterns for all possible TCP-TCP pairs, and cumulating the ranks. To perform this integration, distances were calculated between pairs of proteins in each dataset. For the sequence data, this was based on the number of substitutions. For the expression data and the protein interaction data, this was done by comparing the expression pattern or interaction pattern for each pair of proteins (for details see Methods).

The available expression data sets from AtGenExpress and our newly generated leaf development series were used independently in this analysis. *TCP6, TCP11* and *TCP16* expression could not be detected in either leaves or any other organs, although functions have been published for the latter two (Takeda et al., 2006; Viola et al., 2011). This can be due to very specific expression of these *TCPs* in certain cell types or developmental phases that have not been covered by our analyses. Such cases were excluded from further integrative analyses because no score can be calculated. Likewise, TCPs that exhibited no interaction in the yeast assay were also excluded.

The ten TCP pairs with the best score (lowest rank sum using the integrated sequence, interaction and expression data) were listed for both AtGenExpress and the leaf expression series (Tables 4 and 5). Although the rankings using the two different expression data sets confer high correlation (R=0.74), the tables for top ranked TCP functional redundancy pairs are different. This is due to the strong cut off applied when only counting the top 10 gene pairs. Despite the rigid cut-off three TCP pairs appear in both predicted sets, and almost always in high positions (average position 2.8). These TCP pairs with high predicted functional redundancy capacity are the pairs TCP19-TCP20, TCP13-TCP17 and TCP10-TCP4. Although, all these three cases represent closely related homologues, none of these pairs would have resulted from an analysis based on the highest sequence homology only. Based on this result we suggest that sequence similarity is a strong predictor of functional redundancy, but that available information about expression or protein behavior can be additive and useful. An important advantage of the integration of expression and other functional data into the prediction of functional redundancies is that many potentially redundant TCP pairs could be excluded because either the expression patterns or dimerization behavior were too different. For example, the *jaw*-clade of class II TCP transcription factors includes five members, which means that for any single member there are four potential redundant candidates. It has been shown that these TCPs may share common functions (Palatnik et al., 2003; Koyama et al., 2007). However, this was mainly based on over-expression of dominant-repressive versions of these TCPs (Koyama et al., 2007) or of microRNAs targeting these *TCPs* (Palatnik et al., 2003). Interestingly, these studies indicate that expression patterns and the degree of regulation by *miR319a* differ between the five *jaw*-TCPs, leading to the conclusion that there are differences in their respective functions. For example, TCP4 is closest to TCP3 in protein sequence, but our study that includes expression and protein-protein interaction data indicates that the closest functional homologue of TCP4 is TCP10 instead of TCP3. And indeed, although some of the TCP3 target genes *CUP-SHAPED COTYLEDON 1, 2* and *3 (CUC1,*

CUC2, CUC3) are also up-regulated in cotyledons when expressing a dominant-negative version of TCP10 (TCP10SRDX), and phenotypes upon ectopic expression of *jaw*-TCPs tagged with an SRDX domain look alike (Koyama et al., 2007), *CUC* genes are neither found amongst the TCP4 downstream genes (Schommer et al., 2008), nor in our analyses for the identification of direct TCP10 targets (see below).

Table 4: Top 10 redundancy between TCPs, using integrated AtGenExpress data (Goda et al., 2008).

			Sequence		Protein interaction		Atgenexpress		Type of TCP		Sum
Rank	Proteinl	Protein2	Distance	Rankb	Distance	Rankb	Distance	Rankb	Protein 1	Protein 2	
1	TCP19	TCP20	0.792	41	0	6	0.086	49	typeI	typeI	96
2	TCP13	TCP17	0.735	12	0.176	53	0.07	37	typeII	typeII	102
3	TCP10	TCP4	0.799	47	0.059	12	0.127	82	typeII	typeII	141
4	TCP20	TCP23	0.806	57	0.176	66	0.044	29	typeI	typeI	152
5	TCP10	TCP2	0.874	97	0.059	11	0.1	69	typeII	typeII	177
6	TCP17	TCP24	0.802	53	0.176	59	0.109	79	typeII	typeII	191
7	TCP10	TCP3	0.818	67	0.118	25	0.176	99	typeII	typeII	191
8	TCP20	TCP22	0.806	56	0.176	65	0.104	72	typeI	typeI	193
9	TCP19	TCP23	0.833	79	0.176	62	0.094	62	typeI	typeI	203
10	TCP13	TCP24	0.881	99	0.235	74	0.088	50	typeII	typeII	223

Table 5: Top 10 redundancy between TCPs, using integrated leaf development data.

			Sequence		Protein interaction		Leaf expression		Type of TCP		Sum
Rank	Proteinl	Protein2	Distance	Rankb	Distance	Rankb	Distance	Rankb	Protein 1	Protein2	
1	TCP19	TCP20	0.792	41	0	6	0.042	35	typeI	typeI	82
2	TCP22	TCP23	0.701	4	0	10	0.106	72	typeI	typeI	86
3	TCP2	TCP24	0.779	32	0.059	21	0.052	41	typeII	typeII	94
4	TCP10	TCP4	0.799	47	0.059	12	0.078	57	typeII	typeII	116
5	TCP15	TCP8	0.78	35	0.294	96	0.027	24	typeI	typeI	155
6	TCP13	TCP17	0.735	12	0.176	53	0.145	98	typeII	typeII	163
7	TCP15	TCP20	0.796	45	0.294	95	0.041	34	typeI	typeI	174
8	TCP20	TCP8	0.809	60	0.235	84	0.051	40	typeI	typeI	184
9	TCP15	TCP19	0.81	62	0.294	94	0.082	61	typeI	typeI	217
10	TCP19	TCP8	0.85	87	0.235	82	0.093	64	typeI	typeI	233

Exclusion of functionally distinct but sequence-related copies works also within the class I TCP clade. For example, TCP20 is very closely related to TCP6 and TCP16 in protein sequence, but expression analyses show that a similar function for TCP20 and these two homologues is unlikely. Nevertheless, missing phenotypes in mutant analyses of TCP20 suggests functional redundancy (Li et al., 2005; Herve et al., 2009). A possible candidate for a protein likely to be redundant with TCP20 is TCP19 (Table 4 and Table 5), although it would not have been a candidate extracted from protein sequence analysis exclusively. Crossing knockout lines for this and similar cases of redundant gene pairs and comparing the phenotype with crosses from TCPs with higher sequence similarity but lacking predicted redundancy potential could ultimately prove the power of our approach. A case known from the literature in which a seemingly highly

redundant gene family could be dissected into separate functions by a similar approach, is the gene family encoding for the malic enzyme (Maurino et al., 2009). Here, six gene copies could be functionally separated by extensive expression and enzyme activity analyses.

Continuous expression of microRNA-resistant TCP10 leads to arrested leaf development

Based on our analysis, the closest redundant homolog to TCP4 seems to be TCP10. Because the pair TCP4-TCP10 is predicted to be among the three most likely candidates for redundancy (see above), we decided to assess the hypothesized strong functional redundancy between the two class II TCP transcription factors TCP4 and TCP10 by analyzing the phenotype upon ectopic expression of microRNA resistant *TCP10,* and by comparing this phenotype with previously published data for a microRNA resistant version of *TCP4* (Palatnik et al., 2003; Schommer et al., 2008). We introduced a mutated *TCP10 (TCP10m)* into a constitutive GR-expression vector. The *pCaMV35S::TCP10m-GR* vector allowed *TCP10* to be expressed without the transcript being targeted for *miR319a*-mediated RNA degradation. This construct was transformed into the *jaw*-D genetic background. Continuous induction of TCP10m-GR nuclear import by DEX resulted in arrest of the shoot apical meristem early during vegetative growth, with the formation of only a few or no leaf primordia (Figure 3A and 3B). When DEX-treatment of TCP10m-GR plants started six days after germination, they showed intermediate phenotypes: more leaves were formed, but the leaves were smaller, non-serrated and further leaf initiation was arrested shortly after this DEX-induction (Figure 3C and 3D).

This overexpression phenotype resembles the phenotypes shown upon expression of a microRNA resistant version of *TCP4* (Palatnik et al., 2003; Schommer et al., 2008), suggesting that TCP4 and TCP10 may share similar functions in young seedlings. To further investigate this possibility, we determined target genes of TCP10 and compared them to the genes known to act downstream of TCP4.

Genome-wide identification of TCP10 target genes

TCP10 target genes were identified by inducing 21 days old TCP10m-GR plants with DEX and Cycloheximide, followed by harvesting of material for microarray expression analyses. Transcripts from TCP10m-GR/*jaw*-D and control *jaw*-D plants that were treated the same way were analyzed at time points 0, 2 and 4 hours of induction, and biological replicates were used for each time point. The obtained material was analyzed for genome-wide transcriptome changes on the AGRONOMICS1 microarray platform (Rehrauer et al., 2010). Differentially expressed genes between TCP10m-GR and *jaw*-D plants were compared at time points 0, 2 and 4. At time point 0, *TCP10* itself was found to be over-expressed in TCP10m-GR plants, verifying the functionality of the *CaMV35S* promoter. The only other gene that is differentially expressed is *AT1G02930,* showing that the introduction of the transgene has hardly any effect, as expected. After two hours of induction 39 genes were significantly up- and 135 genes were down-regulated. At four hours after DEX induction 102 genes were up- and 11 were down-regulated. In total, 270 genes were found to be differentially expressed when TCP10m-GR was activated. Only 21 of the genes were

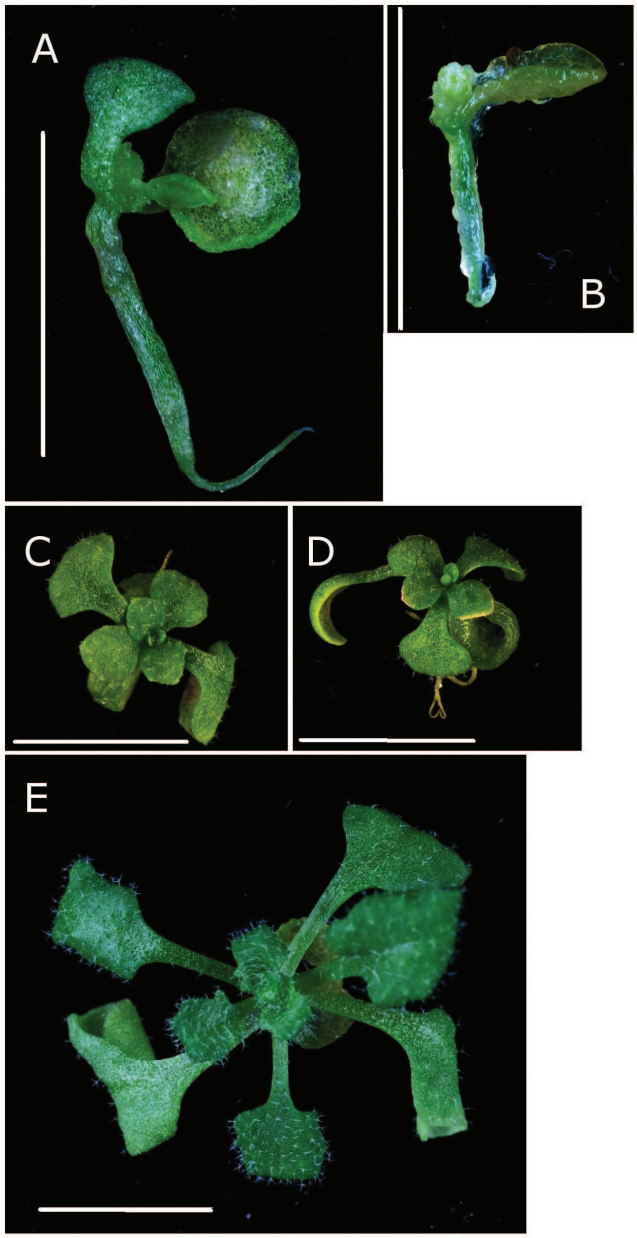


Figure 3: Functional complementation of the jaw-D phenotype by TCP10m-GR. *Plants were grown for three weeks on MS-medium with or without DEX treatment for different periods. Induction of TCP10m-GR by DEX leads to over-compensation (A-B) when DEX was given continuously after germination. When induced six days after germination, first leaves appeared normal (C-D) but plants remained small and eventually died. Jaw-D control (E). The white bar on each picture is 1 cm long.*

induced at both time points (Appendix I). This experiment shows that expression of a gene normalizes despite the presence of a protein synthesis blocker, either by proteins that were present prior to the induction, by regulating microRNAs, or by extant translational activity.

We analyzed the TCP10 target gene list for over-representation of gene ontology annotations with the Cytoscape BiNGO application (Maere et al., 2005). Here, genes responding to jasmonic acid (GO-ID 753), gibberellin (GO-ID 739) and salicylic acid stimuli (GO-ID 751) were overrepresented, indicating a function of TCP10 in hormone regulation. Although not

significantly overrepresented, we also found a high number of cell wall modifying enzymes, including expansin-like proteins, cellulose-like proteins and xyloglucan endotransglucosylase/hydrolases, which can represent additional roles for TCP10 in the regulation of cell wall processes during growth. One such target gene is *TOUCH4,* a gene encoding for a xyloglucan endotransglucosylase/hydrolase which has recently been identified to be involved in cell wall modifications during shade avoidance (Sasidharan et al., 2010). It remains to be shown whether this gene has the same function during normal leaf development. Although the overexpression phenotype of *TCP10* and *TCP4* both lead to an arrest of the shoot apical meristem, the effect on the cell cycle seems not to be direct, as the only cell cycle gene represented in the TCP10 target gene list is *CycP1* and there is no cell cycle gene present in the TCP4 (Schommer et al., 2008) target gene list. The same was already postulated by Efroni and colleagues, as their analysis of differential leaf development in *jaw*-D mutants showed that leaf maturation rather than cell cycle is being affected by the *jaw*-TCPs (Efroni et al., 2008). Interestingly, a recent publication shows that LANCEOLATE activity in tomato is partially dependent on gibberellic acid (Yanai et al., 2011). As gibberellic acid was also shown in the target gene analysis for TCP10, we suggest that the function of the Arabidopsis *jaw*-TCPs is also dependent on gibberellic acid, providing a link between jasmonate synthesis and regulation and gibberellic acid metabolism. Another hormone TCP10 function may be associated with is ethylene, which can be inferred by the high number of ethylene responsive genes, amongst which are eight ethylene response factors (*ERF/AP2*). Ethylene is a plant hormone involved in many plant developmental processes (Doubt, 1917; Lieberman, 1979; Tanimoto et al., 1995; Achard et al., 2003), like fruit ripening, floral development and leaf abscission, but also in stress responses (Abeles and Abeles, 1972). Unfortunately, not much is known about the *ERF/AP2* transcription factors targeted by TCP10. Three out of these eight *ERF/AP2* transcription factors exhibit increased expression in older leaves and four have a decreased expression in older leaves, implying that these factors may have specific functions during leaf development (Figure 4). Based on this observation it is tempting to speculate that TCP10 regulates late leaf development also via the regulation of these ethylene responsive transcription factors.

TCP10 and TCP4 both regulate leaf senescence by targeting jasmonate related genes

Comparison between TCP4 and TCP10 targets lead to an overlap of 12 genes (Palatnik et al., 2003; Schommer et al., 2008) (Figure 5); among these is the chloroplast *LIPOXYGENASE2 (LOX2)* gene that has been found to be responsible for the low jasmonate content in *jaw*-D mutants (Schommer et al., 2008). In contrast to the described effect of TCP4 on *LOX2,* TCP10 induction in the GR line decreases *LOX2* expression. The putative TCP4 target genes were identified based on the analysis of steady state expression levels, whereas the TCP10 target genes are based on direct expression changes upon induction of this transcription factor. This may be the reason why there are differences in the effect of TCP4 and TCP10 on these common target genes. To test this we compared the steady state expression of those target genes that are common between TCP4 and the TCP10 data set of our analysis. For this, TCP10m-GR/*jaw*-D and *jaw*-D

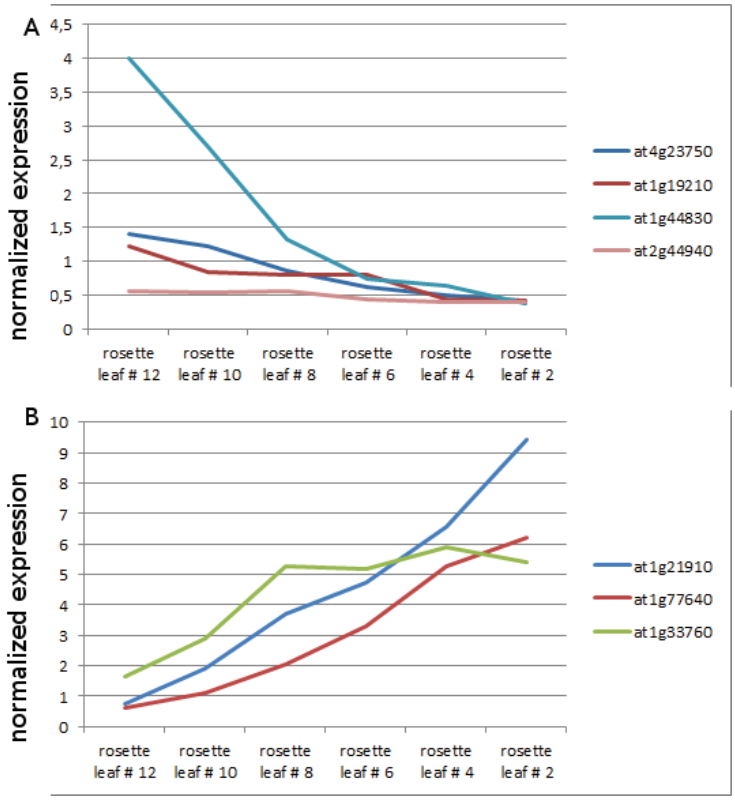


Figure 4. Expression of seven DREB ERF/AP2 transcription factors that are TCP10m-GR targets during Arabidopsis leaf development. *Given are mean-normalized expression values according to AtGenExpress (Goda et al., 2008) of the target genes in rosette leaves of 17 day old plants, whereas rosette leaf #12 is the youngest and leaf #2 is the oldest. (A) Genes that are lower expressed in older leaves. (B) Genes that are higher expressed in older leaves.*

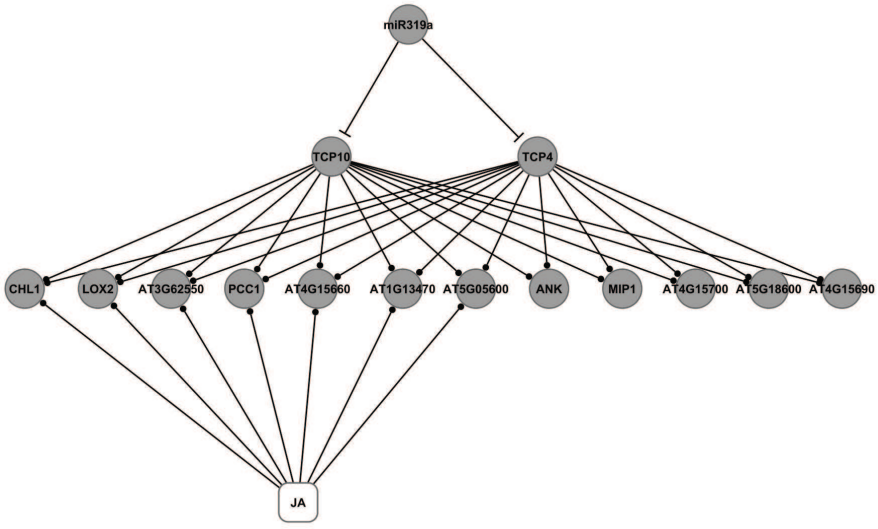


Figure 5: Schematic overview of common target genes of TCP4 and TCP10 and their response to jasmonic acid (JA) treatments (according to AtGenExpress). *Nodes represent genes and proteins (green) or hormones (white), edges represent regulatory effects of TCP10 and TCP4, and of jasmonate on target genes. We do not use a specification of activating or inhibiting activities for TCP10 and TCP4 because the direct effects of TCP4 on its target genes is not known. Network was built using Cytoscape 2.6.1 (yfiles hierarchical clustering, with modifications).*

plants were grown continuously on medium with and without DEX and material was harvested at the same developmental time when the GR induction experiments were conducted. *LOX2* expression was significantly higher in TCP10m-GR plants grown on DEX than on plants grown without (ANOVA and Fisher LSD test, p=0.01) (Figure 6) resembling the long term effect of mutated TCP4 described earlier (Schommer et al., 2008). This also means that the long term effects of increased TCP10 activity differ from the short term effects immediately after a sudden increase of TCP10 activity. Furthermore, seven out of twelve genes that are both TCP10 and TCP4 targets respond to jasmonate treatment according to AtGenExpress (Goda et al., 2008) (Figure 7). The overrepresentation of jasmonate responsive targets in both TCP10 and TCP4 (GO-ID 9753, response to jasmonate stimulus, p = 0.0006) target gene lists reveals a role for both in the modulation of jasmonate response.

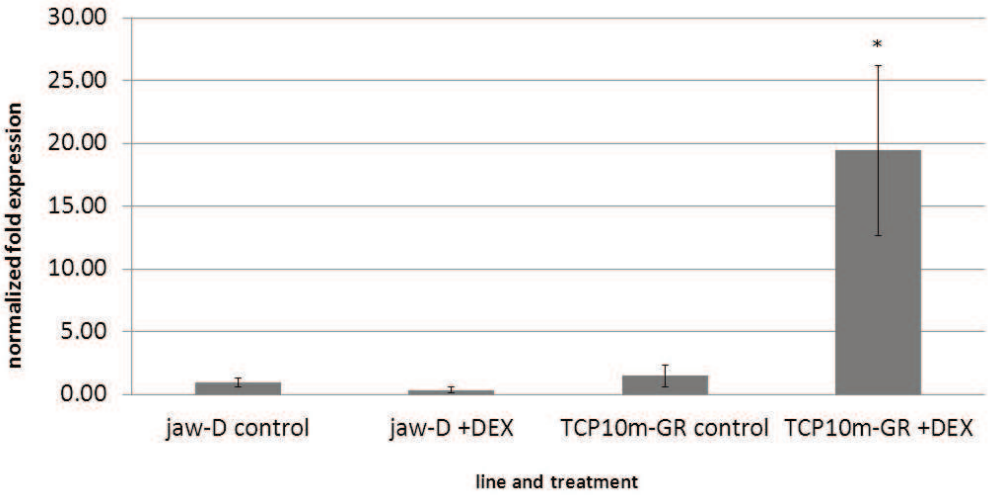


Figure 6. Steady state effect of TCP10m-GR induction by DEX on LOX2 expression. Expression values are normalized for jaw-D control. *TCP10m-GR and jaw-D plants were grown on MS medium with and without 10 µM DEX and harvested after 3 weeks.*

One of the other targets shared by TCP4 and TCP10 is *CHLOROPHYLLASE 1 (CHL1)*, which has previously been described to be involved in chlorophyll degradation and defense responses (Kariola et al., 2005). It has been discussed whether CHL1 is involved in defense or senescence pathways, but it seems that both pathways share this enzyme (Hörtensteiner, 2006; Park et al., 2007; Schenk et al., 2007). *CHL1* is also down-regulated upon application of the ethylene precursor ACC according to the AtGenExpress data set. Together with the up-regulation of seven of the eight ethylene responsive *ERF/AP2* family transcription factors by TCP10, a role for the regulation of ethylene-mediated senescence can be foreseen for TCP10. Earlier publications showed that proteins and transcription factors can be involved in both defense and senescence pathways (Robatzek and Somssich, 2001; Chen et al., 2002; Ülker et al., 2007), indicating that the two biological processes share common regulatory pathways.

In conclusion, we demonstrated that it is possible to create knowledge on gene function and identify genetic redundancies by integrating complementary genomic data sets, namely sequence homology, expression patterns and protein interaction data. Using additional experiments, we validated our approach by comparing the direct target gene list of TCP10 with potential TCP4

targets and extracted a small but significant number of target genes that are supposed to be responsible for the effects of these class II TCPs on leaf development and senescence, encompassing control over hormonal pathways of both jasmonates and ethylene, and downstream transcriptional transducers (Figure 5).

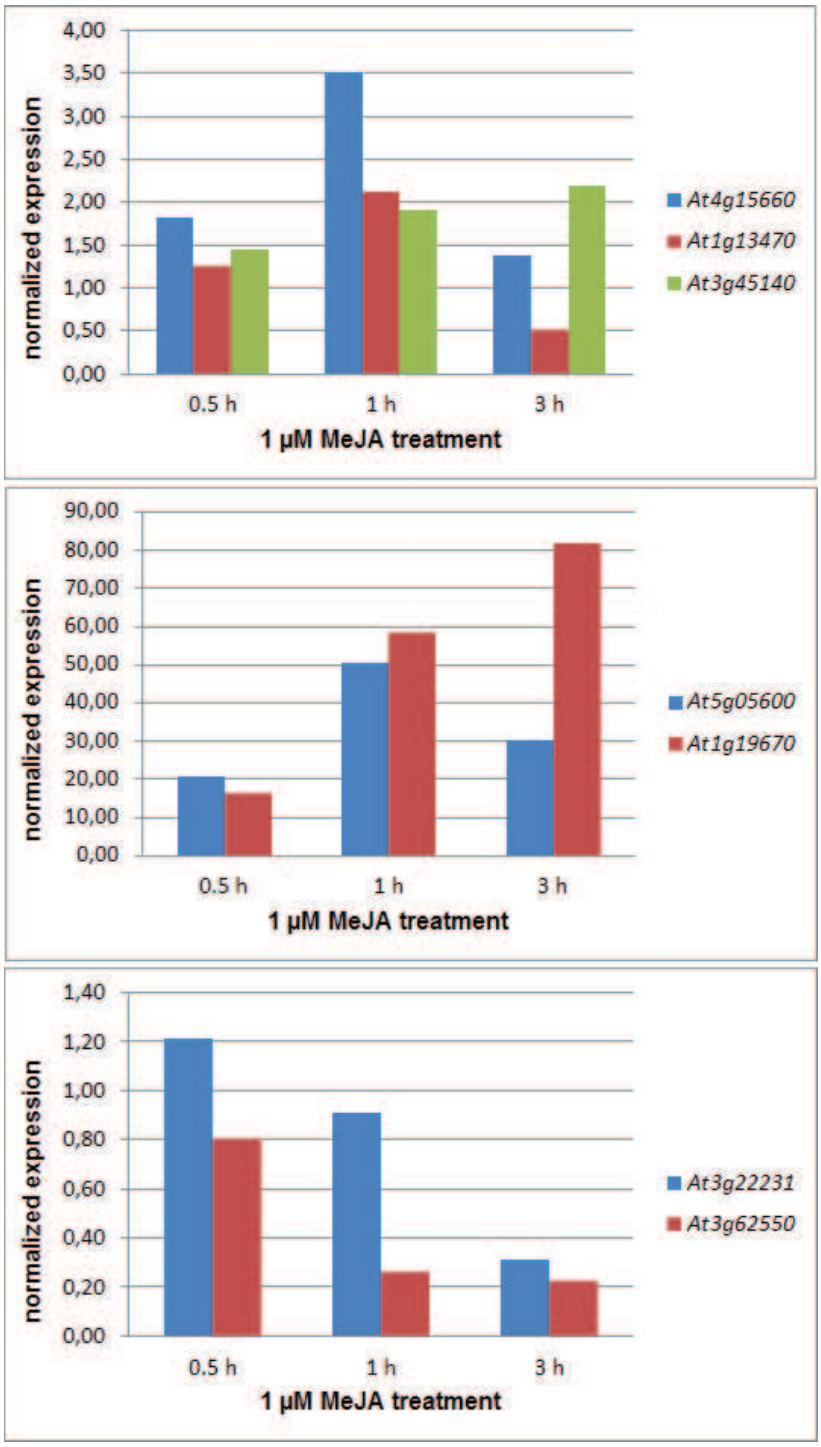


Figure 7. Jasmonate inductivity of common targets genes for TCP4 and TCP10. *The seven genes that, according to AtGenExpress (Goda et al., 2008), react to jasmonate induction are divided into (A) slightly up-regulated, (B) highly up-regulated, and (C) down-regulated. All data was normalized to mock treatment at the same time points.*

Chapter 3

Comprehensive target gene identification unveils a role for the Arabidopsis TCP20 transcription factor in jasmonic acid metabolism and leaf development

Selahattin Danisman, Froukje van der Wal, Stefan de Folter, Aalt-Jan van Dijk, Jose M. Muino, Lucas Cutri, Marcelo C. Dornelas, Gerco C. Angenent, and Richard G.H. Immink

submitted to *Plant Physiology*

Abstract

TCP (TEOSINTE BRANCHED1/CYCLOIDEA/PCF1) transcription factors control developmental processes in plants. The 24 TCP transcription factors encoded in the Arabidopsis genome are divided into two classes, class I and class II TCPs, which are proposed to act antagonistically. We identified direct target genes of the Arabidopsis class I TCP20 protein in leaf development based on a glucocorticoid receptor induction assay and genome-wide expression studies. The TCP20 target genes include a significant number of jasmonate synthesis and response genes. Also class II TCP proteins under control of the microRNA *JAGGED AND WAVY* (*JAW*) control genes involved in jasmonate signaling, although in an antagonistic manner. A *tcp20* knockout mutant does not show phenotypical alterations during leaf development, but mutation of *TCP9,* which is a class I *TCP* gene under direct control of TCP20, results in the formation of larger pavement cells during early leaf developmental stages. Altogether these results point to an antagonistic function of class I and class II TCP proteins in the control of leaf development and maturation via the jasmonate signaling pathway.

Introduction

TCPs (TEOSINTE BRANCHED1/CYCLOIDEA/PCF1) are plant specific transcription factors that are involved in growth related processes, such as branching, floral organ morphogenesis, and leaf growth (for review see Martin Trillo et al, 2010). The Arabidopsis genome encodes for 24 *TCP* transcription factor genes, which based on sequence homology are divided into two classes: class I and class II *TCPs.* Functional analysis of the Arabidopsis class II BRANCHED1 (BRC1) and BRC2 proteins, both closely related to the TCP founder protein TEOSINTE BRANCHED1 (TB1) from maize (Doebley et al., 1997), demonstrated that these genes are involved in suppressing axillary bud outgrowth (Aguilar-Martinez et al., 2007). Another subclass of the class II TCPs contains the genes *TCP2, TCP3, TCP4, TCP10* and *TCP24*, which are all targets of the microRNA *miR319a/JAW* (Palatnik et al, 2003). Simultaneous down-regulation of these five TCPs by ectopic expression of *miR319a/JAW* in *jaw*-D plants results in abnormal curvature and excessive growth of leaves. Conversely, expression of a hyper-activated form of *TCP4* from endogenous regulatory sequences results in decreased cell proliferation and smaller leaves (Sarvepalli and Nath, 2011). *JAW*-regulated expression of *TCP4* is also important for proper development of petals and stamens in the Arabidopsis flower, because expression of a *JAW*-resistant *TCP4* under the control of an *APETALA3*-promoter disrupted the development of these flower organs. Likewise, expression of wild type *TCP4* under the same promoter disrupted petal and stamen development only in the background of a *miR319a* knockout (Nag et al., 2009). Down-regulation of the Armadillo BTB Arabidopsis protein (ABAP1), a protein that is associated with TCP24, increases cell division rates, while overexpression resulted in reduced leaf growth (Masuda et al., 2008). Altogether these examples of class II TCP functions suggest that they play a prominent role in suppressing organ growth processes, and recent research suggests that they do this via the control over hormonal pathways (Efroni et al.2008, Schommer et al. 2008, Yanai et al. 2011) (this thesis, Chapter 2).

In contrast to class II TCPs, much less functional information is available for class I TCPs. Two studies focus on the class I gene *TCP20.* Li and colleagues (2005) described a targeted chromatin immunoprecipitation (ChIP) assay, in which TCP20 was found to bind regulatory sequences of *CYCLIN B1* (*CycB1*), *PROLIFERATING CELL NUCLEAR ANTIGEN* (*PCNA*), and ribosomal genes. Based on these targets it was suggested that TCP20 stimulates the cell cycle and growth of organs (Li et al., 2005). Because the *tcp20* mutant does not show an obvious phenotype, Herve and colleagues (2009) used an alternative approach to gain functional information about TCP20. They generated plants ectopically expressing the TCP20 protein, which was tagged with an EAR transcriptional repressor domain. The *35S:TCP20-EAR* construct caused pleiotropic growth effects and genome-wide expression analysis revealed a plethora of genes differentially expressed in the *TCP20-EAR* plants compared to wild type. Remarkably, there is no overlap in target genes identified in the two studies on TCP20 and both approaches lack a comprehensive list of target genes shown to be directly regulated by TCP20.

Not only growth but also other plant processes seem to be regulated by *TCP* genes. *Jaw*-D plants show, apart from their leaf phenotype, leaf-like petals and late entry into leaf senescence

(Schommer et al., 2008). This late senescence behavior of *jaw*-D plants is caused by alteredjasmonic acid levels. Furthermore, it was shown that class II TCPs, specifically TCP4, directly influence jasmonic acid biosynthesis by regulating the expression of *LIPOXYGENASE2* (*LOX2*) (Schommer et al., 2008). LOX2 catalyzes the reaction from α-linoleic acid to 13(S)-hydroperoxylinolenic acid, which represents one of the first steps of jasmonic acid synthesis in plants (Vick and Zimmerman, 1983).
Molecular analyses revealed that the predicted consensus DNA binding sites of the two TCP classes are partly overlapping. The consensus binding site of class I TCPs is GGNCCCAC, which includes the predicted binding site for TCP20 (GCCCR), whereas class II TCPs bind DNA motifs of the sequence GTGGNCCC (Kosugi and Ohashi, 2002; Li et al., 2005). TCPs of the two different classes are believed to share common targets and the regulation of these targets could be the result of competition between inducing and inhibiting TCP activities (Li et al., 2005). Nevertheless, such common target genes for class I and class II TCPs remain to be identified.

Here we report the analysis of TCP20 function(s), using genome-wide expression analysis upon induction of TCP20 protein function to identify its target genes. We identified *LOX2* among other genes as a common target of TCP20 and TCP4, where TCP20 inhibits and TCP4 induces *LOX2* expression. Although we did not observe phenotypic alterations in *tcp20* mutant plants, at least one of its targets, the class I *TCP9* gene, plays a role in cell size determination during early stages of leaf development. Hence, we propose a model in which the class I TCP proteins TCP20 and TCP9 act at least partially antagonistic to the class II *jaw*-TCPs, which via *LOX2* and jasmonate signaling leads to a coordinated balance between cell proliferation and cell maturation during Arabidopsis leaf development.

Material and Methods

Plant material. Seeds of *Arabidopsis thaliana tcp20* knockout plants (SALK_016203.45.25) and *tcp9* knockout plants (SALK_143587.56.00) were obtained from the Nottingham Arabidopsis Stock Center (NASC).

Plant growth and media. Plant material was either grown on rock wool or on Murashige-Skoog (MS) medium, depending on the experimental set-up. When seeds were sown out on MS-medium, they were gas-sterilized first. Seeds were sterilized by placing an open Eppendorf vial containing the seeds into a glass bowl alongside a jar containing 20 mL Bleach. After addition of 3 mL 37% Hydrochloric acid (fuming) to the Bleach, the glass bowl was closed tightly and kept closed for one to two hours. At the end of the sterilization, seeds were taken out swiftly.
For Dexamethasone induction experiments, 50 mL of ½ MS medium (2,3 g/L) with Agar (6 g/L) was poured per plate and, after polymerization, a sterilized nylon mesh (mesh size: 200 µm) was placed on the medium (Passarinho et al., 2008). 30 to 50 seeds of both the GR-line as well as wild type Columbia-0 were sown out per plate.

Constructs. For the glucocorticoid induction experiments, the *TCP20* coding sequence without stop codon was amplified using the primers (5'-ATGGATCCCAAGAACCTAAATCGT-3') and (5'-ACGACCTGAGCCTTGAGAATC-3') and cloned into pCR8/GW/TOPO to obtain a GATEWAY entry vector. A GATEWAY destination vector suitable for the expression of genes of interest fused to the coding region of the rat Glucocorticoid Receptor (GR) domain was obtained by removing the *AGL11* coding region from vector NOB221 (Kindly provided by Martin Kater). For this purpose the BamHI and NcoI restriction enzymes were used. Subsequently, the digested vector was blunted, followed by introduction of the GATEWAY conversion cassette (Invitrogen, Carlsbad, CA, USA) upstream of the GR coding region and downstream of the CaMV35S promoter. This complete expression cassette was cloned as an AscI/PacI fragment into the binary vector pGD121 (de Folter et al., 2006), resulting in the GATEWAY compatible GR destination vector pARC146. As we wanted to introduce the final *TCP20-GR* construct into a *tcp20* SALK-line, we ensured antibiotic selection by replacing the Kanamycin-resistance cassette of pARC146 with a BASTA-resistance cassette taken out of pB7WG2 (Karimi et al., 2002). In a first step, pARC146 was partly digested with HindIII and a 4.4 kb fragment containing the GR-GATEWAY cassette was recovered. The plasmid pB7WG2 was cut with KpnI and a 7 kb fragment containing the BASTA-resistance cassette was ligated with the 4.4 kb fragment recovered from pARC146, resulting in CZN671. Subsequently, the *TCP20-GR* expression vector was generated by an LR reaction between CZN671 and the before mentioned *TCP20* entry clone.
To obtain a vector for GREEN FLUORESCENT PROTEIN (GFP)-tagged *TCP20* expressed at endogenous levels a *TCP20* genomic fragment was cloned. The genomic fragment was amplified up to the stop codon and including 2466 bp of promoter sequence (primers used were 5'-CACCCTATGATGCATGCCACTCTCG-3' and 5'- ACGACCTGAGCCTTGAGAATC-3') , and cloned into the GFP GATEWAY destination vector pMDC204 (Curtis and Grossniklaus, 2003). This resulted in the expression vector *gTCP20-GFP* (CZN064).

Transformation of Arabidopsis. The *35S:TCP20-GR* (CZN652) and *gTCP20-GFP* (CZN064) constructs were transformed into homozygous *tcp20* knockout plants. For transformation, Arabidopsis was grown on soil till the primary inflorescences emerged. These were cut to promote growth of secondary inflorescences and to increase the number of flowers. The binary constructs were transformed into *Agrobacterium tumefaciens* strain C58C1 (pMP90). Transformation of plants was conducted by floral dip (Clough and Bent, 1998). After transformation plants were kept in growth chambers till seed-set. Seeds were selected on ½MS + Agar (8 g/L) plates containing 25 µg/mL Phospinotricine (PPT/Basta) for the plants expressing the *TCP20-GR* construct, and 30 µg/mL Hygromycin for the TCP20-GFP transformants. After two weeks rooting green seedlings were transferred to soil and grown till seed set. The following T2 generation was checked for expression of the transgene by RT-PCR and in case of TCP20-GFP by Confocal Laser Scanning Microscopy (CLSM).

