Competition versus redundancy in the regulation of ERFIb transcription factors in *Arabidopsis thaliana*

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Summary

Transcription factors belonging to the AP2/ERF gene family are of great importance to control stress related processes in plants and were already successfully used to improve stress robustness in important crop plants like tobacco or rice. The ERFIb group is part of the AP2/ERF family in *Arabidopsis thaliana* and consists of the transcription factors Rap2.4a-Rap2.4h. This work investigates the role of these genes in terms of regulatory similarities and differences upon abiotic stress as well as the interplay between the transcription factors.

Bioinformatical resources were used to analyse the ERFIb expression in different tissues of *A. thaliana*. Low expression abundances were present for most of the ERFIb genes in callus cells, seedling, shoot and roots, whereas Rap2.4b was highly expressed in callus cells, shoots and roots. Moreover, Rap2.4b was mostly co-expressed with Rap2.4d. The analysis of development related ERFIb expression, demonstrated Rap2.4b and Rap2.4d as highly expressed TFs during nearly all stages. Co-response analysis between all ERFIb genes displayed for Rap2.4b and Rap2.4d the highest co-expression upon abiotic stress in roots.

To investigate transcriptional stress responses *in planta*, the expression of all ERFIb genes was analysed after different cold and heat stress treatments. There, similarities and differences in induction kinetics between ERFIb genes could be demonstrated.

The possibility of compensation or competition within the ERFIb transcription factors was analyzed. Expressional profiles of ERFIb genes were analyzed by qPCR after the individual knock-down and transient overexpression. The combination of both datasets allowed establishing regulatory patterns within the ERFIb family.

The relationship between Rap2.4a and Rap2.4h was demonstrated by means of competitive regulation of 2-Cys Peroxiredoxin (2CPA) promoter. Upon mild stress both transcription factors antagonistically regulate the 2CPA promoter activity. Accordingly, 2CPA activity could be fine-tuned by Rap2.4a and Rap2.4h upon mild-stress. Upon tough conditions this regulation is replaced through chloroplast singlet oxygen mediated retrograde signaling pathways.

In summary the ERFIb genes are highlighted for further specific investigations regarding improvement of stress tolerances in *A. thaliana*.

Zusammenfassung

Transkriptionsfaktoren der AP2/ERF-Genfamilie sind von großer Bedeutung für die Kontrolle stress-relevanter Prozesse in Pflanzen und wurden bereits erfolgreich zur Optimierung von Stresstoleranzen bei bedeutenden Nutzpflanzen wie z.B. Tabak oder Reis eingesetzt. In *Arabidopsis thaliana* besteht die zur der AP2/ERF-Familie gehörende ERFIb-Gruppe aus den Transkriptionsfaktoren Rap2.4a bis Rap2.4h. Die vorliegende Arbeit untersucht die Rolle dieser Gene im Hinblick auf regulatorische Gemeinsamkeiten und Unterschiede bei abiotischem Stress sowie die Wechselwirkungen zwischen den Transkriptionsfaktoren.

Die Expression der ERFIb-Gene wurde mithilfe bioinformatischer Ressourcen in verschiedenen Gewebetypen von *A. thaliana* überprüft. Für die meisten dieser Gene, konnte ein niedriges Expressionsniveau in den meisten Gewebetypen festgestellt werden. Rap2.4b wurde in den Calli von Zellkulturen, in den Blättern wie auch in den Wurzeln hoch exprimiert. Zudem co-exprimierte Rap2.4b fast ausschließlich mit Rap2.4d. Die Analyse der Genexpression in verschiedenen Entwicklungsstufen zeigt, dass Rap2.4b und Rap2.4d in nahezu jeder dieser Stufen hochreguliert werden. Weitergehende Analysen der Co-Regulation zwischen den ERFIb-Genen, zeigten für Rap2.4b und Rap2.4d sehr hohe Werte bei abiotischem Stress in den Wurzeln.

Um Stressantworten auf Transkriptebene *in planta* zu untersuchen, wurde die Expression aller ERFIb-Gene nach dem Einsatz verschiedener Arten von Kälte- und Hitzestress analysiert. Hierbei konnten Gemeinsamkeiten wie auch Unterschiede bezüglich der Induktionskinetik zwischen den ERFIb Genen nachgewiesen werden.

Die Möglichkeit von Kompensation oder Kompetition innerhalb der ERFIb-Gruppe wurde ebenfalls untersucht. Mittels qPCR wurden Expressionsprofile der Transkriptionsfaktoren nach dem Knock-down sowie der Überexpression einzelner Rap2.4 Gene ermittelt. Die Kombination beider Datensätze ermöglichte die Etablierung von regulatorischen Mustern.

Die kompetitive Beziehung zwischen Rap2.4a und Rap2.4h wurde anhand der Regulation des Promotors von 2-Cys Peroxiredoxin A (2CPA) demonstriert. Beide Transkriptionsfaktoren regulieren die 2CPA-Aktivität bei milden Stresskonditionen antagonistisch. Bei ansteigendem Stresslevel wird diese Regulation durch chloroplastidäre, retrograde Signalwege ersetzt.

Insgesamt erweisen sich die ERFIb-Gene als mögliche Kandidaten zur Verbesserung von Stresstoleranzen in *A. thaliana* und sollten diesbezüglich zukünftig gezielt auf ihr Potential untersucht werden.

List of abbreviations

2CP	2-Cys Peroxiredoxin
2CPA	2-Cys Peroxiredoxin A (At3g11630)
3-AT	3-amino-1,2,4 triazole
A	absorbance
aa	amino acids
ABA	abscisic acid
ABF	ABRE binding factor
ABI	Aba insensitive
ABRE	ABA responsive element
ANOVA	analysis of variance
AP2	Apetala 2
APx	ascorbate peroxidase
AsA	ascorbic acid
ATP	Adenosine triphosphate
bp	base pairs
bHLH	basic helix-loop-helix
BPM	BTB/POZ-MATH
BSA	bovine albumin serum
bZIP	basic region leucine zipper
C-	carboxyl-
CBF	C-repeat-binding factor
cDNA	complementary DNA
CE	coupling element
CO_2	carbon dioxide
Col-0	Arabidopsis thaliana ecotype Columbia-0
CPD	disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricycle-[3.3.1.13,7]decan}-4-yl)phenyl phosphate
CRT	C-repeat binding factor
Ct	treshold-cycle
DEL1	DP-E2F-LIKE1
DIG	digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotid triphosphates

List of abbreviations

Dof	DNA binding with one finger
DRE	drought response element
DREB	drought-responsive element binding factor
DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
EDTA	ethylene diamine-N tetra acetic acid
EREBP	ethylene responsive element binding proteins
ERF	Ethylene responsive factor
FR	far-red
GABI	Genomanalyse in biologischen System Pflanze
GDB	GCC-box binding domain
GUS	β-glucuronidase
H_2O_2	hydrogen peroxide
HR	hypersensitive response
HRP	horse-radish peroxidase
kb	Kilo base pairs
KD	Knock-down
KO	Knock-out
LHC	Light harvesting complex
MADS	MCM1 AGAMOUS DEFICIENS SRF
MAPK	mitogen activated protein kinase
Min	minute
mM	millimolar
MS	Murashige and Skoog media
MYB	myeloblastosis
N	number
NAC	NAM, ATAF, and CUC
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium chloride
NCBI	national center for biotechnology information
NF-Y	nuclear factor Y
NPQ	non-photochemical quenching
LB	Laura Bertani medium
$_1O^2$	singlet oxygen
O_2^-	superoxide anion
OH^-	hydroxyl radical
PBS	Phosphate buffered saline

PCR	polymerase chain reaction
PRX	peroxiredoxins
PS	photosystem
qPCR	quantitative real time polymerase chain reaction
RAV	related to ABI3/VP1
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
r_s	Spearman´s non-parametric measure of correlation
sAPx	stromal ascorbate peroxidase (At4g08390)
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TA	Annealing temperature
TAE	Tris acetic acid EDTA
TAF	TATA binding protein associated factor
TAIR	The Arabidopsis Information Resources
tAPx	thylakoid-bound ascorbate peroxidase (At1g77490)
TE	Tris-EDTA
TF	transcription factor
Tris	Tris(hydroxymethyl)-aminomethane
UPR	unfolded protein response
UTR	untranslated region
UV	ultra violet
V	volt
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide
Y1H	yeast-one-hybrid
YPD	Yeast Extract-Peptone-Dextrose

1 Introduction

1.1 Gene expression

Gene expression is a two-step event. At first, genetic information stored in DNA is transcribed into RNA (transcription). The following processes translate the nucleotide sequence into protein (translation). These steps are directly connected in prokaryotes, but are spatially and temporally separated in eukaryotes where transcription is maintained in the nucleus. After modification of RNA (processing) and subsequent transport into cytosol, the translation is initiated. Thereafter, folding, post-translational modification and targeting take place.

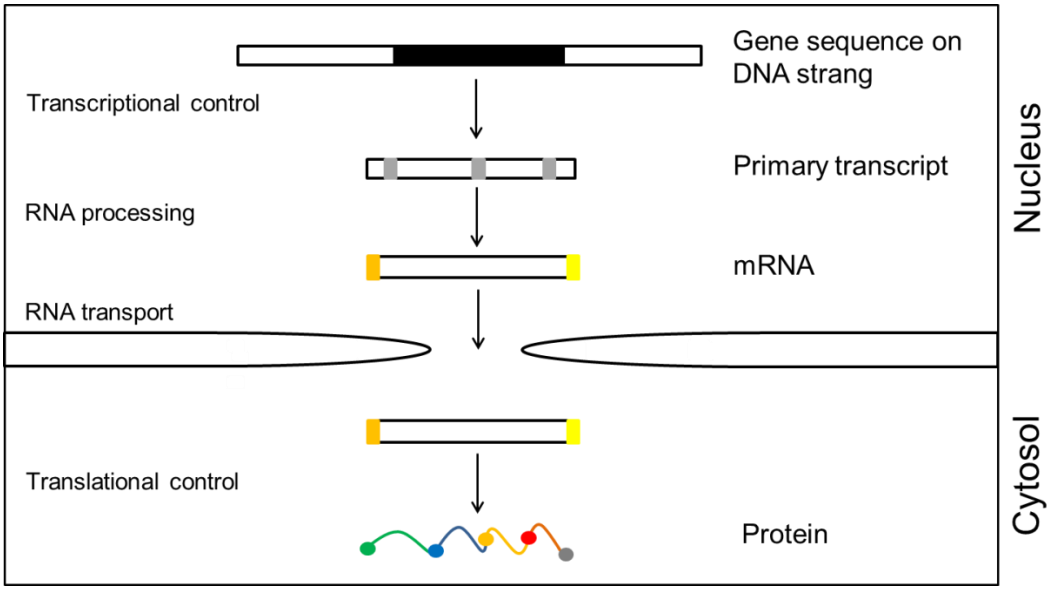

Fig. 1: Simplified scheme of gene expression process; Transcription controls the gene expression in the nucleus and generates a primary transcript, the precursor messenger RNA (pre-mRNA). The pre-mRNA is modified during RNA processing, including the removal of introns (splicing), 5´capping and 3´polyadenylation. The mature messenger RNA (mRNA) in then exported to the cytosol through nuclear pores. During the translation process the mRNA is decoded by ribosomes to synthesize polypeptides. The polypeptide folds into the appropriate functional structure; modified after Maniatis and Reed (2002).

The initiation of transcription happens on the promoter of a gene and is one of the most important steps in expressional regulation (Wray et al. 2003). Here, transcription factors earn a crucial role.

1.2 Transcription factors

Transcription factors (TFs) are *trans*-acting elements; they adjust gene expression through specific binding to DNA sequences (*cis*-acting elements) in promoters of putative target genes (Fig. 2) (Riano-Pachon et al. 2007). TFs can activate or suppress gene expression and regulate the interplay between different signaling cascades (Riano-Pachon et al. 2007; Xu et al. 2011). Modulation of TF activity can affect the whole transcriptome and initiate multiple changes in physiology and morphology caused by altered cell division and regulation of metabolic pathways (Matsui and Ohme-Tagaki 2010).

TFs can be separated in general and regulatory types (Mehterov et al. 2012). General TFs, like for example the TATA-box binding proteins, are crucial to activate transcriptional events (Lee and Young 2000). In combination with RNA polymerase II, they establish the core complex of each transcriptional process (Hampsey 1998). TFs can bind up- or downstream of this core complex and function as inducible or constitutive factors (Fig. 2b). They modulate the initiation of transcription through interaction with members of the core complex (reviewed in Wray et al. 2003). TFs with regulatory preference administrate gene-specific and/or tissue-specific functions. A good example is the mammalian TF Pax-6, which is a key player in the eye- as well as pancreatic morphogenesis (Kammandel et al. 1999). Dependent on the developmental stage it is expressed at different time points with varying expression levels in appropriate tissues (Kammandel et al. 1999). TFs also mediate the transcriptional activity of target genes upon changes due to different stimuli (reviewed in Saibo et al. 2009).

Therefore, it is of great importance to functionally characterize TFs and understand their role in regulatory networks (Riano-Pachon et al. 2007).

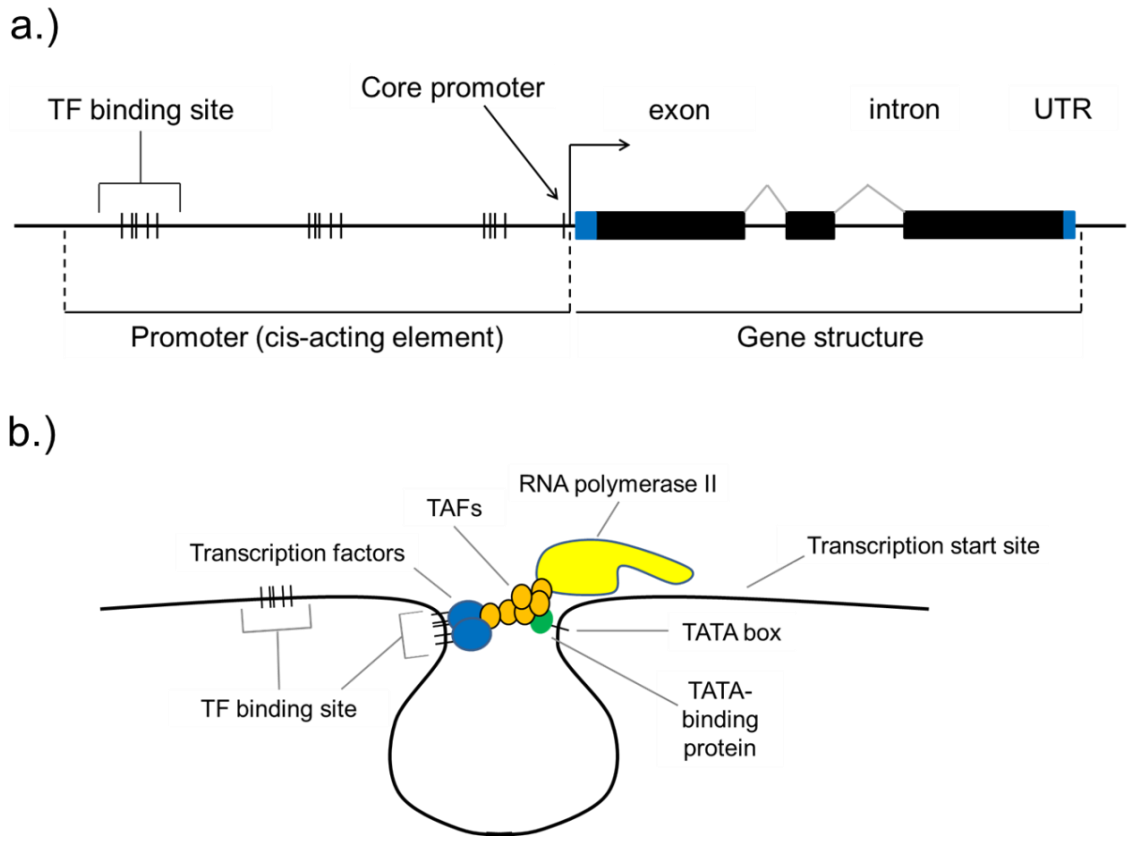

Fig. 2: a.) Simplified structure of a eukaryotic gene, indicated is the relative position of the exons and intron in the gene structure, the core promoter region and transcription factor binding sites in the upstream promoter region **b.) Schematic exposure of working promoter,** indicated are TATA binding proteins and TATA binding protein associated factors (TAFs) as general transcription factors, which form the transcriptional core together with the RNA polymerase II. The regulatory transcription factor binds to the transcription factor binding site in the promoter region and interacts with the core complex to finally initiate gene transcription; modified after Wray et al. (2003).