Confocal Imaging. CLSM of living plant tissue was conducted with a Leica SPE DM5500 upright microscope, using Leica AF 1.8.2 software (Leica, http://leica-microsystems.com). Preparation of inflorescences was done as described before (de Folter et al., 2007).

Scanning Electron Microscopy (SEM). Whole 9-day old seedlings were fixed in 4% paraformaldehyde under vacuum for 24 h and dehydrated through an ethanol series dried under CO2 in a Balzer´s critical point drier, mounted in metallic stubs with carbon conductive adhesive tape, coated with colloidal gold and observed at 20kV using a LEO 435 VP scanning electron microscope, at the University of Sao Paulo (ESALQ-NAP/MEPA). Cell drawings were based on SEM pictures and were analyzed using ImageJ software (http://rsbweb.nih.gov/ij/index.html) and STATISTICA 6.0 (StatSoft, 2001).

Induction experiments. Glucocorticoid induction experiments were conducted 14 days after germination of the plants. Growing the plants on nylon meshes allowed us to transfer the plants into induction media swiftly and without damaging the roots. The induction medium consisted of 2,3 g/L MS, 1% (w/v) sugar, 10 µM Dexamethasone, and 10 µM Cycloheximide (Passarinho et al., 2008). Samples were harvested before treatment and at different time points after start of the treatment.

RNA isolation and qRT-PCR. RNA was isolated using the QIAGEN RNeasy RNA isolation kit according to the manufacturer's protocol. DNase treatment took place on-column, following the protocols from the manufacturer. M-MuLV Reverse Transcriptase (RT) from Promega was used for cDNA synthesis. First, a mix of poly-dT primer and dNTPs was added to 500 ng DNA-free RNA in a volume of 12 µL. This solution was kept in ambient temperature for 2 minutes before 1 µL RT was added. After addition of the RT, samples were incubated at 25 degrees Celsius for 15 minutes and transferred to 42 degrees Celsius for 50 minutes. Here, reverse transcription took place and was stopped by heat treatment at 70 degrees Celsius for 15 minutes. The cDNA made this way was used for quantitative Realtime PCR (qRT-PCR) using the SYBR green mix from BioRad. The reference genes used for all analyses were a SAND family gene *AT2G28390* and the TIP41-like gene *AT4G34270,* both determined superior reference genes (Czechowski et al., 2005). The primers used are given in Table 1.

Microarray analysis. DNA-free RNA from the induction assays was analysed using Affymetrix Tiling 1.0R arrays. The expression data from Tiling 1.0R arrays were pre-processed using the RMA algorithm (Irizarry et al., 2003). The probe annotation was obtained from athtiling1.0rcdf (Naouar et al., 2009) , it contains only probes derived from TAIR7 genes, probes representing non-unique sequence were masked, only probes that are common to all described variants of a transcript were considered, probes representing intronic regions, or regions spanning intron/exon junction were removed. T-test assuming equal variance were sued to test significance. FDR was controlled using the BH method (Benjamini and Hochberg, 1995). Differential regulation of

genes was determined by substracting the log2 expression values of induced versus uninduced samples in the first step, and substracting the resulting values of wild type seedlings from the TCP20-GR seedlings' log expression values. The obtained differential expression values represented the difference of target gene activation or repression between TCP20-GR and wild type plants, respectively. Differentially regulated target genes were those that showed a differential expression value of more than +2 or less than -2. Only genes that were found differentially regulated in both biological replicates were taken up into the target gene list. The microarray data was deposited at the Gene Expression Omnibus (GEO) under the number GSE29012.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) experiments mainly followed the protocol described previously (de Folter et al., 2007; Kaufmann et al., 2010). TCP20-GFP seedlings were grown in liquid ½ MS medium on a horizontal shaker (30rpm) for 4 days in a 16h light-8 hour dark regime. After this time, 3 g seedling material was harvested by pouring the liquid MS medium through a sieve and then the seedlings were fixated with 37% formaldehyde. Immunoprecipitation was conducted using a GFP antibody coupled to magnetic beads. The magnetic beads were used to precipitate the antibody-protein-GFP complexes. The enrichment of TCP20 binding regions was compared between the immunoprecipitate and 1:1000 diluted input material. Promoter elements that were not expected to be bound by TCP20 were used as negative control.

MEME analysis of over-represented motifs. MEME (Bailey et al., 2006) was applied to perform motif discovery with the set of TCP20 target genes obtained in 14 days old seedlings as input. For this, the 1kb upstream sequence according to TAIR9 was used (note that 1000 nt is the recommended upper length limit for MEME input) and statistically significant motifs in those sequences were obtained with MEME. Here, a motif is a sequence pattern that occurs repeatedly in a group of related protein or DNA sequences. MEME represents motifs as position-dependent letter-probability matrices which describe the probability of each possible letter at each position in the pattern. We searched for motifs in those sequences until no additional significant motifs were obtained, using default MEME parameters otherwise. This resulted in the identification of 20 motifs. MAST (Bailey et al., 2006) was applied to obtain motif occurrences.

Yeast one-hybrid analysis. Binding of TCP4 and TCP20 to promoter sequences was analyzed in a yeast-one hybrid system based on the Matchmaker™ Gold Yeast Two-Hybrid System (http://www.clontech.com). In this system, binding events are detected by resistance against the antibiotic Aureobasidin A. The yeast strains used for this analysis were PJ69-4A for the TCP dimer collection and PJ69-α for the promoter reporter construct. The reporter construct is based on the plasmid pAbAi (http://www.clontech.com). This plasmid was made GATEWAY compatible by ligating a GATEWAY-C cassette into the SmaI-site, resulting in CZN1018. The reporter

construct for the *LOX2* promoter included a 2,006 bp fragment upstream of the transcriptional start site. Autoactivation tests for the promoter were conducted in the range from 0 to 500 ng/mL Aureobasidin A. For the *LOX2* promoter, autoactivation was detected up to 100 ng/mL. For the screen with TCP dimers, an Aureobasidin A concentration of 135 ng/mL was used. After growing the yeast for 2-3 days on selective medium in 20 degrees Celsius, mating of TCP dimers and reporter constructs was initialized on SD complete medium over night. The mated yeast was transferred onto selection medium selecting for both vectors of the yeast assay. After having grown for 2-3 days, yeast able to grow on the selective medium was transferred to Aureobasidin containing plates. Plates were incubated at 20° Celsius and scored after 5-7 days.

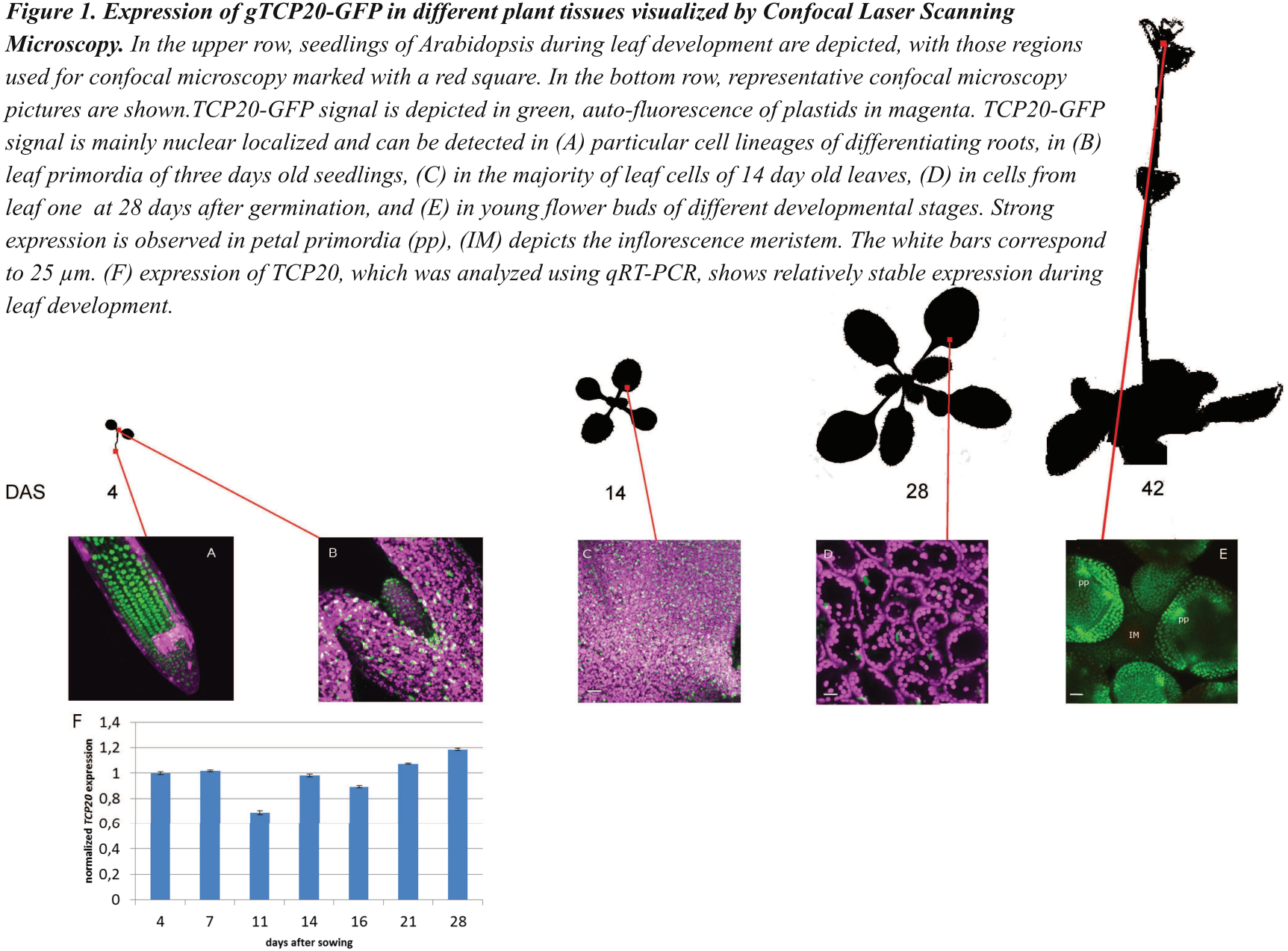
Methyljasmonate treatment. Plants were treated with 1µM MeJA dissolved in water with 0.1% Tween-20, whereas the control treatment only included water with 0.1% Tween-20. The solutions were applied by spraying the plants until they were dripping wet. The two treatments were kept apart because of the high volatility of methyl-jasmonate. Samples were taken just before, 15 minutes after and 6 hours after treatment.

Results

TCP20 is expressed throughout Arabidopsis development

Plants expressing a genomic version of *TCP20* tagged with a GREEN FLUORESCENT PROTEIN encoding sequence (gTCP20-GFP) were analyzed at different time points after germination to obtain detailed information about the temporal and spatial expression pattern of TCP20. Fluorescence was detected by confocal laser scanning microscopy (CLSM) as shown in Figure 1. Expression was observed in a broad number of organs, like young leaves, mature leaves, differentiating root cells and young floral buds (Figure 1A-E). In agreement with its function as transcription factor, TCP20-GFP signal was strongest in nuclei. Strong TCP20-GFP signal could be detected in cells of the differentiating root, in particular in cortex cells (Fig. 1A). In developing leaves, we detected TCP20-GFP signal from the first leaf primordia of seedlings three days after germination (Figure 1B), and in the first leaf of two weeks (Figure 1C) and up to four weeks old plants (Figure 1D). We also detected TCP20-GFP during floral development in all cells of young floral buds (Figure 1E); however, no signal was obtained in the inflorescence meristem. In stage 3/4 flower buds, TCP20 expression is strong and peaks in the early petal primordia, suggesting a function for TCP20 in petal initiation. RT-PCR analyses of wild type plants at different stages of leaf development verified the observation that *TCP20* is expressed throughout leaf development (Figure 1F). As we were interested in the role of TCP20 in leaf development, all further experiments were conducted on seedlings and leaves during vegetative development.

Figure 1. Expression of gTCP20-GFP in different plant tissues visualized by Confocal Laser Scanning Microscopy. *In the upper row, seedlings of Arabidopsis during leaf development are depicted, with those regions used for confocal microscopy marked with a red square. In the bottom row, representative confocal microscopy pictures are shown.TCP20-GFP signal is depicted in green, auto-fluorescence of plastids in magenta. TCP20-GFP signal is mainly nuclear localized and can be detected in (A) particular cell lineages of differentiating roots, in (B) leaf primordia of three days old seedlings, (C) in the majority of leaf cells of 14 day old leaves, (D) in cells from leaf one at 28 days after germination, and (E) in young flower buds of different developmental stages. Strong expression is observed in petal primordia (pp), (IM) depicts the inflorescence meristem. The white bars correspond to 25 µm. (F) expression of TCP20, which was analyzed using qRT-PCR, shows relatively stable expression during leaf development.*



TCP20 function during leaf development

For as far analyzed, mutants of class I TCPs show few phenotypic alterations, and for *TCP20* no mutant phenotypes have been reported in previous studies (Li et al., 2005). We analyzed a *tcp20* T-DNA insertion line (Figure 2) focusing on leaf development using the LeafAnalyser technology (Weight et al., 2008). Also here leaves did not show any obvious growth alterations, possibly because of functional redundancy with other class I TCPs. Subsequently, we decided to screen for genes under direct control of TCP20 to obtain insight into its molecular function. A glucocorticoid inducible system (Aoyama and Chua, 1997) was applied to identify direct targets of TCP20 in leaves. The coding region of TCP20 without stop codon was fused at the 3'-end to a sequence encoding the glucocorticoid receptor (GR) and placed under control of a constitutive CaMV35S promoter. This *35S:TCP20-GR* construct was introduced into *tcp20* knockout plants to avoid competition in target gene binding between endogenous TCP20 and the TCP20-GR protein. Dexamethasone (DEX) induction was performed on 14 days old seedlings in the presence of the protein synthesis blocker Cycloheximide (CYC). All above-ground parts of 20 seedlings per biological replicate were harvested just before and eight hours after DEX induction. As a control, wild type plants of the same age were treated the same way and up- and down-regulated genes were identified making use of Arabidopsis whole-genome tiling arrays. As the variance between biological replicates was high, we decided to analyze expression changes only, and subsequently confirm potential target genes by RT-PCR and ChIP analyses. Expression changes between the two time points were determined for both TCP20-GR and wild type plants. Genes with an expression difference of Log2 between TCP20-GR and wild type samples were considered differentially expressed. Because the protein synthesis inhibitor cycloheximide (CYC) has been used in the treatments, we expect a strong enrichment for direct targets of TCP20 among the differentially expressed genes. In this way we defined a list of 278 potential direct target genes of TCP20 (Appendix II).

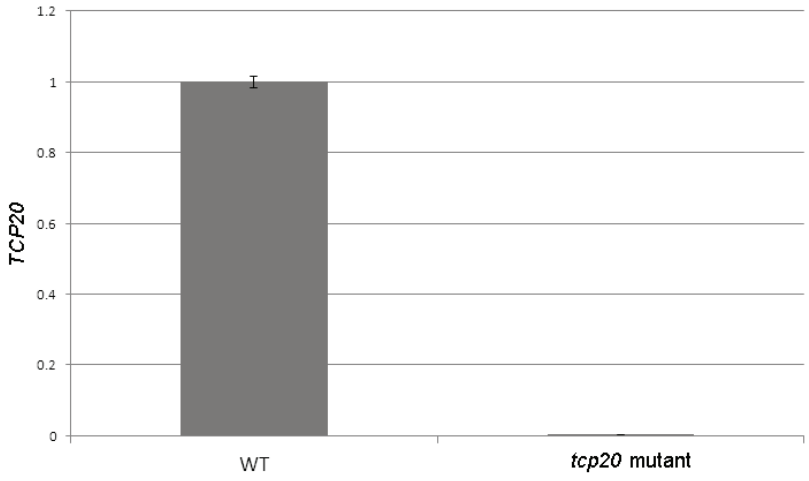


Figure 2. TCP20 expression in tcp20 knockout plants.
Depicted is a qRT-PCR analysis of TCP20 expression in tcp20 knockout mutants vs wild type Col-0 plants. TCP20 is strongly downregulated in tcp20 knockout plants.

Quantitative Realtime PCR (RT-PCR) was used to validate the microarray experiment. We selected seven of the 278 identified putative targets and performed RT-PCR. Alongside these genes, which were up- or down-regulated in the microarray experiment, we selected four additional genes which were categorized as non-regulated by TCP20 according to the microarray experiment. The RT-PCR data showed strong correlation (R^2 =0.87) between the RT-PCR and microarray data for the chosen genes (Figure 3). Furthermore, we noticed a more pronounced expression difference between wild type and TCP20-GR plants in the RT-PCR assay, revealing that the selected cut-off for the micro-array data leads to the identification of genes with a substantial and distinct expression difference, being good candidates for direct TCP20 targets.

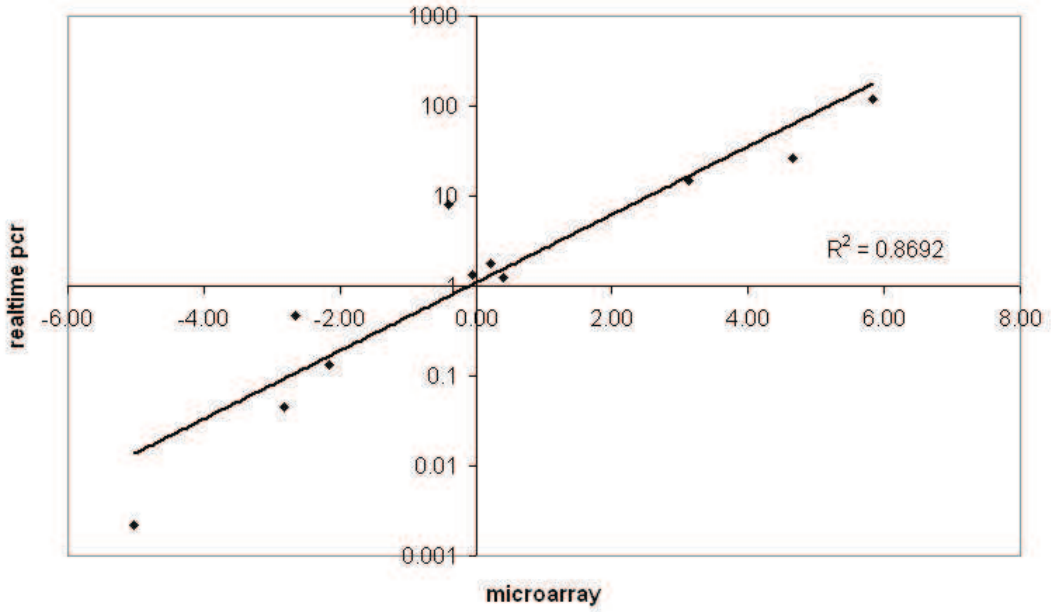


Figure 3. Verification of microarray analysis. *TCP20-GR microarray results were plotted against the RT-PCR data for 11 chosen genes. The two data sets have a correlation of R^2=0.87.*

We further verified our dataset by comparing the direct target gene list with the steady state differences between wild type and *tcp20* knockout plants. For this we used the non-induced wild type and TCP20-GR microarray data because the TCP20-GR construct was transformed into the homozygous *tcp20* mutant and hence, non-induced TCP20-GR plants resemble *tcp20* knockout plants. Genes that are up-regulated in the GR-induction experiments were expected to be down-regulated in the *tcp20* knockout plants in comparison to the wild type situation. Of the 278 genes differentially regulated in the GR induction experiment, 114 were differentially regulated in knockout plants. Of these 114 genes, all genes that were up-regulated in the GR induction experiment were down-regulated in *tcp20* knockout plants, and vice versa. This finding indicates that the expression of a significant fraction of all TCP20 target genes found in the GR analysis is directly dependent on TCP20 absence or presence. Subsequently, we compared the putative target gene list with those reported in previous studies (Li et al., 2005; Herve et al., 2009), but could not find any overlap. This was not surprising; different plant growth conditions were applied, different tissues were sampled, and material was harvested at different time points during

development. For the GR experiments reported here, leaves were treated for a short time period with DEX and CYC, which is expected to result in enrichment for direct TCP20 targets. In contrast, Herve and colleagues analyzed roots and hypocotyls after longer induction times of TCP20 tagged with the EAR transcriptional repressor domain, which most likely results in the identification of numerous indirect and root-specific targets (Herve et al., 2009).
As a next step we identified potential pathways under the control of TCP20 by analyzing the target gene list for over-represented gene ontology categories. This was done with the Cytoscape BiNGO tool (Maere et al., 2005). Overrepresented genome annotations include responses to stimuli, lipid metabolism, chromatin organization, and nicotianamine synthesis. Interestingly, closer analysis of the BiNGO data revealed, amongst others, genes for jasmonic acid synthesis (GO-ID 9695) and genes responding to jasmonic acid stimuli (GO-ID 9753) to be significantly overrepresented. One gene of specific interest was *LIPOXYGENASE2 (LOX2)*, as it had been shown to be controlled by class II TCPs from the CIN-clade (amongst others, TCP4) in earlier publications (Schommer et al., 2008) (chapter 2, this thesis). *LOX2* is an important jasmonic acid synthesis gene (Creelman and Mullet, 1997), which is down-regulated by TCP20 after eight hours of induction by DEX. We assessed the dynamics of TCP20-mediated repression of this gene by using shorter induction periods. For this, we induced 14 days old TCP20-GR and wild type plants, and harvested material after two and four hours of DEX treatment. Using three pools of 20 plants for every line and time point, we found that *LOX2* was down-regulated already two hours after induction (Figure 4A), which strongly suggests that *LOX2* is a direct target of TCP20 regulation.

Do TCP20 and TCP4 compete for the control of LOX2 expression?
To answer this question we first analyzed binding of the individual TCP4 and TCP20 proteins to a 2 kb *LOX2* promoter fragment in a yeast-one hybrid assay. Binding could be detected for TCP4 (Figure 4B), as was expected based on previous studies (Schommer et al., 2008; Aggarwal et al., 2010); though, no binding was found for the single TCP20 protein. Because TCPs have been shown to bind DNA as dimers (Cubas et al., 1999; Aggarwal et al., 2010), TCP20 may need a dimerization partner to bind to the *LOX2* promoter. From previous yeast-two hybrid studies we know that TCP20 forms heterodimers with the proteins TCP6, TCP7, TCP8 TCP14, TCP15, TCP21, TCP22 and TCP23 (chapter 2, this thesis). We do not know if TCP20 forms homodimers because of auto-activation capacity for TCP20 when cloned in the GAL4-BD vector. Also dimerization between TCP20 and the CIN-clade TCP proteins such as TCP4 could not be tested because of the auto-activation by the latter proteins. We analyzed the binding capacities of the identified TCP20 dimers to the *LOX2* promoter in a yeast one-hybrid assay, revealing that six out of the eight TCP20 dimers have some binding capacity to *pLOX2,* with TCP20-TCP22 as strongest binder (Figure 4B).
The *LOX2* promoter contains both putative class II TCP and class I TCP binding sites as depicted in Figure 4C and the yeast one-hybrid analyses revealed possible binding by both TCP4 and TCP20, though for the latter protein only as heterodimer. We then applied a MEME search to perform *de novo* motif discovery for TCP20 binding, using the target gene list for 2 week old

seedlings and the TAIR9 1000bp upstream sequences (Table 2). Of the 20 statistically significant motifs we obtained, three were represented in the 1000 bp upstream *LOX2* promoter. Motif 16 (TGGGCC), which is closest to the putative TCP20 binding sites in literature (Kosugi and Ohashi, 2002; Li et al., 2005), was not found in the 1000 bp upstream region but 2.8 kb upstream of the transcriptional start site. We then used Motif 16 and the three potential binding motifs of TCP20 in the 1kb upstream region of the *LOX2* promoter to perform a targeted chromatin immunoprecipitation assay (ChIP). We analyzed precipitated material from five biological replicates of gTCP20-GFP plants for enrichment of the putative binding sites, using GFP antibodies coupled to magnetic beads for precipitation (de Folter et al., 2007; Kaufmann et al., 2010). The precipitated material was analyzed in a qRT-PCR experiment (primer are given in Table 1). As a negative control we used three sites in the *LOX2* promoter that are 5' upstream of the putative TCP binding sites and additionally the promoter of the SAND family gene *AT2G28390,* which was not identified by us or in any of the published TCP20 target gene studies. Significant enrichment and hence TCP20 binding could be shown for motif 16 in the *LOX2* promoter (ANOVA, p=0.025) (Figure 4D). This motif has not been shown to be bound by TCP4 in earlier studies, and hence the direct competition for a binding site between class I and class II TCPs that has been suggested in earlier studies (Li et al. 2005) could not be verified.

Interrelation between TCP4 and TCP20

The expression of *LOX2* is under antagonistic control of TCP4 and TCP20. *LOX2* expression itself changes strongly during leaf development; whereas it is lowly expressed in early leaf development, it shows a very high expression during late leaf development, when the leaf is going through senescence. We hypnotized that the antagonistic regulation of *LOX2* expression by two TCP transcription factors may be reflected in the expression patterns of the two transcription factors, and the relationship of the two expression patterns to each other. In short, are TCP20 and TCP4, who work antagonistically, also expressed antagonistically? We analyzed this possibility by correlating *TCP4* to *TCP20* expression during Arabidopsis development. For this, we used data from AtGenExpress (Goda et al., 2008) and analyzed correlation using STATISTICA 6.0. The analysis showed that *TCP4* and *TCP20* expression are negatively correlated (R= -0.41, p=0.02) (Figure 5A). Neither in published work on TCP4 nor in our TCP20-GR data set there is any indication that the two transcription factors directly influence each other's expression, however, an indirect dependency between the two genes is conceivable. We suggest that the agent connecting *TCP4* and *TCP20* expression may be jasmonate. Although *TCP4* seems not to react on jasmonate treatment, *TCP20* does. *TCP20* expression decreases after 6 hours of methyl-jasmonate treatment, reflecting a long-term response to methyl-jasmonate (Figure 5B-D). The decrease of *TCP20* expression after jasmonate treatment provides a possible link explaining the interdependency of *TCP4* and *TCP20* expression.

Table 1: *Primers used for both qRT-PCR analyses of transcripts differentially regulated in TCP20-GR induction experiments, and for the analysis of TCP20-ChIP binding to the LOX2 promoter. Given are forward and reverse primers for all genes and fragments analysed. In the case of the primers used for ChIP-RT-PCR analyses, position of the binding site and length of fragment analysed are given.*

Promoter fragment	Product length (bp)	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
pLOX2neg-2400	157	CTCATATATCAAAGGAATTTACAATAT	GAATATTTAACCCAATTTCGAATTCA
pLOX2neg-1600	142	GCAGGTAATGTGGGAGATTAAAA	CTCATCTTATAAATATATAGAGAGGT
pLOX2neg-800	117	GCATTTCGTTTTTCCATTTACTTAA	CTTTCTTCATACTTAAAACTCATAAC
Motif 16	142	CATGAGATGTTTTAACCGTTCTAAGT	ATTAACAATTGTTTGGCTATATACTCC
Motif 1	131	CCCACTTTTCTCAAGAAGATCATT	CTCAACATACCATTTTATCACGT
Motif 2	135	CGTAACTCTTGGATATTTTTAAAC	ATGAAGTGGATATGAGAGGA
TCP4 BS1	96	TGTTACACATTACTTGATGTTTTC	TTCTGCGCTTTGTTGTCTAAATAG
TCP4 BS2	93	CCACCTAAAACTAGAGGATATGATTTCT	ATGGCCGGTTCCTCCAAAACTAG
pTCP9	63	TTGGGCCTAGCCCTCAACAAT	GTCCAGCTCAGGCCCACATT
TCP4 BS3 + motif 3	99	GATGAAAAGAGCTGCATTCT	CATCACATGCAATATCGGGTAAGGTCA
AGI	**Common gene name**	**Forward primer (5'→ 3')**	**Reverse primer (5'→ 3')**
At1g19640	JMT	GTAACATAATATCTCTAGGCAGAAGA	CTATGTTGGAGATGGACAAGAGACT
At1g32640	MYC2	CTCCAAATCAAGAACCAGCTCGAGGAA	CCAACCAATTATCTTCACTTCAATCT
At1g76680	OPR1	GCTATCTGATTGACCAGTTCATGAA	CTTAGCAACTGCATCGACTATTTCTA
At2G28390	SAND-family protein	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC
At2g44810	DAD1	GCCACGCTGACTCATCTCCCTAAT	CCATGTCTCTCAAACTGTGAACACCT
At2g46370	JAR1	CAGAGATCAAGTTCAATACGTCTTT	GATGGGACAGTAATACGGTTGCTTA
At3g15030	TCP4	TTCGGAAGGATTCAGAGACTAGTGG	AGGAGTAGGAGGAGCGAACAGAAAC
At3g25760	AOC1	GAAGAAAGGTGAAAGATTCGAAGCTA	CTGTTGAAGCTTGACCTGTCCGTA
At3g27010	TCP20	TTAGGCTTGTCTCAAGAAGGGAATG	GCATATGGTGAAGAACCCTACCTTG
At3g45140	LOX2	GATGCCCCAGTTCTCATTAACAGGG	CGGGTCTAGTTTGCTTATTAACGGC
At2g45680	TCP9	TGCCGTCGTTTTCAATGTCTTTAGC	TTCTCCCTCTTCCTTCAACCTTCG
At4G34270	TIP41-like protein	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA

Table 2: *Results of the MEME-analysis based on the TCP20 target gene list. Given are motif numbers, sequence of the motif as MEME-output, the width of the motif, the number of occurences in the target gene list, the log-likelihood ratio, and the statistical significance of the motif.*

motif	regular expression	width	sites	llr	e-value
1	[CGA]A[AG][AG][AG]AAAAA[AG]A[GA]AA[AG]AA[AG]A[AG]	21	150	1637	3,50E-107
2	[TG]TTTT[TC]TT[TC]TTTT[TG]T	15	198	1826	3,90E-73
3	[TA][AT][TC]AT[AG]T[AG][TC]ATATATATATATATATATA[TC][AT][TC]	29	31	529	6,00E-31
4	[CGTAA][TA][TAC][ATG][TA][CT][TA][CTG][CTG][TA][TCA][CT]T[TC][TC][CT]TCT[CT][TC][TC][TC][CT]TC[TC][TC][TC]	29	85	996	1,10E-25
5	[GCT]G[GAT][CA][GTC][GTC][CTA]G[GA][CAT][GT][GA][ATC][GAT][CTA][TAC][ACGTG][GA][GC][GA]G[AC][AGT][GC][AT][GA][GA][ATC][GT][GA][GAC][AG][AG][CGA][GAT][GCA][AC][AG][GAC][CGT]G	41	54	775	6,20E-24
6	[CG]A[AC]AA[AGC]AAAAAAAAA	15	89	921	1,60E-15
7	[TG]CCACGT[GC]	8	44	479	3,90E-08
8	G[TG][GA][GTC][GC][AT]C[ACG][CTG][AG][CGT][GAC][CT][TC][TAG][GAC][CAG][CAG]C[AG][TC]G[TC][GTC][TG][CT][AGC]A[CT][CG][TC][AG][CTAGA][ACG][CGT][CG][GC][TC][CG][TA][CG]	41	15	328	2,10E-03
9	[AG]AT[GA]T[GT][CG][ATC][TAG]G[TG][TA][GA]G[ACG][AC][AT][CTG][AG][AC]A[AT]T[ACGTC][AG][AC][ACT][GAC][ACGTC]T[AT][GA][AT][AG][TC][TG][TA][TCG][TC][GC][ACT][ACG]G[GT][GT][AGT][CG][CA][GT]G	50	5	209	1,50E-02
10	[AG][GA][CG]CC[AC][AT][TA][TAC]A[AT]GGCCCA[TG][AT]A[AC]	21	17	268	1,60E-01
11	G[AT]CA[TG][CT][TA]G[AG][GT][TG][GC][GC][CGT][TC]C[CA][TA]T[TC]T[CA]ACT[GC]C[TG]A[GA][GA][CA]CC[ACT][TG][TC]GT[GC][GC][TA][GT]G[CT][GC]TC[CGT][CG]	50	3	162	1,20E+00
12	[CA][CA][GT][CA][CG]G[CT]T[AT][CG]T[AT][CT]A[AT][AT]TTCA[TA][TC][AC]GAC[AT]GAGTCAT[TC]T[TG][CT][AC]AA[CT]TT[GT][TG]A[AG]AC	50	6	231	3,10E-03
13	[CT]T[CG]GTG[AG]A[TACGA]C[AT]C[GA][AC][GT]	15	17	244	3,90E-02
14	[GT][AG][AG][AG][CG]G[AT][GC]A[GAT]C[TG][TC][CTG][GCT][ACG][CAT][CGT]C[TG][AT][GC][AC][AG][AT][GC][AG][GT][ACG][GT][TA]G[AC][GCT][ACG][GAT][GT][TA][ACT][TC][CG][CG][CA][CG][GCT][CGT][CG][GA][GC][CG]	50	8	254	3,80E-01
15	[GA][AT]GA[GA]A[GT][AG][GA]A[GA]A[AG][AG][GA][TA][TG][GA][AT]GG	21	27	377	1,20E+00
16	TGGGCC	6	40	410	1,20E+02
17	[CT][AC][ACT][TC][CG][ATG][CA][TCA][CT][ACT][CT][TC]C[ATG][AT]C[AGT][TA]T[CTG][CT]T[CA][TC][AT][CGT]A[AT][AC]T[AC][TCG][CA]CA[TG][CT]TC[ACT][AC][CAT][TC][CT][CT]	45	10	263	8,50E+01
18	[GA][AT][CAG][GC]AT[GC]TT[AT][AC][ACG][GT][CT][AC][TG][TA][AT][GC][AT][TA][GC][TA]CG[AG][ACT][AG][ACGTA][ACT]G[GAT][AG][AG][GC][CT][TA][TG]C[AC][AT][GT][GA]T[CG][GT]T	47	8	229	1,80E+03
19	[TG]T[TC][CTA][ACT][TCG]TTG[AG][ATC]G[AG]A[GT][AT][GTA][GAT][TC][CA][CA][AC][GT]CC[CGT]A[AC][CAG][AG][GT][AC][CA][TA][TA][ACT][GA][ACG][AC][AC][CT][CT][AC]A[CT][AC]C[CG]TC	50	8	245	1,20E+03
20	[GA][AGT]TCAACAAC[CG]AC[GT][GAT]G[CG][CT][AC][CGA]A	21	11	197	5,20E+01

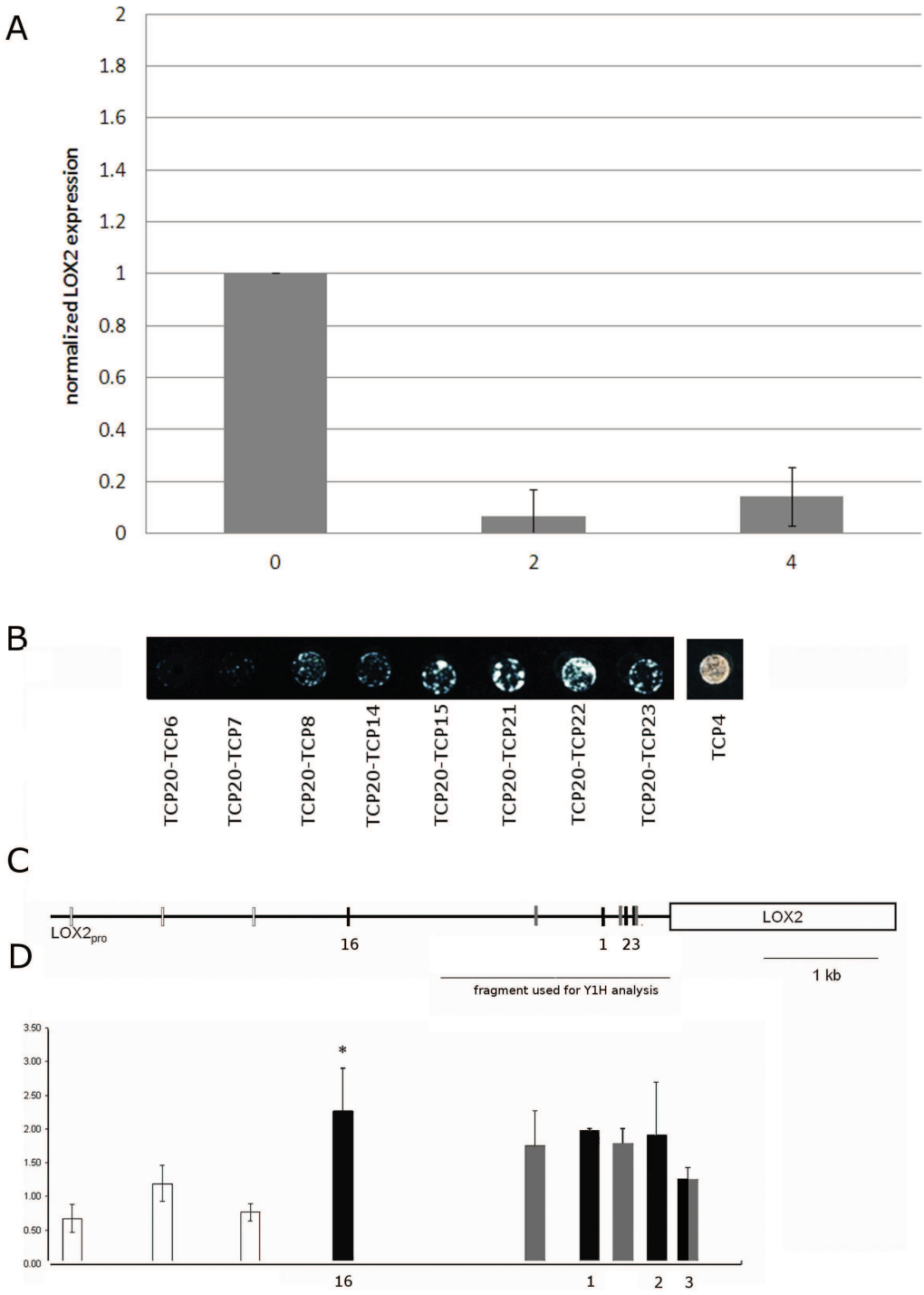


Figure 4. Direct control of LOX2 expression by TCP20. *(A) Realtime RT-PCR analysis of the inhibition of LOX2 expression in TCP20-GR; samples were taken 2 hours and 4 hours after induction with Dexamethasone (DEX) and Cycloheximide (CYC).*
(B) Schematic representation of putative TCP binding sites in the promoter of LOX2. The representation features 5,5 kbp upstream of the first LOX2 exon. The region includes six putative TCP binding sites. Class II TCP binding sites as defined in earlier publications (Schommer et al., 2008) are depicted in grey, putative TCP20 binding sites in black, the negative controls are depicted in white. (C) Realtime RT-PCR of TCP20-GFP ChIP analyses in which the 1000 times diluted input DNA sample is used as a control. Motif 3 could not be detected independent from the neighbouring TCP4 binding site. Binding site enrichment for motif 16 was shown to be significant (ANOVA with subsequent Dunnet-test, p=0.025).

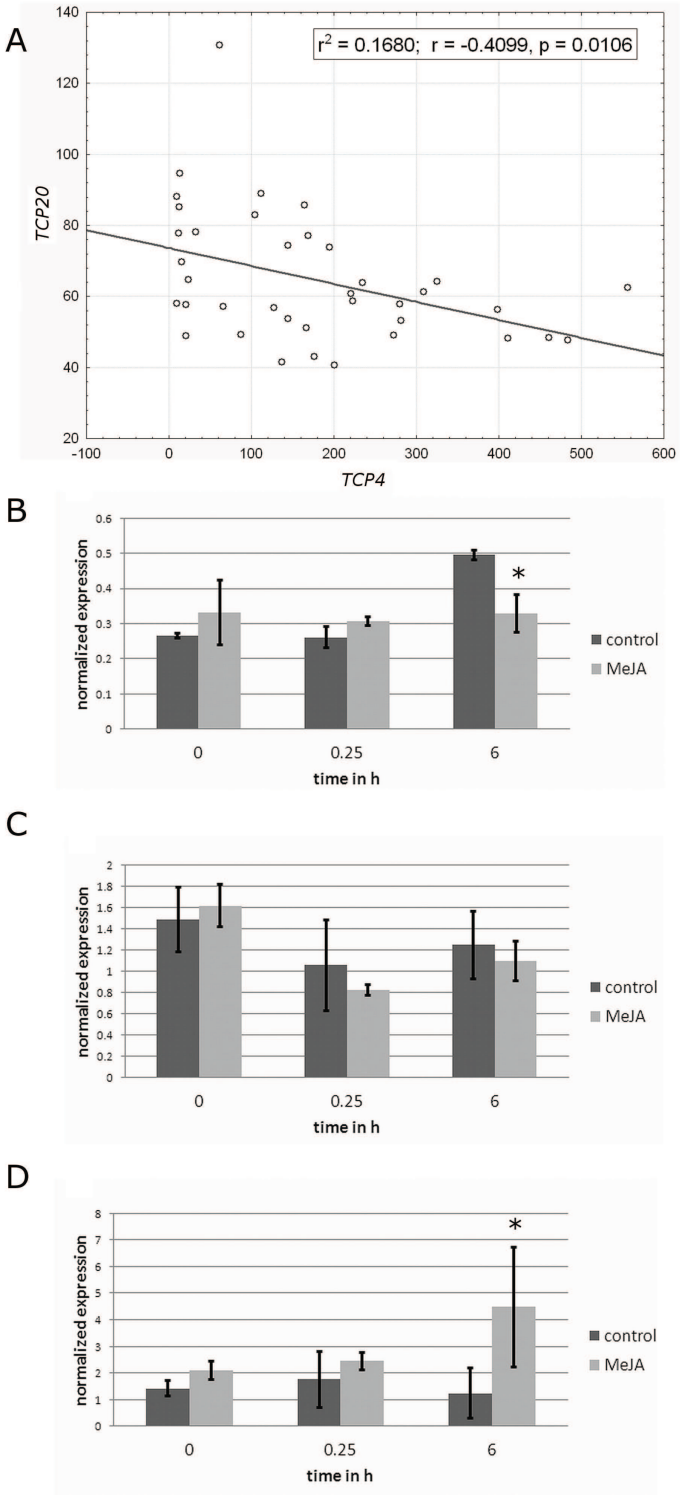


Figure 5. Relation between TCP4 and TCP20 during Arabidopsis development. *(A) statistical analysis of the correlation of TCP20 to TCP4 expressions during Arabidopsis development. Indicated on the graph are correlation coefficient (-0.42) and p-value (0.02). (B-D) Responsiveness of TCP20, TCP4, and LOX2 to MeJA treatment. Wild type plants were treated with 1 mM methyl-jasmonate and samples were taken just before, and 15 minutes and six hours after treatment. Response of (B) TCP20, (C) TCP4 and (D) LOX2 transcripts were measured. Asterisks depict significant expression changes of treated samples in comparison to control.*

TCP9 acts downstream of TCP20

Besides the JA biosysnthesis and response genes various other putative TCP20 targets were identified (Appendix II). Knowing that closely related transcription factors often control each other and are connected in a transcriptional network, our attention was drawn to the class I *TCP9* gene. *TCP9* expression appeared to increase strongly after eight hours of TCP20-GR induction by DEX and CYC (Figure 6A). Further analyses showed that the *TCP9* promoter was bound by the TCP20 protein in a ChIP analyses (Figure 6B). To decipher the function of this class I TCP gene in more detail, a T-DNA insertion line was acquired for *TCP9*. Selected homozygous *tcp9* knockout plants were analyzed for phenotypic effects. Cells in young leaves of *tcp9* mutant plants appeared to be bigger than in wild type plants (Mann-Whitney U test p=0.0015) (Figure 6C-E). Furthermore, *tcp9* knockout plants have significantly shorter roots than wild type seedlings 14 days after germination (Student's t test, p= 0.000009) (Figure 6F-G). Further molecular analysis shows elevated expression of *LOX2* in four day old *tcp9* mutant seedlings (Figure 6H) but not in two week old leaves (Figure 6I). Although there is yet not much known about the molecular role of TCP9, co-expression analysis using the ATTED-II tool (Obayashi et al., 2007) reveals that *TCP9* is co-expressed with several genes involved in both jasmonate synthesis and response (Figure 7). Based on this knowledge, we hypothesize that the obtained phenotypes are at least partially due to TCP9-controlled jasmonate metabolism, as methyl jasmonate is known to repress cell proliferation (Pauwels et al., 2008) and to inhibit root growth (Staswick et al., 1992). These observations and the results on TCP20 targets imply that TCP20 controls JA-related processes directly, and via the activation of *TCP9,* creating robust control over the JA signaling cascade.

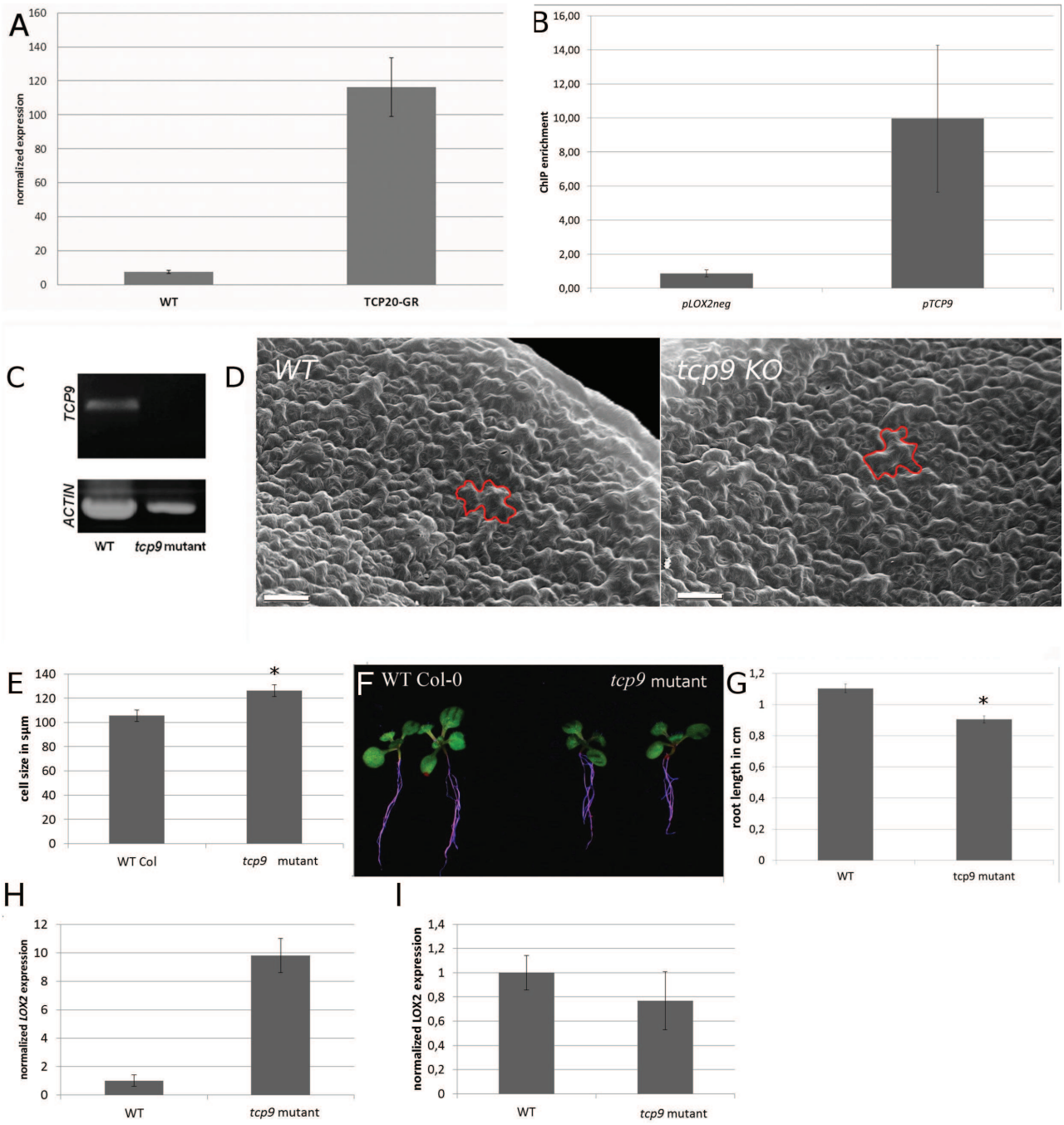


Figure 6. TCP20-dependent TCP9 regulation. *(A) TCP20 directly activates TCP9 expression upon Dexamethasone (DEX)/Cycloheximide (CYC) treatment of TCP20-GR plants. Depicted histogram shows difference in TCP9 induction between TCP20-GR and wild type plants 8h after induction with DEX and CYC. (B) TCP20 binding to TCP9 regulatory sequences in ChIP-qPCR analysis. Enrichment was compared between TCP9 promoter and an average of three unbound regions of the LOX2 promoter. (C) shows the knockout of TCP9 expression in tcp9 knockout plants. (D) Scanning electron microscopy of wild type and tcp9 knockout leaves, respectively, show enlarged cells in young tcp9 leaves. (E) Average cell sizes of wild type and tcp9 knockout leaf cells, calculations were based on cell drawing from ~10 leaf SEM pictures per line. (F) representative picture of wild type and tcp9 knockout plants that were grown on vertical standing petri dishes. (G) tcp9 mutants have significantly shorter roots than wild type plants, as found in three replicate analyses containing 20 plants per line. (H-I) LOX2 expression in tcp9 knockout plants compared to wild type and tcp20 knockout plants. LOX2 expression is significantly increased in (H) four day old seedlings (I) but not in the first leaf of two week old seedlings.*

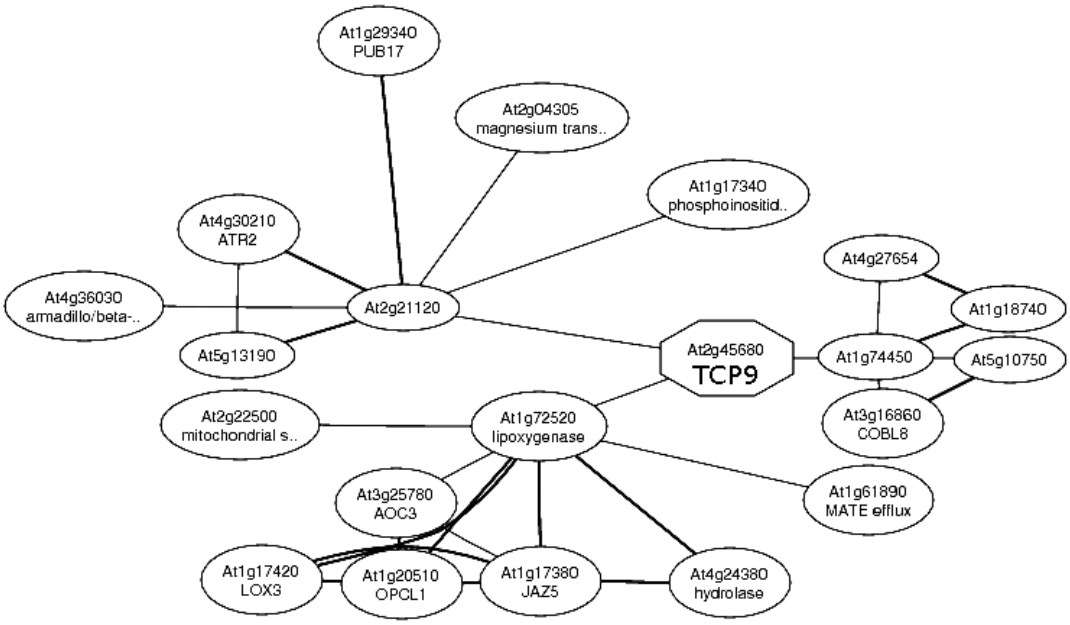


Figure 7. Transcripts co-expressed with TCP9 according to ATTED-II analysis. *The close co-expression with the jasmonate synthesis and response genes At1g72520, AOC3, JAZ5, OPCL1, and LOX3 are of specific interest for the relationship between TCP9 and jasmonate signaling.*

Discussion

The Arabidopsis genome encodes 24 TCP (TEOSINTE-BRANCHED, CYCLOIDEA, and PCF1) transcription factors that are divided into two classes (Cubas et al., 1999). This transcription factor family was first described about twelve years ago and although the role of various members in growth related processes has been deciphered, our understanding of their functioning is far from complete (Martín-Trillo and Cubas, 2010). Here we report on roles for the class I TCP transcription factors TCP20 and TCP9 in leaf development based on genome-wide target gene analysis for TCP20 and mutant analyses.

Regulation of cell cycle genes by TCP proteins: direct or indirect?

In a first step towards elucidating the function of the TCP20 protein, we conducted genome-wide target gene analyses using a glucocorticoid inducible system combined with microarray analyses. In previous studies TCP20 had been entitled to be involved in the regulation of the cell cycle (Li et al., 2005; Herve et al., 2009), especially since Li and colleagues were able to demonstrate TCP20 binding to the promoters of *Cyclin B1 (CycB1)* and *PROLIFERATING CELLULAR NUCLEAR ANTIGEN (PCNA)* (Li et al., 2005). Surprisingly, both genes did not appear in our target gene list and no other cell cycle related genes were identified as being direct TCP20 targets in two week old leaves. A recent study in which the TCP20 protein was fused to an EAR transcriptional repressor domain and expressed ectopically, showed effects on *CycB1* expression,

but not on *PCNA (*Herve et al., 2009). Furthermore, transcription factors and genes playing a role in cell wall modification were over-represented in the genes targeted by TCP20::EAR, but not cell cycle genes. In general, the premise that TCP transcription factors are regulators of cell proliferation is based on *tcp* mutant phenotypes. Plants impaired in different class II *TCP* genes revealed roles in diverse growth processes, such as axillary meristem outgrowth (Aguilar-Martinez et al., 2007), floral symmetry by differential growth of petals (Luo et al., 1996), and leaf growth (Palatnik et al., 2003; Efroni et al., 2008). These observed phenotypes were associated with alterations in cell division capacity or division rate. However, thorough investigation of cell division patterns and transcript changes during leaf development in the *jaw*-D mutant suggests that the class II TCP-dependent regulation of the cell cycle is indirect (Efroni et al., 2008). Based on our target gene analysis and the work performed by Herve and colleagues (2009) this also seems to be the case for the class I TCP20 protein.

The question arising from these observations is: what are the intermediates between TCP transcription factors and the regulation of the cell cycle? In the TCP20 target gene list, the jasmonic acid (JA) signaling pathway stands out, as a significant number of JA biosynthesis and response genes are targeted by TCP20. From the earliest publications jasmonates have not only been identified in wound-response and defense against pathogens (Bell and Mullet, 1991), but also in developmental programs (Wilen et al., 1991) and the suppression of the cell cycle (Pauwels et al., 2008). It is therefore possible that TCP20 controls cell proliferation and growth indirectly via jasmonate. Apart from jasmonate, other plant hormones could act as intermediates between TCPs and growth. Recently, it was found that the TCP1 protein is involved in brassinosteroid synthesis (Guo et al., 2010), and brassinosteroids are key signaling molecules in growth regulation (Kauschmann et al., 1996). Furthermore, TCP3, a member of the class II TCPs regulated by *miR319a,* is involved in auxin signaling (Koyama et al., 2010), which is a hormone that acts synergistically with brassinosteroids in growth regulation (Hardtke, 2007). In the case of TCP20, control of cell cycle genes seems not to be its only function, as its expression is wide-spread and neither limited to, nor excluding proliferating cells. Various genes encoding cell wall biogenesis and modifying proteins were related to TCP20 activity previously, suggesting a role for this transcription factor in the control of cell elongation (Herve et al., 2009). Although not over-represented, we also identified genes related to cell elongation among the TCP20 targets, such as a putative *xyloglucan endotransglycosylase/hydrolase* and a gene from the *EXPANSIN-like* gene family. Altogether, we hypothesize that TCP20 may not directly control cell cycle and growth, but that it is a prerequisite for growth related processes by regulating JA biosynthesis and enzymes involved in cell wall integrity and cell expansion.

Class I and class II TCPs antagonistically regulate LOX2 expression

LOX2, which encodes for a lipoxygenase involved in early steps of jasmonate synthesis (Vick and Zimmerman, 1983), is inhibited by induction of the TCP20-GR protein. Previously, TCP4 was shown to control jasmonate synthesis via regulation of *LOX2* expression as well (Schommer et al., 2008). When *TCP4* is down-regulated in *jaw*-D plants (Palatnik et al., 2003), *LOX2* expression

and JA content in leaves are very low (Schommer et al., 2008). As a consequence, in these leaves the cell proliferation stage is extended and furthermore, JA-induced senescence is delayed. This antagonistic regulation of *LOX2* by the class I TCP20 protein and the class II TCP4 protein confirms the previous postulated concept that class I and class II TCP transcription factors can regulate the same genes but with opposite effects (Li et al., 2005). However, the theory of antagonistic functions of class I and class II TCPs was based on similar putative binding sites of the two classes and hence, direct competition for binding was proposed. We were not able to show that TCP20 and TCP4 bind at the same sites of the *LOX2* promoter, indicating that other molecular mechanisms may play a role in the discovered antagonistic relationship. We observed that the ratio of *TCP4* to *TCP20* expression is negatively correlated during whole Arabidopsis development, with relative high *TCP4* expression during later stages of leaf development, when also *LOX2* is significantly expressed. The negative correlation between *TCP4* and *TCP20* is likely to be at least partially the outcome of an indirect regulation of *TCP20* expression levels by TCP4-controlled jasmonate levels. Based on this observation it is tempting to speculate that *LOX2* expression during development is dependent on temporal and spatial shifts in the ratios of TCP4 and TCP20. We propose that the arrest of cell proliferation at a certain moment in developing wild type leaves, a crucial step in leaf maturation, is at least partially dependent on JA levels, and that these levels are under the control of the balancing TCP activities.

Class I TCPs are involved in leaf development

In this study we showed that genome-wide target gene analyses revealed insight into the function of TCP20 despite the absence of a *tcp20* knockout phenotype. We were not only able to identify jasmonate synthesis and signaling components as direct targets of TCP20, but we also uncovered *TCP9* as a direct target gene and transducer of TCP20-mediated *LOX2* control. A phenotypic effect missing in *tcp20* knockout plants was detected in *tcp9* knockout plants. In *tcp9* mutants, cells of young leaves are significantly larger than in wild type leaves. However, cells of two week old *tcp9* mutants are not distinguishable from wild type anymore, indicating that the effect of TCP9 on leaf growth is limited to early leaf development and is compensated by yet unknown mechanisms at later stages. Correspondingly, *LOX2* expression in *tcp9* mutants is up-regulated only in young seedlings, but not in two weeks old leaves. Later, TCP4-mediated *LOX2* expression leads to a higher jasmonate content in leaves, and thus to leaf cell differentiation and leaf maturation, as discussed above. The fact that young leaves of *tcp9* knockout plants exhibit significantly larger cells than leaves of wild type plants, provides us with another evidence for the antagonistic roles of class I and class II TCPs in the control of jasmonate synthesis and leaf maturation, as the *tcp9* phenotype is the direct opposite of the *jaw*-D cell size phenotype (Efroni et al., 2008). Although *TCP9* is a direct target of TCP20, its steady state expression is not strongly changed in the *tcp20* mutant, which could be an explanation for the lack of the *tcp9* phenotype in the *tcp20* mutant and suggests that *TCP9* is controlled by another factor that is redundantly acting together with TCP20.

We believe that TCP20 and TCP9 form a coherent inhibitory feed-forward loop in *LOX2* control

(Mangan and Alon, 2003). Such coherent feed-forward loops represent delay elements in transcriptional networks, and allow the integration of different stimuli in the expression of a target gene (Mangan et al., 2003). An interesting case of a feed-forward loop in leaf development has recently been published, in which the transcription factor ORE1 positively regulates age-induced cell death in Arabidopsis via activation of *EIN2* and repression of the *EIN2*-repressor *miR164* (Kim et al., 2009). Depending on the exact logic of the TCP20-*TCP9-LOX2* loop, both TCP20 and TCP9 can integrate different developmental stimuli into the *LOX2* promoter. Hormones can fulfill the role of stimuli in this case, as we have already shown a role for jasmonate in the regulation of TCP20. Furthermore, *TCP9* was recently shown to be targeted by genes from the brassinosteroid pathway (Yu et al., 2011), thus providing us with the second stimulus *LOX2* may respond to, and linking brassinosteroids to jasmonate regulatory pathways. Brassinosteroids have been shown to negatively regulate jasmonate signaling already in the past (Campos et al., 2009; Ren et al., 2009), and TCP9 is a good candidate for being involved in the crosstalk between these hormones. This also explains the apparent absence of a *tcp20* knockout phenotype, as *LOX2* inhibition is still maintained by TCP9 when *TCP20* is absent.

In summary, our work shows evidence for the involvement of the class I TCP transcription factors TCP20 and TCP9 in Arabidopsis leaf growth. They control leaf cell sizes and maturation at least partly via the jasmonate signaling pathway during leaf development, and they do this antagonistically with class II TCPs. Future work should elucidate in more detail the position of these TCPs in signalling cascades that drive leaf development, and disentangle how the type I and II TCPs mutually, and possibly antagonistically play a role in the various processes taking place from leaf initiation to maturation.

Chapter 4

Transcriptional coordination between leaf cell differentiation and chloroplast development established by TCP20 and subgroup Ib bHLH transcription factors

Megan Andriankaja*, Selahattin Danisman*, Hannes Claeys, Aleksandra Skirycz, Gerco C Angenent, Richard GH Immink, Dirk Inzé

*these authors contributed equally to the work presented

Abstract

Cell differentiation and chloroplast biogenesis are separate, but coordinated processes during early leaf development. Chloroplast development creates a high demand for iron, and as a consequence sufficient iron has to be provided during early leaf development. However, iron in too high of quantities becomes toxic to the plant, thus plants have evolved a complex network of iron uptake and regulation. Several of these iron transporters and transcriptional regulators of iron homeostasis have been identified in roots since roots are responsible for the uptake of iron from the soil. However, many of these factors have also been shown to play a role in iron homeostasis, and the subgroup Ib of basic helix-loop-helix transcription factors (bHLH038, bHLH039, bHLH100, bHLH101) belong to this category. Here, we show that these transcription factors are up-regulated when leaves transit from primary to secondary leaf morphogenesis, and that they are under negative control of the TEOSINTE-LIKE1, CYCLOIDEA, PCF1 (TCP) transcription factor TCP20. By comparing the transcriptional profiles of genes involved in early leaf development, and those differentially regulated in a TCP20-GR inducible line, it appears that TCP20 may play a role in regulating iron homeostasis in leaves via the subgroup Ib bHLH transcription factors and directly via the control of several iron transporters and nicotianamine synthase (NAS) genes. Single mutants for each of the subgroup Ib *bHLHs* showed smaller leaves, both under *in vitro* conditions and for soil-grown plants. This associates TCP20 with the control of early leaf development and provides evidence that TCP20 is responsible for coordinating leaf development processes through the transcriptional regulation of the subgroup Ib *bHLHs* genes.

Introduction

Leaf development comprises, amongst others, the two phases of primary and secondary morphogenesis (Donnelly et al., 1999). During primary morphogenesis the leaf grows largely through cell proliferation while during secondary morphogenesis the leaf cells stop dividing and begin their post-mitotic expansion (Donnelly et al., 1999). Secondary morphogenesis is a highly dynamic process that does not take place in all leaf cells simultaneously (Efroni et al., 2008), rather it begins at the leaf-tip and continues towards the leaf base. This means that there is a population of expanding and proliferating leaf cells at any given time point during the early stages of secondary morphogenesis (Avery, 1933). Apart from the onset of cell expansion, secondary morphogenesis also includes the initiation of chloroplast differentiation and the establishment of the photosynthetic machinery (Chatterjee et al., 1996; Keddie et al., 1996) (Andriankaja et al. unpublished data). Despite the fact that cell expansion and chloroplast development are independent developmental processes, their regulation is tightly coordinated; though how precisely is still unknown (Hou et al., 1993; Reiter et al., 1994).
Chloroplast development demands iron because of its crucial role in the photosynthetic electron transport chain (Nishio and Terry, 1983). However, as iron toxicity can also be a serious problem for plant growth and survival (Guerinot and Yi, 1994), iron homeostasis is a highly regulated process in plants involving a plethora of components. Two known players are the IRON-REGULATED TRANSPORTERS 1 and 2 (IRT1 and IRT2), which are the major iron transporters in the root (Henriques et al., 2002; Vert et al., 2002), and their regulator FE-DEFICIENCY INDUCED TRANSCRIPTION FACTOR 1 (FIT1) (Colangelo and Guerinot, 2004). FIT1 and other transcription factors have mainly been found to respond to iron deficiency in stress assays (Olsen, 1981; Walker and Connolly, 2008). One group of transcription factors also found to be transcriptionally up-regulated by iron-deficiency stress are the subgroup Ib basic helix-loop-helix transcription factors *bHLH038, bHLH039, bHLH100* and *bHLH101* (Wang et al., 2007). BHLH038 and bHLH039 have been found to interact with FIT1, and when overexpressed the two bHLH/FIT combinations lead to constitutive expression of *IRT1* and of *FERRIC CHELATE OXIDASE (FRO2)*, another gene important in iron uptake (Yuan et al., 2008). Iron homeostasis was mainly investigated with the aim of understanding iron uptake and iron toxicity (Palmer and Guerinot, 2009). Thus, homeostasis in inter- and intracellular spaces of leaves are less well characterized, although early experiments already showed that iron transport takes place in leaves (Eddings and Brown, 1967). This transport is mediated by compounds such as Nicotianamine (NA), which is a chelator of Fe2+ and Fe3+ during transport, as free iron is likely to react and cause ROS damage (Stephan and Scholz, 1993; von Wirén et al., 1999). Lack of NA has been shown to restrict chlorophyll synthesis (Böhme and Scholz, 1960; Douchkov et al., 2005).
In previous experiments, the bHLH transcription factors *bHLH038, bHLH039, bHLH100* and *bHLH101* were found to be transcriptionally up-regulated at the transition from primary to secondary leaf morphogenesis (Andriankaja, unpublished data).

Similarly to these *bHLH* transcription factors, some *TEOSINTE-LIKE 1, CYCLOIDEA* and *PCF1 (TCP)* transcription factors are also differentially regulated during these stages. TCP transcription factors are plant specific transcription factors that are involved in controlling both proliferation and differentiation in leaves (Cubas et al., 1999; Palatnik et al., 2003; Aguilar-Martinez et al., 2007; Nag et al., 2009). Based on sequence homology the TCP transcription factor family can be divided into two classes: class I and class II TCPs. In this study we show that the class I TCP transcription factor TCP20 regulates iron homeostasis at different levels, including transcriptional regulation of the subclade Ib *bHLH* transcription factors, and that these transcription factors are important for iron homeostasis during secondary leaf development.

Material and Methods

Plant material. Seeds of Arabidopsis *tcp20* knockout plants (SALK_016203.45.25) were obtained from the Nottingham Arabidopsis Stock Center (NASC). TCP20-GR plants were described earlier (chapter 3, this thesis).

Plant growth conditions. Arabidopsis *thaliana (*L.) Heyhn. ecotype Columbia-0 (Col-0) seeds were sown on half strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) in 150 mm diameter plates with a density of 2 seeds per square. The plates were then stratified at 4 degrees Celsius for 2 days, after which they were placed in growth rooms kept at 22 degrees Celsius with 16 hour day/8 hour night cycles. For dexamethasone induction experiments, 50 mL of ½ MS medium (2,3 g/L) with Agar (6 g/L) was poured per plate and, after polymerization, a sterilized nylon mesh (mesh size: 200 µm) was placed on the medium (Passarinho et al., 2008). 30 to 50 seeds of both the GR-line as well as wild type Columbia-0 were sown out per plate. Plants were grown in LD conditions.

Norflurazone treatment. Plants were grown on half strength MS on meshes with 20 µm diameter pore sizes. On day 8 the meshes were transferred to either fresh MS plates or MS containing 5 µM norflurazon (Sigma-Aldrich) (Oelmüller and Mohr, 1986). Plants were then harvested at day 9 (24 hours after transfer) and day 10 (48 hours after transfer). Third leaves from the plants were microdissected as described in Skirycz et al. 2011.

GR induction assays. Glucocorticoid induction experiments were conducted 14 days after germination of the plants. Growing the plants on nylon meshes allowed us to transfer the plants

onto induction media swiftly and without damaging the roots. The induction medium consisted of 2,3 g/L MS, 1% (w/v) sugar, 10 µM Dexamethasone, and 10 µM Cycloheximide (Passarinho et al., 2008). Samples were harvested at different time points after start of the treatment.

RNA isolation and qRT-PCR. RNA was isolated using the QIAGEN RNeasy RNA isolation kit according to the manufacturer's protocol. DNase treatment took place on-column, following the protocols from the manufacturer. M-MuLV Reverse Transcriptase (RT) from Promega was used for cDNA synthesis. First, a mix of poly-dT primer and dNTPs was added to 500 ng DNA-free RNA in a volume of 12 µL. This solution was kept in ambient temperature for 2 minutes before 1 µL RT was added. After addition of the RT, samples were incubated at 25 degrees Celsius for 15 minutes and transferred to 42 degrees Celsius for 50 minutes. Here, reverse transcription took place and was stopped by heat treatment at 70 degrees Celsius for 15 minutes. The cDNA made this way was used for quantitative Realtime PCR (qRT-PCR) using the SYBR green mix from BioRad. The reference genes used for all analyses were the SAND family gene *AT2G28390* and the TIP41-like gene *AT4G34270,* both determined superior reference genes (table 1). (Czechowski et al., 2005).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) experiments mainly followed protocols described previously (de Folter et al., 2007; Kaufmann et al., 2010). TCP20-GFP seedlings were grown in liquid ½ MS medium on a horizontal shaker (30rpm) for 4 days in a 16h light-8 hour dark regime. After this time, seedlings were harvested by pouring the liquid MS medium through a sieve and then 3 g plant material was fixated with 37% formaldehyde. Immunoprecipitation was conducted using a GFP antibody coupled to magnetic beads. The magnetic beads were used to precipitate the antibody-protein-GFP complexes. The enrichment of TCP20 binding regions was compared between the immunoprecipitate and 1:1000 diluted input material. The primers used for Realtime PCR analysis of enrichment are given in table 1. Promoter elements that were not expected to be bound by TCP20 were used as negative control.

Table 1: Primers used for this study

Gene	Fwd primer	Rev primer
SAND family gene	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC
TIP41-like	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA
bHLH038 CDS	GGT ACG AGT ATC GGG TCA AAG AGA CT	CTT GGA CCA TCA CTT CGT TGT CAC CA
bHLH039 CDS	GAAGCTAAGCATTCCTGCGACGGTT	GGT GGC TGC TTA ACG TAA CAT TCA GT
bHLH100 CDS	CAGTTTCTTCGACTAGGCTCAGTGA	TCC GAG TAA AAG AGT CGC TCT CCA TGA GA
bHLH101 CDS	GCGAACGAGACCGCCGTAGAAAACT	GTT GAA GTT CTT GCT TCT GCT CTG GT
bHLH038 promoter fragment 1	GTA TGG GAC GAG GAA GTA AAG ACG	CAT TGA AAG ATG ACT CAT GCG AGT GA
bHLH038 promoter fragment 2	CTA GTT CAT GCG TGG AAC ATA GGC T	GTC TCT ATA GGG CAT AGG TTG GCA
bHLH039 promoter	GCC AGT CTA CTT GTG ACT AGA CCT T	GGA CGT AGG TCG ACA CAT GCC A
bHLH100 promoter	GTA TCT GTG ATT ATT GTG GGT TGT A	TCC TCA TTC TAT TTA CGC CTC TTT A
bHLH101 promoter fragment 1	ATC TCG GGA CGT TTA ATT TGA TTT TTC ACA	GGA TCG TTA ACA ATG CTC TTT TAC CTT C
bHLH101 promoter fragment 2	GAA TGA TGA TGA CTG CTG AGG CGT T	CGC GGT CTT AGG TCA ACG GCA
SAND family protein promoter	CGC GTG GGT TTG TTT TGA AAA TTG T	TGT AAG TGC ATA AGA TGA GTC TAA TTT

Results

Subgroup Ib bHLH transcription factor expression are under the control of TCP20 and tightly linked with photomorphogenesis and cell differentiation

Previous experiments, which looked to the morphological and transcriptional events occurring during early leaf development just following abaxial/adaxial specification, revealed that these stages of development are composed of dramatic shifts in cell proliferation, cell expansion, and also photomorphogenesis (Andriankaja et al. unpublished data). Within a 24-hour period between day 9 and day 10 the third leaf of Arabidopsis had shut down cell proliferation in the distal third of the leaf and simultaneously the leaf started photosynthesizing in the same region. The subgroup Ib basic helix-loop-helix transcription factors *bHLH038, bHLH039, bHLH100* and *bHLH101* exhibited some of the strongest changes in transcript levels at this stage. All four transcripts underwent significant up-regulation between days nine and ten, indicating that these play a potential role in regulating the onset of the transition from cell proliferation to expansion and/or photomorphogenesis (Figure 1A-B). As mentioned, the two processes occur simultaneously: cells transition from proliferating to expanding, and photomorphogenesis initiates. In an attempt to identify whether the subgroup Ib bHLH transcription factors were involved in these processes, we transferred plants to norflurazon (NF), a chemical inhibitor of phytoene desaturase, which was previously shown to inhibit the transition to cell expansion and the onset of photomorphogenesis (Andriankaja et al. unpublished data). The *bHLHs* were not up-regulated in young leaves of plants treated with NF, thus when the transitions are blocked bHLHs are no longer activated, indicating they are tightly linked to the onset of cell expansion and photomorphogenesis in young leaves (Figure 1C).

Apart from their up-regulation during the transition from primary to secondary morphogenesis during leaf development, we also showed that subgroup Ib *bHLH* transcription factors are strongly down-regulated upon induced activity of TCP20 (chapter 3, this thesis). All four *bHLH* transcription factors are down-regulated 16 times on average in microarray experiments. These microarray results were confirmed by repeating the GR induction assays and analysing transcript levels in a Realtime RT-PCR experiment. 14 days old plants expressing TCP20-GR in the *tcp20* knockout background were induced and material was harvested shortly before, and two and four hours after induction. At time point 0 of the induction analyses, TCP20-GR plants were expected to act similarly to *tcp20* knockout plants, as the TCP20-GR construct was transformed into this background. Accordingly, subgroup Ib *bHLH* expression was two (*bHLH101, bHLH100*) to eleven times (*bHLH039*) higher in the TCP20-GR *tcp20* than in wild type plants in the absence of Dexamethasone, indicating that the *tcp20* knockout background expresses these transcription factors at an elevated level (Figure 2A). Correspondingly, the subgroup Ib *bHLH* transcription factors were up-regulated in four day old seedlings of *tcp20* knockout plants (Figure 2B). Induction of TCP20 with Dexamethasone led to a decrease in expression of all four *bHLH* transcription factors already two hours after induction. Thus revealing the inhibitory role TCP20 plays in transcriptionally regulating the four subgroup Ib *bHLH* transcription factors. To confirm

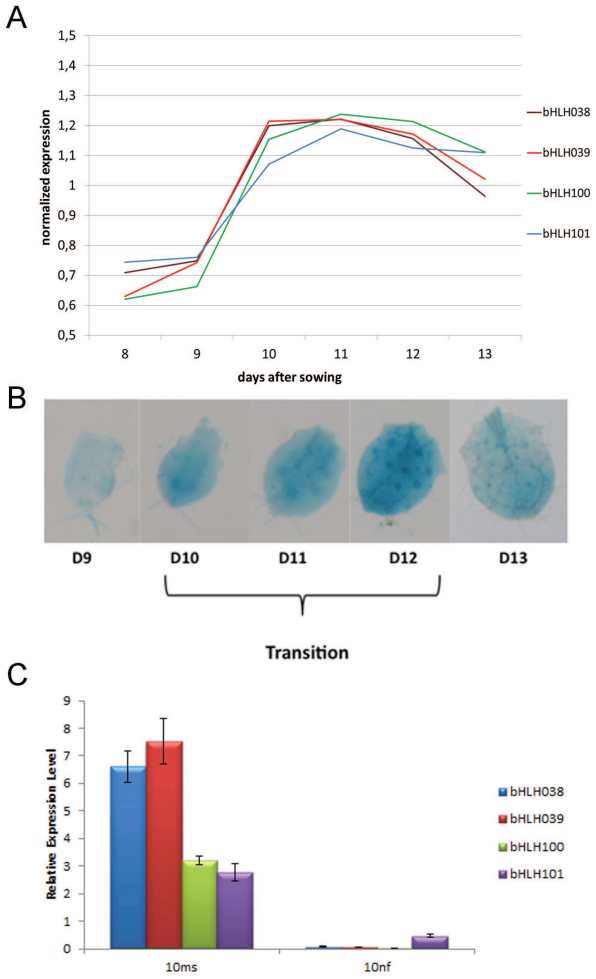


Figure 1. Expression of bHLH transcription factors during the transition from proliferative to expansive growth in leaves. *A) shows the microarray analyses of the four subgroup Ib bHLH transcripts between day 8 and 13 of leaf development, exhibiting a clear change in expression between day nine and day ten. B) shows the expression of bHLH100 based on a transcriptional bHLH100 promoter-GUS fusion. Increase in GUS activity is clearly visible at day ten and repression is seen at day 13. C) Expression of subgroup Ib bHLH transcription factors in leaves treated with NF in comparison with control. Expression is greatly reduced when plants are treated with the photomorphogenesis inhibitor NF.*

that these bHLHs are direct targets of TCP20, the promoters of subgroup Ib *bHLH* transcription factors were tested by targeted chromatin immunoprecipitation (ChIP) analysis and all four promoters were two- to three times enriched (Figure 2C). These results show that *bHLH38, bHLH39, bHLH100*, and *bHLH101* are direct targets of TCP20 and are specifically induced during the transition from cell division to expansion and the onset of photomorphogenesis in young developing leaves.