1.2.1 Transcription factors in plants

During various cycles in plant development, different stress types like drought, high salinity or cold affect the crop yield and plant growth (Epstein et al. 1980; Boyer et al. 1982). As sessile organisms, plants cannot omit harmful situations caused by the wide range of particular or combined environmental impacts. To process signals from outside, plants evolved signaling trails and highly dynamic transcriptomic networks (Rasmussen et al. 2013), which are crucial to respond towards different stimuli as well as developmental processes (Riano-Pachon et al. 2007). Here, TFs earn a crucial role in mediating signals and controlling gene transcription.

Referring to The Arabidopsis Information Resources (TAIR, http://arabidopsis.org), the *Arabidopsis thaliana* genome possesses more than 27000 genes, whereas approximately 2000 genes (8%) encode for transcription factors (Riano-Pachon et al. 2007). This demonstrates a relatively high ratio of TFs towards genes if compared to organisms like *Drosophila melanogaster* (Riechmann et al. 2000) and, moreover, illustrates the high regulatory potential. Concerning the ratio of TF genes per genome *A. thaliana* can be best compared to humans (Venter et al. 2001).

TFs can be grouped into appropriate families, for example, depending on the type and number of available DNA-binding domains (Luscombe et al. 2000; Riechmann et al. 2000; Riano-Pachon et al. 2007). Riechmann and co-workers (2000) described approximately 30 transcription factor families in Arabidopsis. Noteworthy, nearly 1000 transcription factors are exclusively encoded in plants and characterized through plant-specific DNA binding domains (Riechmann et al. 2000). Transcription factor families like AP2-ERF, NAC, Dof or WRKY are only available in plants (Matsui and Ohme-Tagaki 2010). Recent progress revealed stereoscopic structures of DNA binding domains, for instance, from AP2-ERF or NAC TFs (Allen et al. 1998; Ernst et al. 2004), allowing the estimation of spatial interaction of appropriate TFs with the DNA helix (Allen et al 1998).

1.2.2 Apetala2/Ethylene responsive transcription factors

The discovery of Apetala2-ethylene responsive factors (AP2-ERF) started 1983. There, Koornneef and colleagues (1983) reported about the realization of genetic maps, where mutant plants were used as markers for crossing and subsequent estimation of crossover frequencies. Among these mutant plants, the Apetala mutants displayed disturbed flower morphology (Fig. 3), like missing petals or reduced petals and large sepals. Hence, these mutants were abbreviated as *ap-1* and *ap-2;* their position was located on chromosome 1 and 4, respectively (Koornneef et al. 1983).

Fig. 3: Phenotypes of Col-0 wt and apetala2 mutant from Bowman et al. (1989); the mutant displays a disturbed flower development; Bar = 1 mm.

Bowman and colleagues (1989) analyzed in detail the *ap-2* mutant and discovered that the phenotype is temperature sensitive. Besides, they concluded that the mutated gene is important for initiation and differentiation of flower organs.

In 1994 Jofuku and co-workers isolated the *ap-2* insertion mutant using T-DNA insertional mutagenesis. There, the transformed line T10 showed high segregation towards the phenotype described for *ap-2* (Bowman et al. 1989) and was designated *ap2-10* (Jofuku et al. 1994). Upon further genetic linkage analysis with the *ap2-10* line, the AP2 gene could be isolated and the appropriate genomic sequence was determined (Jofuku et al. 1994). Northern blots demonstrated that the AP2 gene is expressed in various tissue parts and important for seed as well as flower development (Jofuku et al. 1994). It was also concluded that AP2 is a transcriptional regulator. Furthermore, the analyzed AP2 peptide contained 2 copies of a 68-amino acid (aa) repeat that was designated as AP2 domain and each repeat contained an 18 aa conserved core (Jofuku et al. 1994). Since both core regions may form α-helical structures, it was suggested that they could take part in protein-protein interactions (Jofuku et al. 1994).

In 1995 Ohme-Takagi and Shinshi reported about a novel type of DNA binding proteins in tobacco whose mRNA level was induced by the phytohormone ethylene. Hence, they were designated ethylene responsive element binding proteins (EREBPs). Furthermore, these proteins interacted with certain DNA region present in pathogen-related and ethylene-sensitive genes (Ohme-Takagi and Shinshi 1995). This region was previously defined as the short sequence GCC and was named GCC-box (Ohme and Shinshi 1990; Eyal et al. 1993; Hart et al. 1993). Additionally, a putative DNA binding domain, with a size of ~60 aa, was found in the conserved region of EREBPs (Ohme-Takagi and Shinshi

1995). In the year 1995 Weigel compared the aa sequences of EREBPs (Ohme-Takagi et al. 1995) with the sequence of AP2 gene (Jofuku et al. 1994). Noteworthy, he demonstrated a close relation between the sequences and speculated about the putative ability of AP2 genes to bind DNA (Weigel 1995). Furthermore, Weigel discussed putative differences in DNA-binding capability, possibly caused through non-constitutive aa substitution in the AP2 domain as demonstrated through different phenotypes between *ap2-1* and *ap2-10* (Jofuku et al. 1994; Weigel 1995)

Utilizing nuclear magnetic resonance spectroscopy, Allen and co-workers (1998) described the three-dimensional structure of the GCC-box binding domain (GBD) in AtERF1. This protein corresponds to EREBP in tobacco (Allen et al. 1998). The binding domain consisted of a 3-stranded anti-parallel ß-sheet and α-helix structure; the interaction with the major groove of DNA double helix was mediated through aa residues in the ß-sheet (Fig. 4) (Allen et al. 1998).

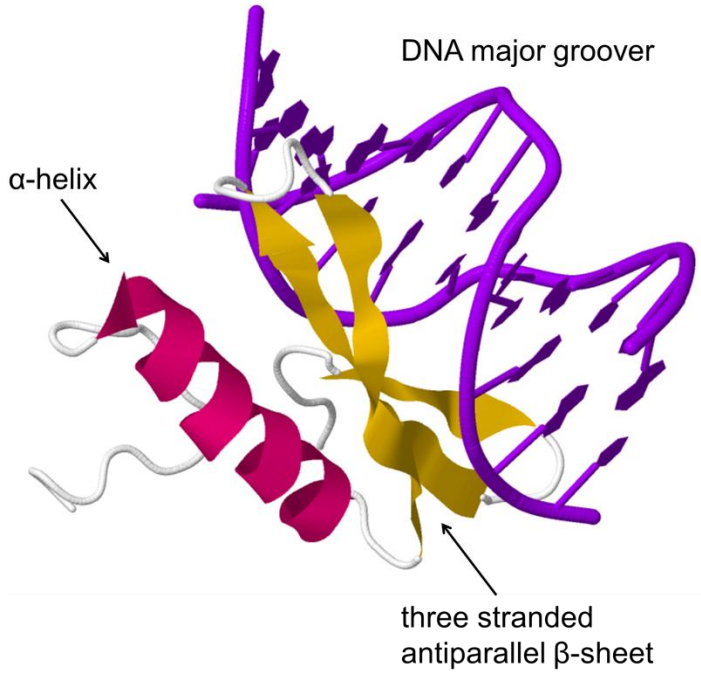

Fig. 4: Interaction of GCC-box binding domain in AtERF1 and the major groove of the DNA double helix mediated through aa residues in the β-sheet; the 3D structure of the GCC-box binding domain of AtERF1 from *A. thaliana* in complex with the target DNA fragment was determined by heteronuclear multidimensional NMR in combination with simulated annealing and restrained molecular dynamic calculation.; according to Allen et al. (1998); visualized with Jmol viewer version 13 (Jmol: an open-source Java viewer for chemical structures in 3D. http://www.jmol.org/).

In the year 2000, Riechmann and co-workers undertook the attempt to identify and organize putative transcription factors available in the Arabidopsis genome according to the DNA binding domain. Among others, he defined 3 groups with the biggest proportion, namely Apetala 2/ethylene responsive element binding proteins (AP2/EREBP),

MYB(R1)R2R3 and basic helix-loop-helix (bHLH). Totally 144 genes were assigned to the AP2/EREBP gene family, which consists of the AP2- and EREBP subfamily as well as RAV-like genes (Riechmann et al. 2000).

At that time reports about the function of genes containing an AP2 domain pointed towards importance in the regulation of developmental processes (Elliott et al. 1996; Moose and Sisco 1996; Chuck et al. 1998; van der Graaff et al. 2000; Banno et al. 2001; Chuck et al. 2002) and embryo development (Boutilier et al. 2002). In addition, involvement in phytohormone signaling (Alonso et al. 2003; Hu et al. 2004; Ohme-Takagi and Shinshi 1995) was demonstrated. Besides, participation in biotic (Yamamoto et al. 1999) and abiotic stresses (Stockinger et al. 1997; Liu et al. 1998; Dubouzet et al. 2003) as well as metabolomic maintenance were revealed (Aharoni et al. 2004; Broun et al. 2004; Zhang et al. 2005).

Strikingly, the intensive investigation of putative AP2 domains outside plants, revealed the evolutionary origin of AP2/ERF genes. Magnani and co-workers (2004) suggested the possibility that during evolution a HNH-AP2 endonuclease was horizontally transferred from bacteria or virus into plants. Alternatively, it has been postulated that HNH-AP2 endonucleases were transported from chloroplasts into nucleus after endosymbiosis of cyanobacteria and subsequent emergence of chloroplasts (Magnani et al. 2004). Since endonucleases behave like transposons (Chevalier and Stoddard 2001), they could have dispersed in the genome and provoked the emergence of AP2/ERF genes in plants (Magnani et al. 2004).

1.2.3 ERFIb transcription factors

In a more advanced approach based on amino acid sequence similarity within the AP2 domain, a phylogenetic assignment was performed and 146 transcription factors were annotated in the AP2/ERF superfamily (Nakano et al. 2006). 3 families belong to the superfamily, namely AP2, RAV (related to ABI3/VP1; Kagaya et al. 1999) and ERF. The AP2 and RAV family members have 2 AP2 domains, whereas proteins within the ERF gene family are characterized through a single AP2 domain (Nakano et al. 2006). Furthermore, the ERF family could be divided in two subfamilies, namely C-repeat binding factor/drought response element binding factor (CBF/DREB) and ethylene responsive transcription factor (ERF) subfamily as well as corresponding groups (Sakuma et al. 2002; Nakano et al. 2006). Hence, ERFIb transcription factors are part of the ERF subfamily and belong to the subgroup A-6 (Fig. 5).

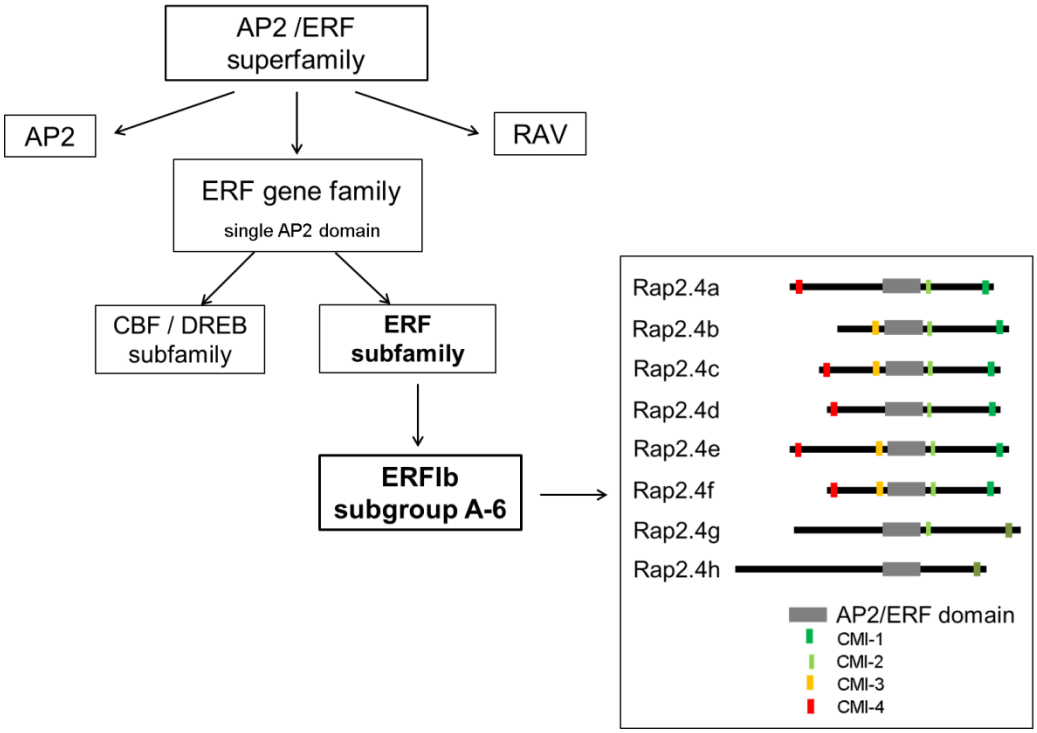

Fig. 5: The ERFIb transcription factor family is a subgroup of the ERF subfamily; modified after Nakano et al. (2006).

This subgroup consists of eight members, designated Rap2.4a-h (Related to Apetala 2) (Fig. 5) (Nakano et al. 2006; Mizoi et al. 2012). Rap2.4 transcription factors are characterized through a single, highly conserved AP2 domain but different C- and N-termini (Nakano et al. 2006). Notably, the conserved motifs CMI1-4 were also used to specify phylogenetic relationships (Nakano et al. 2006). The functions of CMI-1, CMI-2 and CMI-4 are unknown. The CMI-3 motif partly reassembles the HNH domain-like region and reflects the evolutionary origin of AP2/ERF genes via horizontally transferred HNH-AP2 endonucleases from bacteria or virus into plants (Magnani et al. 2004; Nakano et al. 2006).

1.2.3.1 Function of ERFIb genes

The closest relatives of ERFIb proteins are genes associated to the subgroup A-5 (Nakano et al. 2006). The subgroup A-5 contains stress-inducible genes like Rap2.1, which participates in CBF mediated cold responses and functions down-stream of DREB1A (Fowler et al. 2002; Maruyama et al. 2004). Another gene, namely DEAR1, acts as repressor of DREB proteins, which control cold and pathogen responses (Tsutsui et al. 2009). Several associated members contain the functional ERF-associated amphiphilic repression (EAR) motif (Mizoi et al. 2012). Hence, a negative regulation of DREB1 and DREB2 pathways through members of this subgroup is plausible (Mizoi et al. 2012).

Several reports exist concerning the functions of ERFIb genes. Rap2.4a regulates the expression of 2-Cys Peroxiredoxin A (2CPA) (Shaikhali et al. 2008), a key enzyme in the chloroplast antioxidant system, which eliminates the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2) (König et al. 2002). Rap2.4b can bind the ethylene-responsive GCC-box and the dehydration-responsive element (DRE). The Rap2.4b transcript level was down-regulated by light but up-regulated by salt and drought stress (Lin et al. 2008). The transcription factor is also supposed to regulate hypocotyl elongation in a light dependent manner (Lin et al. 2008). Furthermore, Rap2.4b and Rap2.4d are regulated by BTB/POZ-MATH (BPM) proteins as a part of the ubiquitination pathway (Weber et al. 2009). Iwase et al. (2011) showed that Rap2.4b is important for devolution of cell dedifferentiation. In addition, its expression is induced by wounding (Iwase et al. 2011a and b). Furthermore, Rap2.4a and Rap2.4d are induced in promoting callus formation (Iwase et al. 2011b). Rap2.4d seems to possess a putative plastid localization sequence (Schwacke et al. 2007). Hence, ERFIb TFs not only participate in abiotic stress responses but also in control of developmental processes.

Although several functional abilities are described, less is known about the connection within the eight transcription factors themselves. TFs from the same of related family can compete among each other (Gitter et al. 2009; De Vos et al. 2011) or compensate the loss of function of another related transcription factor, especially if a sequence homology consists (Hollenhorst et al. 2001; Gitter et al. 2009).

1.3 Interplay between transcription factors

A putative competition or compensation is feasible between ERFIb genes. Moreover, the relatively high number of 8 transcription factors is a prerequisite for at least compensational features.

1.3.1 Compensation between transcription factors

Zhang and colleagues (2003) revealed compensation by functional redundancy between stress responsive TGA transcription factors, which are important to gain systemic acquired resistance (SAR). Noteworthy, the missing SAR response in a triple-mutant could be complemented by introduction of another TGA transcription factor (Zhang et al. 2003).

Mu and co-workers (2013) demonstrated partially redundant roles for several NF-Y transcription factors in Arabidopsis. They are important in processes like gametophyte development or seed germination. The appropriate transcription factors are co-expressed during developmental stages (Mu et al. 2013). Hence, the lack of transcript in double-mutants was compensated by up-regulation of appropriate NF-Y transcription factor (Mu et al. 2013).

1.3.2 Competition between transcription factors

Schindler and colleagues (1992) described the competition between the basic/leucine zipper (bZIP) transcription factors TGA1 and G-Box in terms of interacting with the hex-motif G-box-like element.

Several partially redundant WRKY transcription factors are maintaining responses towards different pathogens by multiple variants of protein-protein interactions and subsequent formation of homocomplexes and heterocomplexes (Xu et al. 2006). They are able to regulate pathways by cross-talk, individual as well as antagonistic roles (Xu et al. 2006).

Shi et al. (2011) described several SHINE transcription factors, which contain single AP2 domains that belong to a small clade within the AP2/ERF family (Aharoni et al. 2004). The genes are redundant in their role to establish the surface pattering of flower organs in

Arabidopsis. The knock-out of a single gene was compensated by other SHINE transcription factors on mRNA level and firstly a triple-mutant displayed significant phenotype (Shi et al. 2011).

Flower development in Arabidopsis is regulated by MADS-domain transcription factors (Jofuku et al. 1994; Riechmann et al. 1996; Bowman et al. 1989; Favaro et al. 2003). Different MADS transcription factors can form various complexes to interact with their target genes (Smaczniak et al. 2012). The co-existence of such complexes is supposed to compete for, to some extent, a similar DNA-binding motif (Fig. 6) (Smaczniak et al. 2012).

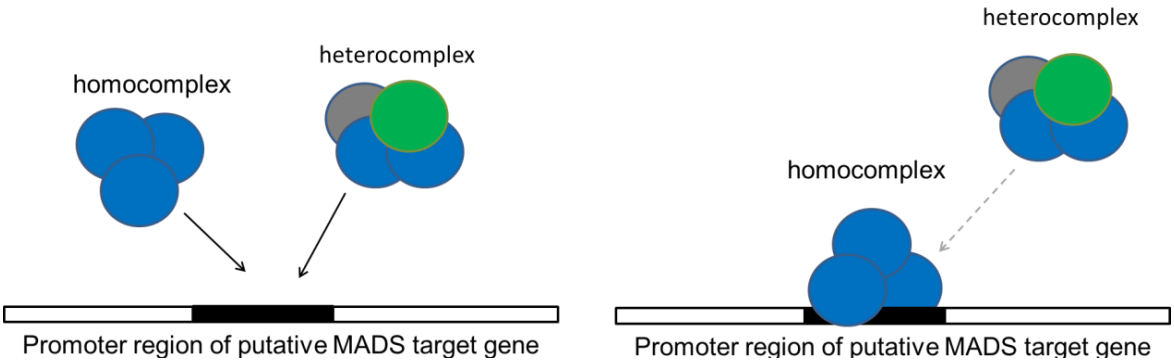

Fig. 6: Competition for target binding by homo- and heterocomplexes of MADS transcription factors in promoter regions of MADS target genes. MADS TFs are able to form homocomplexes and heterocomplexes through protein-protein interactions, these simultaneously existing protein-complexes can compete for the same binding motif to regulate it differentially. Modified after Smaczniak et al. (2012).

Competition between the MYB transcription factors WEREWOLF and CAPRICE was described by Song and colleagues (2011). These two transcription factors trigger between the nonhair and hair cell fate of root epidermis cells in Arabidopsis where already a small difference in transcript amounts of both genes decided about the cell fate in this dosage-dependent regulation (Fig. 7) (Song et al. 2011).

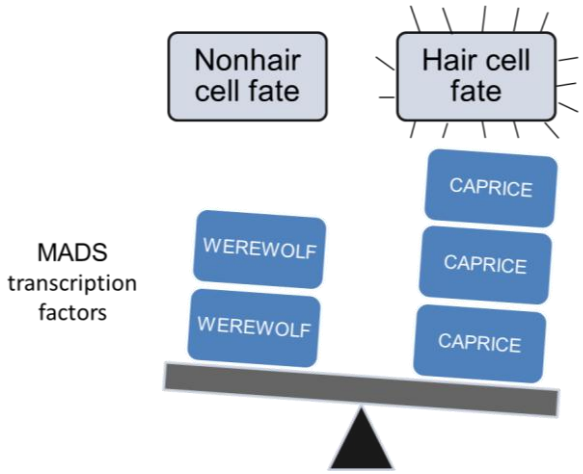

Fig. 7: The cell fate of root epidermis cell is defined in a dosage-dependent manner by WEREWOLF and CAPRICE transcription factors. The balance between the transcript levels of both transcription factors decides about the fate of root epidermis cells. WEREWOLF determines root epidermis cells without root hairs and CAPRICE appropriate cell with hairs, respectively. Modified after Song et al. (2011).

The atypical E2F transcription factor DP-E2F-LIKE1 (DEL1) controls the transition from the mitotic cycle into the endocycle (Berckmans et al. 2011). At this juncture, the transcription factors E2Fb and E2Fc regulate the DEL1 mRNA abundance through competition for a single E2F binding site in the DEL1 promoter region (Berckmans et al. 2011). Thus, E2Fb functions as activator and E2Fc as repressor of transcription (Berckmans et al. 2011).

One extraordinary example of competition between AP2/ERF TFs for the C-repeat (CRT) element binding site in the stress-responsive wheat Cor410b promoter was demonstrated by Eini and co-workers (2013). Here, 6 ERF TFs and 1 DREB/CRF TF were interacting with the CRT element according to Y1H experiments. A quite complex regulation regarding activation and repression could be demonstrated: the ERF TFs TaERF6 and TaERF5a compete for CRT binding with another ERF TF or DREB/CRF TF present among interacting proteins (Eini et al. 2013). Moreover, TaERF6 was shown as putative repressor of TaERF4 and TaERF5 members, since appropriate genes were down-regulated after induction of TaERF6 (Eini et al. 2013).

1.4 Tolerance towards abiotic stress in plants

Plants are perpetually exposed to different abiotic stresses. Drought, salinity, heat and cold stress as well as fluctuating light conditions constrain plant growth and negatively affect productiveness of crop plants (Xu et al. 2011). Bray and co-workers (2000) reported

that drought and salt stress affect more than 10% of cultivatable land. As a consequence, the expected yield of relevant crop plants like rice of wheat is strongly reduced (> 50%). However, it is crucial of gain robustness towards such stresses. In most cases plants have to cope with a combination of several stresses (Chinnusamy et al. 2004). Nevertheless, the time and duration of stress define the response and subsequent acclimatization of plants upon stressful conditions.

This extensive process can affect the plant differentially and impair, for instance, leaf or root tissue development on cellular and molecular levels. The variety of possible changes extinguishes the plants performance to handle with suboptimal environmental occurrences (Farooq et al. 2009). Such processes can affect the leaf area (Samson and Herzog 2007), root growth stimulation (Karaba et al. 2007), sugar accumulation (Taji et al. 2002) or the relative water content (Yang and Miao 2010). Elevated levels of ROS are abolished by enhanced activity of antioxidant enzymes (Apel and Hirt 2004). The phytohormone abscisic acid (ABA) triggers leaf stomata closure and thereby controls transpirational water loss (Schroeder et al. 2001). Ethylene is able to impair the ABA mediated stomata closure (Tanaka et al. 2005), presuming a putative opposite regulation. Molecular responses to abiotic stress involve signal transduction, gene expression and changes in the metabolom to finally improve stress sustainability (Apel and Hirt 2004; Agarwal et al. 2006).

1.4.1 Chloroplast antioxidant system

In tissue of photosynthetic organs like plants, chloroplasts produce the overwhelming part of ROS, at which the reaction centres of PSI and PSII being the main generation sites (Pitzschke et al. 2006; Valero et al. 2009). Especially under stress-full conditions the ROS level can be misbalanced and increased generation of H_2O_2 and singlet oxygen ($^1O_2$) may arise in these organelles (Takahaski and Murata 2008) and cause oxidative defects (Asada 1999; Ishikawa and Sigeoka 2008). Plants evolved various enzymatic as well as non-enzymatic backup systems to cope with continuous ROS generation (Maurino and Flügge 2008). For example, detoxification of H_2O_2 can be regulated by non-enzymatic antioxidants such as ascorbic acid (AsA) and glutathione (Foyer et al. 1983; Meister and Anderson 1983). Moreover, antioxidant enzymes within chloroplasts are involved in ROS reduction (König et al. 2002; Maruta et al. 2010). Here, H_2O_2 can be decomposed to water by ascorbate peroxidases (APX) in the ascorbate-dependent water-water cycle (Asada 1999; Maruta et al. 2010). Among these enzymes the soluble APX (sAPx) is located in the chloroplast stroma and the thylakoid bound APX (tAPx) is anchored to the membrane

(Ishikawa and Shigeoka 2008). Chloroplast targeted peroxiredoxins (Prxs) can reduce H_2O_2 in an ascorbate-independent reaction (Baier and Dietz 1999; Baier et al. 2000). The Prxs are arranged in 4 different types in accordance with available biochemical features and reaction process (Horling et al. 2003). In Arabidopsis chloroplasts two quite similar 2-CysPrxs, namely 2-CysPrxA and 2-CysPrxB as well as 2 other, namely PrxQ, PrxII E are present (Horling et al. 2003).

1.4.2 ROS production under abiotic stress like drought or high salinity

Under all conditions, ROS molecules are continuously generated as side-products of the electron transport chain in photosynthesis and respiration (Blokhina and Fagerstedt 2010). Upon unfavorable conditions the chloroplast is the major site for ROS generation, especially H_2O_2 (Foyer et al. 1994). Under non-stress conditions, the cellular ROS level and the redox-status are strictly maintained by antioxidant. The most prominent are ascorbate (Groden and Beck 1979) and glutathione (Law et al. 1983; Meister and Anderson 1983). Moreover, the enzymatic antioxidant system, for example, the water-water cycle in chloroplasts (Asada 1999; Rizhsky et al. 2003), is important for effective ROS abolishment.

Two major stress types for plants are drought (Boyer et al. 1982) and high salinity (Epstein et al. 1980). They can trigger and elevate ROS production beyond the normal levels. For example, Arabidopsis plants react towards elevated salt concentrations with stomatal closure to curtail water loss; at once the CO_2 influx is limited (Stepien and Johnson 2009). The internal CO_2 concentration decreases and decelerates carbon reduction by the Calvin cycle (Stepien and Johnson 2009). Subsequently, the availability of $NADP^+$ as electron acceptor in photosynthesis in decreased (Hsu and Kao 2003). The elevated cyclic electron flow in photosystem I (PSI) triggers an increased electron leakage, whereupon dioxygen (O_2) is used as an alternative electron acceptor causing $O_2^{-\cdot}$ generation (Mittler 2002). The toxicity of high salt concentrations may also interrupt the photosynthetic electron transport and further enhance electron leakage to $O_2^{-\cdot}$ (Borsani et al. 2001). Moreover, depletion in internal CO_2 concentration could decrease the efficiency of the Calvin cycle and cause enhanced H_2O_2 production in peroxisomes through photorespiration (Wingler et al. 2000). Extensive salt stress can lead to aggregation of hydroxyl radicals (OH^-) in thylakoids (Cruz de Carvalho 2008). Hydroxyl radicals have an eminently strong ability to react with nearly every relevant biomolecule (Dat et al. 2000)

like for example the DNA (Gutteridge and Halliwell 1989). Upon drought stress ROS are produced in a similar fashion: Stomata are closed to avoid water loss; the internal CO_2 level is reduced and triggers H_2O_2 production in peroxisomes due to the negatively impaired Calvin cycle (Abogadallah 2010). Due to limitations in $NADP^+$ amount $O_2^{-\cdot}$ is generated through increased electron leakage in PSI what provokes reduction of O_2 (Noctor et al. 2002).