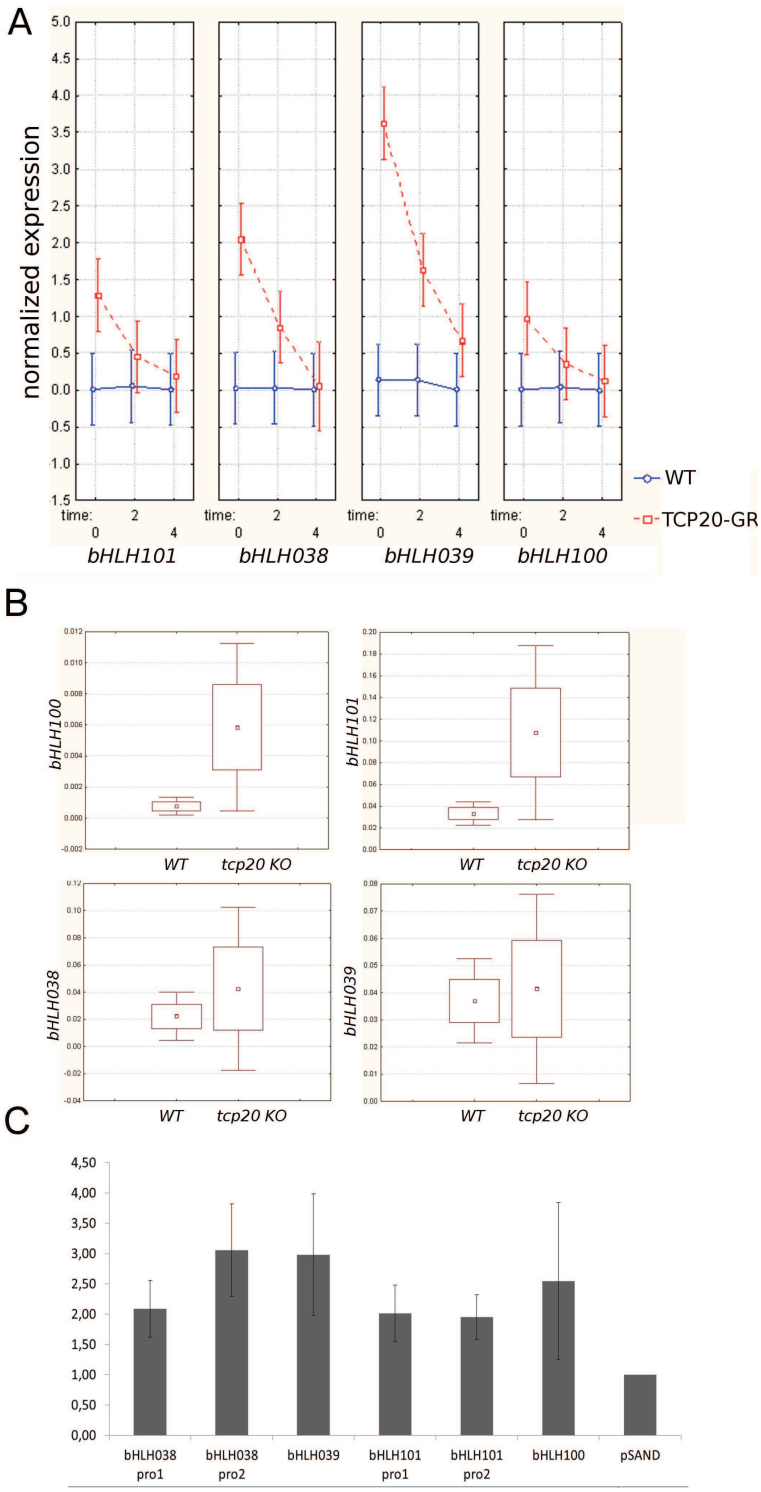


Figure 2. Control of bHLH expression by TCP20. *A) shows the results of the GR induction experiment, where the four bHLH transcripts show elevated expression before Dexamthasone (DEX) induction in the TCP20-GR tcp20 plants, and where the expression decreases rapidly after DEX treatment. B) exhibits the expression of the four transcripts in four day old tcp20 knockout plants, also here transcript levels are higher in the tcp20 knockout plants than in the wild type. C) Binding of TCP20 to the promoters of the four bHLH genes, as measured by a targeted chromatin immunoprecipitation assay (ChIP). The promoters of bHLH038 and bHLH101 are represented with two different promoter fragments that both contain putative TCP20 binding sites. Binding to the putative binding sites is compared to binding to the promoter of the At2g28390 gene, which should not be bound by TCP20.*

Table 2: TCP20 target genes that respond to iron deficiency according to Buckhout et al. 2009

Locus Identifier	Gene
AT1G09240	NAS3
AT1G23020	FRO3
AT1G47400	unknown protein
AT1G48300	unknown protein
AT1G56430	NAS4
AT2G41240	bHLH100
AT2G42750	DNAJ heat shock N-terminal domain-containing protein
AT3G18290	BTS
AT3G56360	unknown protein
AT3G56970	bHLH038
AT3G56980	bHLH039
AT4G19690	IRT1
AT5G04150	bHLH101
AT5G05250	unknown protein
AT5G13740	ZIF1
AT5G53450	ORG1

Iron-deficiency in response to onset of photomorphogenesis regulated by TCP20 and subgroup Ib bHLHs.

The subgroup Ib *bHLH* transcription factors are not only specifically up-regulated during the early transitions of leaf development, but they were also previously found to be involved in iron-deficiency response (Wang et al., 2007; Vorwieger et al., 2007). Interestingly, genes involved in iron homeostasis were also found in the TCP20 target gene list (chapter 3, this thesis). The TCP20 target gene list was compared with previously published lists of genes responding to iron deficiency (Buckhout et al., 2009) and 14 out of 56 genes (25%) that are differentially regulated by iron deficiency were also found in the TCP20 target gene list (Table 2). Interestingly, *FERRIC REDUCTION OXIDASE 2* and *3 (FRO2,FRO3)* and *IRON RESPONSIVE TRANSPORTER 1 (IRT1)*, all targets of bHLH038 and bHLH039, were also amongst these (Yuan et al., 2008). Also included on the TCP20 target gene list were the two *NICOTIANAMINE SYNTHASE* genes *NAS3* and *NAS4,* implying that TCP20 regulates iron transport within tissues as well. Although all other iron homeostasis genes were down-regulated, *NAS3* expression was not, suggesting that *NAS3* and *NAS4* have different functions in response to TCP20 induction. Although we did not analyse binding or activation of iron homeostasis genes by TCP20 as thoroughly as it was done for subgroup Ib *bHLH* transcription factors, the over-representation of iron homeostasis genes in the TCP20 target gene list implies a general role for TCP20 in iron homeostasis.

Because only above ground parts were used for target identification in the TCP20 inducible over-expression line, it is possible that the assay had identified target genes of root specific TCP20 action. Hence, we checked the expression of all 14 target genes plus *bHLH038* and *bHLH100* using AtGenExpress (Goda et al., 2008) and found most transcripts to exhibit peaks in roots, but

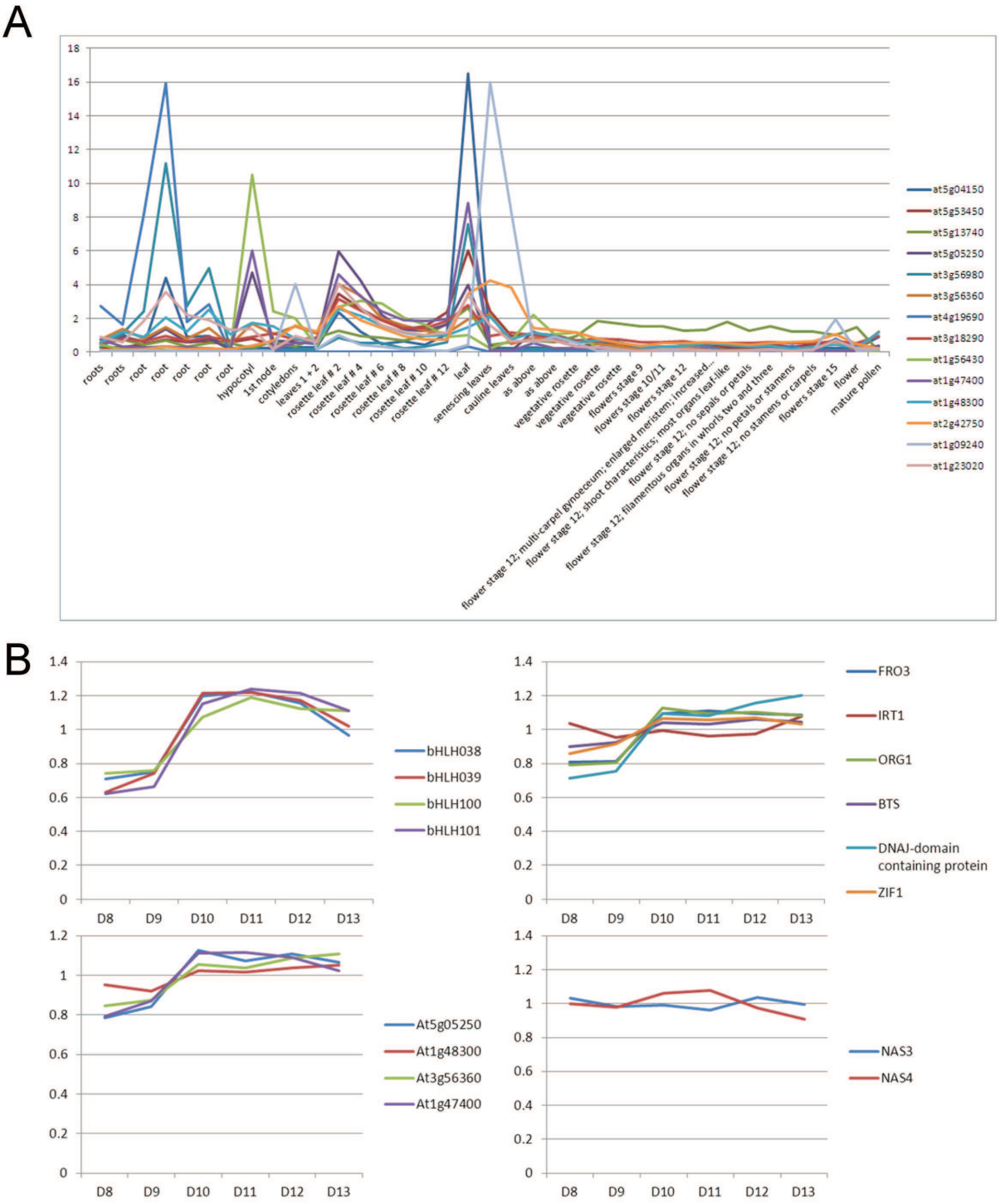


Figure 3. Expression of TCP20 targets that are regulated by iron deficiency. *A) Represents the expression patterns of all transcripts during Arabidopsis development (from Genevestigator). Most transcripts show expression in both roots and leaves. B) Up-regulation of iron responsive TCP20 targets in leaf development assay. With the exception of the nicotianamine synthases (NAS3 and NAS4) and IRT1 all transcripts show an increase in expression between days nine and ten.*

they also exhibited expression peaks during leaf development (Figure 3A). Interestingly, almost all of them showed higher expression in older leaves (rosette leaf #2) than in younger leaves (rosette leaf #4, #6 etc.) indicating a differential expression during leaf development, and plausibly, a role in leaf development. These genes were also up-regulated during the transition from cell proliferation to expansion and during the onset of photomorphogenesis, with the exception of *IRT1, NAS3* and *NAS4* (Figure 3B) (Andriankaja et al. unpublished data). Four of the genes that are found to be both TCP20- and iron-regulated, have no known functions yet: these are the genes *AT1G47400, AT1G48300, AT3G56360* and *AT5G05250.*

Down-regulation of subgroup Ib helix-loop-helix transcription factors leads to reduced leaf growth and delayed starch accumulation

As subgroup Ib bHLH transcription factors affect iron homeostasis during leaf growth, an effect on leaf size was expected when knocking out one or more of these transcription factors. Wild type and knockout mutants for the *bHLH* transcription factors were grown *in vitro* and on soil to assess potential leaf growth phenotypes. In these analyses, we observed that three of the four single mutants had smaller leaves and rosettes than wild type plants both *in vitro* and in soil grown conditions (Figure 4A and B). *Bhlh100* and *bhlh101* showed the strongest phenotypes with a 30% reduction in leaf area in vitro and a 30% reduction in rosette area in soil (Figure 4A and B). The *bhlh38* mutant also showed a smaller rosette area on soil, however was unaffected in leaf size when grown in vitro (Figure 4A and B). In general, the mutants were more reduced in size when grown on nutrient-poor soil compared to nutrient-rich media, which hints towards their involvement in nutrient use efficiency in regulating vegetative growth.

As TCP20 was shown to regulate the expression of subgroup Ib *bHLHs,* we assumed that over-expression of TCP20 will lead to sufficient knock-down of the target genes, and hence to a similar phenotype. When growing TCP20-GR plants on soil and treating the plants twice a week with 10µM Dexamethasone from germination onwards, final rosette sizes were on average 17.2% smaller, confirming our theory (Figure 4C). Wild type plants did not show any response to the DEX treatment. This phenotype was not visible when growing TCP20-GR plants on MS medium supplemented with the same concentration DEX, suggesting that the rich medium complements the physiological iron deficit brought by *TCP20* overexpression. To show that the leaf growth phenotype observed for *bHLH* knockout plants results from a disturbance in photosynthesis, we looked for starch accumulation as indicator of photosynthesis. Using Lugol's iodine we detected no starch accumulation in eight and nine day old *bhlh101* knockout plants (the mutant with the strongest growth reduction), in contrast to wild type plants. These indicate that bHLH101 has functions earlier than at transition (Figure 1D).

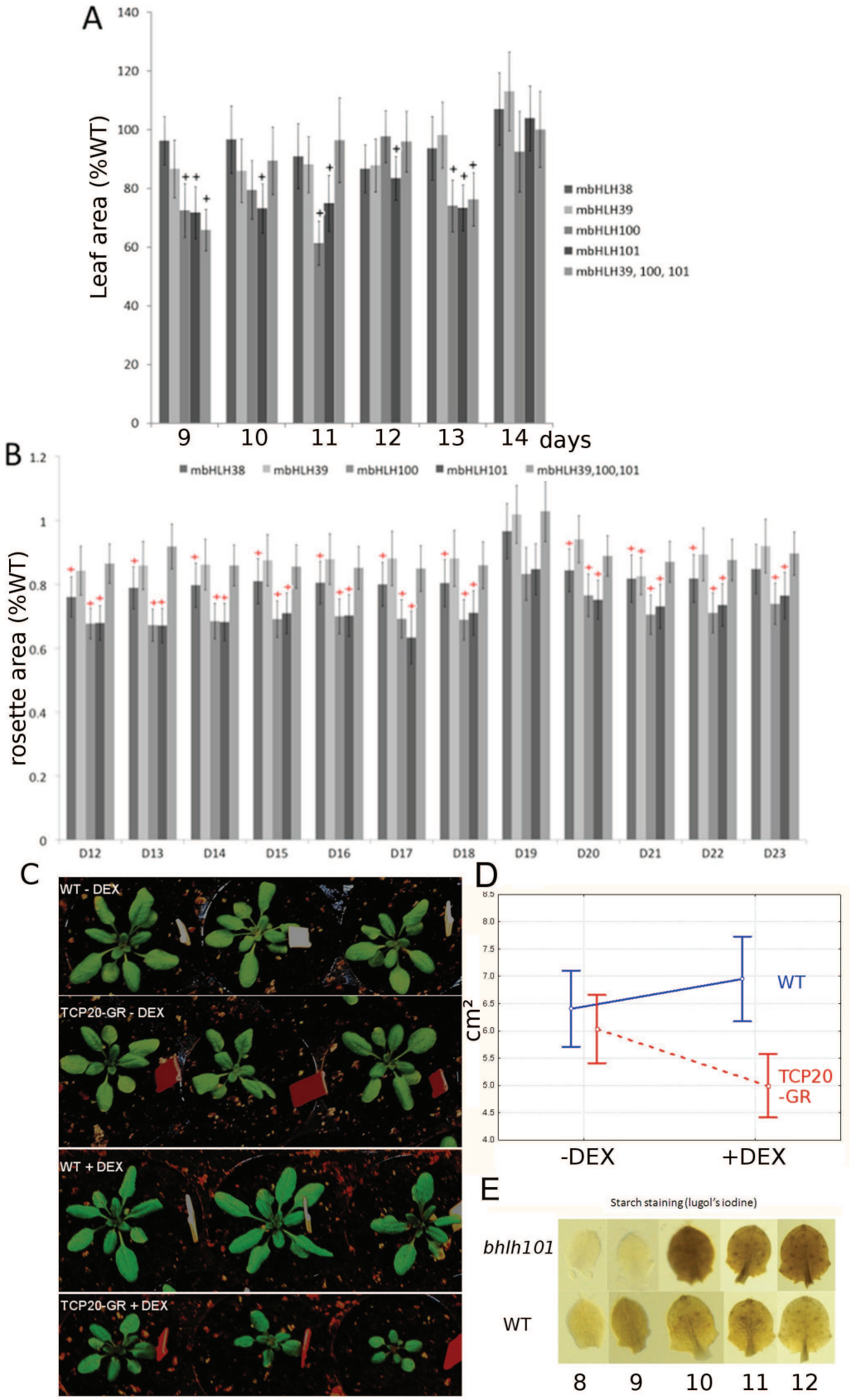


Figure 4. bHLH mutant phenotypes. *In A) leaf area measurements of bhlh mutants compared to wild type plants grown in vitro. bhlh101 mutants have significantly smaller leaves throughout the time series except at day 14. B) comparison of rosette areas between bhlh mutant plants and wild type plants grown on soil. C) representative picture of wild type and TCP20-GR plants grown without and with DEX D) graph demonstrating that TCP20-GR (red) plants treated with DEX display smaller rosettes than wild type plants (blue). E) starch accumulation in bhlh101 plants starts later than in wild type plants.*

Discussion

Iron in leaf growth

Iron is an essential micronutrient involved in various crucial processes in living beings. Up to 79% of the iron in sugar beet leaves is associated with chloroplasts (Terry and Low, 1982), demonstrating the relevance of iron for photosynthesis. So far, iron uptake in the roots was a main topic under investigation in iron homeostasis studies, alongside dealing with the toxicity of iron (Palmer and Guerinot, 2009). In these investigations important players have been identified, such as *IRON RESPONSIVE TRANSPORTER* (*IRT1* and *IRT2*) (Henriques et al., 2002; Vert et al., 2002), the *FERRIC OXID REDUCTASES (FRO2, FRO3*) (Wu et al., 2005), and some of the transcriptional regulators of these genes (FIT, bHLH038, bHLH039) (Colangelo and Guerinot, 2004; Wang et al., 2007; Yuan et al., 2008). Not only this work, but also other sources imply that at least some of these genes also play a role in iron homeostasis of other organs, like leaves and flowers (Mukherjee et al., 2006; Klatte et al., 2009). Especially transport within the leaves is not very well described, although early experiments showed already that iron transport in leaves is crucial (Eddings and Brown, 1967). It is known that nicotianamine is one of the components in leaves needed for the safe transport of iron within tissues (Stephan and Scholz, 1993; von Wirén et al., 1999; Pich et al., 2001). A lack of nicotianamine leads to a disturbance of iron homeostasis in tobacco and subsequently to intervenial chlorosis in leaves and abnormally shaped and sterile flowers (Takahashi et al., 2003).

In our analyses we showed that the TCP20 target genes and subgroup Ib basic helix-loop-helix transcription factors *bHLH038, bHLH039, bHLH100* and *bHLH101* are involved in early photomorphogenesis at a stage when the leaf is beginning to photosynthesize and is developing from an organ comprised of fully proliferating cells to an organ containing expanding cells.

Coordination between leaf growth and photomorphogenesis

Although leaf growth and chloroplast development are separate processes, coordinated regulation has been shown in different cases (Hou et al., 1993; Reiter et al., 1994; Li et al., 1995; Grevelding et al., 1996). In this work we propose that TCP20 is involved in the coordination of the two processes by inhibiting both leaf maturation and photomorphogenesis at early stages of leaf development. Earlier studies suggested that TCP20 plays a role in early leaf development (Li et al., 2005; Herve et al., 2009) (this thesis, Chapter 3). TCP20 control over iron homeostasis is very tight, as both transcriptional regulators of iron homeostasis and direct iron trafficking is affected in TCP20-GR induction assays. Although most affected genes are differentially expressed in leaves we cannot exclude that they are targets of TCP20 in roots as well.

TCP transcription factors have been found to control multiple facets of plant growth, like axillary meristem outgrowth (Aguilar-Martinez et al., 2007), flower symmetry (Luo et al., 1996) and leaf development (Palatnik et al., 2003; Crawford et al., 2004). In recent studies, TCP control over leaf growth has been narrowed down to maturation and senescence processes (Chapter 3, this thesis) (Efroni et al., 2008; Schommer et al., 2008; Sarvepalli and Nath, 2011). According to these

studies, TCPs regulate leaf maturation via hormonal pathways, especially via the jasmonate pathway. Jasmonates were identified to play a role in both wound-response as well as development (Bell and Mullet, 1991; Wilen et al., 1991), and their developmental effects are apparent in diverse processes such as: leaf senescence (He et al., 2002), root growth (Staswick et al., 1992), and petal development (Brioudes et al., 2009). Recently, evidence has been provided that jasmonate can suppress the expression of *FRO2* and *IRT1,* which strengthens the link between jasmonate pathways and iron homeostasis (Maurer et al., 2011). In an earlier publication, the Dof-domain transcription factor OBF-binding protein 3 (OBP3) was found to induce the expression of three target genes *OBP3-RESPONSIVE GENES (ORG1-ORG5)* (Kang et al., 2003). *ORG2* and *ORG3* encode for the bHLH transcription factors bHLH038 and bHLH039, respectively, which are found to be target genes of TCP20 together with *ORG1*. Similarly to *TCP20, OBP3* itself responds to jasmonate treatment. This means that both transcription factors TCP20 and OBP3 transduce hormonal and developmental signals into the regulation of iron homeostasis (Figure 5). An intriguing question to be answered in the near future is whether the TCP20 function is leaf specific, or if this transcription factor has similar functions in roots and probably flowers as well.

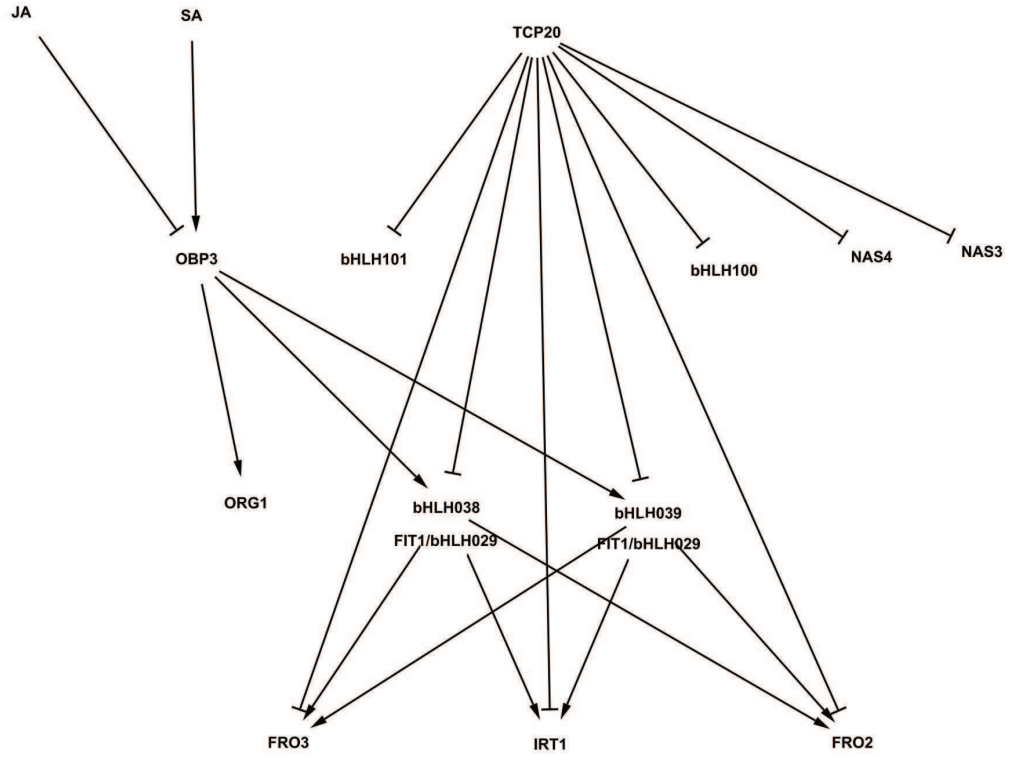


Figure 5. Diagram representing the control of TCP20 over iron homeostasis in leaf development. *TCP20 controls iron homeostasis on different levels: the transcriptional level with the bHLH transcription factors, and on the transport level, within tissues via the nicotianamine synthases and into tissues via the control of iron mediated transporters and ferric reduction oxidases.*

Chapter 5

Identification of novel transcription factors involved in cell cycle regulation during Arabidopsis leaf growth

Selahattin Danisman, Froukje van der Wal, Gerco C. Angenent and Richard G.H. Immink

Abstract

Transcriptional control of the cell cycle is an important regulatory mechanism of leaf growth and development. So far, many transcriptional regulators of the cell cycle remain unidentified. We conducted a large-scale yeast one-hybrid analysis to identify potential transcriptional regulators of three genes involved in the cell cycle and its control: *WEE1, DEL1* and *E2Fa.* In this analysis we found 71 transcriptional regulators binding to the promoters of one or several of the selected target genes. One of the identified regulators is MYC1, a known regulator of endoreduplication in trichomes that binds regulatory sequences of *DEL1* and is co-expressed with the *DEL1* gene, an important regulator of endoreduplication in plants. Further validation and analysis of the regulatory associations found in this analysis should help to identify those transcription factors that are biologically relevant in the control of the core cell cycle genes.

Introduction

Leaf cells undergo a differential pattern of cell division and cell elongation during leaf development, in which the organization of the cell cycle plays a central role. The cell cycle consists of four temporally distinct phases that a cell goes through one or several times during its lifetime: the DNA synthesis (S), the mitosis (M) and the two intercalating gap (G1 and G2) phases. During the gap phases, cell cycle checkpoints ensure the correct timing of the cell cycle. Several of these checkpoints have been identified, fulfilling different functions in the control of cell proliferation (Humphrey and Enoch, 1995). The cell cycle is driven by sequential activation of Cyclin-dependent kinases (CDK)/Cyclin complexes (for a review, see (Arellano and Moreno, 1997)). CDKs are regulated by kinases that phosphorylate conserved residues. One such kinase is WEE1, which negatively regulates CDK activity by phosphorylation of Tyr-15 and Thr-14 (Berry and Gould, 1996). In Arabidopsis, the WEE1 kinase controls CDK activity at the DNA integrity checkpoint between G2 and M phases, inhibiting the cell cycle when DNA damage occurs (De Schutter et al., 2007).

Between G2 and M phases, the cell cycle can switch to endoreduplication as an alternative to the mitotic cell cycle. At endoreduplication cell division does not take place after duplication of the chromosomes, and the resulting cells have a doubled DNA content upon each endoreduplication cycle; they are polyploid. Endoreduplication has been associated with increased cell growth (Sugimoto-Shirasu and Roberts, 2003), for example endoreduplicated leaf cells were shown to be larger than “normal” leaf cells (Melaragno et al., 1993). At the molecular level, endoreduplication is triggered by the anaphase promoting complex/cyclosome (APC/C) that eliminates M phase components (Lilly and Duronio, 2005). In Arabidopsis, CCS52A2 is one of the activators of the APC/C (Fülöp K, 2005). *CCS52A2* itself is under the control of the atypical E2F transcription factor DP-E2F-LIKE 1 (DEL1) (Liebman et al., 1979; Dimova and Dyson, 2005). Although *del1* knockout plants show increased endoreduplication, leaf size is not immediately affected.

Although some information is available, we are still far from a comprehensive view of the transcriptional control of the cell cycle (Berckmans and De Veylder, 2009). One of the best described cases is the regulation of genes expressed at the G1-to-S phase by E2F and DP transcription factor dimers (Kosugi and Ohashi, 2002). E2F transcription factors are highly conserved regulators of the cell cycle in eukaryotes, and have been first identified to fulfill this role in mammals (Lukas et al., 1996; Lavia and Jansen-Dürr, 1999). In Arabidopsis six *E2F* genes and two copies of *DP* genes, which encode for the dimerization partners of E2F proteins, have been identified. Detailed analysis of E2F functions showed that they have a large influence on many aspects of the cell cycle. They are important for the balance between proliferation and differentiation of the cell, and the regulation of endoreduplication (De Veylder, 2002). Other identified transcriptional regulators of the cell cycle belong to various transcription factor families; amongst which are members from the MYB, APETALA2 (AP2) and TEOSINTE-like/CYCLOIDEA/PCF1 (TCP) transcription factor families, although the mode of molecular

action of these regulators remains largely unknown. Nevertheless, the known interactions most likely represent only the tip of the iceberg and a lot of transcriptional regulators of the cell cycle remain to be identified. In this study, we attempted to identify new and unknown potential transcriptional regulators of *DEL1, WEE1* and *E2Fa* by a comprehensive yeast-one hybrid assay analyzing 1393 Arabidopsis transcription factors (Castrillo et al. 2011). For a few selected cases subsequent follow-up analyses were performed to confirm the protein-DNA interactions and to obtain evidence for the biological relevance of the identified interactions.

Material and Methods

Plant growth and media. Wild type Columbia-0 plants and transgenic reporter lines were grown on soil under long-day conditions and 20 degrees Celsius.

Constructs. A construct for transient protoplast transfections was made by inserting a sYFP2 encoding open reading frame (orf) (Kremers et al., 2006) flanked by a XbaI and SacI restriction enzyme site at the 5' and 3'ends, respectively, into pGD120 (Immink et al., 2002). This resulted in the vector CZN574, which was made GATEWAY- compatible by inserting the Gateway conversion cassette from Invitrogen into the XbaI digested and blunted vectors. The obtained destination vector was named CZN576. Genes encoding for transcription factors of interest and lacking the stop codon were cloned into this destination vector by the Gateway LR reaction. Besides the YFP-destination vector (CZN576), another destination vector was made containing a VP16 activation domain combined with the sYFP2 flurophore. This vector was generated by amplifying the VP16 activation domain out of a VP16-overexpression vector (kindly provided by Barbara Berckmans) using the primers GTCGACATGGACCTGTTGGTCGA and AGATCTCCCCCCAAAGTCGTCAA, providing the fragment with SalI and XbaI restriction sites. This fragment was inserted into the opened pGD120-sYFP2 vector mentioned above (CZN574). After cutting the resulting vector with XbaI and blunting its ends using Klenow, the C1 GATEWAY box was inserted and the vector was transformed into *E.coli* DB3.1 cells. This VP16-YFP destination vector was named CZN1791. The reporter destination vector was constructed by amplifying the TagRFP open reading frame out of the pGD120-TagRFP construct CZN481, using the forward primer PDS2651 (5'-CGCTGCAGTTTTTATTTTTAATTTTCTTTCAAATACTTCCACATGAGCGAGCTGATTAAGG-3'; including a sequence representing the AMV leader) and the reverse primer (PDS2652: 5'-CGCTCGAGTTACATAATAACACATTTAGTTTTAGACTTCTTCTTCTTCTTCTTACTTCCGCCTCCCTTGTGCCCCAGTTTGCT-3'; including a sequence encoding for a CaaX-tag at the C-terminal end of the TagRFP). The product of this reaction was inserted into pGEM-T (Promega). In a next step, a C1 GATEWAY-box was inserted in front of the AMV leader, which could be used to insert the promoters of *WEE1* and *DEL1.* These were obtained with the primers 5'-CCCACATTTTAGAATTAATCAAAC-3' and 5'-CGAGCTAAAATTTGGGAATAGTC-3' for *WEE1* and 5'-GCAGAAGCAGAAACTGAGAG-3' and

5’-CCAAAATAATACATTTTCAATCAATA-3’ for *DEL1.* This whole cassette was finally placed into the binary vector pGD121 (De Folter et al., 2006). As a positive control, a *35S-TagRFP-CaaX* construct was made by amplifying *TagRFP* out of a *TagRFP*-destination vector using the primers 5’-CGCTGCAGTTTTTATTTTTAATTTTCTTTCAAATACTTCCACATGAGCGAGCTGATTAAGG-3’ and 5’-CGCTGCAGTTTTTATTTTTAATTTTCTTTCAAATACTTCCACATGAGCGAGCTGATTAAGG-3’ and 5’-CGCTCGAGTTACATAATAACACATTTAGTTTTAGACTTCTTCTTCTTCTTCTTACTTCCGCCTCCCTTGTGCCCCAGTTTGCT-3’, in which the second primer contains the CaaX-box sequence. The resulting 0.8 kb TagRFP-CaaX fragment was cloned into pGEM-T and subsequently into pGD120 for protoplast transfection. The coding sequence of the putative transcriptional regulator *MYC1* was isolated out of cDNA using the primer pair 5’-ATGTCTTTGACAATGGCTGA-3’ and 5’-ATG AAA GAT ACA AAT CGC CCA-3’.

Transformation of Arabidopsis. The reporter constructs were transformed into wild type Arabidopsis. For transformation, Arabidopsis was grown on soil till the primary inflorescences emerged. These were cut to promote growth of secondary inflorescences and to increase the number of flowers. The binary reporter constructs were transformed into *Agrobacterium tumefaciens* strain C58C1 (pMP90). Transformation of plants was conducted by floral dip (Clough and Bent, 1998). After transformation, plants were kept in growth chambers till seed-set. Seeds were selected on ½MS + Agar (8 g/L) plates containing 30µg/mL Kanamycine. The following T2 generation was checked for expression of the transgene by RT-PCR and by Confocal Laser Scanning Microscopy (CLSM).

Yeast one-hybrid analysis. Binding of transcriptional regulators to promoter sequences was analyzed in a yeast-one hybrid system based on the Matchmaker™ Gold Yeast Two-Hybrid System (http://www.clontech.com). In this system, binding events are detected by resistance against the antibiotic Aureobasidin A. For a more detailed description of the method, see Chapter 3, this thesis. In essence, a GATEWAY cassette was cloned into the Aureobasidin A reporter construct from the manufacturer, leading to the vector CZN1018. Promoter fragments of *DEL1, E2Fa* and *WEE1* were isolated out of genomic DNA with the primers CCCACATTTTAGAATTAATCAAAC-3’ and 5’-CGAGCTAAAATTTGGGAATAGTC-3’ for *pWEE1,* primer 5’- AACCAGCCTCTGCCTACATT -3’ and 5’- TGTTGGGATAGATGTGGAGG -3’ for *pE2Fa,* and 5’- GCAGAAGCAGAAACTGAGAG-3’and 5’-CCAAAATAATACATTTTCAATCAATA-3 for *pDEL,* resulting in promoter fragments of 432bp, 1183bp, and 762 bp length, respectively. These promoters were transferred into CZN1018 by a LR reaction and transformed into the yeast strain PJ69-4α (James et al 1996).

Protoplast isolation and Transfection. Protoplast isolation mainly followed the steps described by Bücherl and colleagues (Bücherl, 2010). Protoplasts were isolated out of leaves from 3-4 week old seedlings. After cutting the leaves with a sharp scalpel, they were incubated in a petri dish containing enzyme solution with 0.2% Macerozyme R10 and 0.4% Cellulase dissolved in TEX buffer (Denecke et al., 1995). Incubation took place over night, in darkness and at room temperature. The next day, protoplasts were isolated in repeated W5 washing steps, and transfected using a 40% polyethylene glycol solution. Transfection was stopped after 10 minutes by adding W5. The transfected protoplasts were analyzed with confocal microscopy after at least six hours cultivation, or alternatively, the next day after 24 hours of incubation. In both cases, protoplasts were kept in dark at room temperature.

Confocal microscopy. CLSM of living plant tissue and protoplasts was conducted with a Leica SPE DM5500 upright microscope, using Leica AF 1.8.2 software (Leica, http://leica-microsystems.com).