1.4.3 Role of plant transcription factors in abiotic stress

One major regulator of abiotic stresses and a systemic supervisor of gene expression is the phytohormone ABA (Cutler et al. 2010), which facilitates plants to manage with decreased water availability (Kim et al. 2010). The ABA-coupled signaling network consists of the AREB/ABF regulons (ABA-responsive element-binding protein/ABA-binding factor) and the MYC/MYB regulon (Abe et al. 1997; Busk and Pagés, 1998; Saibo et al. 2009). The CBF/DREB regulon as well as the NAC and ZF-HD regulon are induced uncoupled from ABA signals (Nakashima et al. 2009; Saibo et al. 2009). The combination of ABA-coupled and -uncoupled pathways, are transduced through ERF family members (Yamaguchi-Shinozaki and Shinozaki 1993; Kizis and Pagés 2002). In stress reactions the responses are often cross-talks between existing regulons. Lee and co-workers (2009) reported the interaction of DREB2C with the bZIP transcription factors ABF3 and ABF4. In parallel, DREB1A and DREB2A cooperate with ABF2. The interactions conduct activation of ABA responsive genes (Lee et al. 2010).

1.4.3.1 Exemplarily role of the CBF/DREB regulon in abiotic stress response

DREB TFs have a single AP2 DNA binding domain (Magnani et al. 2004). They trigger several genes involved in stress responses and, hence, control stress sensitivity (Xu et al. 2011). DRE binding proteins like CBF1 (CRT binding factor1) or DREB1A (Stockinger et al. 1997; Liu et al. 1998) impair the expression of genes regulated via the DRE sequence motif (5'-TACCGACAT-3') (Yamaguchi-Shinozaki and Shinozaki 1993). The two main subgroups of the DREB subfamily, DREB1 and DREB2, mediate different pathways upon cold and drought stress. Homologs of the major DREB2 TFs, DREB2A and DREB2B (Liu et al. 1998), were also found in cereals (Nakashima et al. 2009). The expression of these genes is tissue-specific and depends on duration of stress treatment. For example, AtDREB2A is enriched in leaves and roots under standard conditions (Liu et al. 1998).

The transcription factor DREB2C was expressed in late embryo stage and in cotyledons of young seedlings (Lee et al. 2010). Upon cold stress treatment at 4°C, the AtDREB1 transcript level was increased in less than 10 min (Liu et al. 1998). CBF transcript was induced after 30 min at 4°C and the expressional peak was reached after 1 h (Medina et al. 1999). Salt and drought stress induced the transcription of AtDREB2A and AtDREB2B: but the transcript levels were not remarkably changed upon ABA application and cold stress treatment (Liu et al. 1998; Nakashima et al. 2000). Besides, neither addition of ABA nor low temperatures induced the transcript level of DREB2C (Lee et al. 2010). Overexpression of DREB1B/CBF1 or DREB1A/CBF3 in Arabidopsis plants resulted in advanced robustness towards cold, drought and salt stress (Jaglo-Ottosen et al. 1998; Kasuga et al. 1999). Overexpression of DREB1A/CBF3 caused accumulation of sugars and proline under control conditions (Gilmour et al. 2000). The α-amino acid proline can stabilize proteins and subcellular structures upon osmotic stress (van Rensburg et al. 1993). Microarray analysis of transcriptomic changes in Arabidopsis overexpressing a constitutively active from of DREB2A (without its negative regulatory domain), revealed the function of DREB2A in multiple stress pathways, including salt-, drought-, and heat-responses (Sakuma et al. 2006). These reports suggest that especially ERF TFs are of essential role to properly regulate the expression of relevant genes to enhance the plants robustness.

1.4.3.2 Transcription factor activation through ROS mediated signal transduction

Transcription factors, belonging to the AP2/ERF family, control the ability of plants to cope with abiotic stresses (Liu et al. 1998; Fujita et al. 2004; Hu et al. 2006; Lin et al. 2008; Zhu et al. 2010). Under normal conditions the cellular ROS level is within normal ranges but can change quickly if stress occurs (Apel and Hirt 2004) and orchestrate various stress responsive pathways (Mittler et al. 2006). Hence, ROS may function as a sensor of environmental changes and trigger transcription factors. It can be presumed that H_2O_2 generated inside chloroplasts may change the redox poise and induce signaling cascades to regulate the expression of responsive genes in the nucleus (Casano et al. 2001). The major part of proteins and most likely TFs are nuclearly encoded and further targeted to chloroplasts (Abdallah et al. 2000). Hence, a possibility to adjust stress related pathways and trigger appropriate TFs could be the redox-mediated communication between the chloroplast and the nucleus (Apel and Hirt 2004). Such regulation could be transduced by changes in the redox-poise, whereupon TFs could activate or repress the right genes to adapt the plant to drought, salinity or cold stress.

Several reports describe that the enhanced generation of chloroplastic H_2O_2 or $^1O_2$ affects the whole transcriptome and up-regulates the mRNA levels of several chloroplast targeted ERF TFs (op den Camp et al. 2003; Laloi et al. 2007; Balazadeh et al. 2012; Maruta et al. 2012; Mehterov et al. 2012). Of note, the possibility exists that the activation of TFs can even be different depending on of the ROS species, for instance, $^1O_2$ or H_2O_2 (op den Camp et al. 2003; Laloi et al. 2006; Maruta et al. 2012). To this end, the mechanism of TF activation by ROS is not fully understood yet.

A possibility may be redox-mediated regulation through signal transmitters. TFs could be activated by protein kinases, which were initially triggered by H_2O_2 (Miao et al. 2007). Pitzschke and colleagues (2009) reported about redox sensitive MAPK kinases, which can activate transcription factors. It should be noted that AP2 TFs were present among regulated genes (Pitzschke et al. 2009). It was presumed that TFs could also interact with H_2O_2 responsive elements in target gene promoters and, thus, regulate the expression (Foyer and Noctor 2005). H_2O_2 may also directly oxidize TFs in either the cytosolic compartment or in the nucleus and change their activity (Balazadeh et al. 2012).

H_2O_2 was discussed as signal transducer due to its putative ability to diffuse out of chloroplasts (Ivanov 2000). Intact chloroplasts from spinach leaves were subjected to different light intensities. Subsequently H_2O_2 was detected with AmplexRed assays and spin trapping EPR spectroscopy (Mubarakshina et al. 2010), which enable the detection and identification of ROS species (Mojovic et al 2005). Both methods demonstrated that H_2O_2 produced in chloroplasts upon increasing light intensities, could be localized outside the organelles (Mubarakshina et al. 2010). Nevertheless, instead of simple H_2O_2 diffusion through the chloroplast envelope membrane, it is more likely that H_2O_2 may pass through aquaporins (Bienert et al. 2007; Mubarakshina et al. 2010). Chloroplasts have a highly efficient antioxidant system what rather limits the diffusion of chloroplast-generated H_2O_2 over long distances (Mubarakshina et al. 2010).

New approaches for ROS signaling were reviewed by Mittler et al. (2011). Mitochondria from animal's heart muscle cells convert the appropriate signals in waves of ROS-induced-ROS-releases (Zhou et al. 2010). A similar signaling process between plant cells can be assumed via waves originating from ROS-induction and subsequent ROS-generation over long ranges (Mittler et al. 2011). The antioxidant system in plants is highly evolved and offers high buffering capacities for ROS detoxification (Baier et al. 2005): Signaling over long ranges could be explained by constant ROS generation, for example, though the NADPH oxidase RbohD (Miller et al. 2009), and auto-propagating of ROS

signals along a signaling trail (Mittler et al. 2011). Finally, the arrival of such ROS waves could initiate appropriate signaling cascades to modulate TF activity.

Proof for direct effect of H_2O_2 on a TF exists in *E. coli*. There, the OxyR TF mediates oxidative stress response (Zheng et al. 1998). The TF is redox-sensitive and can only initiate gene expression in its active form, which is present upon increasing H_2O_2 level (Zheng et al 1998). During oxidative stress covalent modifications of cysteine thiol groups in OxyR protein structure are accomplished (Zheng et al. 1998). Recently, a redox-regulated AP2 TF, namely Rap2.4a, was described (Shaikhali et al. 2008). It changes the oligomerization status depending on the cellular redox poise and activates the 2CPA promoter only as a dimer (Shaikhali et al. 2008).

1.5 Usability of AP2 transcription factors in crop improvement

A further proof for the importance of AP2-dependent regulation is the wide approach of genetic engineering in crop plants. For example, the gene DREB1A from Arabidopsis significantly improved the tolerance of important economic plants like tobacco (Kasuga et al.2004) or rice (Oh et al. 2005) towards abiotic stresses when introduced via transformation. Thereafter, the plants were more resistant towards drought, salt or low temperatures (Kasuga et al. 2004; Oh et al. 2005). The overexpression of AtCBF1 in potato decreased the susceptibility to cold stress (Pino et al. 2008). Gao et al. (2009) reported that transgenic cotton overexpressing the GhDREB gene had a higher tolerance to drought, salinity and cold stress but displayed no differences in phenotype to control. Overexpression of the tomato ERF gene TERF1 in rice (Gao et al.2008) and tobacco (Zhang et al. 2005) improved resistance to drought and high salinity, respectively. Overexpressing of AP37 in rice enhanced robustness to salt and drought stress (Oh et al. 2009). Furthermore, under stressful drought conditions the grain yield production was significantly higher (> 16%) if compared to controls (Oh et al. 2009). Stronger expression of the ERF genes SNORKEL1 and SNORKEL2 increased the internode elongation in rice and enabled deep water adaption (Hattori et al. 2009). These findings demonstrate the potential of AP2 genes to improve abiotic tolerance in crops without negative influence on phenotype.

1.6 Aim of this study

AP2 transcription factors are of great importance in Arabidopsis and other plant species. This is supported through the large number of AP2/ERF transcription factors in the Arabidopsis genome as well as through the dedicated scientific effort to address functions of AP2 genes. To date, various reports described AP2 genes as mainly involved in stress accomplishment. The subgroup Ib within the ERF subfamily consists of the TFs, namely Rap2.4a-h.

The present work aims at investigating and comparing the transcriptional regulation of all 8 TFs. Transcriptional dependencies were analyzed with bioinformatic resources. Further, the transcriptional regulation was examined upon different abiotic stresses as well as the putative potential to improve stress handling. Furthermore, special interest was given to putative compensation or competition between Rap2.4 genes. Transcript abundance modulation was studied in response to transcript deficiency and overexpression to estimate the regulatory dependencies between the TFs and their impact on target genes belonging to the chloroplast antioxidant system. Since ROS is an inherent part upon abiotic stress occurrence, ROS generation in chloroplasts was induced to analyse the impact on TF mRNA levels and to elucidate the possibility of retrograde signaling.

2 Material and methods

2.1 Plant material and growth conditions

2.1.1 Sterile culture of *Arabidopsis thaliana* seedlings

A. thaliana seeds were sterilized for 1 min in 70% (v/v) ethanol and 10 min in 25% (v/v) household bleach (Glorix, Lever Farbergé, the Netherlands). After six-fold washing with sterile water, the seeds were placed on Murashige and Skoog (MS; Duchefa, Haarlam, The Netherlands) medium. Before starting the corresponding experiment, the seeds were stratificated for 24 hours (h) in darkness at 4°C. In general all experiments were performed at 100 µmol photons m^{-2} s^{-1} white light (OsramDulux L 36W / 840 Lumilux Cool White; 4000 K) in a growth chamber under short day conditions (10 h light, 20°C / 14 h dark, 18°C). or in a climate controlled chamber (CU-41L4X; Percival Scientific Inc., Perry, IA, United States) at temperature of 20°C and 100 µmol photons m^{-2} s^{-1} light intensity with 10 h light / 14 h dark photoperiod. Plantlets were grown in Petri dishes up to 15 days (d) depending on the experiment.

MS medium	0.2% (w/v) MS basal salt mixture (Duchefa, Haarlam, The Netherlands) 0.5% (w/v) Phytagel (Sigma, Steinheim, Germany) 1% (w/v) Saccharose pH 5.7, autoclaved

2.1.2 Growth of mature *Arabidopsis thaliana* plants on soil

After stratification at 4°C for 24 h, seeds were germinated in pots containing Arabidopsis substrate composed of 42.4% (v/v) P-soil (Einheitserde, Sinntal-Altengronau, Germany), 42.4% (v/v) T-soil (Einheitserde, Sinntal-Altengronau, Germany) and 15.2% (v/v) perlite (Perligran G; Knauf Perlite, Dortmund, Germany).

Tab. 1: Arabidopsis substrate ingredients and parameters as specified by the manufacturer

	Ingredients	Organic substrate	pH ($CaCl_2$)	Salt KCl [g/l]	N [mg/l] $CaCl_2$	P_2O_5 [mg/l] CAL	K_2O [mg/l] CAL
P-soil	white peat, clay	75%	5.8	1.5	150	150	210
T-soil	White peat, clay	75%	5.8	2.5	310	300	420
Arabidopsis Substrate	**white peat, clay, perligran G**	**50%**	**5.8**	**1.7**	**194.5**	**189**	**267**

2.2 Isolation of DNA from plant material

2.2.1 Isolation of DNA for PCR

Single leaves from 2 week old *A. thaliana* seedling were homogenized in 200 µl Rapid Extraction Buffer (REB). After extraction with 200 µl phenol-chloroform-isoamyl alcohol (25:24:1), samples were centrifuged at 13000 rpm for 20 min at room temperature (RT). The upper phase was transferred into 200 µl isopropanol. After incubation for 24 h at -20°C, DNA was sedimented by centrifugation at 13000 rpm for 20 min. After washing the pellet with 200 µl 70 % (v/v) ethanol and centrifugation at 13000 rpm for 4 min, the DNA was resuspended in 40 µl sterile water and stored at -20°C.

REB buffer
50 mM Tris / HCl pH 8.0
25 mM EDTA
250 mM NaCl
0.5% (w/v) SDS

2.2.2 DNA isolation for Southern blot and copy number determination

Genomic DNA from T-DNA insertion lines and Col-0 wild-type plants was extracted using the DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany) as recommended by the supplier. In general, the plant material was homogenized in liquid nitrogen using mortar and pestle. Cells were lysed by addition of lysis buffer AP1 and incubation at 65°C for 10 min. During this step RNA was cleaved since the lysis buffer contained RNase A. After addition of buffer P3, polysaccharides and proteins were salt-precipitated during incubation on ice for 10 min. After centrifugation at 4000 rpm for 5 min the lysate was transferred to a QIAshredder column. During an additional centrifugation step at 4000 rpm for 5 min cell

debris and precipitates were removed by filtration and homogenization. Binding buffer AW1 was added to the cleared lysate to facilitate DNA-binding. Afterwards the sample was applied to the DNeasy spin column. Upon centrifugation the DNA bound to the silica-based membrane. Proteins and polysaccharides were removed by additional wash step with buffer AW2. The resulting DNA was eluted in buffer AE or sterile water and stored at -20°C.

2.3 Polymerase chain reaction (PCR)

2.3.1 Standard PCR procedure

Optitaq DNA Polymerase (Roboklon, Berlin, Germany) was used for amplification of DNA in standard PCR procedure. Optitaq DNA Polymerase is enzymes blend containing *Thermus aquaticus* DNA polymerase and *Pyrococcus furiosus* DNA polymerase which optimizes accurateness of amplification. DNA polymerase PCR reactions were performed in 20 µl reaction mixture containing 1 µl DNA (2.4.1) sample, 2 µl 10x pol Buffer C (Roboklon, Berlin, Germany) with included loading buffer, 2 mM dNTPs as well as 0.5 mM forward and reverse primer. General PCR reaction protocol is shown in table 2. The annealing temperatures resulted from the melting temperatures (T_m) of the primer pairs used.

Tab. 2: Standard PCR protocol.

Step	Temperature [°C]	Time [min]	Cycles
1. Initial denaturation	94	5:00	1
2. Denaturation	94	1:00	35
3. Annealing	50-60	1:00	
4. Elongation	72	1:00	
5. Final elongation	72	7:00	1
6. Hold	18	∞	-

2.3.2 Identification of homozygous T-DNA insertion lines by PCR

After DNA isolation from two week old single leaves of T-DNA insertion plants (2.2.1.), heterozygosity or homozygosity for the T-DNA insertion in SALK or GABI lines was detected by genotyping in two different PCR reactions with appropriate primers (Tab. 3).

Tab. 3: List of primers and annealing temperatures used for genotyping of ERFIb T-DNA insertion lines.

Gene	Gene code	Primer	Sequence Forward primer / reversre primer (5´ - 3´)	TA °C
		SALK LBb13	ATTTTGCCGATTTCGGAAC	
		GABI LB	CCCATTTGGACGTGAATGTAGACAC	
Rap2.4a	At1g36060	SALK 091212 LP	TAACATCCGAAGTCGGTGAAC	50
		SALK 091212 RP	GAGTTGATCAACTTCATGGGC	
		SALK 066681 LP	CAAGGTGAGGTTGAGAGCATC	50
		SALK 066681 RP	GTTCCTGGGTAAACGGATCTC	
Rap2.4b	At1g78080	SALK 020767 LP	ATACAGAGCAAAACACCGGTG	50
		SALK 020767 RP	TTCTGTCGTAACCAAACCGAC	
Rap2.4c	At2g22200	SALK 108879 LP	TCCATATCTTTTGGGCTTCG	52
		SALK 108879 RP	CGAAGCTTAACTTCCCAAACC	
Rap2.4d	At1g22190	SALK 139727 LP	GTGTATCGGTGAGGCTGAGAG	52
		SALK 139727 RP	GTCCTCCTCCGGTAGTTTCAC	
		SALK 091654 LP	TTATCACCGATTGAAACGAATG	52
		SALK 091654 RP	ATTTCATACGGGTCGGATCTC	
Rap2.4f	At4g39780	SALK 100678 LP	GCTGACGAGAAACAAATCGTC	50
		SALK 100678 RP	TCTCATCAAATCCAGAATCCG	
		SALK 138494 LP	GCTGACGAGAAACAAATCGTC	50
		SALK 138494 RP	CATCCCAATCAATCTCCACAG	
Rap2.4g	At1g64380	GABI 819C03 LP	TGAATAAATTGAAGGGTATGCAAG	55
		GABI 819C03 RP	CTGTTTTGCGGCAATCTTATC	
Rap2.4h	At4g13620	GABI 469C03 LP	TGAGAGAGCATCCCAAATGAG	50
		GABI 469C03 RP	TTGGCAGAGCGTAGATCTGAG	

2.3.2.1 Provided *Arabidopsis thaliana* mutant plants

Mutant plants deficient in 2CPA, 2PB or both 2CP genes were kindly provided by Prof. Dr. Francisco Javier Cejudo (Instituto de Bioquımica Vegetal y Fotosıntesis, Universidad de Sevilla and CSIC, Avda Americo Vespucio 49, 41092-Sevilla, Spain). Conditional fluorescent in blue light (*flu)*-mutant plants were kindly provided by Prof. Dr. K. Apel (Boyce Thompson Institute for Plant Research, 533 Tower Road, Ithaca, New York 14853-1801, USA).

2.4 Separation of DNA by agarose gel electrophoresis

Amplified PCR products or fragments from enzymatic restriction reactions were separated electrophoretically on 1.2% (w/v) TAE agarose gels. Agarose was melted in 1 x TAE buffer and supplemented with 0.5 µg ml^{-1} ethidium bromide, which intercalates in DNA. The EtBr-DNA complexes show strong fluorescence if exposed to UV light at 312 nm. The results were documented with the INTAS Gel iX-Imager (INTAS, Göttingen, Germany).

50X TAE buffer	40 mM Tris-acetate pH 7.5
	1 mM EDTA

2.5 Purification of PCR products by gel extraction

Agarose gel pieces containing DNA fragments of interest were excised under UV light with a clean, sharp scalpel. The DNA was extracted using the Roboklon PCR Clean-up Kit (Roboklon, Berlin, Germany) or the Invisorb Fragment CleanUp Kit (Stratec, Birkenfeld, Germany) according to manual. Basically the DNA was solubilized by melting the agarose gel in a buffer with high salt concentration and bound to a silica membrane. Subsequently, the DNA was washed and eluted by low salt buffer or sterile water.

2.6 Bacterial growth conditions

The used bacterial strain *E. coli* DH5α was grown at 37°C in liquid Laura Bertani (LB) medium or on solid LB agar plates supplemented with appropriate antibiotics.

LB medium	1% (w/v) tryptone
	1% (w/v) NaCl
	0.5% (w/v) yeast extract (Carl Roth, Karlsruhe, Germany)
	for solid medium additionally 1.5% (w/v) Agar
	adjusted to pH 7.0, autoclaved

The *Agrobacterium tumefaciens* strain GV3101 (pMP90) was grown at 28°C in liquid Yeast Extract Broth (YEB) medium or on solid YEB agar plates containing rifampicin (150

µg ml^{-1}), gentamycin (25 µg ml^{-1}) and appropriate antibiotics according to the used plasmid, such as spectinomycin for pCR8/GW and pMDC7 (100 µg ml^{-1}).

YEB medium	0.5% (w/v) Peptone (Carl Roth, Karlsruhe, Germany) 0.1% (w/v) yeast extract (Carl Roth, Karlsruhe, Germany) 0.5% (w/v) beef extract (Carl Roth, Karlsruhe, Germany) 0.5% (w/v) sucrose 0.5% (w/v) $MgCl_2$ for solid medium additionally 1.5% (w/v) Agar autoclaved

2.6.1 *Escherichia coli* manipulations

2.6.1.1 Transformation of *E. coli*

Chemically competent E. coli DH5α cells were transformed with appropriate vectors by the heat shock method. The plasmid was added after defreezing the cells on ice for 10 min. Following a second incubation on ice for 30 min, the cells were heated to 42°C for 30 s and transferred back on ice for 2 min. 500 µl preheated liquid LB medium (2.6) was added and the cells were incubated at 37°C for 2 h. Selection of positive transformants containing the recombinant plasmid happened on selective LB agar plates.

2.6.1.2 *E. coli* colony-PCR

A single colony of transformed *E. coli* cells was transferred into 20 µl of PCR mix (2.3.1). The general PCR protocol described in tab. 2 2 was used; for cell lysis the duration of initial incubation was changed to 10 min.

2.6.1.3 Plasmid isolation from *E. coli*

Plasmid DNA isolation from *E. coli* was performed using the Roboklon Plasmid Miniprep DNA Purification Kit (Roboklon, Berlin, Germany) or the Invisorb Spin Plasmid Mini Two Kit (Stratec, Birkenfeld, Germany) according to manual. Plasmid DNA was eluted in elution buffer and stored at -20°C.

2.7 Plasmid manipulations

2.7.1 Ligation of PCR-products into pCR8/GW/TOPO entry plasmid

For cloning purposes the Gateway system compatible pCR8/GW/TOPO plasmid (see Appendix; figure 64) (Invitrogen, Carlsbad, United States) was used to generate entry plasmids. After PCR amplification, DNA fragments were cloned following the manual. 1µl salt solution was added to 1 µl fresh PCR product. Water was added to a final volume of 5 µl. To initiate the TOPO reaction 1 µl pCR8/GW/TOPO vector was added, the solution was mixed gently and incubated over night at RT. Primers used for amplification of the PCR products are listed in table 4. The resulting constructs were introduced into chemically competent DH5α *E. coli* cells by heat shock transformation (2.6.1.1). The correct insert orientation in the entry plasmid was determined by colony PCR (2.6.1.2), with the left primer specific for the plasmid and the right primer for the PCR product, respectively. After plasmid isolation (2.6.1.3), additional restriction analysis with appropriate restriction enzymes was performed to verify the successful insert.

Tab. 4: List of primers and annealing temperatures used for amplification of Rap2.4 sequences for TOPO cloning procedures.

Gene	gene code	Sequence Forward primer / reverse primer (5´ - 3´)	TA °C
Rap2.4a	At1g36060	ATGGCGGATCTCTTCGGTG GATTGGGCTTCAATTTTCTCG	50
Rap2.4b	At1g78080	ATGGCAGCTGCTATGAATTTG TCGATTGGGATTCGATTCTAGCT	50
Rap2.4c	At2g22200	ATGGAAACTGCTTCTCTTTCTTTC AGAATTGGCCAGTTTACTAATTG	50
Rap2.4d	At1g22190	ATGACAACTTCTATGGATTTT ATTTACAAGACTCGAACACT	55
Rap2.4e	At5g65130	ATGGCTTTAAACATGAATGCT GAAGAGTTTCTCTATAGCGTC	55
Rap2.4f	At4g39780	ATGGCAGCCATAGATATGTTC AGATTCGGACAATTTGCTAATC	55
Rap2.4g	At1g64380	ATGGAAGAAAGCAATGATAT ATTGGCAAGAACTTCCCAA	55
Rap2.4h	At4g13620	ATGATCACACCAATACACAC AGAAGAATGAGGAAATGAGAGA	50

2.7.2 LR reaction

The Gateway compatible pMDC7 plasmid (Curtis et al. 2003; Appendix figure 65) served as destination vector. It includes the chimeric factor VXE allowing an estradiol induced gene overexpression. To perform the recombination reaction between the entry plasmid and the destination vector, 50-100 ng entry plasmid was added to 150 ng pMDC7 vector. The volume was supplemented with TE-buffer to 8 µl. After short vortexing 2 µl LR Clonase II enzyme mix (Invitrogen, Carlsbad, United States) was added. After second vortexing the reaction mixture was incubated for 24 h at 25°C. The reaction was stopped by adding 1 µl of Proteinase K solution (Invitrogen, Carlsbad, United States) and incubation at 37°C for 10 min.

TE-buffer	10 mM Tris / HCl pH 8.0
	1 mM EDTA

2.7.3 Cleaving double-stranded DNA with restriction enzymes

Restriction of 1 µg isolated plasmid DNA was performed in total volume of 20 µl at 37°C for 30 min using FastDigest restriction enzymes (Thermo Fisher Scientific, Massachusetts, USA) according to manual. The fragment pattern was documented after agarose gel electrophoresis (2.4).

2.8 Sequencing of DNA

To ensure error free DNA sequences in cloning procedures, 1 µg of purified plasmid DNA was diluted in total volume of 30 µl dH_2O. The samples were sequenced at GATC (GATC Biotech AG, Konstanz, Germany) using the chain-termination method on an 11x ABI 3730xl system. The obtained results were analyzed by comparison with genomic sequences from the Arabidopsis Information Resource database (TAIR; http://arabidopsis.org/) using the align DNA sequences function in SerialCloner 2.6 software (http://serialbasics.free.fr/).

2.9 Southern blot analysis

2.9.1 Enzymatic DNA restriction for Southern blot analysis

10 µg of genomic DNA was cleaved with 10 µl HindIII FastDigest enzymes (Thermo Fisher Scientific, Massachusetts, United States) in a total volume of 300 µl with 1x FastDigest buffer (Thermo Fisher Scientific, Massachusetts, USA) at 37°C for 2 h. The restricted DNA was precipitated by adding 1/10 volume 3 M sodium acetate pH 5.2 and 3 volumes of 96% (v/v) ethanol. The sample was incubated at -20°C overnight. After centrifugation at 13000 rpm for 15 min, the pellet was washed with 500 µl 70% (v/v) ethanol. For sample resuspension, 20 µl TE-buffer (2.7.2.) were added following incubation for 10 min at 55°C and 100 rpm in a mixing block (MB-102, Bioer, Hangzhou, P.R. China). DNA was stored at -20°C.

2.9.2 DIG-labeling of hybridization probe

The probe was synthesized in a PCR reaction using the Digoxigenin (DIG) Probe Synthesis Kit (Roche, Penzberg, Germany) and the 35S probe primer pair (35S-F (GGTCTTGCGAAGGATAGTGG), 35S-R (GGTGGAGCACGACACACTT)) following manufacturers' instruction and the standard PCR protocol (2.3.1.). 1 µg genomic DNA from homozygous T-DNA insertion plants served as template, DIG-dUTPs present in the PCR DIG Probe Synthesis Mix were incorporated by the polymerase chain reaction and enabled the probe labeling. An unlabeled PCR product served as control. Since no DIG-dUTPs were present, the electrophoretic migration on an agarose gel was faster compared with the labeled probe, emerging a smaller product size.

10x PCR DIG	2 mM dATP, dCTP and dGTP
Probe Synthesis Mix	1.3 mM dTTP
	0.7 mM DIG-11-dUTP

2.9.3 Southern transfer

Enzymatically fragmented DNA was separated on a 1.2% (w/v) TAE agarose gel (2.4.) after 5 h electrophoresis at 80 V. Prior to blotting procedure the double-stranded DNA was denaturated by incubating the gel twice for 15 min in denaturing solution. After washing

with distilled water the gel was incubated twice in neutralization buffer for 15 min each. Nylon Amersham Hybond-N membrane (GE Healthcare, Buckinghamshire, UK) served for the nucleic acid transfer and was prepared by incubation in distilled water for 10 min as well as in Nucleic acid transfer buffer for 20 min. To setup the capillary DNA transfer, all 3MM blotting papers (GE Healthcare, Buckinghamshire, UK) were soaked in Nucleic acid transfer buffer. The pretreated agarose gel was placed top down on two layers of Whatman 3MM blotting paper. To avoid a drying-out, the ends of the Whatman 3MM blotting paper were placed in a reservoir filled with Nucleic acid transfer buffer and served as wick. The transfer membrane was placed on the gel and covered with three layers Whatman 3MM blotting paper. The blot installation was completed by a paper towel stack of 5 cm height, covered with a plate and a 500 g weight plate. The DNA was transferred to the membrane by capillary action overnight at RT. The nucleic acid was fixed to the membrane by UV crosslinking at 254 nm and a dosage of 0.120 Joules cm^{-2}.