Results and Discussion

Comprehensive yeast one-hybrid analyses revealed 71 new putative transcriptional regulators for three cell cycle genes

Upstream regulatory sequences of the cell cycle genes *WEE, E2Fa* and *DEL1* were inserted into the yeast one-hybrid Aureobasidin-reporter vector CZN1018, and yeast one-hybrid analyses were performed using the Arabidopsis REGIA transcription factor collection (Paz-Ares and Consortium 2002, Castrillo et al. 2011). We identified 71 transcription factors binding to the three cell cycle promoters (table 1-3). Binding events were scored positive when they identified in 3 out of 4 replicates. The transcription factor family with most representatives was the MYB family, from which 13 proteins were identified as binding factors. MYB-domain transcription factors comprise a huge family of 130 genes in Arabidopsis (Jiang et al., 2004) that were found to have functions in a plethora of biological processes in plants (Dubos et al., 2010). Amongst others, also the cell cycle has been found to be under the control of MYB transcription factors, e.g. in the case of DUO POLLEN1 (DUO1) (Brownfield et al., 2009), CELL DIVISION CYCLE 5 (CDC5) (Lin et al., 2007); and FOUR LIPS and MYB88 (Lai et al., 2005), of which the latter three are represented in the REGIA collection, but not amongst the direct regulators of the three genes analyzed in this study. Of the 13 MYB domain transcription factors found to bind the target DNA in our analyses, two have been shown to control the cell cycle in earlier publications: these are LATERAL ORGAN FUSION1 (LOF1) (Lee et al., 2009) and MYB59 (Mu et al., 2009). Several phenotypic alterations were observed in *lof1* mutants, including cauline leaves that are fused to the base of axillary stems. The double mutant *lof 1 lof2* exhibits an enhanced organ fusion phenotype (Lee et al., 2009). Subsequent analyses showed that LOF1 regulates cell division and expansion at the boundary between cauline leaves and axillary branches (Lee et al., 2009). The MYB-domain transcription factor MYB59 has been shown to control the cell cycle during root

***Table 1**: list of transcription factors binding pWEE1 in a REGIA transcription factor collection yeast one-hybrid screen. Given are the AGI codes of binding transcription factors, their common gene names as far as known, and the transcription factor families these factors belong to*

AGI	Gene name	Family
At1g18330	REVEILLE 7 (RVE7)	Myb-like
At1g19000		Homeodomain-like
At1g26310	CAULIFLOWER (CAL/AGL10)	MADS
At1g30650	WRKY14	WRKY
At1g31310		Myb-like
At1g48000	MYB112	MYB
At1g56280	DROUGHT-INDUCED 19 (DI19)	zinc-finger
At1g63910	MYB103	MYB
At1g74080	MYB122	MYB
At1g74930		ERF/AP2
At1g79580	SOMBRERO (SMB)	NAC-domain
At2g33290		SET-domain
At2g41240	bHLH100	bHLH
At2g43000	NAC042	NAC-domain
At3g06490	MYB108	MYB
At3g10590		Homeodomain-like
At3g14020		CCAAT-binding
At3g15030	TCP4	TCP
At3g16500	PHYTOCHROME-ASSOCIATED PR(	AUX/IAA protein
At3g17100		bHLH
At3g23250	MYB15	MYB
At3g27785	MYB118	MYB
At3g27810	MYB21	MYB
At3g57230	AGAMOUS-like 16 (AGL16)	MADS
At4g01980		transposable element ger
At4g04450	WRKY42	WRKY
At4g18450		ERF/AP2
At4g25560	MYB18	MYB
At4g36920	APETALA 2 (AP2)	AP2/EREBP
At4g38900		bZIP
At5g01200		Homeodomain-like
At5g05410		ERF/AP2
At5g06950		bZIP
At5g07690	MYB29	MYB
At5g07700	MYB76	MYB
At5g35550	TRANSPARENT TESTA 2 (TT2)	MYB
At5g41570	WRKY24	WRKY
At5g46760		bHLH
At5g59780	MYB59	MYB

Table 2: *list of transcription factors binding pE2Fa in a REGIA transcription factor collection yeast one-hybrid screen. Given are the AGI codes of binding transcription factors, their common gene names as far as known, and the transcription factor families these factors belong to*

AGI	Gene name	Family
At1g06070		bZIP
At1g21340		Dof
At1g25440		B-box zinc finger
At1g32870	NAC13	NAC
At1g55960		Polyketide cyclase/dehydrase and lipid transport superfamily
At2g16770	bZIP23	bZIP
At2g24790	CONSTANS-LIKE 3 (COL3)	B-box zinc finger
At2g30470		B3 domain
At2g33290		SET domain
At2g34440	AGAMOUS-like 29 (AGL29)	MADS
At2g38880		CCAAT-binding
At2g41240	bHLH100	bHLH
At2g43000	NAC042	NAC
At3g04420	NAC048	NAC
At3g06490	MYB108	MYB
At3g10590		Homeodomain-like
At3g14020		CCAAT-binding
At3g16500	PHYTOCHROME-ASSOCIATED	AUX/IAA protein
At3g17100		bHLH
At3g18010	WUSCHEL-RELATED HOMEOB	WOX
At3g24650	ABA-INSENSITIVE 3 (ABI3)	B3 domain
At3g27810	MYB21	MYB
At3g49950		GRAS
At3g55370		Dof
At3g57230	AGAMOUS-like 16 (AGL16)	MADS
At3g60530	GATA4	GATA
At4g01980		transposable element gene
At4g18450		ERF/AP2
At4g32730	MYB3R-1	MYB
At4g36920	APETALA 2 (AP2)	AP2/EREBP
At5g06950		bZIP
At5g07690	MYB29	MYB
At5g24800	bZIP9	bZIP
At5g35550	TRANSPARENT TESTA 2 (TT2)	MYB
At5g39610	NAC6/ORESARA1 (ORE1)	NAC
At5g41570	WRKY24	WRKY
At5g49450	bZIP1	bZIP
At5g59780	MYB59	MYB

Table 3: *list of transcription factors binding pDEL1 in a REGIA transcription factor collection yeast one-hybrid screen. Given are the AGI codes of binding transcription factors, their common gene names as far as known, and the transcription factor families these factors belong to*

AGI	Gene name	Family
At1g21340		Dof
At1g26780	LATERAL ORGAN FUSION 1 (LOF1)	MYB
At1g26960	HOMEOBOX PROTEIN 23 (HD23)	HD-Zip
At1g32870	NAC13	NAC
At1g48000	MYB112	MYB
At1g56280	DROUGHT INDUCED 19 (DI19)	zinc-finger
At1g74080	MYB122	MYB
At1g74930		ERF/AP2
At1g79580	SOMBRERO (SMB)	NAC
At2g18670	bZIP23	bZIP
At2g16770		RING/U-box
At2g33290		SET domain
At2g41240	bHLH100	bHLH
At2g43000	NAC042	NAC
At3g04420	NAC048	NAC
At3g05700		Drought-responsive family
At3g06490	MYB108	MYB
At3g10590		Homeodomain-like
At3g14020		CCAAT-binding
At3g15030	TCP4	TCP
At3g16500	PHYTOCHROME-ASSOCIATED PROT	AUX/IAA protein
At3g17100		bHLH
At3g20640		bHLH
At3g27785	MYB118	MYB
At3g27810	MYB21	MYB
At3g44350	NAC061	NAC
At3g49950		GRAS
At3g51910		Hsf
At3g55370		Dof
At3g57230	AGAMOUS-like 16	MADS
At3g57390	AGAMOUS-like 18	MADS
At3g60530	GATA4	GATA
At4g00480	MYC1	bHLH
At4g01980		transposable element gene
At4g23750	TARGET OF MONOPTEROS 3 (TMO3	ERF/AP2
At4g25560	MYB18	MYB
At4g36920	APETALA 2 (AP2)	MADS
At4g39250	RAD-like 1	Homeodomain-like
At5g01200		Homeodomain-like
At5g06950		bZIP
At5g07690	MYB29	MYB
At5g07700	MYB76	MYB
At5g24800	bZIP9	bZIP
At5g26990		Drought-responsive family
At5g35550	TRANSPARENT TESTA 2 (TT2)	MYB
At5g46760		bHLH
At5g49450	bZIP1	bZIP
At5g59780	MYB59	MYB

growth, and *myb59* mutants exhibit elongated primary roots.
Target gene analyses have shown that MYB59 regulates, amongst others, the cell cycle gene *CyclinB1,* which may be one of the access points of MYB59 into the cell cycle (Mu et al., 2009). Of the 11 other MYB genes that had been found in our analyses, none had been identified in the control of cell cycle genes previously. For example, the MYB gene MYB122 that was found to bind both the promoters of *DEL1* and *WEE1,* controls the production of indolic glucosinolates in roots and rosette leaves (Gigolashvili et al., 2007). In general, most regulatory proteins found were unknown as cell cycle regulators.
Another family of transcription factors that is strongly represented family in the list of potential regulators are the NAC-domain transcription factors. Similar to the MYB-family most transcription factors that were found to bind the cell cycle promoters had not been identified to play a role in cell cycle regulation earlier. An interesting exception is SOMBRERO/NAC33, which previously has been shown to inhibit cell division in root cap cells (Willemsen et al., 2008; Bennett et al., 2010). Future analysis will reveal if SOMBRERO controls the cell cycle via the regulation of its potential target genes *WEE1* and *DEL1,* as implied by this yeast one-hybrid analyses, or not.
Some other regulators that caught our attention were TCP4 and bHLH100. TCP4 and its homologues, also known as the *jaw*-TCPs, have an effect on the cell cycle when knocked down simultaneously (Palatnik et al., 2003; Efroni et al., 2008). The question remains if the cell cycle is the main direct target of *jaw*-TCPs, and several analyses hint to the fact that it is not (Efroni et al., 2008; Schommer et al., 2008) (this thesis, chapter 2). However, the fact that TCP4 binds to the promoters of WEE1 and DEL1 suggests that these genes may be targets of TCP4 at specific time points during Arabidopsis development. The basic helix-loop-helix transcription factor gene *bHLH100* had previously only been found to be up-regulated at iron-deficiency, together with its homologues *bHLH038, bHLH039*, and *bHLH101* (Wang et al., 2007). Additionally, the homologues *bHLH038* and *bHLH039* were found to be involved in iron homeostasis (Yuan et al., 2008). In our studies, we found all four bHLH genes to be up-regulated at the transition phase of leaf development, when cells at the leaf-tip stop proliferating and start expansive growth; and we linked this to an increased iron demand during photomorphogenesis, which takes place in this period (Chapter 4, this thesis). Future target gene analyses will have to show if iron homeostasis is the only function these transcription factors control, or if the cell cycle is also a target of these transcription factors, a question raised by the fact that bHLH100 binds to the promoters of *WEE1* and *E2Fa.* Lack of BHLH100 binding to these promoters may be the reason of the mutant phenotypes observed earlier (Chapter 4, this thesis), however, this connection will have to be explored in future analyses.

In depth analyses of expression patterns identifies transcription factors that may control cell cycle genes

We analyzed co-expression between potential transcriptional regulators and their respective target genes to narrow down the number of candidate genes for transcriptional regulation of the three

cell cycle genes under study. The co-expression analysis was conducted using the Gene Co-Expression Analysis Toolbox (GeneCAT) (Mutwil et al., 2008). Those regulators that were most closely co-expressed with their target genes, were analyzed in detail for co-expression using the CoExViewer application of the Atted-II website (www.atted.jp) (Obayashi et al., 2007). The *WEE1* gene was found to be highly co-expressed to the MYB-like hydroxyproline-rich

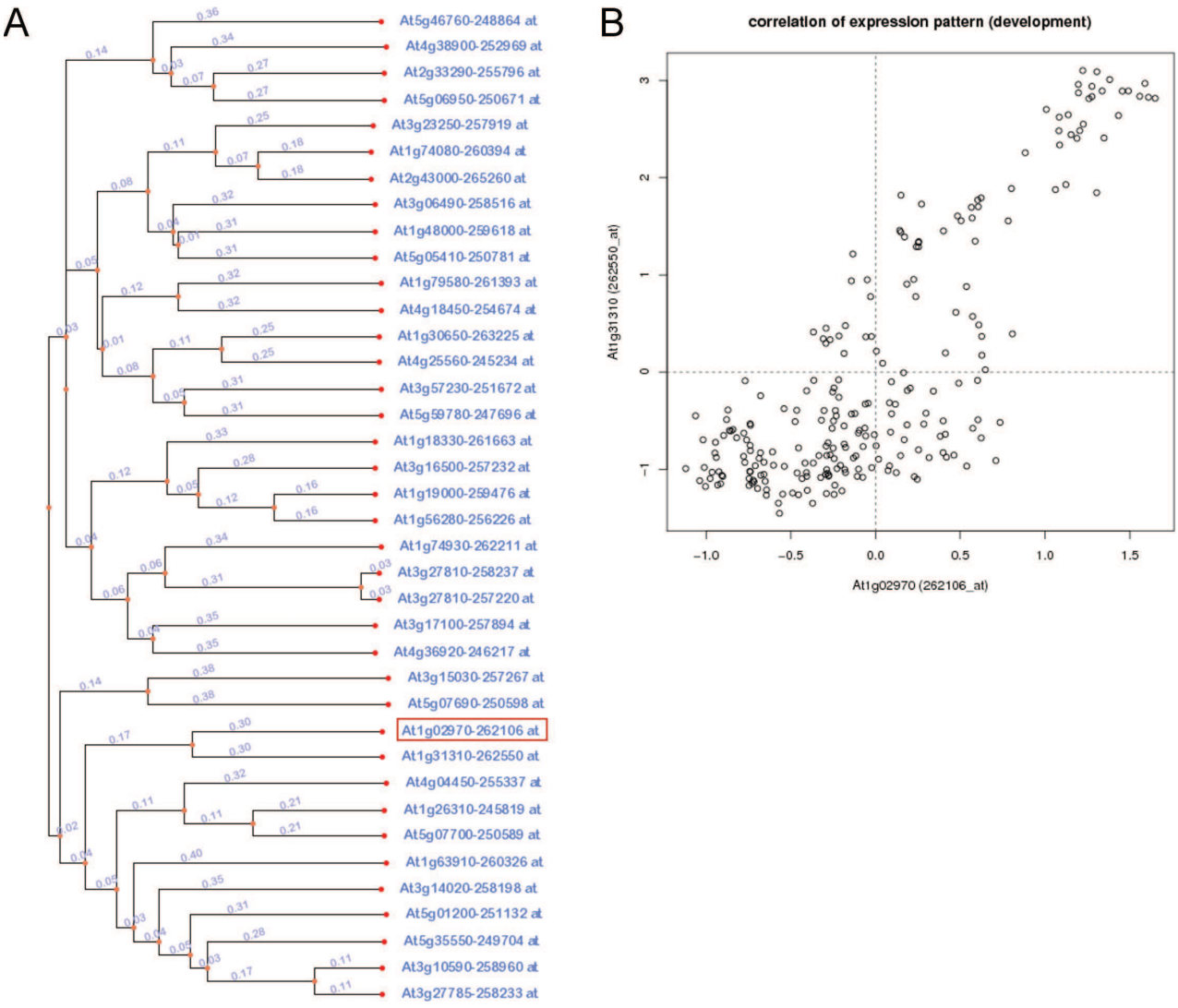


Figure 1. Co-expression of potential regulators with WEE1. *A) WEE1 coexpression (marked red) with its transcriptional regulators, according to GeneCAT (Mutwil et al., 2008). B) co-expression between WEE1 (x-axes) and its potential regulator At1g31310 (y-axes) during Arabidopsis development, as given by Atted CoExViewer (Obayashi et al., 2007). Each data point represents the expression in a certain tissue of developmental stage.*

glycoprotein family gene *At1g31310* (Figure 1), for which no function has been described yet. Further investigations are needed to elucidate its function, and to find out if its function also involves control of WEE1 expression.

A more informative connection was found between *E2Fa* and its closest co-expressed potential activator MYB3R-1 (Figure 2A, B). This transcription factor was recently identified to regulate

cytokinesis in Arabidopsis embryos during the first divisions after fertilization (Haga et al., 2007). Plants without *MYB3R-1* and its close homologue *MYB3R-4* exhibited several growth defects, including multinucleate cells, gapped walls and cell wall stubs. Further investigation showed that G2/M phase genes are down-regulated in *myb3r1 myb3r4* double knockout mutants, and that they control these genes via the syntaxin gene family protein KNOLLE (Lauber et al., 1997; Haga et

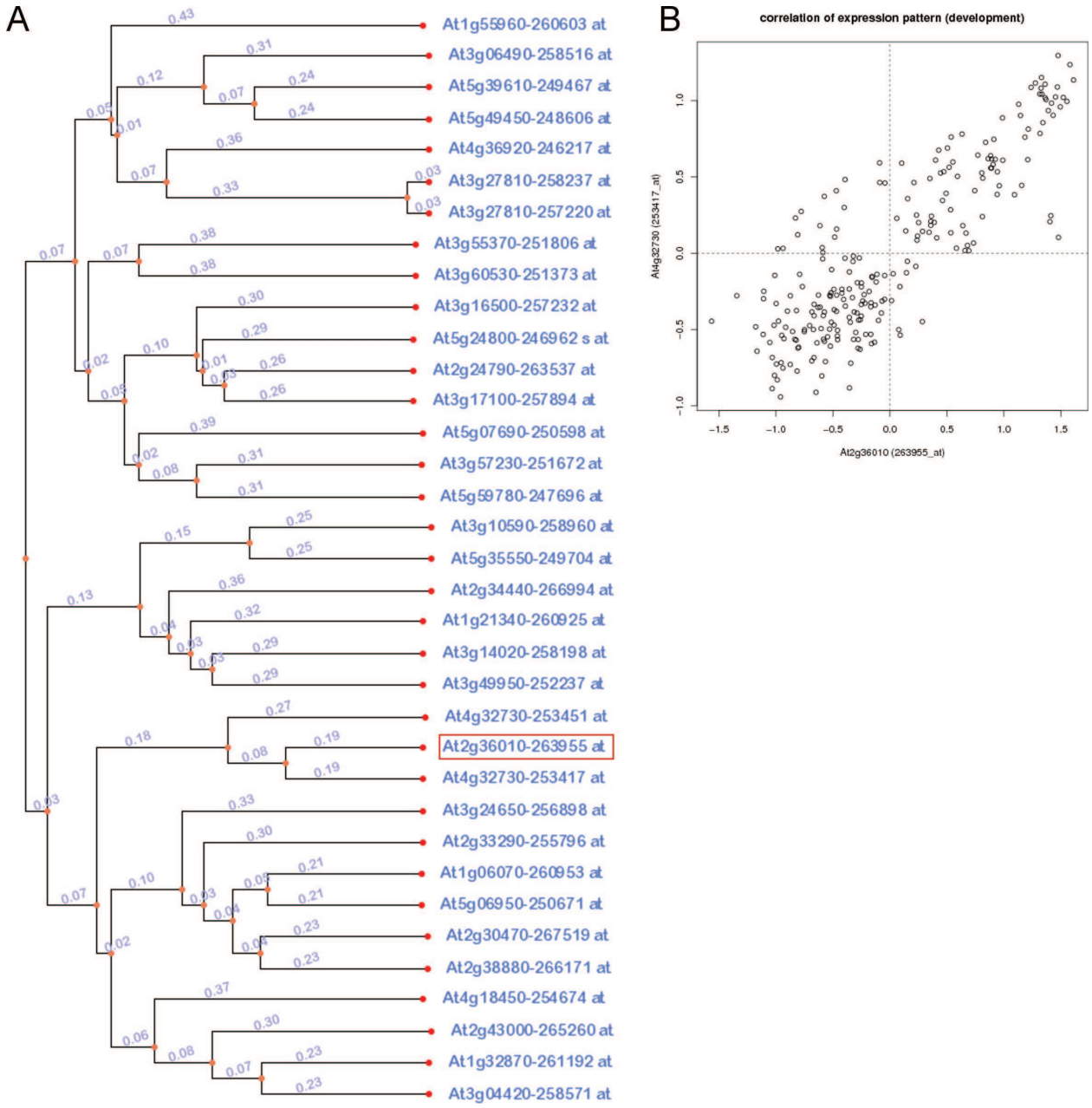


Figure 2. Co-expression of potential regulators with E2Fa. *A) E2Fa coexpression expression (marked red) in comparison with its transcriptional regulators, according to GeneCAT. At4g32730, which is most highest co-expressed with E2Fa, is presented twice in the tree, because it is represented by two probes in the GeneCAT database B) co-expression between E2Fa (x-axes) and its potential regulator At4g32730 (y-axes) during Arabidopsis development, as given by Atted CoExViewer.*

al., 2007). Because there is no microarray data available for *DEL1* expression in GeneCAT, we explored the At-TAX tool for co-expression analysis (Laubinger et al., 2008). We compared the expression patterns of all binding transcription factors with *DEL1* and found in multiple correlation analyses *MYC1* to be the highest co-expressor of *DEL1* (r^2=0.7295, p=0.0008) (Figure 3A, B). *MYC1* encodes for a transcription factor with a basic helix-loop-helix domain (bHLH) and is also known as *bHLH012.* It has shown to interact, similar to other bHLH transcription factors, with MYB-domain transcription factors such as TRANSPARENT TESTA GLABRA 1 (TTG1) (Zimmermann et al., 2004). Similarly to the functions of TTG1 in plants (Baudry et al., 2004; Gonzalez et al., 2008; Maes et al., 2008), MYC1/bHLH012 has been found to be involved in trichome initiation (Morohashi and Grotewold, 2009) and anthocyanin production (Hichri et al., 2010). MYC1 had not been described before to be involved in cell cycle regulation, although analysis of co-expressed genes using Atted (Obayashi et al., 2007) revealed two cyclin-dependent kinases and twelve cyclins to be highly co-expressed with MYC1. In line with these observations, genes involved in the cell cycle were over-represented (p=1.28•10-6) when analyzing the top 300 *MYC1* co-expressed genes for over-represented gene ontologies with the Cytoscape BiNGO application (Maere et al., 2005). Trichomes are hair-like structures on the plant epidermis that develop in a specific spatial pattern. Trichome development is tightly associated to endoreduplication, resulting in elevated ploidy levels ranging from 4C up to 64C (Melaragno et al., 1993). The fact that MYC1 binds to the promoter of *DEL1,* which has been found to regulate endoreduplication in Arabidopsis (Liebman et al., 1979; Dimova and Dyson, 2005), suggests that this Y1H binding event represents a biologically relevant regulatory association. A definite proof of this relationship would be the isolation of the *DEL1* promoter in chromatin immunoprecipitation (ChIP) experiments isolating targets of MYC1 in trichomes, or by analyzing *DEL1* expression after induced *MYC1* expression or induced MYC1 action, e.g. using a glucocorticoid inducible system (Sablowski and Meyerowitz, 1998; Passarinho et al., 2008).

Identified potential regulatory associations need to be validated in further experiments

In our yeast one-hybrid assay with promoters of three cell cycle genes as bait, we found 71 transcription factors, of which some have been shown to be involved in cell cycle regulation previously. Most of the transcription factors found have not been described in cell cycle regulation previously, and hence might represent either false positives or novel regulators of their respective target genes. Furthermore, many known cell cycle regulators have not been found in this assay, probably because they do not regulate the specific target genes directly or they are involved in the regulation of other cell cycle genes. We can also not exclude that some of these TFs did not bind to our targets because of experimental shortcomings of the yeast one-hybrid technology leading to false negative results. In previous studies it has been shown that yeast two-hybrid methods, applied for the identification of protein-protein interactions, resulted in the discovery of different sets of protein-protein interactions, and that none of the used yeast two-hybrid methods discovered more than about 40% of a positive reference set of protein-protein interactions (Chen et al., 2010). Alternatively, the Y1H reporter vector did not contain the right

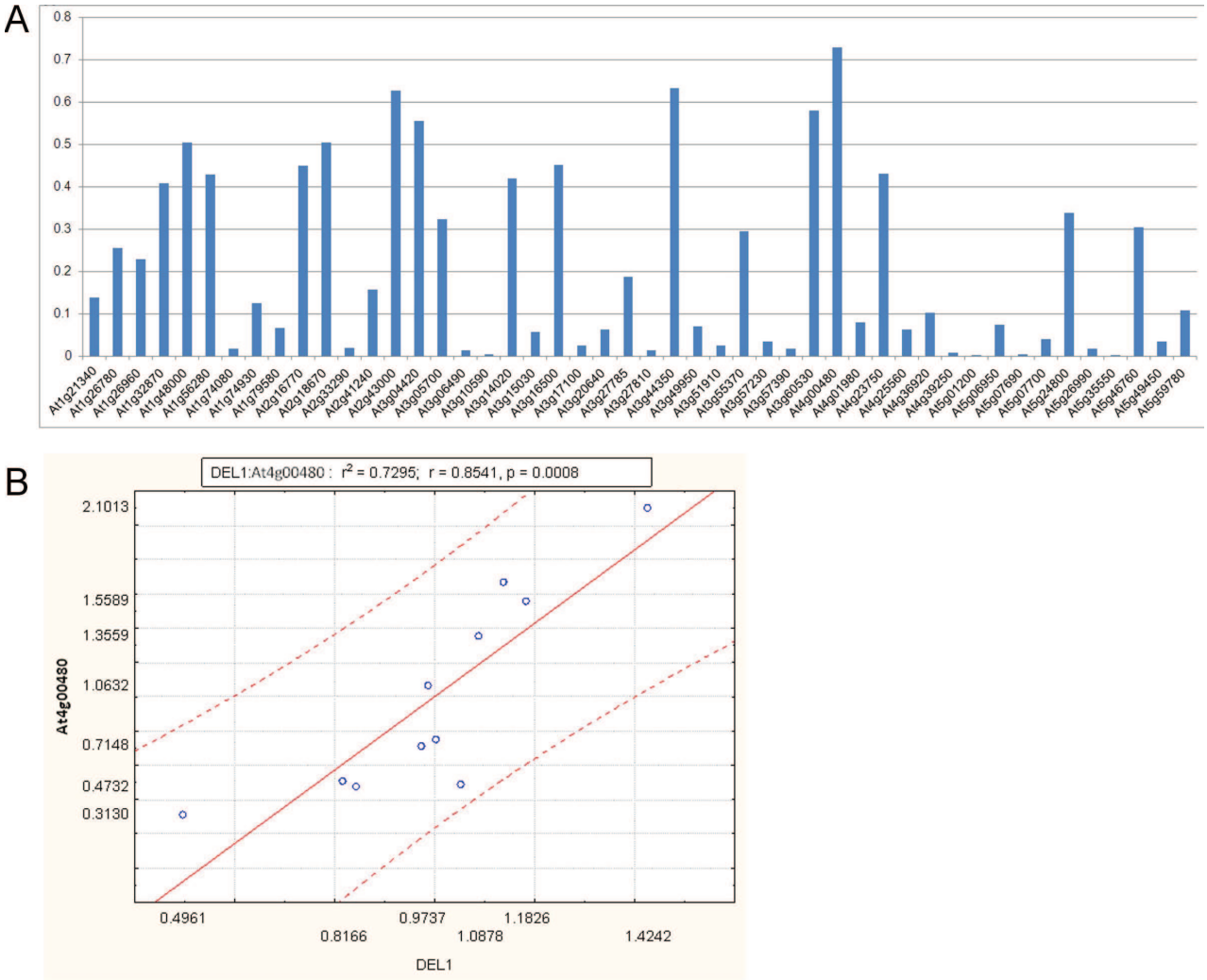


Figure 3. Co-expression of potential regulators with DEL1, analyzed with Microsoft Excel 2010 and STATISTICA 6.0. *A) R²-values of co-expression between all binding genes and DEL1, after extracting their expression values from At-TAX (Laubinger et al., 2008). At4g00480 has the highest r² of 0.7295 (p=0.0008). B) Co-expression between At4g00480 (y-axes) and its potential target gene DEL1 (x-axes). Mean-normalized expression values for both genes were extracted from At-TAX and analyzed using STATISTICA 6.0.*

promoter fragment or the distance between the binding site and the minimal promoter was not optimal for reporter gene activation in yeast. It is therefore likely that the interactions discovered in this study are only a subset of all possible transcription factor-target gene interactions. Further validation of the interactions and the determination of the mode of transcriptional regulation (suppression or activation) are needed. Helpful tools for these experiments are transactivation assays (Asai et al., 2002) or transcription factor centered studies like chromatin immunoprecipitation (ChIP) (de Folter et al., 2007; Kaufmann et al., 2010) or glucocorticoid response (GR) inducible assays (Aoyama and Chua, 1997).

In an attempt to validate some of the found interactions we developed a transactivation system that allows analysis of target gene activation including determination of the amount of transcription factor needed for the transactivation. For this system, both the target gene and the transcription factor are tagged with fluorophores. The target gene reporter is stably transformed

into plants and the potential transactivators are transiently transfected in a protoplast-based assay. The fluorophores were sYFP2 (Kremers et al., 2006) for the effectors and TagRFP (Merzlyak et al., 2007) for the reporters. We isolated the genomic promoters of *WEE1* and *DEL1* and inserted them into a reporter construct encoding for TagRFP. Because YFP2 signal tends to bleed through into the tagRFP detection channel, we tagged the tagRFP reporter with a CaaX-domain (Hancock et al., 1991; Kloc et al., 1991). The CaaX-domain was used to locate the tagRFP signal to the membranes and avoid reporter signal in the nucleus, where the transcriptional effectors and accompanying YFP signals were expected. To verify the membrane localization of the TagRFP-CaaX reporter we transfected TagRFP-CaaX under the control of a constitutive promoter (*35S:TagRFP-CaaX*) into Arabidopsis wild type protoplasts. Here, TagRFP signal was detected in both the cell membrane as well as the nuclear envelope (Figure 4A). The *pTargetGene-TagRFP-CaaX* reporters were transformed into Arabidopsis plants and stable signal was detected in the T2 generation of transformed *pDEL1* and *pWEE1* reporter plants (Fig. 4B-C). Secondly, we tagged the transcription factors under study not only with YFP2, but also with a VP16 activation domain (Chasman et al., 1989), as transcription factors repressing target gene expression would not be detected in the transactivation assay. Protoplasts were isolated of *pWEE1* and *pDEL1* reporter plants, and transfected with *MYC1*, tagged with VP16-YFP or YFP, only. As a positive control we used *E2Fb* which is known to bind the *DEL1* promoter and to activate *DEL1* transcription (Lieven de Veylder, personal communication). Although both effectors gave yellow fluorescent signal and transfection efficiencies were reasonable, we were not able to detect a significant transactivation event in either of the two transfected reporter lines. To our surprise, in almost all cases when the effectors were expressed, YFP signal was not only detected in the nucleus, but also in the rest of the protoplasts, which could potentially mask the detection of a transactivation event (Figure 4D). Also in the case of the positive control, represented by a DEL1 reporter line transfected with *E2Fb-YFP*, we could only detect E2Fb-YFP fluorescent signal.

One of the problems of the transactivation system we developed was caused by the choice of fluorescent tags. The excitation spectrum of YFP2 exhibits a broad base towards higher wavelengths and bleeds through into the tagRFP excitation spectrum (Kremers et al., 2006; Merzlyak et al., 2007). We tried to solve this problem by translocating the reporter to membranes. However, all effector constructs showed not only expression in the nucleus but also in the cytoplasm, potentially interfering with the detection of transactivation. It is possible that some transcription factors fulfill roles in the cytoplasm as well or they need a dimerization partner to enter the nucleus, but in any case the extranuclear expression of YFP2 hinders detection of potential tagRFP signal. A better strategy would have been to switch the fluorophores, using YFP2 for the reporter gene and TagRFP for the effectors, eventually still retaining the CaaX-box as additional safety to the system. The usage of slowly maturing fluorophores for the effectors would also be a possibility to delay the effector signal in a way that allows the reporter to appear before the whole cell is filled with effector signal. Apart from the choice of fluorophores, the necessity to transform the target gene reporter stably into plants represents a serious time lag

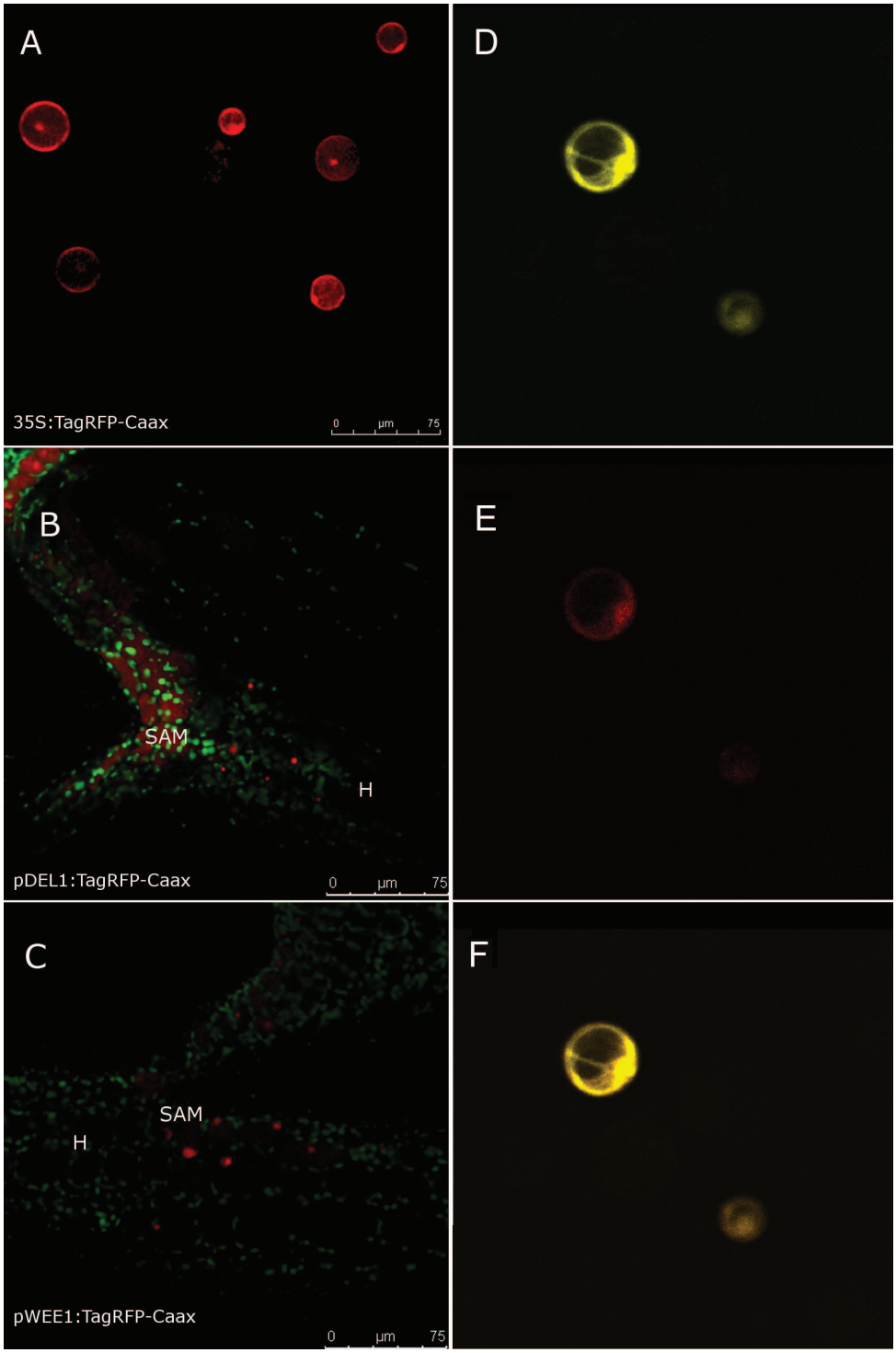


Figure 4. Imaging of TagRFP reporter gene constructs and transactivation analysis. *A) positive control, 35S-TagRFP-CaaX transfected into protoplasts leads to strong accumulation of red signal in protoplast membranes as well as in the nuclear envelops; B) Imaging of a 4 days old seedling stably expressing pDEL1-TagRFP-CaaX or C) pWEE1-TagRFP-CaaX. TagRFP signal is depicted in red, chloroplasts in green. The red dots represent the accumulation of TagRFP-CaaX protein in the nuclear envelope of cells at the base of the cotelydons. H designates the hypocotyl, SAM the shoot apical meristem. (D-F) representative pictures of a transactivation assay, in this case E2Fb transfected protoplasts of DEL1 reporter plants, showing the amount of (D) E2Fb-YFP signal and the obvious bleed-through into the (E) TagRFP channel. (F) The overlap does not show any obvious difference between the two channels, representing the strong masking effect of the YFP-signal.*

in the performance of transcription factor studies. Transiently transfecting the reporter gene is a possible solution to the problem, but this evokes a further problem by increasing false negative results, as a missing signal can be explained by a lack of transactivational relationship but also by a non-successful co-transfection of the reporter construct. A control gene on the reporter construct, represented by a third fluorophore or another non-invasive selection marker under a stable promoter might be introduced to cope with this problem. Transactivation systems exist for some time (Asai et al., 2002), but in many cases they are not quantitative. The transactivation system presented in this work was an attempt to establish a quantitative system with read outs for the amounts of transcription factors and transcriptional response by using fluorophores for both effectors and reporters. Unfortunately, due to technical difficulties and a lack of time we did not succeed in the development of a robust system to verify our yeast one-hybrid results.
Conclusively, a large set of putative regulators of three important cell cycle genes have been identified that provide a valuable resource for follow up experiments in order to unravel the transcriptional regulation of the cell cycle.

Chapter 6

Conclusions and Perspectives:
TCPs - not masters but henchmen of growth?

The historical henchman (*pl*. henchmen) is described as 'a squire or page of honour to a person of rank' in the Oxford dictionary (http://oxforddictionaries.com/). In terms of molecular networks, a henchman would be a subordinate transducer of signals sent by master regulators. TEOSINTE-like/CYCLOIDEA/PCF1 (TCP) transcription factors seem to take on this role in plant development.

Processes controlled by TCP transcription factors

Previously, TCPs have been found to alter growth phenotypes in a number of plant species, including two of the three founder genes of the *TCPs (*Doebley et al., 1995; Luo et al., 1996; Kosugi and Ohashi, 1997). Based on the phenotypes of the *teosinte branched 1* and *cycloidea* mutants, and the fact that PCF1 binds cell cycle genes, soon the hypothesis was formulated that TCP transcription factors are direct regulators of the cell cycle (Cubas et al., 1999; Li et al., 2005). In depth analyses of *jaw*-D mutants led to first doubts against this hypothesis (Efroni et al., 2008), and we found cell cycle genes even to be under-represented in genome-wide target gene analyses for the two Arabidopsis TCP transcription factors TCP10 and TCP20 (Chapters 2 and 3, this thesis). Instead, both TCPs have among others significantly higher numbers of target genes involved in hormone metabolism and response, and cell wall biosynthesis and modification. Already in earlier studies, TCP4, which is a member of the so called class II TCPs, has been found to regulate the expression of *LIPOXYGENASE2 (LOX2),* a gene encoding for an enzyme that catalyses a major step in jasmonate biosynthesis (Schommer et al., 2008). In the *jaw*-D mutant the microRNA *miR319a* is overexpressed, and hence five class II *TCPs* that are *miR319a* targets, including *TCP4,* are knocked down (Palatnik et al., 2003). Consequently, jasmonate contents are very low (Schommer et al., 2008). *Jaw*-D plants show a plethora of growth defects, the most obvious of which are the highly serrated leaves (Palatnik et al., 2003), which are the result of prolonged cell proliferation during leaf growth (Efroni et al., 2008). Furthermore, *jaw*-D plants grow slower, exhibit delayed senescence, and their flowers display abnormal petals (Palatnik et al., 2003; Schommer et al., 2008). As jasmonates are known to be involved in all these processes (Staswick et al., 1992; Schommer et al., 2008; Zhang and Turner, 2008; Brioudes et al., 2009), we hypothesize that jasmonates positioned downstream of the *miR319a* regulated *TCPs* play a major role in the specification of the *jaw*-D phenotype. In this thesis, TCP10 and the class I TCP20 protein have also been found to control jasmonate synthesis and response. Moreover, other TCPs are involved in hormonal regulation as well: TCP3 is involved in auxin signalling (Koyama et al., 2010), TCP1 in brassinosteroid synthesis (Guo et al., 2010), and the TCP9 gene responds to brassinosteroids (Yu et al., 2011) and its expression is associated with jasmonate synthesis (Chapter 3, this thesis).