Nucleic acid transfer buffer (20x SSC)	3 M NaCl 0.3 M Trisodium citrate Adjusted to pH 7.0
1x Denaturing Solution	0.5 M NaOH 1.5 M NaCl
1x Neutralizing Solution	0.5 M Tris 3 M NaCl Adjusted to pH 7.5 with concentrated hydrochloric acid

2.9.4 Southern blot hybridization

The hybridization solution was gained by dissolving one portion of DIG Easy Hyb granules (Roche, Penzberg, Germany) in 64 ml double distilled water for 5 min at 37°C in a water bath. The DIG labeled *35S* probe was denatured by incubation for 5 min at 95°C and instantly cooled on ice. 40 µl of denatured probe were added to 20 ml of hybridization solution, adjusted to hybridization temperature. The hybridization temperature was determined according to GC level and probe / target homology calculated by the equation: T_m = 49.82 + 0.41 (% G+C) – (600 / l) – 20°C, where l is displayed through the probe length in bp. Hybridization temperature T_{opt} is 20 - 25°C below the T_m value. The membrane was pre-hybridized in 25 ml DIG Easy Hyb solution for 30 min at 50°C. The

pre-hybridization solution was exchanged with the hybridization solution containing the DIG labeled probe. Hybridization was performed overnight at T_{opt}. To remove inadvertent hybrids the membrane was washed two times in preheated High-Stringency Wash I solution at 68°C for 15 min and twice in preheated High-Stringency Wash II solution at 68°C for 15 min.

High-Stringency Wash I	0.5x SSC 0.1% SDS
High-Stringency Wash II	0.1x SSC 0.1% SDS

2.9.5 Probe detection

Southern blot detection was done using the CPD-Star, ready-to-use solution (Roche, Penzberg, Germany), which includes the chemiluminescent alkaline phosphatase substrate disodium 2-chloro-5-(4-methoxyspiro {1,2-dioxetane-3,2´-(5-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate. After hybridization and stringency washes the membrane was rinsed with DIG Wash buffer at RT for 5 min. Blocking solution was added to the membrane followed by 2 h incubation at RT with gentle agitation. To prepare the blocking solution Blocking reagent (Roche, Penzberg, Germany) was diluting 1% (w/v) in Maleic acid buffer in a microwave. The membrane was then incubated in the antibody solution for 30 min at RT, followed by washing for 15 min with DIG wash-buffer and equilibration for 5 min in Detection buffer. For signal detection, the membrane was put with the DNA side upside on a hybridization bag. After applying 1 ml of CDP-*Star,* ready-to-use solution, the membrane was incubated for 5 min at RT. During the incubation the alkaline phosphatase dephosphorylated dioxetane, resulting in light emission at 466 nm. The chemiluminescence signal was documented with the ImageQuant LAS 4000 mini system (GE Healthcare, München, Germany) after 5 min detection time.

Antibody solution	add 37.5 mU ml^{-1} Anti-digoxigenin-AP, Fab fragments (Roche, Penzberg, Germany) in 1x blocking solution
Detection buffer	1 M Tris-HCl, pH 9.5 1 M NaCl
DIG wash-buffer	maleic acid buffer 0.3% Tween 20 (v/v)

Maleic acid buffer	100 mM maleic acid 150 mM NaCl

2.10 Gene expression analyses

2.10.1 RNA isolation

Prior to RNA isolation, 10-15 seedlings were pooled and immediately harvested in liquid nitrogen. Plant material was homogenized using a Retsch shaking-mill (Retsch, Haan, Germany) and 2 mm glass beads for 30 sec and a frequency of 30 Hz. RNA was isolated using the GeneMATRIX Universal RNA Purification KIT (EURx, Gdansk, Poland) according to the manufacturer's instructions. During the isolation procedure the samples were homogenized by usage of the homogenization spin-columns. Additionally, DNA was removed by binding to homogenization resin. The RNA was selectively bound to the silica-based membrane in the RNA binding column. In the final step the RNA was eluted and stored at -80 °C. RNA purity was checked spectrophotometrically by determining the A_{260} / A_{280} ratio (NanoPhotometer P-300, Implen, München, Germany).

2.10.2 First strand cDNA synthesis

The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, United States) was used for cDNA synthesis. A total reaction volume of 25 µl total was used with 1 µg RNA, 1 x RT Buffer, 8 mM dNTP mix, 1 x RT Random Primers and 1 µl (50 U) MultiScribe reverse transcriptase. The PCR program listed in table 5 was set up to perform the reaction. For quantitative real-time PCR measurements, cDNA was diluted in RNase-free sterile water to a concentration of 30 ng / µl.

Tab. 5: PCR protocol for cDNA synthesis.

Step	Temperature [°C]	Time [min]	Cycles
1. Primer extension	25	10	1
2. Reverse transcription	37	120	1
3. Inactivation	85	5	1
4. Hold	18	∞	-

2.10.3 Quantitative real-time PCR

2.10.3.1 Primer design

Primer pairs for Real-time PCR were designed using QuantPrime software (Arvidsson et al. 2008; http://www.quantprime.mpimp-golm.mpg.de). The standard settings were used for primer design. If the gene was not intron free, at least one primer spanned the exon border to prevent amplification of genomic DNA. Primer pairs with the highest rank score and best test results were chosen. Real-time PCR primers are listed in appendix, table 16. After performed real-time PCR reaction, specificity of primer pairs was checked by melting curve analysis, showing a single peak for each. Additional analysis by gel electrophoresis showed with a single PCR product with appropriate size.

2.10.3.2 Signal detection

Quantitative real-time PCR was performed on a CFX96 thermo cycler (Bio-Rad Laboratories, München, Germany) Usage of the fluorescent dye SYBR Green allowed quantifying the transcript amount. Sybr Green fluoresces when bound to double-stranded DNA, where the DNA-dye complex absorbs blue light at 497 nm and emits green light at 520 nm. The fluorescent signal was detected at the end of the amplification step and is expressed as the threshold value (Ct). This value described the cycle number, where the measured fluorescence signal was higher than the background fluorescence. To set-up the reaction, blank qPCR Master Mix (2x) (Roboklon, Berlin, Germany) was used according to manual. The reaction volume of 10 µl included 1 x qPCR Master Mix, 3 µl cDNA sample and 600 nM of forward and reverse primer. PCR was performed according to the cycling protocol in table 6. All reactions were performed in three technical replicates. All reactions were performed in two biological replicates, each representing an individual RNA isolation.

Tab. 6: Quantitative real-time PCR protocol.

Step	Temperature [°C]	Time [min]	Cycles
1. Initial denaturation	94	10:00	1
2. Denaturation	94	0:30	39
3. Annealing	60	0:30	
4. Elongation	72	0:30	
5. Melting curve analysis	65-95 / 0.5 increase	0:05	1

2.10.3.3 Standardization

Gene expression data has to be normalized against one or more housekeeping genes; their expression should be constitutive under normal and stressful conditions in all analyzed tissues. Here Actin 7 (At5g09810) and F-box (Czechowski et al. 2005), served as housekeeping genes, relative quantification of gene expression ratio was calculated according to the $R = 2^{-\Delta\Delta Ct}$ method (Pfaffl 2004). The value 2 describes the PCR efficiency for the used primer pair and the formula $^{-\Delta\Delta Ct}$ depicts the equation ΔCt value sample –ΔCt value housekeeping gene. The calculations were performed with the Bio-Rad CFX Manager software (Bio-Rad Laboratories GmbH, München, Germany).

2.10.3.4 Copy number determination

Copy number of T-DNA insertions was performed in a qPCR-based approach (Yang et al. 2012). Usage of modified $2^{-\Delta\Delta Ct}$ method allowed faster and easier results than Southern-blot analysis. The copy number was calculated from the ratio of $E_{\text{transgene control}}^{\Delta Ct \text{ transgene control (control – sample)}}$ / $E_{\text{endogenous control}}^{\Delta Ct \text{ (control-sample)}}$ multiplied by 2. Peroxiredoxin Q served as endogenous control since it was reported as single one copy in the *A. thaliana* genome (Horling et al. 2003). The homozygous T-DNA insertion line GABI-KAT 469C03 with single T-DNA insertion (www.gabi-kat.de) served as transgene control. Primer pairs were manually designed, whereas amplified products had the same bp length and did not bias the resulting Ct values. Genomic DNA served as template and was isolated from appropriate T-DNA insertion lines, accurate quality was controlled by spectrophotometer by spectrophotometric determination of A_{230} / A_{260} ratio as well as by electrophoresis. For copy number determination the DNA quantity was adjusted to the same amount of ng per µl. Real-time PCR was performed according to the protocol listed in table 6.

2.11 *Agrobacterium tumefaciens* manipulations

2.11.1 Transformation of *A. tumefaciens*

A. tumefaciens strain GV3101 (pMP90) (Koncz and Schell 1986) was transformed using the freeze-thaw method (Weigel and Glazebrook 2002). 0.2 ml competent cells were mixed with 1 µg plasmid DNA and incubated on ice for 15 min. The cells were frozen in liquid N_2 for 5 min and further transferred into a 37°C water bath for additional 5 min. 1 ml YEB was added and the cells were incubated for 2-4 h at 28°C. The cells were plated on

YEB plates containing 1.5% (w/v) agar, rifampicin (150 µg ml^{-1}), gentamycin (25 µg ml^{-1}) and spectinomycin (100 µg ml^{-1}), and incubated at 28°C for 2 d. Colonies were plated on fresh YEB plates and incubated at 28°C for 24 h before transformed Agrobacteria were used for plant transformation.

2.11.2 Plasmid isolation from *A. tumefaciens*

Plasmids from *A. tumefaciens* were isolated with the Invisorb Spin Plasmid Mini Two Kit (Stratec, Birkenfeld, Germany). 10 ml of appropriate liquid over-night culture were used as well as additional lysozyme (10 mg/ml) treatment for 10 min at 37°C after pellet resuspension.

2.12 Transient transformation of *Arabidopsis thaliana* seedlings

2.12.1 Preparation of suspension for infiltration

A. thaliana seedlings were grown sterile on MS plates for 12 d and transiently transformed based on the p19 co-expression system (Voinnet et al. 2003). 5 ml YEB medium containing the appropriate antibiotics were inoculated with *A. tumefaciens* strain GV3101 (pMP90), harboring the appropriate pMDC7 constructs and strain GV3101 (pMP90) carrying the 35S CaMV driven p19 protein of tomato bushy stunt virus respectively. The cultures were grown at 28°C and 180 rpm overnight. The culture was transferred into 100 ml of YEB medium with the same antibiotics and further incubated until OD_{600} reached minimum 0.5. Before co-infiltration, both Agrobacteria cultures were merged with a ratio of 60% pMDC7 and 40% p19 respectively. Cells were pelleted by centrifugation for 15 min at 3000 rpm at room temperature and resuspended in 40 ml Activation buffer. After 1 h incubation at RT, *A. thaliana* seedlings were vacuum infiltrated.

Activation buffer	10 mM MES / KOH pH 5.6
	10 mM $CaCl_2$
	150 µM Acetosyringon

2.12.2 Transient transformation procedure

MS plates containing 12 d old Arabidopsis seedlings were filled with 40 ml transformation suspension and vacuum infiltrated 6 times for 1.5 min respectively. After the procedure, the seedlings were transferred on fresh MS plates containing cefotaxime (100 µg ml^{-1}) and appropriate estradiol concentration under sterile conditions. The plantlets were harvested after 1-2 d of incubation in Percival under standard conditions.

Transformation solution	5% sucrose (w/v) 0.02% Silwet L-77 (LEHLE SEEDS, Texas, United States), added directly before transformation

2.12.3 Transformation by floral dip

Transformation of *A. thaliana* was performed by floral dip method (Bechtold et al. 1993; Clough et al. 1998). Two pots per construct, each with 5 *A. thaliana* plants, were grown in the greenhouse under long day conditions until flowering stage. For dipping the inflorescences should exhibit a length of 10 cm. To prepare the transformation suspension, 5 ml YEB medium containing the appropriate antibiotics was inoculated with transformed Agrobacteria and grown overnight at 28°C and 180 rpm. Next day the suspension was transferred into 400 ml YEB medium supplemented with the appropriate antibiotics and incubated at 28°C and 180 rpm until OD_{600} reached minimum 0.5. The cells were harvested by centrifugation at 3000 rpm for 30 min at RT and resuspended in 400 ml freshly made transformation solution (2.12.2). The transformation suspension was transferred into a 500 ml beaker glass positioned in a desiccator. The aerial parts of the plants were dipped into the suspension with gentle agitation for 10 sec followed by vacuum infiltration for 2 min. The transformed plants were horizontally positioned in a tray and covered with plastic foil to ensure high humidity conditions. After overnight incubation at RT in darkness they were transferred to the green house.

2.12.4 Selection of transformants

Positively transformed Arabidopsis seedlings were obtained by hygromycin B selection according to the description from Harrison et al. (2006). Surface sterilized seeds were resolved in warm MS medium and spreaded on MS plates containing hygromycin B (25 µg ml^{-1}). After 2 d of stratification at 4°C, the plates were transferred for 8 h into Percival to induce germination under standard conditions. All plates were wrapped in aluminum foil and further kept in percival for 5 d. Positive transformants showed elongated hypocotyls and were transferred on soil for further growing in growth chamber under short day conditions. After 2 weeks, the plants were transferred into the greenhouse.

2.13 Crossing Arabidopsis thaliana plants

2.13.1 Crossing procedure

Siliques and open buds or flowers were removed from the inflorescence of the mother plant. Buds on the verge of opening were prepared for further procedure by removing septals, petals and anthers carefully without damaging the stigma. An anther from the father plant was dabbed on the stigma of the emasculated mother plant. If possible several anthers were used. All steps were performed with a pair of fine tweezers Successful crosses generated elongated siliques after two days. Plants from F_1 generation were checked for heterozygosity by genotyping (2.3.2) and further self-crossed. Homozygous F_2 plants were verified by genotyping and used for further analysis.

2.14 Quantification of total protein

The Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, München, Germany) was used to determine total protein level according to manual. It relays on the Bradford protein assay (Bradford, 1976), providing estimation of soluble protein concentration. Protein was added to an acidic solution containing the dye Coomassie Brilliant Blue G-250, which changes the absorption from 465 nm to 595 nm after protein binding. The absorbance was documented with a microplate reader in a total volume of 400 µl after 15 min of incubation at RT. Utilization of a standard curve performed with known concentrations of bovine serum albumin (BSA) allowed estimation of relative protein concentration.

2.15 GUS staining

Plant material was harvest in ice-cold 90% (v/v) acetone on ice. After vacuum infiltration for 10 minutes at RT, the material was fixed at room temperature for 30 min. Acetone was exchanged with Wash buffer and vacuum infiltrated for 10 min. Upon removal of wash buffer, the plant tissue was vacuum infiltrated in staining buffer on ice for 20 minutes. After verifying that all plant tissue sank, the vacuum was released; this step was repeated 3 times. The samples were incubated at 37°C overnight in darkness. To remove chlorophyll, plant tissue was incubated in a mixture of acetic acid, glycerol and 96% (v/v) ethanol (1:1:3) at 80°C and stored at 4°C until analysis.