Apart from hormonal signalling, at least three TCPs (TCP4, TCP10 and TCP20) seem to be involved in cell wall biosynthesis and modification (Chapters 2 and 3, this thesis) (Schommer et al., 2008). Target genes of these transcription factors include cellulose synthase like genes, expansins and xyloglucan endotransglucosylase/hydrolases. Plant cell walls are heterogenous structures that consist of multiple polysaccharide and glycoprotein components, specifically

celluloses and hemicelluloses (Varner and Lin, 1989; Burton et al., 2010), and they contribute to the rigidity and mechanic strength of the plant cell. The composition of cell walls undergoes major changes during development, e.g. during expansive growth of leaf cells (Carpita and Gibeaut, 1993; Cosgrove, 2000). Expansins and xyloglucan endotransglucolyases/hydrolases (XTH) play major roles in these changes (Fry et al., 1992; McQueen-Mason et al., 1992).
The gene *TOUCH4 (TCH4)*, for example, encodes for a XTH, and was found to be up-regulated by TCP10 (Chapter 2, this thesis). XTHs modify cell walls in another manner than expansins but seem to have similarly diverse expression patterns and functions (McQueen-Mason et al., 1993; Rose et al., 2002), such as cell elongation in flower apices (Hyodo et al., 2003) and petiole elongation in shade avoidance reactions (Sasidharan et al., 2010). *TCH4* has been shown to react to environmental stimuli, respond to auxin and brassinosteroid signals, and to be differentially expressed during Arabidopsis development (Xu et al., 1995). According to The Bio-Array Resource for Plant Biology (BAR, http://bar.utoronto.ca/), *TCH4* is most strongly expressed in leaves (Toufighi et al., 2005), but it was detected in all organs, specifically during phases of rapid growth in leaves, trichomes, carpels and siliques (Xu et al., 1995). As TCP10 has been shown to influence leaf development, we hypothesize that it is a major regulator of *TCH4* expression during leaf development.
Another interesting process where XTHs (and expansins) seem to be involved is petal expansion during flower development. Similarly to leaf development, petal development can be divided into cell proliferation and cell expansion phases (for a review see Irish, 2008). XTHs and expansins both seem to act during the rapid expansion of petal cells in roses (Takahashi, 2007), gerbera (Laitinen et al., 2007), and carnation flowers (Harada et al., 2011). If XTHs play a conserved role in petal development, they may have the same function in Arabidopsis. TCP4 regulates Arabidopsis petal growth (Nag et al., 2009), and the increased expression of *TCP20* in petal primordia of young Arabidopsis flower buds (this thesis, Chapter 3) hints at a function of this class I TCP in petal development as well. It is tempting to speculate that the rapid petal growth, which is necessary for correct Arabidopsis flower development and opening, is at least partially regulated by class I and class II TCPs and their influence on expansins and XTHs. Future work on the regulatory association between TCPs and XTHs may provide proof for this hypothesis.

TCPs and leaf development

Leaf development is a highly complex and coordinated process (Van Lijsebettens and Clarke, 1998; Granier et al., 2002), where cells of the same leaf can be proliferating, while others are differentiating to stomata cells or are heavily expanding (Avery, 1933). Because *TCPs* are expressed throughout leaf development and due to the diversity of processes that are on-going at a particular leaf developmental stage, it is very difficult to assign specific TCP functions to a single event in leaf cell development. However, based on the knowledge that exists about target genes of the TCP transcription factor family members TCP3 (Koyama et al., 2010), TCP4 (Palatnik et al., 2003; Schommer et al., 2008), TCP10 and TCP20 (this thesis), we can compile the processes in which TCPs are involved (Figure 1).

While earlier publications differentiate between three phases of leaf development (Donnelly et al., 1999), we expand this and distinguish between five different phases: initiation, proliferation, transition, expansion and senescence.

During leaf initiation, the shoot apical meristem (SAM) produces agglomerations of cells at its flanks that will eventually grow out to become leaves. The plant hormone auxin seems to play a prominent role in this process (Reinhardt et al., 2000), while inhibition of auxin action leads to a disruption of leaf initiation (Scanlon, 2003). The TCP transcription factor TCP3 controls auxin response in leaf development, but also the expression of *ASYMETRIC LEAVES 1 (AS1)*, a MYB domain protein that is involved in leaf initiation (Byrne et al., 2000). TCP3 also controls the microRNA *miR164,* which is a regulator of *CUP-SHAPED COTYLEDON (CUC)* genes (Raman et al., 2008; Koyama et al., 2010). CUC proteins are important for correct leaf initiation because they specify the borders of leaf primordia, and knockout of *CUC* genes leads to fused organs (Aida et al., 1997). Other class II TCPs may also control leaf initiation, as observed when over-expressing microRNA resistant versions of *TCP4* or *TCP10.* These lead to aberrant phenotypes, where either no or only one or two leaves initiate, after which a seedling eventually dies (Palatnik et al., 2003) (Chapter 2, this thesis).

In the proliferation phase of leaf development, especially TCP20 seems to inhibit maturation processes by inhibiting jasmonate expression, iron homeostasis and cell wall modification, and thus guaranteeing that leaves do not differentiate prematurely (Chapters 3 and 4, this thesis). Antagonistically to TCP20, the class II TCPs TCP4 and TCP10 seem to stimulate cell expansion and maturation via hormonal pathways and cell wall modification (Chapter 2, this thesis) (Schommer et al., 2008). Downregulation of these class II TCPs leads to the *jaw*-D phenotype, that can be seen as ever-proliferating leaves (Palatnik et al., 2003; Efroni et al., 2008).

In the transition phase and during leaf cell expansion, the class II TCP transcription factors TCP4 and TCP10 control the modification of cell wall components and jasmonate response pathways. Detailed analyses of *jaw*-D plants revealed that senescence is under the control of the class II TCPs (Palatnik et al., 2003; Schommer et al., 2008). Interestingly, the microRNA *miR164,* that is important for leaf initiation, plays a role in senescence as well (Kim et al., 2009). *MiR164a,* together with ORESARA 1 (ORE1) and ETHYLENE INSENSITIVE 2 (EIN2), regulates age-induced cell death. Because TCP3 has been shown to regulate *miR164a* expression (Koyama et al., 2010) in early leaf development, the question arises if *miR164a* expression during leaf senescence is also under the control of TCP3. This has to be proven yet, using for example stage-specific ectopic expression of *TCP3*.

In all stages of leaf development, TCPs seem to regulate developmental changes via the regulation of plant hormone metabolism and responses. Hormones affected this way include auxins, jasmonates, and ethylene. All these hormones are known for their effects on the cell cycle. It will be a question of future TCP research, if all other phenotypes that are attributed to TCPs, like the axillary bud meristem outgrowth of *teosinte like 1* (Doebley et al., 1995), or the aberrant flower symmetry of *cycloidea* (Luo et al., 1996) and *the jaw*-D petal phenotype (Palatnik et al., 2003), are the outcome of TCP-regulated hormonal changes that affect the cell cycle, either by

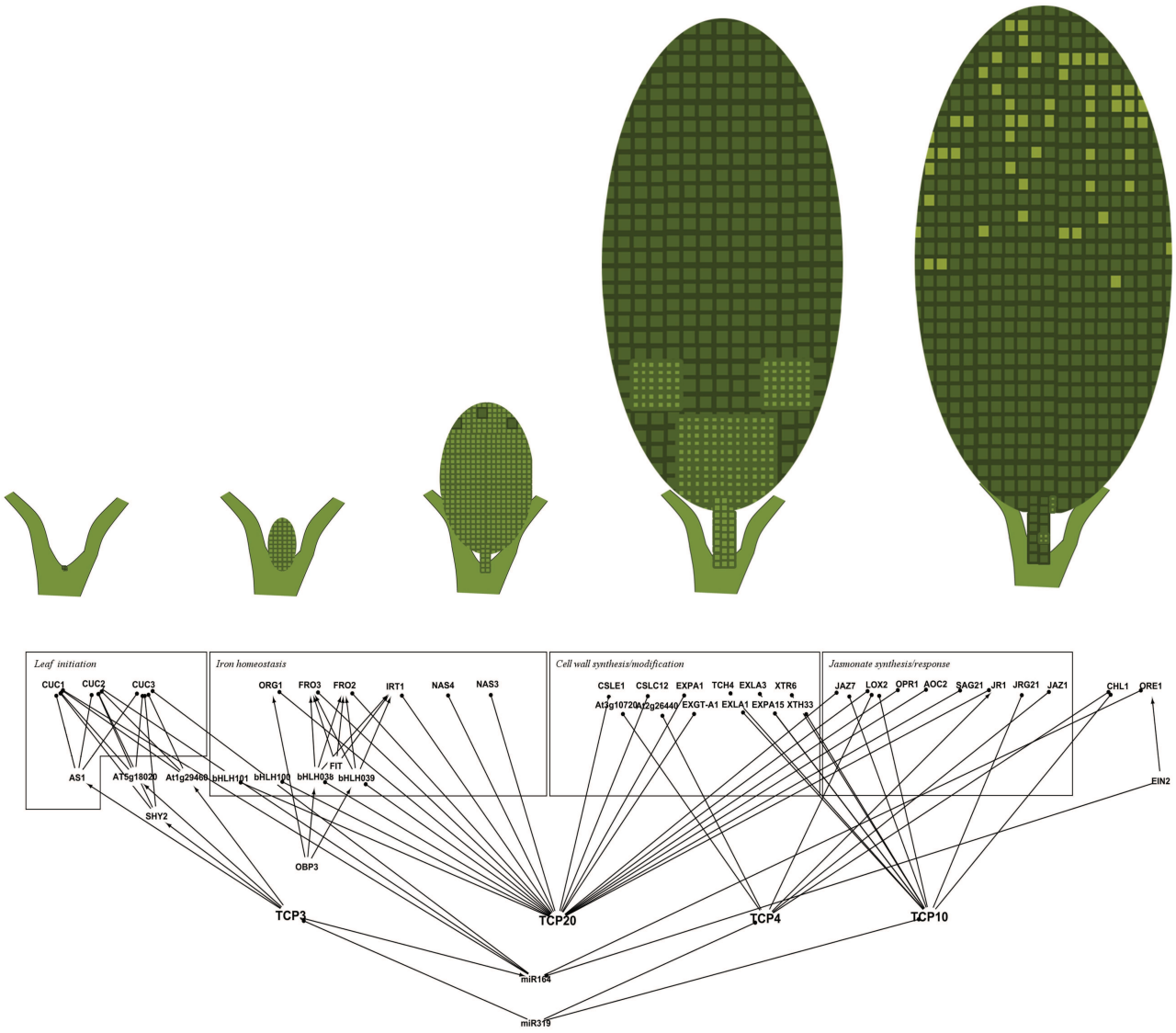


Figure 1. Schematic representation of the processes that TCPs control and their temporal distribution throughout leaf development. *At the top: a schematic leaf in the five different developmental stages: Initiation, proliferation, transition, expansion and senescence. Squares represent cells that first proliferate (resulting in more squares) and then expand (resulting in bigger squares). The colour change from light green – to green – to yellow represent the changes from proliferating to mature cell and from mature cell to an aging leaf cell. At the bottom: gene regulatory networks under the control of TCP transcription factors. In bigger letters the four TCPs which have been included in this model, in smaller letters target genes and co-regulators of the TCPs. For simplicity, target genes are grouped into target gene categories of the same function (squares).*

inhibiting the cell cycle or by promoting further proliferation. Jasmonates, for example, are known to inhibit the cell cycle when applied to cell suspension cultures or when produced endogenously by wounding of the plant (Światek et al., 2002; Zhang and Turner, 2008). The lack of jasmonic acid in *jaw*-D mutants (Schommer et al., 2008), which is the result of missing activation of *LOX2* by class II TCP transcription factors, may be a reason for the ever-proliferating leaf cell phenotype detected in these plants (Efroni et al., 2008). This would explain why so many *tcp* mutants seem to have an effect on the cell cycle although cell cycle genes seem not to be direct targets of TCPs.

Who controls the TCPs?

TCPs exhibit relatively ubiquitous expression which is an indication for a more general role of TCPs in plant development. Nevertheless, misexpression of *TCPs* can lead to abnormal growth phenotypes (Herve et al., 2009; Nag et al., 2009; Viola, 2011), which means that expression patterns and correct regulation of *TCPs* is of relevance for normal plant development. Besides post-transcriptional regulation of *TCP* expression, which has been shown in the case of *miR319a* regulated class II *TCPs* (Palatnik et al., 2003), upstream control of *TCP* expression is also conceivable. But what would be the upstream regulators of *TCPs* that define the developmental time points at which *TCPs* are active? In genome-wide chromatin immunoprecipitation (ChIP) analyses followed by next generation sequencing Kaufmann and colleagues found *TCPs* to be over-represented target genes of the MADS-box transcription factor SEPALLATA3 (SEP3) (Kaufmann et al., 2009). Furthermore, putative TCP binding sites were close to sites where SEP3 was bound, implying activation of *TCP* expression and then co-operation between SEP3 and TCP transcription factors in the control of other target genes (Kaufmann et al., 2009). In contrast to most *TCP* genes, *MADS-box* genes have defined temporal and spatial expression patterns. Based on this knowledge we suggest that TCPs might regulate the growth of organs downstream of the organ-determining signals of MADS domain transcription factors (Dornelas et al., 2011), or in other words, TCPs are the henchmen of their MADS-box masters.

Another mode of regulating TCP action is by association with different dimerization partners. The TCP domain is not only a DNA binding domain, it is also involved in mediating protein-protein interactions with other TCP transcription factors (Kosugi and Ohashi, 2002). We were able to show the existence of a plethora of potential TCP-TCP dimers that can fulfil different functions (Chapter 2, this thesis). Apparently, TCPs prefer to form dimers with TCPs of their own class (Kosugi and Ohashi, 2002) (Chapter 2, this thesis). To what extent this class specific dimerization pattern is of functional importance needs more thorough investigation. It is possible that specific dimers can only form at very restricted time points during development, due to the expression patterns of the two involved *TCPs,* and that this leads to a temporally limited regulation of common target genes. Apart from TCP-TCP dimers, the example of TCP21 and its dimerization partner TIMING OF CAB1 EXPRESSION (TOC1), a pseudo response regulator, shows that TCPs can form complexes with other proteins as well, broadening the horizon of possible regulatory interactions (Pruneda-Paz et al., 2009).

The dimerization of TCP21 with TOC1 serves the regulation of the core circadian clock gene *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* (Wang and Tobin, 1998; Pruneda-Paz et al., 2009). This is not the only association between TCPs and circadian clock components. Another study shows that the TCPs TCP2, TCP3, TCP11 and TCP15 can form dimers with several clock components and that class II TCPs can bind promoters of circadian clock genes (Giraud et al., 2010). Conversely, many *TCP* transcripts themselves follow a circadian expression pattern. There is still little known about the relationship between TCPs and the clock, but it seems as if there is a close functional link between them. One possibility is that TCPs are downstream transducers of signals sent from the clock. *TCP21,* for example, is specifically strongly expressed in leaves and follows a circadian expression pattern. This suggests that TCP21 is not only involved in the regulation of *CCA1,* but that it might also fulfil the role of a downstream transducer of circadian signals into leaf growth.

Perspectives of TCP research

TCPs are actively involved 'on the workbench' of branching, floral symmetry regulation and leaf growth. Yet, to fully understand TCP-mediated control of these developmental processes, and particularly leaf development, which is the topic of this thesis, more information is needed on TCPs and their target genes. Especially the functions of class I TCPs are poorly understood, as redundancy hampers attempts to characterize them. Often this redundancy is only partial and subtle changes in growth or development can be observed in single mutants, e.g. *tcp9*, as demonstrated in Chapter 3. However, since the majority of the single knockouts do not show obvious defects, alternative ways have to be taken to understand their functions. The classical way is to obtain double or multiple knockouts of closely related *TCP* genes. As there are 13 class I *TCPs* in the Arabidopsis genome, close analyses of expression patterns and other molecular and physical properties of the encoded proteins can help identifying *TCP* genes sharing common functions, as described in Chapter 2, and knocking out those *TCPs* specifically may be more successful than simply impairing *TCP* gene pairs with closest sequence homology. Furthermore, direct target gene identification using glucocorticoid inducible TCP proteins shows promising results and can be applied for more TCP proteins. The target genes identified can be analysed for their expression in single and multiple *tcp* knockout plants, whereas chromatin immunoprecipitation can help to verify physical and specific DNA binding, and hence support the elucidation of TCP functionality. Additionally, studies have been published following an approach in which TCPs are tagged to the transcriptional repressing EAR- or transcriptional activating VP16 domains (Herve et al., 2009; Sarvepalli and Nath, 2011; Viola et al., 2011). These are valid approaches as well, but especially when expressing these constructs under a constitutive promoter, phenotypic effects are likely to be indirect and pleiotropic, which makes it difficult to interpret the results and place the TCPs in pathways they are naturally involved in.

Another approach that may shed light on TCP functions is to unravel the *in vivo* protein-protein interactions of TCPs amongst each other and with other proteins. It is unknown yet if dimerization is necessary for TCP function, although association with TOC1 helps TCP21 to bind

the *CCA1* promoter (Pruneda-Paz et al., 2009), and TCP20 binds in a yeast assay to the *LOX2* promoter in combination with other class I TCPs (Chapter 3, this thesis). Determination of *in vivo* binding partners by protein immunoprecipitation and mass spectrometry (Hubner et al., 2010; Kaufmann et al., 2011), or by in *vivo* imaging (Hébert et al., 2006) will bring us one step further in unravelling the importance of complex formation for TCP functioning.

A topic of TCP research that has not been a main focus in this thesis is the widespread presence of this family in the plant kingdom and the repercussions of this fact for TCP functions. As mentioned in Chapter 1, TCPs can be found in all higher plants, but they are also found in the freshwater algae *Cosmarium sp.* (Navaud et al., 2007). A first question arising is whether the functions of TCPs are conserved between different species. Several examples show that this is at least the case for some family members. Firstly, a strong knockout of the *CINCINNATA (CIN)* gene in *Antirrhinum majus*, which has high sequence similarity to the *TCPs* downregulated in the Arabidopsis *jaw*-D plants (Palatnik et al., 2003), leads to severe leaf growth defects (Crawford et al., 2004) similar to the *jaw*-D phenotype. Downregulation of a second closely-related sub-clade of *CIN-like* genes in Arabidopsis leads to divergent leaf growth again, demonstrating that especially CIN-TCPs are important for normal leaf development (Efroni et al., 2008). Similarly, the *lanceolate* mutant in tomato, which displays altered leaf morphogenesis, is also the result of misexpressed *CIN-TCPs* (Ori et al., 2007; Shleizer-Burko et al., 2011). The second evidence for a conserved function of TCPs in plant development comes from the axillary bud meristem outgrowth phenotype of *teosinte branched 1* in maize (Doebley et al., 1995) and the *teosinte branched-like* phenotypes in Arabidopsis (Aguilar-Martinez et al., 2007). Here, the branching function of TCPs is conserved between monocots and dicots, indicating this to be an ancient TCP function. Another TCP that is known to function in several plant species is CYCLOIDEA, which has been shown to influence flower symmetry in different plant families (Citerne et al., 2003; Gubitz et al., 2003; Reeves and Olmstead, 2003; Broholm et al., 2008).

In the above mentioned cases several Arabidopsis TCPs share a function that is represented by the presence of only one gene in another species, indicating that in Arabidopsis and its ancestors duplication events led to increased functional redundancy. In summary, TCPs seem to have conserved functions in different plant species, and most analyses so far have been done in eudicot plant species. Further research in monocots and lower plants will provide a better understanding of the rate of conservation within the TCP transcription factor family (Mondragón-Palomino and Trontin, 2011).

Growth – who is in charge?

TCP transcription factors have been chosen as targets for the work described in this thesis because earlier publications suggested that TCPs are major transcriptional regulators of growth, and in particular of leaf growth (Cubas et al., 1999; Li et al., 2005). Indeed, some *tcp* mutants show growth phenotypes, but in general TCPs seem to be regulating growth not as masters but as downstream transducers of developmental signals. So who controls growth? It is likely that this question tends to simplify the phenomenon of growth. Growth is a multi-factorial process that is

the sum of external and internal factors.
That growth is coordinated endogenously is evident from the fact that individuals of one species display low variations in size of organs, although external factors can affect the overall growth of a plant. In plants, factors that have been identified to be involved in endogenous growth regulation include transcription factors (e.g. Krizek, 1999), plant hormones (e.g. Morinaka et al., 2006), cell cycle components (Dewitte et al., 2007), ribosomal proteins (Van Minnebruggen et al., 2010), and microRNAs (Palatnik et al., 2003).
Apart from these internal factors, exogenous influences affect growth as well: these can be e.g. light conditions (Morelli and Ruberti, 2002), carbon-to-nitrogen availability (Martin et al., 2002), and water availability (van der Weele et al., 2000). The integration of all these different inputs into plant growth requires sophisticated signalling and regulatory networks (Achard et al., 2006), which increases the number of genes and connectivity of the genes involved in the processes underlying growth.
In the course of the EU-AGRONomics (LSHG-CT-2006-037704) project 14 internationally renowned labs joined forces to systematically identify the factors controlling leaf growth in Arabidopsis. The work described in this thesis is part of this project. In four years' time, a lot of data has been generated on Arabidopsis leaf growth and the the integration of different data-sets into a model that explains leaf growth as comprehensively as possible will be challenging. Next to the progress in experimental methods and their use in data generation, computer-based modelling will be of utmost importance for developmental plant biology in the future (Prusinkiewicz, 2004; Rhee et al., 2006). The synergy between experimental and computer-based methods will bring us closer to the understanding of the multi-factorial process we tend to simplify by just naming it 'growth'.

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Summary

Leaves are the main organs for photosynthesis in plants, and hence the development of these organs is under strict control. Leaf development is generally divided into two phases, the proliferation and expansion phases. In the proliferation phase, most leaf cells are undifferentiated and undergo repetitive mitosis. After this phase, cells stop dividing and start to differentiate and grow by expansion, leading to mature leaf cells. Additionally to these two main stages of leaf development, several other stages are important as well. These are the initiation of the leaf from the shoot apical meristem (SAM), the transition phase, in which the leaf cells switch from proliferative to expansive growth, and finally leaf senescence, a stage in which leaves undergo controlled aging and death. All these phases are under the control of both endogenous and exogenous influences. In this work we were interested in a particular class of transcription factors known to control leaf development: the TCP transcription factor family.

In Chapter 1 of this thesis a detailed introduction is given into TCP transcription factors and their supposed role in leaf development. Some members from the TCP transcription factor family are involved in transcriptional control of leaf development in different plant species, including tomato, snapdragon and the model plant Arabidopsis thaliana. The Arabidopsis genome encodes 24 genes of the TCP transcription factor family, which according to sequences were divided into two classes, class I and class II TCPs. Based on the putative consensus binding sites of TCP proteins belonging to these classes, the theory has been postulated that proteins of the two classes compete for binding in the promoters of the same genes, leading to an antagonistic regulation of these targets. In general, not much is known about TCP functions, and almost all knowledge comes from analyses of class II tcp mutants, whose divergent phenotypes have been characterized in single and multiple gene knockouts. Single tcp knockouts often have inconspicuous phenotypes that can be explained by strong functional redundancy within the TCP transcription factor family. The most prominent example is the five TCP transcription factors that are under control of the microRNA miR319a, and whose collective downregulation leads to the JAGGED AND WAVY (JAW) phenotype.

Different ways exist to circumvent problems with genetic redundancy. One way is to cross knockout lines for closely related homologues in order to knock-out complete functions. In the TCP family, where sequences are highly variable outside the so called TCP domain, highest sequence homology is not always a good predictor for functional redundancy, which we could show in Chapter 2. Instead, integration of expression and other functional data can help determining the level of functional redundancy between closely related genes. In Chapter 2, we could show e.g. that from the four TCPs that are closely related to TCP4 the transcription factor TCP10 has the highest overlap in sequence, expression, and protein-protein interaction capacity. Further investigation of TCP10 target genes and comparison with published data on TCP4 targets shows a strong overlap, specifically covering genes that are involved in jasmonate (JA) synthesis and response, indicating common functions and

confirming earlier studies regarding the importance of JA signaling in mediating class II TCP control of leaf development. Another way to analyze gene function of a transcription factor despite genetic redundancy within a gene family, is to identify the genes that are under direct transcriptional control of the transcription factor. For this purpose, several methods are available, like chromatin immunoprecipitation (ChIP) and the use of glucocorticoid induction assays (GR) combined with genome-wide expression studies by micro-array analyses or RNA-seq. We made use of ChIP and GR assays to determine the function of the class I TCP transcription factor TCP20, because no phenotypic alterations could be observed in tcp20 single knockout mutants (Chapter 3). We identified 278 potential direct target genes for TCP20, of which a significant proportion appeared to be again JA synthesis and response genes. Surprisingly, cell cycle genes, which were supposed to be under the control of TCP20, were not found in our study. Though, LIPOXYGENASE2 (LOX2), which is also under the control of the class II TCP transcription factors TCP4 and TCP10, was found, which gave us the possibility to investigate the previously suggested antagonistic control of target genes by class I and class II TCPs. We could not show a direct competition between TCP20 and TCP4 for binding to the same cis-elements in the LOX2 locus, but were able to show binding of both TCP4 and TCP20 to LOX2 regulatory sequences and activation versus repression of LOX2 transcription by TCP4 and TCP20, respectively. This at least partially conformed the theory and suggests that the two classes have antagonistic functions.

Another group of genes that was found to be over-represented in the TCP20 target gene list were genes involved in iron homeostasis of both roots and leaves. In Chapter 4 we describe the analysis of this TCP20-mediated control of the subgroup Ib basic helix-loop-helix transcription factors bHLH038, bHLH039, bHLH100, and bHLH101. This study includes a functional analysis of these genes, which previously had been found to be involved in iron homeostasis and to be specifically up-regulated at the transition stage when leaves switch from mainly proliferative to expansive growth. The experiments showed that these transcription factors are involved in photomorphogenesis, suggesting that they are regulating iron homeostasis during the switch from proliferative to differentiated leaf cells, and that the inhibition by TCP20 may function as a means to suppress cell differentiation during early leaf development.

If TCPs are not involved in the control of the cell cycle during leaf growth, the question remains which transcription factors fulfill this function. A large scale analysis of transcription factor binding to three selected cell cycle promoters is described in Chapter 5. In a yeast one-hybrid assay we found TCPs to be under-represented, as expected. Instead, transcription factors of different families have been identified, some of which previously have not been associated to cell cycle control. Of these families especially the MYB and NAC families of transcription factors stand out. For a large part of the identified transcription factors no function is known from mutant analysis and hence, their involvement in cell cycle regulation, especially in leaf development, should be investigated in future research.

In Chapter 6, the results of the previous chapters are summarized and interpreted. In sum, we analyzed functions for two TCP transcription factors by the identification of their genome-wide target gene spectrum and found both to be involved in hormonal control of leaf

development, but also in cell wall control and iron homeostasis, increasing the number of cellular functions in which TCPs are potentially involved. Additionally, we could not find any indications that core cell cycle genes are direct targets of these transcription factors, despite the growth effects discovered when knocking down several TCP genes. The fact that in these tcp mutants ultimately cell proliferation is affected leads to the assumption that TCP genes indirectly control the cell cycle via downstream targets. For this, especially the hormones under their control are strong candidates because jasmonic acid and other hormones have previously been shown to be involved in both cell cycle control and the regulation of leaf development at different stages. We conclude that the broadly expressed members from the TCP transcription factor family are not the key regulators of growth, but act as co-factors or mediators in this biological process.

Samenvatting

Bladeren zijn belangrijke plantenorganen vanwege hun fotosynthetische capaciteit, en de ontwikkeling van deze organen wordt exact gecoördineerd. Bladontwikkeling kan worden onderverdeeld in twee fasen, de proliferatie- en de expansiefase. In de proliferatiefase zijn de meesten bladcellen ongedifferentieerd en ondergaan herhaaldelijk mitose. Na deze fase stoppen cellen met het delen en beginnen te differentiëren en groeien door expansie, wat leidt tot volwassen bladcellen. Naast deze twee belangrijke fasen van bladontwikkeling zijn een aantal andere processen van belang. Deze zijn de initiatie van het blad primordium vanaf het scheut apicale meristeem (SAM), de overgangsfase, waarin de bladcellen stoppen met delen en beginnen te expanderen, en ten slotte de bladveroudering, een fase waarin bladeren gecontroleerd hun activiteiten afbouwen en uiteindelijk verwelken en afsterven. Al deze fasen zijn onder controle van endogene en exogene signalen. In deze studie zijn zogenaamde TCP (TEOSINTE-like, CYCLOIDEA, PCF1) transcriptiefactoren geanalyseerd vanwege de veronderstelde functie van deze regulatiefactoren in bladontwikkeling.

Hoofdstuk 1 van dit proefschrift is een gedetailleerde inleiding over de TCP transcriptiefactoren en hun vermeende rol in de bladontwikkeling. Uit onderzoek is gebleken dat specifieke leden van deze familie zijn betrokken bij de transcriptionele controle van bladontwikkeling in verschillende plantensoorten, waaronder tomaat, leeuwebek en de modelplant Arabidopsis thaliana. Het genoom van Arabidopsis codeert voor 24 genen behorende tot de TCP familie, die op basis van sequentie homologie in twee klassen kunnen worden verdeeld, de klasse I en klasse II TCP's. Op basis van de vermoedelijke consensus DNA bindingsplaatsen van TCP-eiwitten van de beide klassen is gesuggereerd dat eiwitten van de twee klassen concurreren voor binding aan de promotors van dezelfde genen, wat leidt tot een antagonistische regulering van deze doelgenen. Er is in het algemeen niet veel bekend over functies van TCP genen, en bijna alle kennis komt uit analyses van klasse II tcp mutanten, waar afwijkende fenotypes zijn gekarakteriseerd in enkelvoudige en meervoudige knockouts. Meestal hebben tcp knockouts onopvallende of helemaal geen afwijkende fenotypes. Dit kan worden verklaard door een sterke functionele redundantie binnen de TCP transcriptiefactor familie. Het meest prominente voorbeeld is het zogenaamde JAGGED AND WAVY (JAW) fenotype, dat verkregen werd door het tot overexpressie brengen van microRNA miR319a, waardoor de expressie van vijf TCP genen tegelijkertijd gereduceerd wordt.

Er zijn verschillende manieren bekend om problemen door genetische redundantie bij het bepalen van gen functies te omzeilen. Een manier is om knock-out lijnen voor nauw verwante homologen te kruisen om zodoende volledige functies uit te schakelen. In de TCP familie, waar sequenties relatief variabel zijn buiten het geconsenveerde en zogenaamde TCP-domein, is de mate van sequentiehomologie niet altijd een goede voorspeller voor functionele redundantie. Dit hebben wij laten zien in hoofdstuk 2, waar geanalyseerd is hoe het bepalen van het niveau van functionele redundantie kan worden verbeterd door naast het niveau van sequentiehomologie ook expressie data en andere functionele gegevens mee te nemen. Door

middel van deze method konden we bijvoorbeeld aantonen dat van de vier TCP's die nauw verwant zijn aan TCP4 de transcriptiefactor TCP10 de hoogste overlap qua sequentie, expressie, en eiwit-eiwit interactie capaciteiten heeft. Nader onderzoek van TCP10 doelgenen en vergelijking met gepubliceerde gegevens over TCP4 doelgenen bevestigt deze sterke overlap in functie. Vooral genen die betrokken zijn bij jasmijnzuur (JA) synthese en signalering zijn gemeenschappelijk doelen van deze twee transcriptiefactoren. Dit bevestigt tevens het in eerdere studies gesuggereerde belang van klasse II TCPs in de regulatie van JA signalering in relatie tot bladontwikkeling. Een andere manier voor de functionele analyse van transcriptiefactoren, die geschikt is om de genetische redundantie te omzeilen, is het identificeren van de doelgenen die onder directe transcriptionele controle staan van de transcriptiefactor. Hiervoor kunnen technieken gebruikt worden zoals chromatine immunoprecipitatie (ChIP) en het toepassen van glucocorticoïde inductie assays (GR) in combinatie met genoomwijde expressie studies via bijvoorbeeld micro-array analyses of mRNA sequencering. Wij hebben ChIP- en GR-assays toegepast om de functie van de klasse I TCP transcriptiefactor TCP20 te bepalen. Ook voor dit TCP gen geldt dat er geen fenotypische veranderingen kunnen worden waargenomen in tcp20 single knock-out mutanten (hoofdstuk 3). De analyse heeft geleid tot de identificatie van 278 mogelijke directe doelgenen voor TCP20, waarvan opnieuw een aanzienlijk deel JA biosynthese en response genen bleken te zijn. Verrassend genoeg werden genen betrokken bij de regulatie van de celcyclus, die geacht worden onder de controle van TCP20 te staan, niet gevonden in ons onderzoek. Echter, het JA biosynthese gen LIPOXYGENASE2 (LOX2), dat ook onder controle staat van de klasse II TCP transcriptiefactoren TCP4 en TCP10, werd wel gevonden. Dit gaf ons de mogelijkheid de eerder veronderstelde antagonistische controle van doelgenen door klasse I en klasse II TCP's te onderzoeken. We konden in deze analyse geen directe concurrentie tussen TCP20 en TCP4 voor binding aan hetzelfde DNA motief in het LOX2 locus ontdekken maar wij waren wel in staat om de binding van zowel TCP4 als TCP20 aan LOX2 regulerende sequenties aan te tonen. Daarnaast bleek duidelijk dat TCP4 een activator is van LOX2 terwijl TCP20 de expressie van dit gen onderdrukt. Hiermee is de theorie ten dele bevestigt en dit geeft sterke aanwijzingen dat het klasse I TCP20 eiwit en het klasse II TCP4 eiwit antagonistische functies hebben.

Een andere groep van genen die bleek te zijn oververtegenwoordigd onder de TCP20 doelgenen zijn genen die betrokken zijn in ijzer homeostase van wortels en bladeren. In hoofdstuk 4 beschrijven we de gedetailleerde analyse van deze interactie tussen TCP20 en de subgroep Ib basic helix-loop helix transcriptiefactoren bHLH038, bHLH039, bHLH100, en bHLH101. Deze studie omvat een functionele analyse van deze genen, die eerder waren gevonden betrokken te zijn bij ijzer homeostase. Verder bleek dat de expressie van deze genen een specifiek piek vertoont in bladcellen tijdens het overgangsstadium van groei door proliferatie naar expansieve groei. De experimenten toonden aan dat deze transcriptiefactoren betrokken zijn bij fotomorfogenese, wat betekent dat ze ijzer homeostase kunnen reguleren tijdens de overstap van ongedifferentieerde delende cellen tot gedifferentieerde bladcellen, en dat de remming door TCP20 kan functioneren als en middel om celdifferentiatie te onderdrukken tijdens de vroege ontwikkeling van het blad. Uit bovengenoemd onderzoek aan een aantal TCP's blijkt dat deze niet direkt zijn betrokken bij de controle van de celcyclus

tijdens de bladgroei. De vraag blijft dan bestaan welke transcriptiefactoren deze functie wel uitvoeren. In hoofdstuk 5 is een grootschalige analyse van transcriptiefactor binding aan drie geselecteerde cel-cyclus promotoren beschreven. In deze gist one-hybrid analyses vonden we zoals verwacht nauwelijks binding van cel-cyclus promotoren door TCP's. In plaats daarvan hebben wij transcriptiefactoren van verschillende families geïdentificeerd, waarvan sommige niet eerder in verband zijn gebracht met cel-cyclus controle. Van deze families vielen vooral de MYB en NAC families van transcriptiefactoren op, vanwege de vele hits. Voor een groot deel van de geïdentificeerde transcriptiefactoren is geen functie bekend middels analyse van mutanten. Daarom zal het noodzakelijk zijn om hun veronderstelde betrokkenheid bij de controle van de cel-cyclus verder te onderzoeken in de toekomst.

In hoofdstuk 6 worden de resultaten van de voorgaande hoofdstukken samengevat en geïnterpreteerd, gevolgd door aanbevelingen voor toekomstig onderzoek. In totaal analyseerden we functies voor twee TCP-transcriptiefactoren door de identificatie van hun genoomwijde doelgenen, en wij vonden voor beide dat ze betrokken zijn bij hormonale controle van bladontwikkeling maar ook in de controle van celwand vorming en ijzer homeostase, wat het aantal cellulaire functies waarbij TCP's zijn betrokken verhoogd. Opvallend is dat we geen aanwijzingen konden vinden dat de cel-cyclus genen directe doelwitten zijn van deze TCP transcriptiefactoren zijn, ondanks de groei-effecten die ontdekt werden in bepaalde tcp mutanten. Het feit dat bij deze tcp mutanten, waaronder de JAW mutant waarin TCP10 expressie is gereduceerd, uiteindelijk de celproliferatie wel wordt beïnvloed leidt tot de algemene veronderstelling dat TCP genen indirect de celcyclus controleren via hun downstream doelgenen. In het bijzonder zijn de hormonen die onder hun controle staan sterke kandidaten voor deze bemiddelaar functie van TCP controle over de celcyclus. Voor jasmijnzuur en andere hormonen is namelijk eerder al aangetoond dat ze betrokken zijn bij zowel de cel-cyclus controle als de regulatie van bladontwikkeling in verschillende ontwikkelingsstadia. We concluderen daarom dat leden van de TCP transcriptiefactor familie niet de sleutel regulatiefactoren zijn van groei maar als co-factoren of bemiddelaars in dit belangrijke biologisch proces dienen.

Zusammenfassung

Da das Blatt das Hauptorgan der pflanzlichen Photosynthese ist, unterliegt die Entwicklung dieses Organs einer strengen Kontrolle. Blattentwicklung wird generell in zwei Phasen unterteilt: die Zellteilungs- und Zellexpansionsphasen. Die Blattzellen der Zellteilungsphase sind undifferenziert und vermehren sich in mehreren mitotischen Teilungen. Sobald diese Zellen die Zellteilungen beenden, differenzieren sie zu reifen Blattzellen. Während ihrer Differenzierung expandieren Blattzellen und wachsen auf das Mehrfache ihrer ursprünglichen Volumina heran. Neben diesen zwei Hauptstadien der Blattentwicklung existieren weitere wichtige Zwischenstadien. Diese sind die Phasen der Blattinitiierung, der Übergansphase, in der die Blattzellen die Zellteilungsphase beenden und in die Expansionsphase eintreten, und die Phase der Blattalterung (Seneszenz), in der Blätter und ihre Zellen einem kontrollierten Alterungs- und Sterbeprozess unterworfen werden. All diese Phasen sind unter der Kontrolle endogener und äußerer Faktoren. In dieser Studie untersuchten wir eine spezifische Klasse von Genen, die dafür bekannt sind, Blattentwicklung zu regulieren: die TCP (TEOSINTE-like 1, CYCLOIDEA, PCF1) Transkriptionsfaktoren.

In Kapitel 1 werden TCP Transkriptionsfaktoren und deren Rolle in der Blattentwicklung detailliert vorgestellt. Einigen Mitgliedern dieser Genfamilie wurde schon nachgewiesen, dass sie die Blattentwicklung in verschiedenen Pflanzenarten regulieren, wie z.B. in Tomaten, im Löwenmäulchen und in der Modellpflanze Arabidopsis thaliana. Das Arabidopsis Genom umfasst 24 verschiedene TCP-Gene, die entsprechend ihrer Sequenzen in die Klassen I und II unterteilt werden. Da die putativen Bindestellen der Transkriptionsfaktoren beider Klassen einander ähneln, wurde die Theorie aufgestellt, das Transkriptionsfaktoren beider Klassen um Bindestellen gemeinsamer Zielgene konkurrieren, und das sie diese gemeinsamen Zielgene antagonistisch regulieren.

Über die Funktionen von TCPs ist wenig bekannt, und beinahe alles, was bekannt ist, stammt von Untersuchungen an Klasse II TCP Mutanten, die als Einzel- oder Mehrfachmutanten abweichende Phänotypen zeigen. Die meisten TCP Einzelmutanten haben keine oder nur sehr unauffällige phänotypische Abweichungen. Dies wird dadurch erklärt, dass die TCP-Familie starke funktionelle Redundanz zwischen ihren einzelnen Mitgliedern aufweist. Das bekannteste Beispiel redundanter Funktionen von TCPs ist der JAGGED AND WAVY (JAW) Phänotyp, bei dem fünf TCP-Transkriptionsfaktoren, die unter der Kontrolle der Mikro-RNA miR319a sind, durch Überexpression der Mikro-RNA herunterreguliert werden. Erst der sogenannte „knockdown“ dieser fünf Gene führt zu einem deutlich abweichenden Pflanzenwachstum. Zu den bekanntesten Strategien, genetische Redundanz als Hindernis bei funktionellen Analysen von Genen zu umgehen, gehört das Kreuzen von Mutanten interessanter Gene mit Mutanten nahe verwandter Gene, und somit das Ausschalten ganzer Gengruppen und ihrer Funktionen. Diese Strategie stößt in solchen Genfamilien auf ihre Grenzen, bei denen die Sequenzen außerhalb einer gemeinsamen Domäne hohe Variabilität aufweisen, wie das bei der TCP-Familie der Fall ist. In Kapitel 2 zeigen wir. dass in solchen Fällen Sequenzhomologie nicht immer der beste Hinweis auf funktionell redundante Gene ist.

Stattdessen helfen Expressionsdaten und weitere funktionelle Informationen, diejenigen homologen Gene zu bestimmen, die funktionelle Redundanz aufweisen. Beispielsweise zeigen wir, dass von den vier TCPs, die verwandt sind mit dem Transkriptionsfaktor TCP4, TCP10 die grösste Übereinstimmung in Sequenz, Expressionsmuster und Protein-Protein-Interaktionen aufweist. Weitere Analysen zeigen, dass TCP10 und TCP4 gemeinsame Zielgene im Jasmonsäuremetabolismus haben. Dementsprechend konnten wir zeigen, dass die beiden Transkriptionsfaktoren überlappende Funktionen ausführen und dass frühere Studien über die Rolle von Jasmonsäure in der Vermittlung von Klasse II TCP Kontrolle in der Blattentwicklung korrekt sind.

Eine andere Methode, die in der funktionellen Analyse von Transkriptionsfaktorfamilien angewandt werden kann, um genetische Redundanz als Hindernis bei der Bestimmung von genetischen Funktionen zu umgehen, ist die direkte Bestimmung von Zielgenen eines Transkriptionsfaktors. Hierfür kann man Methoden wie z.B. Chromatinimmunprezipitation (ChIP) oder die Verwendung von Glucocorticoid-Induktionsassays (GR) in Verbindung mit Microarray- oder Sequenzierungstechnologien (RNASeq) verwenden. Wir nutzten GR-Assays zur Bestimmung der Funktionen des Klasse I TCP Transkriptionsfaktors TCP20, nachdem Mutanten für diese Transkriptionsfaktor keine phänotypischen Veränderungen gezeigt hatten (Kapitel 3). In dieser Studie identifizierten wir 278 potentielle Zielgene für TCP20, unter diesen signifikant viele Gene des Jasmonsäuremetabolismus. Überraschenderweise waren Gene des Zellzyklus, von denen ursprünglich angenommen wurde, dass sie unter der Kontrolle von TCP20 sind, nicht in unserer Analyse vertreten. Allerdings war das Gen der LIPOXYGENASE2 (LOX2) vertreten, ein Gen der Jasmonsaüresynthese, dass auch schon als Zielgen der Klasse II TCP Transkriptionsfaktoren TCP4 und TCP10 bekannt war. Dies ermöglichte uns zu untersuchen, ob die Theorie der antagonistischen Kontrolle von gemeinsamen Zielgenen durch Klasse I und Klasse II TCP Transkriptionsfaktoren einer biologischen Realität entspricht. Wir konnten keine direkte Konkurrenz um dieselben Bindestellen zwischen den beiden Transkriptionsfaktoren TCP4 und TCP20 feststellen, aber konnten die Theorie teilweise verifizieren, da sowohl TCP4 als auch TCP20 verschiedene regulatorische Domänen des LOX2 Promotors binden und die Expression des Zielgens in verschiedene Richtungen beeinflussen.

Eine andere Gruppe von Zielgenen, die unter der Kontrolle von TCP20 stehen, sind Gene die dem Eisentransport und der Eisenhomöostase in Wurzeln und Blättern von Pflanzen dienen. In Kapitel 4 beschreiben wir die Subfamilie der Ib basic helix-loop-helix (bHLH) Transkriptionsfaktoren bHLH038, bHLH039, bHLH100 und bHLH101, die zu den Zielgenen von TCP20 gehören. Diese Studie umfasst eine funktionelle Analyse dieser Gene, die zuvor als Gene der Eisenhomöostase identifiziert wurden, und vor allem dann stärker exprimiert wurden, wenn das sich wachsende Blatt ins Übergangsstadium zwischen Zellteilung- und Zellexpansionsphasen eintrat. Wir konnten experimentell nachweisen, dass diese Transkriptionsfaktoren eine Rolle in der Photomorphogenese spielen. Daraus entwickelte sich unsere Annahme, dass die Subfamilie der Ib bHLH Transkriptionsfaktoren eine wichtige Rolle in der Eisenhomöostase während der Photomorphogenese einnehmen, und dass die Repression dieser Gene durch TCP20 eine Kontrollmaßnahme früher Blattentwicklung

darstellt, die eine verfrühte Aufnahme von Eisen in der laufenden Zellteilungsphase unterbinden soll.

Da TCP Transkriptionsfaktoren nicht die erwartete Rolle in der Kontrolle des Zellzyklus während des Blattwachstums spielen, stellte sich die Frage, welche anderen Transkriptionsfaktoren diese Rolle erfüllen. Um dieser Frage nachzugehen, vollzogen wir eine umfassende Yeast-1-Hybrid Studie, in der wir die Bindekapazitäten von Transkriptionsfaktoren einer Transkriptionsfaktor-Sammlung an Promotoren dreier Zellzyklusgene untersuchten (Kapitel 5). In dieser Analyse konnten wir kaum TCP Transkriptionsfaktoren detektieren, die die Promotoren binden, was unserer Theorie entspricht, dass TCPs keine oder nur geringe direkte Kontrolle über Zellzyklusgene ausüben. Stattdessen waren Transkriptionsfaktoren verschiedener Familien in der Lage, diese Promotoren zu binden. Vor allem MYB und NAC Transkriptionsfaktoren waren überrepräsentiert in der resultierenden Transkriptionsfaktorliste, die mehrere Transkriptionsfaktoren umfasste, die zuvor keine bekannte Funktion in der Zellzykluskontrolle erfüllten. Da die Mehrheit der entdeckten Transkriptionfaktoren noch nicht funktionell charakterisiert sind, wird es eine Aufgabe zukünftiger Studien sein, diese Funktionen in Mutanten- und Zielgenanalysen zu bestimmen.

In Kapitel 6 werden die Ergebnisse der vorherigen Kapitel zusammengefasst und interpretiert. Insgesamt analysierten wir die Funktionen zweier TCP Transkriptionsfaktoren mittels umfassender Zielgenanalysen und wir entdeckten, dass beide Transkriptionsfaktoren in der hormonellen Kontrolle der Blattentwicklung involviert sind, dass sie aber auch Kontrolle über andere biologische Prozesse ausüben, wie z.B. in der Kontrolle der Zellwandzusammensetzung oder in der Eisenhomöostase. Interessanterweise konnten wir kaum direkte Verbindungen zwischen TCPs und dem Zellzyklus feststellen, obwohl die Phänotypen, die im Beginn der TCP-Forschung entdeckt wurden, zu einer entsprechenden Annahme geführt hatten. Da Einzel- und Mehrfach-TCP-Mutanten den Zellzyklus beeinflussen, Zellzyklusgene aber keine direkten Zielgene von TCP Transkriptionsfaktoren zu sein scheinen, gehen wir davon aus, dass der Zellzyklus indirekt von TCPs kontrolliert wird. Vor allem Hormone scheinen die Mittlerrolle zwischen TCPs und dem Zellzyklus einnehmen zu können, da TCPs viele Hormone in Synthese und Metabolismus beeinflussen, und da mehrere Hormone Einfluss auf den Zellzyklus ausüben können. Letztlich beenden wir das Kapitel 6 und diese Arbeit mit der Annahme, dass die ubiquitär exprimierten TCP Transkriptionsfaktoren nicht die Hauptrolle in der Kontrolle von Wachstum spielen, dass sie aber wichtige Nebenprozesse, wie Hormone, Zellwandzusammensetzung und Eisenhomöostase, kontrollieren.

Appendix I

Genome-wide target gene list of TCP10

Target genes of TCP10 as determined in microarray analyses

Genes found to be differentially regulated between TCP10m-GR and *jaw*-D plants after two and four hours of induction. First two columns depict at which time point transcript was significantly different between two lines. Third column depicts AGI, fourth depicts gene description as given by TAIR10.

t2	t4	AGI	gene name
	x	AT1G02360	Chitinase family protein
x		AT1G02610	RING/FYVE/PHD zinc finger superfamily protein
x		AT1G03090	MCCA is the biotinylated subunit of the dimer MCCase, which is involved in leucine degradation. Both subunits are nuclear coded and the active enzyme is located in the mitochondrion. protein_coding (MCCA) (MCCA)
	x	AT1G06135	unknown protein
	x	AT1G06137	unknown protein
	x	AT1G07160	Protein phosphatase 2C family protein
x		AT1G07900	LOB domain-containing protein 1 (LBD1)
x	x	AT1G08830	Encodes a cytosolic copper/zinc superoxide dismutase CSD1 that can detoxify superoxide radicals. Its expression is affected by miR398-directed mRNA cleavage. Regulated by biotic and abiotic stress. protein_coding COPPER/ZINC SUPEROXIDE DISMUTASE 1 (CSD1) COPPER/ZINC SUPEROXIDE DISMUTASE 1 (CSD1)
x		AT1G10140	Uncharacterised conserved protein UCP031279
	x	AT1G10550	Encodes a membrane-localized protein that is predicted to function during cell wall modification.Overexpression of XTH33 results in abnormal cell morphology. It's expression is under epigenetic control by ATX1. protein_coding XYLOGLUCAN:XYLOGLUCOSYL TRANSFERASE 33 (XTH33) XYLOGLUCAN:XYLOGLUCOSYL TRANSFERASE 33 (XTH33)
x		AT1G11260	Encodes a H+/hexose cotransporter. protein_coding SUGAR TRANSPORTER 1 (STP1) SUGAR TRANSPORTER 1 (STP1)
	x	AT1G12520	Copper-zinc superoxide dismutase copper chaperone (delivers copper to the Cu-Zn superoxide dismutase). Localized to the chloroplast. Expressed in roots and shoots. Up-regulated in response to copper and senescence. The AtACC activates all three CuZnSOD activities located in three different subcellular compartments. Contains three domains, central, ATX-1 like and C-terminal. ATX-1 like domain essential for the copper chaperone function of AtCCS in planta. protein_coding COPPER CHAPERONE FOR SOD1 (CCS) COPPER CHAPERONE FOR SOD1 (ATCCS)
	x	AT1G13470	Protein of unknown function (DUF1262)
	x	AT1G13520	Protein of unknown function (DUF1262)
	x	AT1G14540	Peroxidase superfamily protein

t2	t4	AGI	gene name
	x	AT1G14550	Peroxidase superfamily protein
	x	AT1G14730	Cytochrome b561/ferric reductase transmembrane protein family
x		AT1G15125	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
x		AT1G15380	Lactoylglutathione lyase / glyoxalase I family protein
x		AT1G17710	Pyridoxal phosphate phosphatase-related protein
x		AT1G18330	EARLY-PHYTOCHROME-RESPONSIVE1 protein_coding EARLY-PHYTOCHROME-RESPONSIVE1 (EPR1) EARLY-PHYTOCHROME-RESPONSIVE1 (EPR1)
x		AT1G19050	Encodes a member of the Arabidopsis response regulator (ARR) family, most closely related to ARR15. A two-component response regulator protein containing a phosphate accepting domain in the receiver domain but lacking a DNA binding domain in the output domain. Involved in response to cytokinin and meristem stem cell maintenance. Arr7 protein is stabilized by cytokinin. protein_coding RESPONSE REGULATOR 7 (ARR7) RESPONSE REGULATOR 7 (ARR7)
	x	AT1G19210	encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 15 members in this subfamily including RAP2.1, RAP2.9 and RAP2.10. protein_coding
x	x	AT1G19670	Chlorophyllase is the first enzyme involved in chlorophyll degradation. It catalyzes the hydrolysis of the ester bond to yield chlorophyllide and phytol. AtCLH1 lacks a typical signal sequence for the chloroplast. Its expression is induced rapidly by methyljasmonate, a known promoter of senescence and chlorophyll degradation. protein_coding CHLOROPHYLLASE 1 (CLH1) CHLOROPHYLLASE 1 (ATCLH1)
x		AT1G19960	BEST Arabidopsis thaliana protein match is: transmembrane receptors (TAIR:AT2G32140.1)
	x	AT1G20470	SAUR-like auxin-responsive protein family
	x	AT1G21910	encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 15 members in this subfamily including RAP2.1, RAP2.9 and RAP2.10. protein_coding DEHYDRATION RESPONSE ELEMENT-BINDING PROTEIN 26 (DREB26) DEHYDRATION RESPONSE ELEMENT-BINDING PROTEIN 26 (DREB26)
	x	AT1G22470	unknown protein

t2	t4	AGI	gene name
x		AT1G22500	Gene encodes a putative C3HC4-type RING zinc finger factor. it is induced in response to light and ascorbate stimulus. protein_coding ARABIDOPSIS TOXICOS EN LEVADURA 15 (ATL15) ARABIDOPSIS TOXICOS EN LEVADURA 15 (ATL15)
x		AT1G23390	Kelch repeat-containing F-box family protein
x		AT1G25400	unknown protein
x		AT1G28330	dormancy-associated protein (DRM1) protein_coding DORMANCY-ASSOCIATED PROTEIN-LIKE 1 (DYL1) DORMANCY-ASSOCIATED PROTEIN-LIKE 1 (DYL1)
	x	AT1G29500	SAUR-like auxin-responsive protein family
x		AT1G32540	Encodes a protein with 3 plant-specific zinc finger domains that acts as a positive regulator of cell death. protein_coding LSD ONE LIKE 1 (LOL1) LSD ONE LIKE 1 (LOL1)
	x	AT1G33760	encodes a member of the DREB subfamily A-4 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 17 members in this subfamily including TINY. protein_coding
	x	AT1G35140	Encodes PHI1/EXL1 protein_coding PHOSPHATE-INDUCED 1 (PHI-1) PHOSPHATE-INDUCED 1 (PHI-1)
x		AT1G35612	pseudogene of Ulp1 protease family protein transposable_element_gene
	x	AT1G36640	unknown protein
	x	AT1G41855	transposable element gene
x		AT1G43160	encodes a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family (RAP2.6). The protein contains one AP2 domain. There are 7 members in this subfamily. protein_coding RELATED TO AP2 6 (RAP2.6) RELATED TO AP2 6 (RAP2.6)
x		AT1G44350	encodes a protein similar to IAA amino acid conjugate hydrolase. protein_coding IAA-LEUCINE RESISTANT (ILR)-LIKE GENE 6 (ILL6) IAA-LEUCINE RESISTANT (ILR)-LIKE GENE 6 (ILL6)
x		AT1G44830	encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 15 members in this subfamily including RAP2.1, RAP2.9 and RAP2.10. protein_coding
x		AT1G47395	unknown protein
x		AT1G49230	RING/U-box superfamily protein
	x	AT1G51620	Protein kinase superfamily protein
	x	AT1G51913	LOCATED IN: endomembrane system
	x	AT1G51915	cryptdin protein-related

t2	t4	AGI	gene name
		AT1G52400	encodes a member of glycosyl hydrolase family 1, located in inducible ER bodies which were formed after wounding, required in inducible ER body formation protein_coding BETA GLUCOSIDASE 18 (BGLU18) BETA-GLUCOSIDASE HOMOLOG 1 (BGL1)
x		AT1G54020	GDSL-like Lipase/Acylhydrolase superfamily protein
	x	AT1G55790	FUNCTIONS IN: metal ion binding
	x	AT1G58225	unknown protein
x	x	AT1G58420	Uncharacterised conserved protein UCP031279
x		AT1G62610	NAD(P)-binding Rossmann-fold superfamily protein
x		AT1G64220	translocase of outer membrane 7 kDa subunit 2 (TOM7-2)
	x	AT1G65484	unknown protein
	x	AT1G65510	unknown protein
	x	AT1G66400	Encodes a calmodulin-like protein. Regulates nitric oxide levels and transition to flowering. protein_coding CALMODULIN LIKE 23 (CML23) CALMODULIN LIKE 23 (CML23)
	x	AT1G67860	unknown protein
x	x	AT1G67865	unknown protein
		AT1G68360	C2H2 and C2HC zinc fingers superfamily protein
x	x	AT1G68450	VQ motif-containing protein
		AT1G70290	Encodes an enzyme putatively involved in trehalose biosynthesis. Though the protein has both trehalose-6-phosphate synthase (TPS)-like and trehalose-6-phosphate phosphatase (TPP)-like domains, neither activity has been detected in enzymatic assays nor has the protein been able to complement yeast TPS or TPP mutants. protein_coding TREHALOSE-6-PHOSPHATASE SYNTHASE S8 (TPS8) (ATTPS8)
x	x	AT1G70420	Protein of unknown function (DUF1645)
x		AT1G71030	Encodes a putative myb family transcription factor. In contrast to most other myb-like proteins its myb domain consists of a single repeat. A proline-rich region potentially involved in transactivation is found in the C-terminal part of the protein. Its transcript accumulates mainly in leaves. protein_coding MYB-LIKE 2 (MYBL2) ARABIDOPSIS MYB-LIKE 2 (ATMYBL2)
x		AT1G72240	unknown protein
x		AT1G73325	Kunitz family trypsin and protease inhibitor protein
	x	AT1G74670	Gibberellin-regulated family protein

t2	t4	AGI	gene name
	x	AT1G74710	Encodes a protein with isochorismate synthase activity. Mutants fail to accumulate salicylic acid. Its function may be redundant with that of ICS2 (AT1G18870). protein_coding ENHANCED DISEASE SUSCEPTIBILITY TO ERYSIPHE ORONTII 16 (EDS16) ENHANCED DISEASE SUSCEPTIBILITY TO ERYSIPHE ORONTII 16 (EDS16)
x		AT1G74890	Encodes a nuclear response regulator that acts as a negative regulator in cytokinin-mediated signal transduction. Transcript accumulates in leaves and roots in response to cytokinin treatment. protein_coding RESPONSE REGULATOR 15 (ARR15) RESPONSE REGULATOR 15 (ARR15)
x		AT1G76590	PLATZ transcription factor family protein
x		AT1G77210	AtSTP14 belongs to the family of sugar transport proteins (AtSTPs)in volved in monosaccharide transport. Heterologous expression in yeast revealed that AtSTP14 is the transporter specifc for galactose and does not transport other monosaccharides such as glucose or fructose. protein_coding SUGAR TRANSPORT PROTEIN 14 (STP14) SUGAR TRANSPORT PROTEIN 14 (STP14)
	x	AT1G77640	encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 15 members in this subfamily including RAP2.1, RAP2.9 and RAP2.10. protein_coding
x		AT1G78450	SOUL heme-binding family protein
x		AT1G78830	Curculin-like (mannose-binding) lectin family protein
x		AT1G79110	Encodes one of the BRGs (BOI-related gene) involved in resistance to Botrytis cinerea. protein_coding BOI-RELATED GENE 2 (BRG2) BOI-RELATED GENE 2 (BRG2)
x		AT1G79700	Integrase-type DNA-binding superfamily protein
x		AT1G80440	Galactose oxidase/kelch repeat superfamily protein
x		AT1G80920	A nuclear encoded soluble protein found in the chloroplast stroma. protein_coding (J8) (J8)
x		AT2G02710	Encodes a putative blue light receptor protein. protein_coding PAS/LOV PROTEIN B (PLPB) PAS/LOV PROTEIN (PLP)
x		AT2G03090	member of Alpha-Expansin Gene Family. Naming convention from the Expansin Working Group (Kende et al, 2004. Plant Mol Bio). Involved in the formation of nematode-induced syncytia in roots of Arabidopsis thaliana. protein_coding EXPANSIN A15 (EXPA15) EXPANSIN A15 (ATEXPA15)
x		AT2G05440	GLYCINE RICH PROTEIN 9 (GRP9)
x		AT2G15890	maternal effect embryo arrest 14 (MEE14)
x		AT2G17880	Chaperone DnaJ-domain superfamily protein

t2	t4	AGI	gene name
		AT2G18660	Encodes PNP-A (Plant Natriuretic Peptide A). PNPs are a class of systemically mobile molecules distantly related to expansins
x	x	AT2G18700	Encodes an enzyme putatively involved in trehalose biosynthesis. The protein has a trehalose synthase (TPS)-like domain that may or may not be active as well as a trehalose phosphatase (TPP)-like domain. protein_coding TREHALOSE PHOSPHATASE/SYNTHASE 11 (TPS11) TREHALOSE PHOSPHATASE/SYNTHASE 11 (ATTPS11)
x		AT2G20670	Protein of unknown function (DUF506)
x		AT2G20723	snoRNA
x		AT2G21640	Encodes a protein of unknown function that is a marker for oxidative stress response. protein_coding
	x	AT2G22470	Encodes arabinogalactan-protein (AGP2). protein_coding ARABINOGALACTAN PROTEIN 2 (AGP2) ARABINOGALACTAN PROTEIN 2 (AGP2)
x		AT2G24550	unknown protein
x		AT2G24850	Encodes a tyrosine aminotransferase that is responsive to treatment with jasmonic acid. protein_coding TYROSINE AMINOTRANSFERASE 3 (TAT3) TYROSINE AMINOTRANSFERASE 3 (TAT3)
x		AT2G25200	Plant protein of unknown function (DUF868)
x		AT2G25900	putative Cys3His zinc finger protein (ATCTH) mRNA, complete protein_coding (ATCTH) (ATCTH)
	x	AT2G27080	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
	x	AT2G27389	unknown protein
x		AT2G27830	unknown protein
x	x	AT2G28190	Encodes a chloroplastic copper/zinc superoxide dismutase CSD2 that can detoxify superoxide radicals. Its expression is affected by miR398-directed mRNA cleavage. protein_coding COPPER/ZINC SUPEROXIDE DISMUTASE 2 (CSD2) COPPER/ZINC SUPEROXIDE DISMUTASE 2 (CSD2)
x		AT2G28630	Encodes KCS12, a member of the 3-ketoacyl-CoA synthase family involved in the biosynthesis of VLCFA (very long chain fatty acids). protein_coding 3-KETOACYL-COA SYNTHASE 12 (KCS12) 3-KETOACYL-COA SYNTHASE 12 (KCS12)
x		AT2G30600	BTB/POZ domain-containing protein
x		AT2G30766	unknown protein
x		AT2G31945	unknown protein
	x	AT2G32190	unknown protein
x		AT2G33830	Dormancy/auxin associated family protein
x		AT2G34600	jasmonate-zim-domain protein 7 (JAZ7)

t2	t4	AGI	gene name
	x	AT2G34810	FAD-binding Berberine family protein
	x	AT2G35658	unknown protein
	x	AT2G36440	unknown protein
	x	AT2G36690	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
x		AT2G37770	Encodes an NADPH-dependent aldo-keto reductase that can act on a wide variety of substrates in vitro including saturated and unsaturated aldehydes, steroids, and sugars. GFP-tagged AKR4C9 localizes to the chloroplast where it may play a role in detoxifying reactive carbonyl compounds that threaten to impair the photosynthetic process. Transcript levels for this gene are up-regulated in response to cold, salt, and drought stress. protein_coding CHLOROPLASTIC ALDO-KETO REDUCTASE (ChlAKR) CHLOROPLASTIC ALDO-KETO REDUCTASE (ChlAKR)
x		AT2G37950	RING/FYVE/PHD zinc finger superfamily protein
x		AT2G38240	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
x		AT2G39330	jacalin-related lectin 23 (JAL23)
	x	AT2G39518	Uncharacterised protein family (UPF0497)
	x	AT2G39530	Uncharacterised protein family (UPF0497)
x		AT2G39570	ACT domain-containing protein
	x	AT2G40740	member of WRKY Transcription Factor
	x	AT2G41800	FUNCTIONS IN: molecular_function unknown
x		AT2G41940	Encodes a zinc finger protein containing only a single zinc finger. protein_coding ZINC FINGER PROTEIN 8 (ZFP8) ZINC FINGER PROTEIN 8 (ZFP8)
	x	AT2G42060	Cysteine/Histidine-rich C1 domain family protein
x	x	AT2G43290	Encodes calmodulin-like MSS3. protein_coding MULTICOPY SUPPRESSORS OF SNF4 DEFICIENCY IN YEAST 3 (MSS3) MULTICOPY SUPPRESSORS OF SNF4 DEFICIENCY IN YEAST 3 (MSS3)
x		AT2G43530	Encodes a defensin-like (DEFL) family protein. protein_coding
x		AT2G43550	Encodes a defensin-like (DEFL) family protein. protein_coding
x		AT2G44130	Galactose oxidase/kelch repeat superfamily protein
	x	AT2G44370	Cysteine/Histidine-rich C1 domain family protein
x		AT2G44940	encodes a member of the DREB subfamily A-4 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 17 members in this subfamily including TINY. protein_coding
x		AT2G45080	cyclin p3

t2	t4	AGI	gene name
x		AT2G45170	Involved in autophagy. Under nutrient starvation the protein localizes to autophagosomes. protein_coding AUTOPHAGY 8E (ATG8E) AUTOPHAGY 8E (ATATG8E)
	x	AT2G46410	Nuclear-localized R3-type MYB transcription factor. Positive regulator of hair-cell differentiation. Preferentially transcribed in hairless cells. Moves from atrichoblasts into trichoblast via plasmodesmata in a tissue-specific mode. N-terminus and part of the Myb domain are required for this movement, with W76 playing a crucial role. Capability to increase the size-exclusion limit of plasmodesmata. Regulated by WEREWOLF. protein_coding CAPRICE (CPC) CAPRICE (CPC)
x	x	AT2G47180	GolS1 is a galactinol synthase that catalyzes the formation of galactinol from UDP-galactose and myo-inositol. GolS1 transcript levels rise in response to methyl viologen, an oxidative damage-inducing agent. Plants over-expressing GolS1 have increased tolerance to salt, chilling, and high-light stress. protein_coding GALACTINOL SYNTHASE 1 (GolS1) GALACTINOL SYNTHASE 1 (AtGolS1)
x		AT3G02040	senescence-related gene 3 (SRG3)
x		AT3G04720	Encodes a protein similar to the antifungal chitin-binding protein hevein from rubber tree latex. mRNA levels increase in response to ethylene and turnip crinkle virus infection. protein_coding PATHOGENESIS-RELATED 4 (PR4) PATHOGENESIS-RELATED 4 (PR4)
x		AT3G07350	Protein of unknown function (DUF506)
x		AT3G10020	unknown protein
	x	AT3G12580	heat shock protein 70 (HSP70)
	x	AT3G13432	unknown protein
	x	AT3G13433	unknown protein
x		AT3G13450	branched chain alpha-keto acid dehydrogenase E1 beta protein_coding DARK INDUCIBLE 4 (DIN4) DARK INDUCIBLE 4 (DIN4)
	x	AT3G13610	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
	x	AT3G14225	Contains lipase signature motif and GDSL domain. protein_coding GDSL-MOTIF LIPASE 4 (GLIP4) GDSL-MOTIF LIPASE 4 (GLIP4)
x		AT3G15356	Legume lectin family protein
x		AT3G15450	Aluminium induced protein with YGL and LRDR motifs

t2	t4	AGI	gene name
x		AT3G15500	Encodes an ATAF-like NAC-domain transcription factor that doesn't contain C-terminal sequences shared by CUC1, CUC2 and NAM. Note: this protein (AtNAC3) is not to be confused with the protein encoded by locus AT3G29035, which, on occasion, has also been referred to as AtNAC3. protein_coding NAC DOMAIN CONTAINING PROTEIN 3 (NAC3) NAC DOMAIN CONTAINING PROTEIN 3 (ATNAC3)
x		AT3G15630	unknown protein
x		AT3G15760	unknown protein
x		AT3G16870	Encodes a member of the GATA factor family of zinc finger transcription factors. protein_coding GATA TRANSCRIPTION FACTOR 17 (GATA17) GATA TRANSCRIPTION FACTOR 17 (GATA17)
	x	AT3G21520	DUF679 domain membrane protein 1 (DMP1)
x		AT3G22231	Encodes a member of a novel 6 member Arabidopsis gene family. Expression of PCC1 is regulated by the circadian clock and is upregulated in response to both virulent and avirulent strains of Pseudomonas syringae pv. tomato. protein_coding PATHOGEN AND CIRCADIAN CONTROLLED 1 (PCC1) PATHOGEN AND CIRCADIAN CONTROLLED 1 (PCC1)
x	x	AT3G22840	Encodes an early light-inducible protein. protein_coding EARLY LIGHT-INDUCABLE PROTEIN (ELIP1) EARLY LIGHT-INDUCABLE PROTEIN (ELIP1)
x		AT3G23550	MATE efflux family protein
x		AT3G26200	putative cytochrome P450 protein_coding "CYTOCHROME P450, FAMILY 71, SUBFAMILY B, POLYPEPTIDE 22" (CYP71B22) "CYTOCHROME P450, FAMILY 71, SUBFAMILY B, POLYPEPTIDE 22" (CYP71B22)
	x	AT3G28210	Encodes a putative zinc finger protein (PMZ). protein_coding (PMZ) (PMZ)
x		AT3G29670	HXXXD-type acyl-transferase family protein
x		AT3G30775	Encodes a proline oxidase that is predicted to localize to the inner mitochondrial membrane, its mRNA expression induced by high levels of Al and by osmotic stress. The promoter contains an L-proline-inducible element. protein_coding EARLY RESPONSIVE TO DEHYDRATION 5 (ERD5) EARLY RESPONSIVE TO DEHYDRATION 5 (ERD5)
x		AT3G44860	Encodes a farnesoic acid carboxyl-O-methyltransferase. protein_coding FARNESOIC ACID CARBOXYL-O-METHYLTRANSFERASE (FAMT) FARNESOIC ACID CARBOXYL-O-METHYLTRANSFERASE (FAMT)
x		AT3G44970	Cytochrome P450 superfamily protein

t2	t4	AGI	gene name
x	x	AT3G45140	Chloroplast lipoxygenase required for wound-induced jasmonic acid accumulation in Arabidopsis.Mutants are resistant to Staphylococcus aureus and accumulate salicylic acid upon infection. protein_coding LIPOXYGENASE 2 (LOX2) LIPOXYGENASE 2 (LOX2)
x	x	AT3G45960	member of EXPANSIN-LIKE. Naming convention from the Expansin Working Group (Kende et al, 2004. Plant Mol Bio) protein_coding EXPANSIN-LIKE A3 (EXLA3) EXPANSIN-LIKE A3 (ATEXLA3)
	x	AT3G45970	member of EXPANSIN-LIKE. Naming convention from the Expansin Working Group (Kende et al, 2004. Plant Mol Bio) protein_coding EXPANSIN-LIKE A1 (EXLA1) EXPANSIN-LIKE A1 (ATEXLA1)
	x	AT3G46080	C2H2-type zinc finger family protein
	x	AT3G46090	ZAT7
x		AT3G47340	encodes a glutamine-dependent asparagine synthetase, the predicted ASN1 peptide contains a purF-type glutamine-binding domain, and is expressed predominantly in shoot tissues, where light has a negative effect on its mRNA accumulation. Expression is induced within 3 hours of dark treatment, in senescing leaves and treatment with exogenous photosynthesis inhibitor. Induction of gene expression was suppressed in excised leaves supplied with sugar. The authors suggest that the gene's expression pattern is responding to the level of sugar in the cell. protein_coding GLUTAMINE-DEPENDENT ASPARAGINE SYNTHASE 1 (ASN1) GLUTAMINE-DEPENDENT ASPARAGINE SYNTHASE 1 (ASN1)
		AT3G48360	encodes a protein (BT2) that is an essential component of the TAC1-mediated telomerase activation pathway. Acts redundantly with BT3 and BT1 during female gametophyte development and with BT3 during male gametophyte development. BT2 also mediates multiple responses to nutrients, stresses, and hormones. protein_coding BTB AND TAZ DOMAIN PROTEIN 2 (bt2) BTB AND TAZ DOMAIN PROTEIN 2 (bt2)
x	x	AT3G48650	pseudogene, At14a-related protein, similar to At14a (GI:11994571 and GI:11994573) (Arabidopsis thaliana) pseudogene
x		AT3G49790	Carbohydrate-binding protein

t2	t4	AGI	gene name
	x	AT3G50060	Encodes a member of the R2R3 transcription factor gene family. Expressed in response to potassium deprivation and auxin. Involved in lateral root development. Interacts with ARF7 and regulates the expression of some auxin responsive genes. protein_coding MYB DOMAIN PROTEIN 77 (MYB77) MYB DOMAIN PROTEIN 77 (MYB77)
x		AT3G50280	HXXXD-type acyl-transferase family protein
	x	AT3G54040	PAR1 protein
x		AT3G55970	jasmonate-regulated gene 21 (JRG21)
x		AT3G57520	SIP2 encodes a raffinose-specific alpha-galactosidase that catalyzes the breakdown of raffinose into alpha-galatose and sucrose. This enzyme may function in unloading raffinose from the phloem as part of sink metabolism. Although it was originally predicted to act as a raffinose synthase (RS), that activity was not observed for recombinant SIP2. protein_coding SEED IMBIBITION 2 (SIP2) SEED IMBIBITION 2 (AtSIP2)
x		AT3G58070	Putative transcription factor, contains C2H2 domain, regulates aspects of shoot maturation in Arabidopsis thaliana. GIS loss-of-function mutations affect the epidermal differentiation of inflorescence organs, causing a premature decrease in trichome production on successive leaves, stem internodes, and branches. Overexpression has the opposite effect on trichome initiation and causes other heterochronic phenotypes, affecting flowering and juvenile–adult leaf transition and inducing the formation of rosette leaves on inflorescence stems. protein_coding GLABROUS INFLORESCENCE
x		AT3G59940	Galactose oxidase/kelch repeat superfamily protein
x		AT3G61060	phloem protein 2-A13 (PP2-A13)
x		AT3G62550	Adenine nucleotide alpha hydrolases-like superfamily protein
x		AT3G62950	Thioredoxin superfamily protein
	x	AT4G01360	unknown protein
	x	AT4G02170	unknown protein
	x	AT4G03295	snoRNA
x		AT4G03510	RMA1 encodes a novel 28 kDa protein with a RING finger motif and a C-terminal membrane-anchoring domain that is involved in the secretory pathway. Has E3 ubiquitin ligase activity. protein_coding RING MEMBRANE-ANCHOR 1 (RMA1) RING MEMBRANE-ANCHOR 1 (RMA1)
x		AT4G04630	Protein of unknown function, DUF584
x		AT4G04830	methionine sulfoxide reductase B5 (MSRB5)
	x	AT4G08950	EXORDIUM (EXO)

t2	t4	AGI	gene name
	x	AT4G09030	Encodes arabinogalactan protein (AGP10). protein_coding ARABINOGALACTAN PROTEIN 10 (AGP10) ARABINOGALACTAN PROTEIN 10 (AGP10)
	x	AT4G09100	RING/U-box superfamily protein
	x	AT4G11070	member of WRKY Transcription Factor
x		AT4G12470	Encodes AZI1 (AZELAIC ACID INDUCED 1). Involved in the priming of salicylic acid induction and systemic immunity triggered by pathogen or azelaic acid. protein_coding AZELAIC ACID INDUCED 1 (AZI1) AZELAIC ACID INDUCED 1 (AZI1)
x	x	AT4G14630	germin-like protein with N-terminal signal sequence that may target it to the vacuole, plasma membrane and/or outside the cell. protein_coding GERMIN-LIKE PROTEIN 9 (GLP9) GERMIN-LIKE PROTEIN 9 (GLP9)
x	x	AT4G14690	Encodes an early light-induced protein. ELIPs are thought not to be directly involved in the synthesis and assembly of specific photosynthetic complexes, but rather affect the biogenesis of all chlorophyll-binding complexes. A study (PMID 17553115) has shown that the chlorophyll synthesis pathway was downregulated as a result of constitutive ELIP2 expression, leading to decreased chlorophyll availability for the assembly of pigment-binding proteins for photosynthesis. protein_coding EARLY LIGHT-INDUCIBLE PROTEIN 2 (ELIP2) EARLY LIGHT-INDUCIBLE PROTEIN 2 (ELIP2)
x		AT4G15210	cytosolic beta-amylase expressed in rosette leaves and inducible by sugar. RAM1 mutants have reduced beta amylase in leaves and stems. protein_coding BETA-AMYLASE 5 (BAM5) ARABIDOPSIS THALIANA BETA-AMYLASE (ATBETA-AMY)
x		AT4G15660	Thioredoxin superfamily protein
x		AT4G15690	Thioredoxin superfamily protein
x		AT4G15700	Thioredoxin superfamily protein
x		AT4G16000	unknown protein
	x	AT4G17215	Pollen Ole e 1 allergen and extensin family protein
x		AT4G17670	Protein of unknown function (DUF581)
x		AT4G18340	Glycosyl hydrolase superfamily protein
x		AT4G19160	unknown protein
	x	AT4G20000	VQ motif-containing protein
	x	AT4G20006	unknown protein
	x	AT4G23610	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family

t2	t4	AGI	gene name
x		AT4G23750	encodes a member of the ERF (ethylene response factor) subfamily B-5 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 7 members in this subfamily. Monopteros target gene. protein_coding CYTOKININ RESPONSE FACTOR 2 (CRF2) CYTOKININ RESPONSE FACTOR 2 (CRF2)
x		AT4G24230	acyl-CoA-binding protein ACBP3. Localized extracellularly in transiently expressed tobacco BY-2 cells and onion epidermal cells. Binds arachidonyl-CoA with high affinity. Microarray data shows up-regulation of many biotic- and abiotic-stress-related genes in an ACBP3 OE-1 in comparison to wild type. protein_coding ACYL-COA-BINDING DOMAIN 3 (ACBP3) ACYL-COA-BINDING DOMAIN 3 (ACBP3)
x		AT4G25630	encodes a fibrillarin, a key nucleolar protein in eukaryotes which associates with box C/D small nucleolar RNAs (snoRNAs) directing 2'-O-ribose methylation of the rRNA. This gene also encodes a novel box C/D snoRNA, U60.2f in its fifth intron that accumulates in seedlings and that their targeted residue on the 25 S rRNA is methylated. protein_coding FIBRILLARIN 2 (FIB2) FIBRILLARIN 2 (FIB2)
	x	AT4G25810	xyloglucan endotransglycosylase-related protein (XTR6) protein_coding XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6 (XTR6) XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6 (XTR6)
	x	AT4G26200	Member of a family of proteins in Arabidopsis that encode 1-Amino-cyclopropane-1-carboxylate synthase, an enzyme involved in ethylene biosynthesis. Not expressed in response to IAA. protein_coding 1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 7 (ACS7) 1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 7 (ACS7)
x		AT4G27410	Encodes a NAC transcription factor induced in response to dessication. It is localized to the nucleus and acts as a transcriptional activator in ABA-mediated dehydration response. protein_coding RESPONSIVE TO DESICCATION 26 (RD26) RESPONSIVE TO DESICCATION 26 (RD26)
x		AT4G27450	Aluminium induced protein with YGL and LRDR motifs
	x	AT4G30430	Member of TETRASPANIN family protein_coding TETRASPANIN9 (TET9) TETRASPANIN9 (TET9)
x		AT4G32480	Protein of unknown function (DUF506)