Staining buffer	100 mM Potassium-phosphate-buffer; pH 7.0 0.5 M EDTA, pH 8 0.1% Triton X-100 500 mM Ferrocyanid III 500 mM Ferrocyanid IV 100 mM X-Gluc dissolved in 2 ml DMSO
Wash buffer	staining buffer without 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc)

2.15.1 GUS activity quantification

GUS activity was estimated by measuring the absorbance of para-nitrophenol (PNP) at 405 nm. PNP is a chromophoric product resulting from enzymatic cleavage of para-nitrophenyl β-D-glucuronide (PNPG) by β-glucuronidase (GUS) (Aich et al. 2001). For quantification 200 µl extraction buffer was added to 100 mg homogenized plant material. After centrifugation at 13000 rpm for 15 min, the supernatant was transferred to a new 1.5 ml Eppendorf tube and centrifuged at 13000 rpm for 5 min. The supernatant was used for quantification of total protein content (2.14) and GUS activity. GUS quantification was performed in 96 well microtiter plates, 10 µl supernatant was added to 90 µl reaction buffer already provided to appropriate amount of wells. The same amount of wells with reaction buffer only served as control for background absorbance. After centrifugation for 3 min at 3000 rpm, the 96 well microtiter plate was incubated for 2 h at 37°C in darkness. To stop the reaction, 100 µl 400 mM Na_2CO_3 was added to the appropriate wells.

Absorbance was measured with a microplate reader at 405 nm (ε= 18300 Δ Abs L $mol^{-1}cm^{-1}$).

Extraction buffer	10 mM EDTA 0.1% Triton X-100 0.1 M KH_2PO_4 0.1 M K_2HPO_4
Reaction buffer	2 mM EDTA 5% Glycerol 0.1 M KH_2PO_4 0.1 M K_2HPO_4 Adjusted to pH 7.0 2 mM DTT, added immediately before using 10mM β-mercaptoethanol, added immediately before using

2.16 Reactive oxygen species staining

Reactive oxygen species like singlet oxygen ($^1O_2$) or hydrogen peroxide (H_2O_2) originate from reactions in the photosynthetic electron transport chain present in chloroplasts or in the respiratory electron transport chain in mitochondria but are also produced in peroxisomes. Their role as stress parameter, as well as signal molecule, is an important parameter.

2.16.1 NBT staining

Since superoxide anions (O_2^-) have a very short half-life, a treatment with sodium azide (NaN_3) was performed to stop enzymatic reactions abolishing the molecule. Leaf tissue was vacuum infiltrated in 1x PBS buffer with 10 mM NaN_3 for 10 min. To visualize spots of superoxide anions (O_2^-), the leaf tissue was transferred in 1x PBS buffer with 1 mg ml^{-1} Nitro blue tetrazolium Chloride (NBT) and vacuum infiltrated in darkness until a blue coloration was visible (Kawai-Yamada et al. 2004). The reduction of NBT by superoxide results in the artificial dye formazan, responsible for the blue precipitate. Before documentation, the chlorophyll was removed by incubating the plant material at 80°C in a mixture of acetic acid, glycerol and 96% (v/v) ethanol (1:1:3).

10x PBS buffer	0.73 M NaCl
	0.03 M KCl
	0.1 M Na_2HPO_4
	0.02 M KH_2PO_4

2.16.1.1 ImageJ evaluation of NBT staining

To quantify the NBT staining area, ImageJ (magej.nih.gov/ij/download/) software was used. In general, the manual for Area Measurements of a Complex Object was used. Here, the appropriate pictures of stained leaves were converted to grayscale. The threshold was set manually to include the stained area within the leaves. The size of stained area was calculated in relation to whole leaf size.

2.17 Gene expression analysis

Several different online databases were used to elucidate expressional changes and co-expression from the ERFIb transcription factors.

2.17.1 Genevestigator

The web-based Genevestigator (https://www.genevestigator.com/gv/plant.jsp) software suite provides categorized quantitative information about genes contained in large high-quality microarray databases (Zimmermann et al. 2004). It enables exploration of gene expression across a wide range of biological contexts. The first version was established in 2004 and has now been updated to enable faster, more powerful and more diverse types of queries for gene-gene function analysis and network discovery (Zimmermann et al. 2008). The newest version 4.0 was used for analysis.

2.17.2 The A. thaliana Co-Response Database (CSB.DB)

CSB.DB (Steinhauser et al. 2004; http://csbdb.mpimp-golm.mpg.de/csbdb/dbcor/cor.html) gives easy access to the results of large-scale co-response analyses, which are currently based exclusively on the publicly available compendia of transcript profiles. The database was used for co-expression analysis between gene samples. The latest update was performed 2005.

3 Results and discussion

3.1 In *silico* analysis of Rap2.4 gene expression in developmental conditions and abiotic stress in roots

Transcription factors from the same family often compete among each other for binding sites (Gitter et al. 2009; De Vos et al. 2011), but can also compensate the loss of function from another related transcription factor, especially if the sequence similarity of the target is high (Hollenhorst et al. 2001; Gitter et al. 2009). ERFIb transcription factors share an almost identical AP2 domain which mediates DNA interaction (Fig. 8).

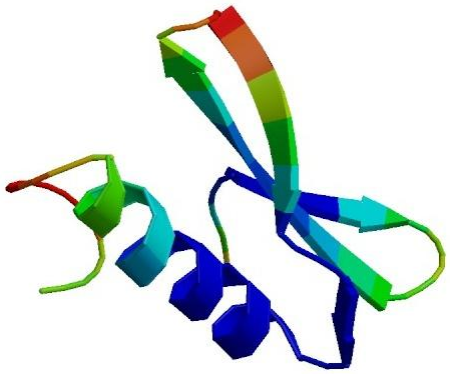

Fig. 8: Example for structure of the AP2 domain from Rap2.4b transcription factor. AP2 domain mediates interaction with the DNA major groove. Exposure according to SWISS-MODEL Workspace (Geux and Peitsch 1997; Schwede et al. 2003; Arnold et al. 2006).

Shaikhali and colleagues (2008) reported that the Rap2.4a knockdown affects the expression of other ERFIb family members. For example, in RT-PCR analysis the Rap2.4b transcript level was elevated in Rap2.4a-KO plants; in contrast the transcript level of Rap2.4c was not affected. Rap2.4d and Rap2.4e mRNA levels were negatively impaired. These different transcriptional changes upon loss of Rap2.4a function displayed a possible dependency between the regulations of the factors.

Here, the question has been addressed whether several ERFIb transcription factors are involved in similar regulatory mechanisms, but with differences in their individual regulating potential. In the first instance, bioinformatic tools for *in silico* analysis were used for differential experimental setups. The Genevestigator database (Hruz et al. 2008)

enables comparison of ERFIb expression in different tissue as well as during the plants life cycle. The comprehensive systems-biology database (Steinhauser et al. 2004) delivers information on the transcript level regulation. Here, especially the possibility of functional correlation between the factors was analyzed in co-expression studies, which are based on calculations of Spearman´s rank correlation coefficient (Steinhauser et al. 2004).

The data sets joined in the databases originate from more than 12000 arrays as well as 780 experiments. More than 8000 arrays (>500 experiments) were performed with ATH1 22K Affymetrix chips (Baginsky et al. 2009) and depict the main informational source derived from microarray experiments. For Rap2.4h no expressional data is available in any microarray database due to the fact that the gene is not available on the ATH1 22K Affymetrix chips.

Czechowski and co-workers (2004) performed a qPCR transcript profiling for over 1400 transcription factors to check for expressional specificity in shoots and roots. Their data sets included Rap2.4h and showed that the transcription is expressed mainly in roots (Czechowski et al. 2004).

3.1.1 Genevestigator based expressional analysis

3.1.1.1 ERFIb expression in different tissue

Tissue-specific ERFIb expression in *A. thaliana* was analyzed *in silico* using the Genevestigator database (Hruz et al. 2008). The Genevestigator database collects data from microarray experiments generated though the scientific society. After verifying a high quality standard, the data is conducted according to annotations (i.e. tissue or stress type). Such proceeded data can then be analyzed via different computational calculations according to user requirements (Hruz et al. 2008).

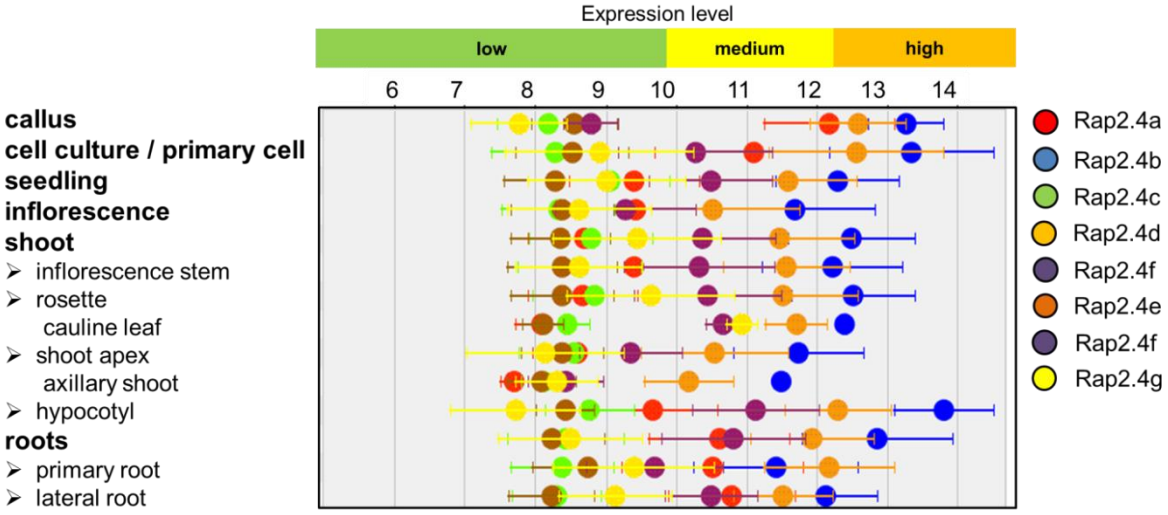

Fig. 9: log2 expression of ERFIb genes in different tissue, signal intensity on a 22k array; modified according to Genevestigator Version 4; Anatomy analysis dataset.

The expression of individual ERFIb differs depending on the tissue and the transcript level. Notably, Rap2.4b displayed a very high expression level in callus formation and primary cell, here in consistence with results shown by Iwase et al. (2011a and b), indicating that Rap2.4b promotes cell dedifferentiation and cell proliferation (Iwase et al. 2011a). Furthermore Rap2.4a and Rap2.4d were supposed to promote callus formation (Iwase et al. 2011b). This was demonstrated through very high Rap2.4a and Rap2.4d expression levels in callus tissue. Rap2.4b showed in the array series (Fig. 9) also high expression level in roots, consistent with reports from Lin et al. (2008). There, Rap2.4b was described as a salt and drought stress inducible gene (Lin et al. 2008). Since in general only roots are in direct contact upon salt treatments (Kreps et al. 2002), a high basal Rap2.4b expression in roots is feasible to mediate a fast response to salinity stress.

In addition to Rap2.4b, Rap2.4d is also expressed at high level in roots. Moreover, the additional differentiation in primary and lateral roots further demonstrated that Rap2.4b and Rap2.4d are also co-expressed is these root tissues at high levels. In shoots, Rap2.4b and Rap2.4d demonstrated the highest expression levels. A more detailed division of shoot tissue showed a very high Rap2.4b expression level in hypocotyls. There, the transcription factor is supposed to regulate hypocotyl elongation in a light dependent manner (Lin et al. 2008).

The predominant high expression level for Rap2.4b and Rap2.4d demonstrated a particular importance and / or co-regulation especially in callus formation, primary cells and root tissue. In contrast, the majority of ERFIb genes, namely Rap2.4c, Rap2.4e, Rap2.4f and Rap2.4g, demonstrated low expression levels in all tissues.

3.1.1.2 ERFIb expression at different developmental stages

The Genevestigator database (Hruz et al. 2008) was also used for a first overview over expression of ERFIb genes during different developmental stages of *A. thaliana.*

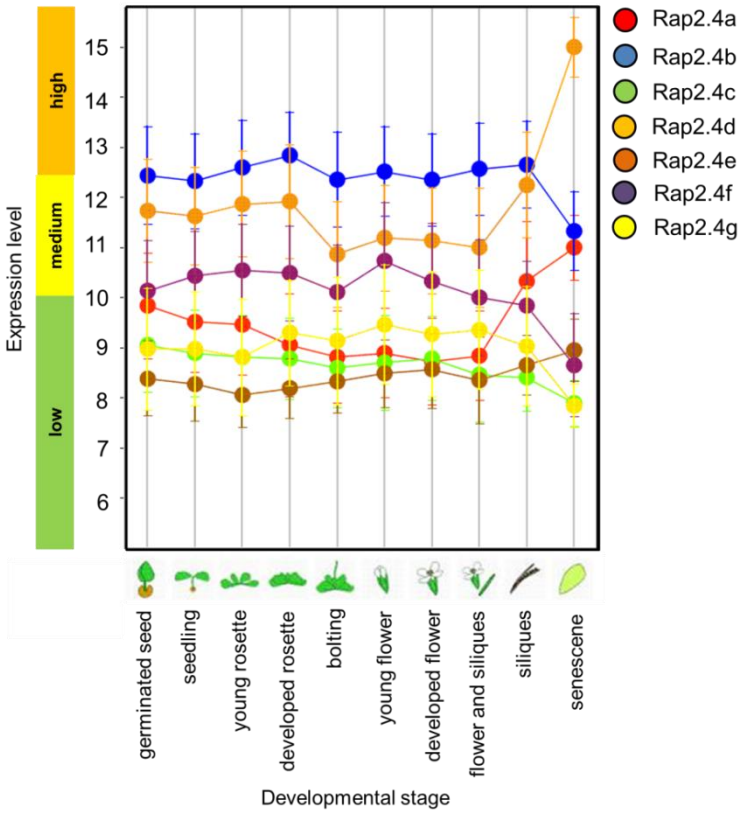

Fig. 10: log2 expression of ERFIb genes during developmental stages, signal intensity on a 22k array; modified according to Genevestigator Version 4; Development analysis dataset.

The data showed expressional differences between the ERFIb genes in the developmental time course. Rap2.4b expression levels were high during all stages of development, except senescence. Rap2.4d was expressed at medium level, whereas the expression was higher during first four stages (germinated seed – developed rosette) when compared to the following four stages (bolting – flower and siliques). In the last two stages (siliques; senescence), the expression was at highest level pointing towards transcriptional activation upon senescence. Rap2.4f was to a greater or lesser extent constitutively expressed in shoots through developmental stages. In case of Rap2.4a, Rap2.4c, Rap2.4e and Rap2.g the expression levels were low in all developmental stages. The only difference occurred was during the senescence stage. Here, the Rap2.4a expression increased, while Rap2.c and Rap2.g transcript rates further decrease.

Rap2.4a and Rap2.4d showed strongest induction in senescence stage presuming putative involvement in senescence related processes. The assumption is supported since several putative Rap2.4a and Rap2.4d target genes are involved in senescence associated pathways (PhD thesis; Jote Bulcha). Notably, Parlitz and co-workers (2011) revealed Rap2.4a among transcription factors up-regulated in dark-induced senescence.