t2	t4	AGI	gene name
x		AT4G35770	Senescence-associated gene that is strongly induced by phosphate starvation. Transcripts are differentially regulated at the level of mRNA stability at different times of day. mRNAs are targets of the mRNA degradation pathway mediated by the downstream (DST) instability determinant. protein_coding SENESCENCE 1 (SEN1) SENESCENCE 1 (SEN1)
x		AT4G36110	SAUR-like auxin-responsive protein family
x		AT4G36670	Major facilitator superfamily protein
x		AT4G37610	BTB and TAZ domain protein. Located in cytoplasm and expressed in fruit, flower and leaves. protein_coding BTB AND TAZ DOMAIN PROTEIN 5 (bt5) BTB AND TAZ DOMAIN PROTEIN 5 (bt5)
x		AT4G38470	ACT-like protein tyrosine kinase family protein
x		AT4G39800	** Referred to as MIPS2 in Mitsuhashi et al 2008. myo-inositol-1-phosphate synthase isoform 1.Expressed in leaf, root and silique. Immunolocalization experiments with an antibody recognizing MIPS1, MIPS2, and MIPS3 showed endosperm localization. protein_coding MYO-INOSITOL-1-PHOSPHATE SYNTHASE 1 (MIPS1) (MI-1-P SYNTHASE)
	x	AT5G05300	unknown protein
x		AT5G05600	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
x		AT5G06690	Encodes a thioredoxin (WCRKC1) localized in chloroplast stroma. Contains a WCRKC motif. protein_coding WCRKC THIOREDOXIN 1 (WCRKC1) WCRKC THIOREDOXIN 1 (WCRKC1)
x		AT5G06870	Encodes a polygalacturonase inhibiting protein involved in plant defense response. PGIPs inhibit the activity of pectin degrading enzymes such as those produced by fungal pathogens. PGIP2 is induced by fungal infection and methyl jasmonate. protein_coding POLYGALACTURONASE INHIBITING PROTEIN 2 (PGIP2) POLYGALACTURONASE INHIBITING PROTEIN 2 (PGIP2)
x		AT5G07440	Encodes the beta-subunit of the glutamate dehydrogenase. The enzyme is almost exclusively found in the mitochondria of stem and leaf companion cells. protein_coding GLUTAMATE DEHYDROGENASE 2 (GDH2) GLUTAMATE DEHYDROGENASE 2 (GDH2)
	x	AT5G10830	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
	x	AT5G11140	Arabidopsis phospholipase-like protein (PEARLI 4) family

t2	t4	AGI	gene name
	x	AT5G13320	Encodes an enzyme capable of conjugating amino acids to 4-substituted benzoates. 4-HBA (4-hydroxybenzoic acid) and pABA (4-aminobenzoate) may be targets of the enzyme in Arabidopsis, leading to the production of pABA-Glu, 4HBA-Glu, or other related compounds. This enzyme is involved in disease-resistance signaling. It is required for the accumulation of salicylic acid, activation of defense responses, and resistance to Pseudomonas syringae. Salicylic acid can decrease this enzyme's activity in vitro and may act as a competitive inhibitor. Expression of PBS3/GH3.12 can be detected in cotyledons, true leaves, hypocot
x		AT5G14120	Major facilitator superfamily protein
x		AT5G15948	Upstream open reading frames (uORFs) are small open reading frames found in the 5' UTR of a mature mRNA, and can potentially mediate translational regulation of the largest, or major, ORF (mORF). CPuORF10 represents a conserved upstream opening reading frame relative to major ORF AT5G15950.1 protein_coding CONSERVED PEPTIDE UPSTREAM OPEN READING FRAME 10 (CPuORF10) CONSERVED PEPTIDE UPSTREAM OPEN READING FRAME 10 (CPuORF10)
x		AT5G15950	Adenosylmethionine decarboxylase family protein
	x	AT5G17350	unknown protein
x		AT5G18600	Thioredoxin superfamily protein
x		AT5G18670	putative beta-amylase BMY3 (BMY3) protein_coding BETA-AMYLASE 3 (BMY3) BETA-AMYLASE 3 (BMY3)
x		AT5G19120	Eukaryotic aspartyl protease family protein
x		AT5G20250	encodes a member of glycosyl hydrolase family 36. Expression is induced within 3 hours of dark treatment, in senescing leaves and treatment with exogenous photosynthesis inhibitor. Induction of gene expression was suppressed in excised leaves supplied with sugar. The authors suggest that the gene's expression pattern is responding to the level of sugar in the cell. protein_coding DARK INDUCIBLE 10 (DIN10) DARK INDUCIBLE 10 (DIN10)
x		AT5G21170	Encodes AKINbeta1, a subunit of the SnRK1 kinase (Sucrose non-fermenting-1-related protein kinase). Involved in regulation of nitrogen and sugar metabolism. protein_coding (AKINBETA1) (AKINBETA1)
x		AT5G21940	unknown protein
	x	AT5G22380	NAC domain containing protein 90 (NAC090)
	x	AT5G22680	FUNCTIONS IN: molecular_function unknown
x		AT5G22920	CHY-type/CTCHY-type/RING-type Zinc finger protein
	x	AT5G24040	Protein of unknown function (DUF295)

t2	t4	AGI	gene name
	x	AT5G24200	alpha/beta-Hydrolases superfamily protein
x	x	AT5G24780	encodes an acid phosphatase similar to soybean vegetative storage proteins. Gene expression is induced by wounding and jasmonic acid. protein_coding VEGETATIVE STORAGE PROTEIN 1 (VSP1) VEGETATIVE STORAGE PROTEIN 1 (VSP1)
	x	AT5G25260	SPFH/Band 7/PHB domain-containing membrane-associated protein family
	x	AT5G26220	ChaC-like family protein
		AT5G36910	Encodes a thionin that is expressed at a low basal level in seedlings and shows circadian variation. Predicted to encode a PR (pathogenesis-related) protein. Belongs to the plant thionin (PR-13) family with the following members: At1g66100, At5g36910, At1g72260, At2g15010, At1g12663, At1g12660. protein_coding THIONIN 2.2 (THI2.2) THIONIN 2.2 (THI2.2)
	x	AT5G37770	Encodes a protein with 40% similarity to calmodulin. Binds Ca(2+) and, as a consequence, undergoes conformational changes. CML24 expression occurs in all major organs, and transcript levels are increased from 2- to 15-fold in plants subjected to touch, darkness, heat, cold, hydrogen peroxide, abscisic acid (ABA), and indole-3-acetic acid. However, CML24 protein accumulation changes were not detectable. The putative CML24 regulatory region confers reporter expression at sites of predicted mechanical stress
x	x	AT5G39580	Peroxidase superfamily protein
x		AT5G41080	PLC-like phosphodiesterases superfamily protein
x		AT5G42530	unknown protein
x		AT5G42830	HXXXD-type acyl-transferase family protein
	x	AT5G43580	
	x	AT5G47850	CRINKLY4 related 4 (CCR4)
x	x	AT5G48850	homologous to the wheat sulphate deficiency-induced gene sdi1. Expression in root and leaf is induced by sulfur starvation. Knockout mutants retained higher root and leaf sulfate concentrations, indicating a role in regulation of stored sulfate pools. protein_coding SULPHUR DEFICIENCY-INDUCED 1 (ATSDI1) SULPHUR DEFICIENCY-INDUCED 1 (ATSDI1)
x		AT5G49360	Encodes a bifunctional {beta}-D-xylosidase/{alpha}-L-arabinofuranosidase required for pectic arabinan modification. Located in the extracellular matrix. Gene is expressed specifically in tissues undergoing secondary wall thickening. This is a member of glycosyl hydrolase family 3 and has six other closely related members. protein_coding BETA-XYLOSIDASE 1 (BXL1) BETA-XYLOSIDASE 1 (BXL1)

t2	t4	AGI	gene name
x		AT5G49448	Upstream open reading frames (uORFs) are small open reading frames found in the 5' UTR of a mature mRNA, and can potentially mediate translational regulation of the largest, or major, ORF (mORF). CPuORF4 represents a conserved upstream opening reading frame relative to major ORF AT5G49450.1 protein_coding CONSERVED PEPTIDE UPSTREAM OPEN READING FRAME 4 (CPuORF4) CONSERVED PEPTIDE UPSTREAM OPEN READING FRAME 4 (CPuORF4)
x		AT5G49450	basic leucine-zipper 1 (bZIP1)
x		AT5G51440	HSP20-like chaperones superfamily protein
	x	AT5G53110	RING/U-box superfamily protein
x	x	AT5G54610	Induced in response to Salicylic acid. Belongs to the ankyrin repeat protein family. protein_coding ANKYRIN (ANK) ANKYRIN (ANK)
x		AT5G55970	RING/U-box superfamily protein
x		AT5G56550	Encodes OXIDATIVE STRESS 3 (OXS3)&#65292
x		AT5G56870	beta-galactosidase 4 (BGAL4)
	x	AT5G57560	Encodes a cell wall-modifying enzyme, rapidly upregulated in response to environmental stimuli protein_coding TOUCH 4 (TCH4) TOUCH 4 (TCH4)
x		AT5G57655	xylose isomerase family protein
x		AT5G61440	Encodes a member of the thioredoxin family protein. Located in the chloroplast. protein_coding ATYPICAL CYS HIS RICH THIOREDOXIN 5 (ACHT5) ATYPICAL CYS HIS RICH THIOREDOXIN 5 (ACHT5)
x		AT5G62360	Plant invertase/pectin methylesterase inhibitor superfamily protein
x		AT5G64190	unknown protein
x		AT5G64905	elicitor peptide 3 precursor (PROPEP3)
x		AT5G65207	unknown protein
x		AT5G67080	member of MEKK subfamily protein_coding MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 19 (MAPKKK19) MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 19 (MAPKKK19)
	x	AT5G67450	Encodes zinc-finger protein. mRNA levels are elevated in response to low temperature, cold temperatures and high salt. The protein is localized to the nucleus and acts as a transcriptional repressor. protein_coding ZINC-FINGER PROTEIN 1 (ZF1) ZINC-FINGER PROTEIN 1 (AZF1)
	x	ATCG00080	PSII I protein protein_coding PHOTOSYSTEM II REACTION CENTER PROTEIN I (PSBI) PHOTOSYSTEM II REACTION CENTER PROTEIN I (PSBI)
	x	ATCG00090	tRNA-Ser pre_trna (TRNS.1) (TRNS.1)

Appendix II

Genome-wide target gene list of TCP20

Target gene list of TCP20 as determined in microarray analyses

Genes found to be differentially regulated after eight hours of DEX induction in comparison between TCP20-GR and wild type Columbia-0 plants. Gene names as given by TAIR10.

AGI	Gene Description
AT1G01580	FERRIC REDUCTION OXIDASE 2 (FRO2)
AT1G02205	ECERIFERUM 1 (CER1)
AT1G03230	extracellular dermal glycoprotein, putative / EDGP, putative
AT1G04040	acid phosphatase class B family protein
AT1G04400	CRYPTOCHROME 2 (CRY2)
AT1G04430	dehydration-responsive protein-related
AT1G04800	glycine-rich protein
AT1G05560	UDP-GLUCOSYLTRANSFERASE 75B1 (UGT75B1)
AT1G06100	fatty acid desaturase family protein
AT1G06360	fatty acid desaturase family protein
AT1G06475	unknown protein
AT1G07400	17.8 kDa class I heat shock protein (HSP17.8-CI)
AT1G08570	ATYPICAL CYS HIS RICH THIOREDOXIN 4 (ACHT4)
AT1G08900	carbohydrate transmembrane transporter
AT1G09200	histone H3
AT1G09240	NICOTIANAMINE SYNTHASE 3 (NAS3)
AT1G09380	integral membrane family protein / nodulin MtN21-related
AT1G09750	chloroplast nucleoid DNA-binding protein-related
AT1G13300	HYPERSENSITIVITY TO LOW PI-ELICITED PRIMARY ROOT SHORTENING 1 (HRS1)
AT1G13607	Encodes a defensin-like (DEFL) family protein
AT1G13608	Encodes a defensin-like (DEFL) family protein
AT1G13609	Encodes a defensin-like (DEFL) family protein
AT1G15125	S-adenosylmethionine-dependent methyltransferase/ methyltransferase
AT1G17345	auxin-responsive protein-related
AT1G19510	ARABIDOPSIS RAD-LIKE 5 (ATRL5)
AT1G21680	unknown protein
AT1G22710	SUCROSE-PROTON SYMPORTER 2 (SUC2)
AT1G23020	FERRIC REDUCTION OXIDASE (FRO3)
AT1G23110	unknown protein
AT1G23720	proline-rich extensin-like family protein
AT1G24145	unknown protein
AT1G24147	unknown protein
AT1G25425	CLAVATA3/ESR-RELATED 43 (CLE43)
AT1G31580	encodes a cell wall protein (ECS1)
AT1G32560	late embryogenesis abundant group 1 domain-containing protein / LEA group 1 domain-containing protein

AGI	Gene Description
AT1G43970	unknown protein
AT1G45070	transposable element gene
AT1G45201	TRIACYLGLYCEROL LIPASE-LIKE 1 (TLL1)
AT1G45688	unknown protein
AT1G47400	unknown protein
AT1G48300	unknown protein
AT1G50160	unknown protein
AT1G52100	jacalin lectin family protein
AT1G52400	BETA GLUCOSIDASE 18 (BGLU18)
AT1G53690	Protein of unknown function that is homologous to At5g41010, which encodes a non-catalytic subunit common to nuclear DNA-dependent RNA polymerases II, IV and V
AT1G54010	myrosinase-associated protein, putative
AT1G54740	unknown protein
AT1G54820	protein kinase family protein
AT1G55850	CELLULOSE SYNTHASE LIKE E1 (CSLE1)
AT1G56300	DNAJ heat shock N-terminal domain-containing protein
AT1G56430	NICOTIANAMINE SYNTHASE 4 (NAS4)
AT1G56600	ARABIDOPSIS THALIANA GALACTINOL SYNTHASE 2 (AtGolS2)
AT1G59860	17.5 kDa class I heat shock protein (HSP17.6A-Cl)
AT1G61800	GLUCOSE-6-PHOSPHATE/PHOSPHATE TRANSLOCATOR 2 (GPT2)
AT1G62000	unknown protein
AT1G64370	unknown protein
AT1G64400	long-chain-fatty-acid--CoA ligase, putative / long-chain acyl-CoA synthetase, putative
AT1G65610	KORRIGAN 2 (KOR2)
AT1G66100	Predicted to encode a PR (pathogenesis-related) protein
AT1G66270	(BGLU21)
AT1G66940	protein kinase-related
AT1G67360	rubber elongation factor (REF) family protein
AT1G67865	unknown protein
AT1G67870	glycine-rich protein
AT1G67910	unknown protein
AT1G68190	zinc finger (B-box type) family protein
AT1G69160	unknown protein
AT1G69530	ARABIDOPSIS THALIANA EXPANSIN A1 (ATEXPA1)
AT1G71030	ARABIDOPSIS MYB-LIKE 2 (MYBL2)
AT1G71450	encodes a member of the DREB subfamily A-4 of ERF/AP2 transcription factor family
AT1G73260	KUNITZ TRYPSIN INHIBITOR 1 (KTI1)
AT1G73330	ARABIDOPSIS THALIANA DROUGHT-REPRESSED 4 (ATDR4)
AT1G73920	lipase family protein
AT1G74310	ARABIDOPSIS THALIANA HEAT SHOCK PROTEIN 101 (ATHSP101)

AGI	Gene Description
AT1G74430	MYB DOMAIN PROTEIN 95 (MYB95)
AT1G74670	gibberellin-responsive protein, putative
AT1G75380	ARABIDOPSIS THALIANA BIFUNCTIONAL NUCLEASE IN BASAL DEFENSE RESPONSE 1 (ATBBD1)
AT1G75390	ARABIDOPSIS THALIANA BASIC LEUCINE-ZIPPER 44 (AtbZIP44)
AT1G76210	unknown protein
AT1G76680	12-OXOPHYTODIENOATE REDUCTASE 1 (OPR1)
AT1G77760	NITRATE REDUCTASE 1 (NIA1)
AT1G78830	curculin-like (mannose-binding) lectin family protein
AT1G80130	
AT1G80180	unknown protein
AT1G80920	J8 mRNA, nuclear gene encoding plastid protein (J8)
AT2G02990	RIBONUCLEASE 1 (RNS1)
AT2G06850	ENDOXYLOGLUCAN TRANSFERASE (EXGT-A1)
AT2G14635	unknown protein
AT2G16660	nodulin family protein
AT2G17840	EARLY-RESPONSIVE TO DEHYDRATION 7 (ERD7)
AT2G19620	N-MYC DOWNREGULATED-LIKE 3 (NDL3)
AT2G20560	DNAJ heat shock family protein
AT2G21790	RIBONUCLEOTIDE REDUCTASE 1 (RNR1)
AT2G22122	unknown protein
AT2G22860	PHYTOSULFOKINE 2 PRECURSOR (ATPSK2)
AT2G23120	unknown protein
AT2G23130	ARABINOGALACTAN PROTEIN 17 (AGP17)
AT2G25770	unknown protein
AT2G26150	ARABIDOPSIS THALIANA HEAT SHOCK TRANSCRIPTION FACTOR A2 (ATHSFA2)
AT2G27505	unknown protein
AT2G27690	CYTOCHROME P450, FAMILY 94, SUBFAMILY C, POLYPEPTIDE 1 (CYP94C1)
AT2G29090	CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE 2 (CYP707A2)
AT2G29500	17.6 kDa class I small heat shock protein (HSP 17.6B-Cl)
AT2G32870	meprin and TRAF homology domain-containing protein / MATH domain-containing protein
AT2G32880	meprin and TRAF homology domain-containing protein / MATH domain-containing protein
AT2G33330	PLASMODESMATA-LOCATED PROTEIN 3 (PDLP3)
AT2G33570	unknown protein
AT2G33830	dormancy/auxin associated family protein
AT2G33850	unknown protein
AT2G34300	dehydration-responsive protein-related
AT2G34430	LIGHT-HARVESTING CHLOROPHYLL-PROTEIN COMPLEX II SUBUNIT B1 (LHB1B1)

AGI	Gene Description
AT2G34600	JASMONATE-ZIM-DOMAIN PROTEIN 7 (JAZ7)
AT2G34930	disease resistance family protein
AT2G38240	oxidoreductase, 2OG-Fe(II) oxygenase family protein
AT2G38465	unknown protein
AT2G39310	JACALIN-RELATED LECTIN 22 (JAL22)
AT2G39470	PSBP-LIKE PROTEIN 2 (PPL2)
AT2G39800	DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1 (P5CS1)
AT2G40020	unknown protein
AT2G41240	BASIC HELIX-LOOP-HELIX PROTEIN 100 (BHLH100)
AT2G42740	RIBOSOMAL PROTEIN LARGE SUBUNIT 16A (RPL16A)
AT2G42750	DNAJ heat shock N-terminal domain-containing protein
AT2G42840	PROTODERMAL FACTOR 1 (PDF1)
AT2G44940	encodes a member of the DREB subfamily A-4 of ERF/AP2 transcription factor family
AT2G45170	AUTOPHAGY 8E (ATATG8E)
AT2G45660	AGAMOUS-LIKE 20 (AGL20)
AT2G46220	unknown protein
AT2G46550	unknown protein
AT2G47270	transcription factor/ transcription regulator
AT3G03280	unknown protein
AT3G04210	disease resistance protein (TIR-NBS class), putative
AT3G05730	Encodes a defensin-like (DEFL) family protein
AT3G08770	LIPID TRANSFER PROTEIN 6 (LTP6)
AT3G08970	(ATERDJ3A)
AT3G09260	(PYK10)
AT3G09870	auxin-responsive family protein
AT3G12110	ACTIN-11 (ACT11)
AT3G12580	HEAT SHOCK PROTEIN 70 (HSP70)
AT3G14200	DNAJ heat shock N-terminal domain-containing protein
AT3G14210	EPITHIOSPECIFIER MODIFIER 1 (ESM1)
AT3G14720	MAP KINASE 19 (MPK19)
AT3G14990	4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein, putative
AT3G15356	legume lectin family protein
AT3G15650	phospholipase/carboxylesterase family protein
AT3G16400	NITRILE SPECIFIER PROTEIN 1 (NSP1)
AT3G16450	jacalin lectin family protein
AT3G16470	JASMONATE RESPONSIVE 1 (JR1)
AT3G16530	Lectin like protein whose expression is induced upon treatment with chitin oligomers
AT3G16700	fumarylacetoacetate hydrolase family protein
AT3G18290	EMBRYO DEFECTIVE 2454 (EMB2454)
AT3G19615	unknown protein
AT3G20060	UBIQUITIN-CONJUGATING ENZYME19 (UBC19)

AGI	Gene Description
AT3G20370	meprin and TRAF homology domain-containing protein / MATH domain-containing protein
AT3G21352	unknown protein
AT3G25180	CYTOCHROME P450, FAMILY 82, SUBFAMILY G, POLYPEPTIDE 1 (CYP82G1)
AT3G25770	ALLENE OXIDE CYCLASE 2 (AOC2)
AT3G27060	TSO MEANING 'UGLY' IN CHINESE (TSO2)
AT3G27360	histone H3
AT3G27690	PHOTOSYSTEM II LIGHT HARVESTING COMPLEX GENE 2.3 (LHCB2.3)
AT3G28210	Encodes a putative zinc finger protein (PMZ)
AT3G29810	COBRA-LIKE PROTEIN 2 PRECURSOR (COBL2)
AT3G45140	LIPOXYGENASE 2 (LOX2)
AT3G46230	HEAT SHOCK PROTEIN 17.4 (HSP17.4)
AT3G50770	calmodulin-related protein, putative
AT3G51860	CATION EXCHANGER 3 (CAX3)
AT3G51970	ACYL-COA STEROL ACYL TRANSFERASE 1 (ASAT1)
AT3G52720	ALPHA CARBONIC ANHYDRASE 1 (ACA1)
AT3G53230	cell division cycle protein 48, putative / CDC48, putative
AT3G54590	HYDROXYPROLINE-RICH GLYCOPROTEIN (ATHRGP1)
AT3G56360	unknown protein
AT3G56380	ARABIDOPSIS RESPONSE REGULATOR 17 (ARR17)
AT3G56970	Encodes a member of the basic helix-loop-helix transcription factor family protein (BHLH038)
AT3G56980	Encodes a member of the basic helix-loop-helix transcription factor family protein (BHLH039)
AT3G58790	GALACTURONOSYLTRANSFERASE 15 (GAUT15)
AT3G61210	embryo-abundant protein-related
AT3G62200	unknown protein
AT3G62410	CP12-2 encodes a small peptide found in the chloroplast stroma
AT3G62550	universal stress protein (USP) family protein
AT3G62760	Encodes glutathione transferase belonging to the phi class of GSTs (ATGSTF13)
AT3G63160	unknown protein
AT4G00695	unknown protein
AT4G00780	meprin and TRAF homology domain-containing protein / MATH domain-containing protein
AT4G01250	(WRKY22)
AT4G01360	unknown protein
AT4G01640	unknown protein
AT4G02160	unknown protein
AT4G02380	SENESCENCE-ASSOCIATED GENE 21 (SAG21)
AT4G02520	GLUTATHIONE S-TRANSFERASE PHI 2 (ATGSTF2)
AT4G03400	DWARF IN LIGHT 2 (DFL2)

AGI	Gene Description
AT4G05070	unknown protein
AT4G07960	CELLULOSE-SYNTHASE LIKE C12 (ATCSLC12)
AT4G09030	ARABINOGALACTAN PROTEIN 10 (AGP10)
AT4G11250	AGAMOUS-LIKE 52 (AGL52)
AT4G11320	cysteine proteinase, putative
AT4G12400	stress-inducible protein, putative
AT4G13570	HISTONE H2A 4 (HTA4)
AT4G14400	ACCELERATED CELL DEATH 6 (ACD6)
AT4G17245	zinc finger (C3HC4-type RING finger) family protein
AT4G17670	senescence-associated protein-related
AT4G17770	ARABIDOPSIS THALIANA TREHALOSE PHOSPHATASE/SYNTHASE 5 (ATTPS5)
AT4G19660	NPR1-LIKE PROTEIN 4 (NPR4)
AT4G19690	IRON-REGULATED TRANSPORTER 1 (IRT1)
AT4G19840	ARABIDOPSIS THALIANA PHLOEM PROTEIN 2-A1 (ATPP2-A1)
AT4G21320	HEAT-STRESS-ASSOCIATED 32 (HSA32)
AT4G21445	unknown protein
AT4G21680	proton-dependent oligopeptide transport (POT) family protein
AT4G23700	CATION/H+ EXCHANGER 17 (ATCHX17)
AT4G24800	MA3 domain-containing protein
AT4G25100	FE SUPEROXIDE DISMUTASE 1 (FSD1)
AT4G26530	fructose-bisphosphate aldolase, putative
AT4G27440	PROTOCHLOROPHYLLIDE OXIDOREDUCTASE B (PORB)
AT4G27860	integral membrane family protein
AT4G28780	GDSL-motif lipase/hydrolase family protein
AT4G32800	encodes a member of the DREB subfamily A-4 of ERF/AP2 transcription factor family
AT4G34560	unknown protein
AT4G34710	ARGININE DECARBOXYLASE 2 (ADC2)
AT4G34950	nodulin family protein
AT4G35320	unknown protein
AT4G37925	SUBUNIT NDH-M OF NAD(P)H:PLASTOQUINONE DEHYDROGENASE COMPLEX (NDH-M)
AT4G37980	ELICITOR-ACTIVATED GENE 3-1 (ELI3-1)
AT4G39250	ARABIDOPSIS RAD-LIKE 1 (ATRL1)
AT5G01380	transcription factor
AT5G02490	heat shock cognate 70 kDa protein 2 (HSC70-2) (HSP70-2)
AT5G02780	GLUTATHIONE TRANSFERASE LAMBDA 1 (GSTL1)
AT5G04150	(BHLH101)
AT5G04840	bZIP protein
AT5G05250	unknown protein
AT5G05365	metal ion binding
AT5G05600	oxidoreductase, 2OG-Fe(II) oxygenase family protein
AT5G06720	ARABIDOPSIS THALIANA PEROXIDASE 2 (ATPA2)

AGI	Gene Description
AT5G09220	AMINO ACID PERMEASE 2 (AAP2)
AT5G11360	unknown protein
AT5G11790	N-MYC DOWNREGULATED-LIKE 2 (NDL2)
AT5G12020	17.6 KDA CLASS II HEAT SHOCK PROTEIN (HSP17.6II)
AT5G12030	ARABIDOPSIS THALIANA HEAT SHOCK PROTEIN 17.6A (AT-HSP17.6A)
AT5G13740	ZINC INDUCED FACILITATOR 1 (ZIF1)
AT5G16360	NC domain-containing protein
AT5G19530	ACAULIS 5 (ACL5)
AT5G20190	unknown protein
AT5G20630	GERMIN 3 (GER3)
AT5G21940	unknown protein
AT5G22545	unknown protein
AT5G22880	HISTONE B2 (HTB2)
AT5G23820	MD-2-related lipid recognition domain-containing protein / ML domain-containing protein
AT5G24080	protein kinase family protein
AT5G24490	30S ribosomal protein, putative
AT5G25140	CYTOCHROME P450, FAMILY 71, SUBFAMILY B, POLYPEPTIDE 13 (CYP71B13)
AT5G25460	unknown protein
AT5G25980	GLUCOSIDE GLUCOHYDROLASE 2 (TGG2)
AT5G26000	THIOGLUCOSIDE GLUCOHYDROLASE 1 (TGG1)
AT5G26850	unknown protein
AT5G28800	unknown protein
AT5G37260	CIRCADIAN1
AT5G37540	aspartyl protease family protein
AT5G39050	transferase/ transferase, transferring acyl groups other than amino-acyl groups
AT5G43380	TYPE ONE SERINE/THREONINE PROTEIN PHOSPHATASE 6 (TOPP6)
AT5G43570	Predicted to encode a PR (pathogenesis-related) peptide that belongs to the PR-6 proteinase inhibitor family
AT5G46890	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
AT5G48485	DEFECTIVE IN INDUCED RESISTANCE 1 (DIR1)
AT5G48490	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
AT5G48570	peptidyl-prolyl cis-trans isomerase, putative / FK506-binding protein, putative
AT5G51000	F-box family protein
AT5G51440	23.5 kDa mitochondrial small heat shock protein (HSP23.5-M)
AT5G52640	HEAT SHOCK PROTEIN 90.1 (ATHSP90.1)
AT5G53420	unknown protein
AT5G53450	OBP3-RESPONSIVE GENE 1 (ORG1)
AT5G54030	DC1 domain-containing protein
AT5G55970	zinc finger (C3HC4-type RING finger) family protein

AGI	Gene Description
AT5G57220	CYTOCHROME P450, FAMILY 81, SUBFAMILY F, POLYPEPTIDE 2 (CYP81F2)
AT5G58750	wound-responsive protein-related
AT5G59400	unknown protein
AT5G59690	histone H4
AT5G59870	HISTONE H2A 6 (HTA6)
AT5G60680	unknown protein
AT5G61270	PHYTOCHROME-INTERACTING FACTOR7 (PIF7)
AT5G63810	BETA-GALACTOSIDASE 10 (BGAL10)
AT5G64190	unknown protein
AT5G65470	unknown protein

Acknowledgements

I have heard once that a PhD is not the achievement of one person but of a whole work group of people, those people that supported the candidate, and I'd like to agree with that.

First of all I'd like to thank Gerco (the big boss) Angenent. Thanks, Gerco, for accepting me in your group and being a great boss. You gave me the kick right up my bottom when I needed one and the next day you would be just as casual as ever, and I probably learned more from just observing you as a boss and scientist than you could have ever taught me in meetings and discussions. On the work-floor of science the credits for teaching me most goes to Richard (the tall boss) Immink. Richard, I hope it was as great for you to have me as a student as it was for me to have you as a supervisor. It was great that you could share enthusiasm about any of my ideas, no matter what rubbish they turned into. You taught me to be patient, creative, critical and obsessive about a project without forgetting that science is mostly fun! And really, the only time you left me abandoned was on a train station in Basel, Switzerland, an occurrence I will remind you of in years to come! Thank you!

Third in line comes Froukje van der Wal, alias "Wonderwoman". If anybody needs to get anything done in the lab, try Froukje! When you started to help me with my project I realized how great competent help is. You took quite some workload and worries of my chest, and I cannot thank you enough for that. Apart from that it is always great fun to work with such a light-hearted person as you are.

Special thanks goes to all those people who joined me on my path as a PhD student at PRI Wageningen. And indeed I always saw you as my classmates than as colleagues. Thank you Cezary, who turned from the quiet workaholic to a good friend and neighbor I would not want to miss. There is only few people I know with whom I can talk hardcore science in one second, and have an absolute rubbish conversation in the other, and you belong to them. You may feel honored now. Thanks for your help and friendship in and outside the lab. To Jenny, the most chatty person I know: I know you will make it even without my moral support and once you are a big boss somewhere in Ecuador, sent me a postcard! Anneke, my good-morning-co-singer, keep your good spirits and continue to amaze everybody with your good work! Alice, you still have the longest way to go, but I am sure you will go it with glory. Violeta, who chose to work with two rough topics, TCPs and tomatoes. Hang in there! Hui, (what are you working on again?) it was great to have you as direct desk neighbor, especially when you had a nap!

And then there is the many other people from the Plant Developmental Systems Team that need to be mentioned as well: Martijn Fiers, who taught me that you can be chaotic and still a good scientist. Kerstin Kaufmann, a great scientist and person, and for a long time my only German buddy in the lab. Stephan de Folter, thanks for being my supervisor for the first 5 months and helping me in my first steps in the lab. Thanks to Marco Busscher and Michiel Lammers, the superteam of technicians who are able to get you anything (except M-MulV Reverse Transcriptase) within days, no matter when or what you ask them for. I never tried to get anything illegal but I am sure they are able to get it. And then also thanks to: Ruud, Jose, Kim, Jacqueline, Mieke, Rumyana, Andrea, Jose, Steven, Tjitske and Jan.

A lot of people came and left while I was there and I am sure that I forget one or two. Those, whom I remember are: Susy, Guodong, Nayelli, Merche, Martina, Michelina, Lukas, Tom, Long, Peter, Andrea, Wilco, Giusy, Camilla, Priscilla, Lucas, Juliuli, Tetty and Pepe. Thanks to all of you for providing the best working environment one can imagine and being friends when I needed friends, and colleagues when I needed colleagues! I will not forget all the social activities which spiced up my time at the PRI, be it the massive eating at Christmas dinners, being left in the dark with Zombies by Kim et al, or trying to run away from Martijn any time when water was close. Outside of my direct working environment there are a few others that need special thanks. Megan Andriankaja, thanks for being such a good collaborator and I am sure we'll stay in contact once you are in Germany. Aalt-Jan van Dijk, same goes to you except for the Germany-part!

And yes, there is people out there that were not related to my work and that should be mentioned as well. Anneke, Ansa, Mascha, Hakan, Guillaume, Yvette, Philip (I never learned to write your name right), Louise, Laura, Mourao, Totti and all the unnamed I forgot. Thanks to you all for my great time I had in Holland!

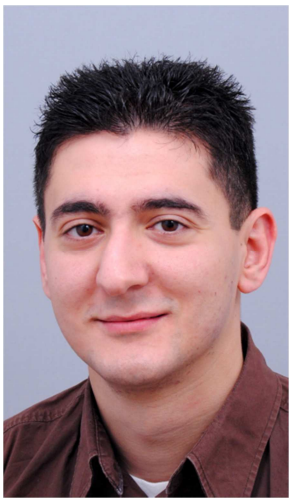
Thanks, last but lost least, to my family, to all of you young and old, who were my moral support during the last years and my biology-free island once I came around to visit you all. Especially I have to thank my mom and dad, whose help during the last few years and all the years before I cannot pay back.

Thank you!

Selahattin Danisman

Bielefeld 2nd of November, 2011

Curriculum vitae



Selahattin Danişman, the fourth child of Sevim and Möhyettin Danişman, was born in Wesel (Germany) at the 28th of February, 1982. With five he moved to Siegen where he went to and finished school at the Fürst-Johann-Moritz Gymnasium in the year 2001. Never that far away from being a nerd, he discovered his passion for biology only late in his school career. After school he went to study Biology at Bielefeld University. In his first year at Bielefeld University he confidently announced that he would never work with plants. Soon thereafter he discovered that you should be more careful with predictions. He started working on the little weed *Arabidopsis thaliana* and finished his studies 2007 with a dissertation on the involvement of a small glycine-rich RNA-binding protein (*At*GRP7) in the induction of flowering in Arabidopsis (see Streitner et al. 2008). After that he went on to do his PhD at the Plant Research International at Wageningen University in the Netherlands. Here he studied the involvement of TCP transcription factors in leaf development in the course of the EU-AGRONomics project. Currently he is a postdoc at Bielefeld University working on microRNAs, RNA-regulation and their effect on flowering time, and the application of synthetic biology approaches in plants. When he is not in a lab or behind a computer (so quite rarely) he engages in sports, specifically Capoeira Angola, a blend of martial arts, dance and play of Afro-Brazilian origin.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Selahattin Danisman
Date: 19 December 2011
Group: Plant Developmental Systems, Wageningen University & Research centre

1) Start-up phase	date
► **First presentation of your project**	
Interaction of TCP transcription factors in Arabidopsis thaliana	Sep 12, 2007
► **Writing or rewriting a project proposal**	
Project proposal: Involvement of TCP transcription factors in the regulation of leaf growth in Arabiopsis thaliana	2007
► **Writing a review or book chapter**	
list title if applicable	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7,5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Wageningen	Sep 13, 2007
PhD joint retreat 2008, Wageningen	Oct 02-03, 2008
EPS PhD student day, Leiden	Feb 26, 2009
PhD joint retreat 2010, Cologne (Germany)	Apr 15-17, 2010
► **EPS theme symposia**	
EPS theme 1 symposium 'Developmental Biology of Plants', Wageningen	Oct 11, 2007
EPS theme 1 symposium 'Developmental Biology of Plants', Leiden	Jan 30, 2009
EPS theme 1 symposium 'Developmental Biology of Plants', Wageningen	Jan 28, 2010
► **NWO Lunteren days and other National Platforms**	
EPW meeting Lunteren, The Netherlands	Apr 07-08, 2008
EPW meeting Lunteren, The Netherlands	Apr 06-07, 2009
EPW meeting Lunteren, The Netherlands	Apr 19-20, 2010
EPW meeting Lunteren, The Netherlands	Apr 04-05, 2011
► **Seminars (series), workshops and symposia**	
Internal seminar "PRI seminars"	2007-2011
► **Seminar plus**	
► **International symposia and congresses**	
EU Agronomics meeting in Golm, Germany	Feb 18-22, 2008
EU Agronomics meeting in Warwick, UK	Feb 09-10, 2009
EU Agronomics meeting in Bern, Switzerland	Feb 03-06,2010
EU Agronomics meeting in Tubingen, Germany	Feb 22-24, 2011
► **Presentations**	
Talk given on EU Agronomics meetings	Feb 09-10, 2009
Talk given on EPS theme symposia	Jan 28, 2010
Talk given on PhD joint retreat 2010, Cologne	Apr 15-17, 2010
Talk given on International PhD School on Plant Development, Regenz, Germany	Oct 06-08, 2010
Talk given on EPW meeting Lunteren, The Netherlands	Apr 04-05, 2011
► **IAB interview**	Dec 02, 2009
► **Excursions**	
Subtotal Scientific Exposure	*16,4 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Mathematics and Biology Course in Paris	Jul 02-16, 2007
CPS Workshop "Transient expression technology" in Leeds	Jul 16-21, 2007
Confocal Light Microscopy Course in Amsterdam	Jun 02-06, 2008
Plant Bioinformatics, Systems and Synthetic Biology Summer School, Nottingham	Jul 27-31, 2009
International PhD School on Plant Development, Regenz, Germany	Oct 06-08, 2010
► **Journal club**	
participation at journal club	2007-2011
► **Individual research training**	
Subtotal In-Depth Studies	*15,2 credits**

4) Personal development	date
► **Skill training courses**	
WGS course: Scientific writing	Jan 14-Mar 13, 2010
WGS course: Career Perspectives	Oct 19-Nov 16, 2010
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3,4 credits**

TOTAL NUMBER OF CREDIT POINTS*	**42.5**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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