3.1.2 Co-response analysis with the comprehensive systems-biology database (CSB.DB)

Co-response or co-expression analysis can help to elucidate biological functions of barely investigated genes (Gachon et al. 2005; Persson et al. 2005). Analysis of the best co-responses among changing transcript levels, enables hypotheses on functional gene-gene correlations (Steinhauser et al. 2004). Moreover, expressional correlation indicates gene co-functionality in shared pathways (Persson et al. 2005; Hirai et al. 2007; Humphry et al. 2010). Often highly co-expressed gens are involved in the same biological process and provide knowledge about gene function (Usadel et al. 2009; Bhardwaj and Lu 2009), as demonstrated in several publications (Lisso et al. 2005; Usadel et al. 2005; Horan et al. 2008).

The comprehensive systems biology database (CSB.DB; Steinhauser et al. 2004) was used for co-response analysis between gene samples. Conditional pair wise gene-to-gene co-response queries for single genes (sGQ) and multiple genes (mGQ) were generated. They were applied to data matrices with transcript abundances of various developmental stages as well as abiotic stresses. The datasets were divided in transcript data from aboveground organs and root tissue. The expressional data originated from 22K oligonucleotide Affymetrix microarrays prepared from the AtGenExpress consortium (Steinhauser et al. 2004) (Tab. 7). The abiotic stress series combines experiments addressing all major stress stimuli. Array data sets were evaluated as follows: zero and negative transcript intensities were skipped as non-valid as well as genes with > 5% missing values.

Tab. 7: Matrix types used for co-expression analysis in CSB.DB database.

Matrix	Description	Stress	Tissue	Valid genes
agte0100	developmental series		whole Col-0 seedlings	12200
atge0200	abiotic stress series	cold (4°C) osmotic stress (300 mM mannitol) salt stress (250 mM NaCl) drought stress	Col-0 seedlings, aboveground organs	13197
atge0250	abiotic stress series	genotoxic stress oxidative (10 µM methyl viologen) heat (38°C) UV-B wounding	Col-0 seedlings, roots	15377

For comparison, the non-parametric Spearman´s rank correlation coefficient (r_s) was calculated. A value of +1 means a perfect positive correlation while a value of -1 represents a perfect negative correlation, respectively. The p-value describes the probability of correlation coefficient and ranges from zero to one. In case of a small p-value, the observed correlation is less likely to be random. In general a p-value of < 0.05 is considered to be significant and has been taken as a minimal value for analysis.

3.1.2.1 Multiple gene query co-response

Co-responses between ERFIb transcription factors were generated using the transcript co-response analysis with the available matrix types according to table 7. Data were available for the transcription factors Rap2.4b, Rap2.4c, Rap2.4f and Rap2g. However, data availability for particular transcription factors differed. For example, Rap2.4a was present only in the developmental series with very limited amount of co-responsive genes. Rap2.4f was not available in the developmental data set. This demonstrates difficulties in microarray signal detection for Rap2.4a or Rap2.4f as caused by low signal values. Especially for transcription factors it is likely that the detection sensitivity is limited (Horak and Snyder 2002; Baginsky et al. 2009). Expression intensity might be insufficient to detect relevant changes at the lower end of the dynamic range of the transcriptome in low abundance genes (Draghici et al. 2006). Nevertheless, Rap2.4a was included in analysis to gain information about co-response with other ERFIb genes.

3.1.2.1.1 Multiple gene co-response between ERFIb genes during development and upon stress in aboveground organs

Tab. 8: ERFIb multiple gene query analysis with the developmental series (top) and abiotic stress series in aboveground organs (bottom). As an indicator for correlation, the Spearman´s non-parametric measure of correlation was calculated. Small p-value indicates strong correlation.

Rank	Gene 1	Gene 2	Spearman r_s	p-value
1	2.4d	2.4g	0.42	4.68e-04
2	2.4b	2.4d	0.24	5.13e-02
3	2.4b	2.4g	0.08	5.18e-01

Rank	Gene 1	Gene 2	Spearman r_s	p-value
1	2.4b	2.4d	0.44	4.05e-04
2	2.4d	2.4f	0.30	1.91e-02
3	2.4b	2.4f	0.30	1.98e-02

p-value	< 0.001	0.01 - 0.001	0.05 - 0.01

The queries for co-response between the available ERFIb genes displayed only low Spearman´s correlation in all cases. The highest correlation occurred between Rap2.4b and Rap2.4d with an r_s value of 0.42 and 0.44. The correlation between the other ERFIb genes were low in all cases (<0.3). The results from both co-response analyses indicate that the expression of these transcription factors is most likely not linked during developmental processes.

3.1.2.1.2 Multiple gene co-response using the abiotic stress series in roots

The dataset for abiotic stress in roots inherits transcriptional activity for the highest number of available ERFIb genes, namely Rap2.4a, Rap2.4b, Rap2.4d, Rap2.4f and Rap2.4g.

Tab. 9: ERFIb multiple gene query analysis with the abiotic stress matrix. As an indicator for correlation, the Spearman´s non-parametric measure of correlation was calculated. Small p-value indicates strong correlation.

Rank	Gene 1	Gene 2	Spearman r_s	p-value
1	2.4b	2.4d	0.7	7.24e-10
2	2.4d	2.4g	0.49	5.13e-05
3	2.4b	2.4f	0.38	2.14e-03
4	2.4b	2.4g	0.29	2.09e-02
5	2.4a	2.4f	0.24	5.85e-02
6	2.4a	2.4b	0.16	2.04e-01
7	2.4d	2.4f	0.07	5.72e-01
8	2.4f	2.4g	0.01	8.85e-01
9	2.4a	2.4d	0.007	9.53e-01
10	2.4a	2.4g	-0.16	2.31e-01

p-value	< 0.001	0.01 - 0.001	0.05 - 0.01

The co-response analysis of transcriptional changes upon abiotic stress in roots demonstrated the strongest positive correlation between Rap2.4b and Rap2.4d (r_s= 0.7). Rap2.4d and Rap2.4g showed only weak correlation (r_s= 0.49). The remaining co-response results were comparatively low between other ERFIb genes.

The multiple gene query analysis revealed positive correlation between Rap2.4b and Rap2.4d. The fact that the correlation was stronger in roots (r_s = 0.7) than in aboveground organs (r_s = 0.44) indicated that both genes are expressed differentially in plant tissue. In case of abiotic stress in roots a similar response is feasible, possibly activated through the same type of abiotic stress.

Weaker co-response values were indicated for Rap2.4b and Rap2.4d with Rap2.4g. Rap2.4f transcript regulation positively correlated with Rap2.4b and Rap2.4d upon abiotic stresses in aboveground organs. In all cases the co-response was only weak, suggesting a less rigid relation between these Rap2.4 genes under the conditions analyzed.

3.1.2.1.3 Single gene query co-response

Single gene query allows identification of genes, which are co-regulated with the gene of interest. Upon subsequent data analysis, the co-regulated genes can be sorted with MapMan (Thimm et al. 2004) in hierarchical categories (BINs) according to the function of the gene product.

3.1.2.1.4 Evaluation of the positive top 100 co-response genes with Rap2.4b and Rap2.4d in developmental series

The high co-response values from multiple query analysis between Rap2.4b and Rap2.4d in the developmental series and in abiotic stress series in roots, demanded for a more extensive analysis in terms of co-response with other genes. For the Rap2.4 genes the matrices developmental series (12000 genes) and abiotic stress in roots (15377 genes) were available.

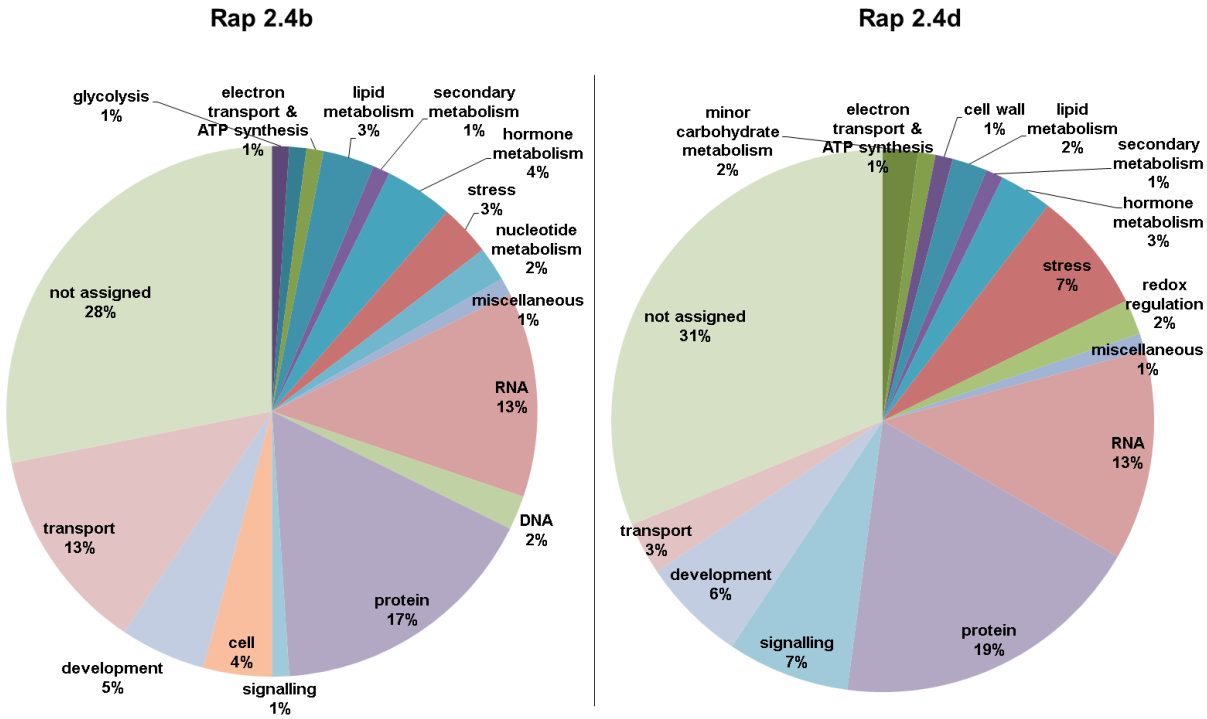

Fig. 11: Co-response for Rap2.4b and Rap2.4d with single gene query analysis and the matrix type's developmental series (atge0100); exposure according to MapMan hierarchical categorization.

The evaluation of top 100 co-responsive genes for the Rap2.4 genes in developmental series showed most hits for categories such as development (2.4b 5%; 2.4d = 6%), protein (2.4b 17%; 2.4d = 19%) and RNA (2.4b 13%; 2.4d = 13%). Rap2.4d had higher values for the signaling (2.4b 1%; 2.4d = 7%) and stress (2.4b 3%; 2.4d = 7%) categories. The category with genes associated to transport processes showed higher percentage value for Rap2.4b (2.4b = 13%; 2.4d = 3%). Categories with values >10%, were assigned as major categories which indicated for Rap2.4b the group's protein, transport and RNA. For Rap2.4d the protein and RNA categories could be assigned. For both Rap2.4 genes the category protein was the biggest one.

A more detailed view into the protein categories, displayed for Rap2.4b and Rap2.4d nearly equal ratios between genes involved in posttranslational modification through

phosphorylation and genes involved in degradation via the ubiquitination pathway (for comparison see appendix table 17 and tab. 18 18).

For Rap2.4b the category transport contained genes responsible for sugar, amino acids but also auxin and ethylene transport. This correlation is supported since Rap2.4b mediates ethylene signaling (Lin et al. 2008).

3.1.2.1.5 Detailed analysis of the category RNA and associated genes for Rap2.4b and Rap2.4d in developmental series

For Rap2.4b and Rap2.4d the category RNA was examined in detail, since the category was a major one and correlation with other transcription factors should reveal the functional regulation of these Rap2.4 genes more precisely.

Tab. 10: Rap2.4b single gene query in developmental series; **genes strongly correlated and assigned into the category RNA according to MapMan**. As an indicator for correlation, the Spearman´s non-parametric measure of correlation was calculated. Small p-value indicates strong correlation; the p-value is < 0.001.

Function	Gene	Description	r_s	Rank
RNA.regulation of transcription AP2/EREBP	at5g05410	DREB2A (DRE-BINDING PROTEIN 2A)	0.730	1
RNA.processing	at4g32850	nPAP (NUCLEAR POLY(A) POLYMERASE)	0.692	4
unclassified	at3g17611	rhomboid family protein / zinc finger protein-related	0.667	11
Global transcription factor group	at3g27260	GTE8 (global trascription factor group E8)	0.653	14
unclassified	at5g16680	RING/FYVE/PHD zinc finger superfamily protein	0.636	25
bZIP transcription factor family	at3g10800	putative bZIP transcription factor (bZIP28)	0.629	28
putative transcription regulator	at2g18090	PHD finger family protein	0.625	32
putative transcription regulator	at5g15020	SNL2 (SIN3-LIKE 2)	0.619	37
MYB domain transcription factor family	at2g16720	myb domain protein 7 (MYB7)	0.619	38
unclassified	at5g19420	Regulator of chromosome condensation (RCC1)	0.592	80
RNA.processing.RNA helicase	at5g13010	EMB3011	0.592	82
G2-like transcription factor family GARP	at4g37180	myb family transcription factor	0.592	82

Closer examination of co-responding genes from the RNA category with Rap2.4b (Tab. 10), showed DREB2A (drought response element binding) on first position (r_s = 0.73). DREB2A induces gene expression upon drought and salt stress (Sakuma et al. 2006). It is also involved in heat stress response, however, with different induction rates (Vainonen et al. 2012). The expression of Rap2.4b was described to be salt and drought stress induced (Lin et al. 2008). Moreover, Rap2.4b is also able to bind the ethylene-responsive GCC-box (G-box) and the dehydration-responsive element (DRE) (Lin et al. 2008) which mediates drought stress responses like DREB2A. This correlation proved functional similarity. The nuclear poly(A) polymerase (PAP4) also strongly correlated with Rap2.4b (r_s = of 0.69); this protein is localized in the nucleus (Meeks et al. 2009) like Rap2.4b (Lin et al. 2008) and mediates mRNA 3´ end formation. The protein of unknown function / rhomboid family protein (At3g17611) strongly correlated with Rap2.4b and was recently

described as hydrogen peroxide (H_2O_2)-inducible gene (Inze et al. 2011). Moreover G2-like, MYB and bZIP transcription factors were among co-responding genes.

Tab. 11: Rap2.4d single gene query in developmental series; **genes strongly correlated and assigned into the category RNA according to MapMan**. As an indicator for correlation, the Spearman´s non-parametric measure of correlation was calculated. Small p-value indicates strong correlation; the p-value is < 0.001 for all correlations.

Function	Gene	Description	r_s	Rank
C2H2 zinc finger family	at1g27730	ZAT10 (salt tolerance zinc finger)	0.905	1
bZIP transcription factor family	at1g42990	AtbZIP60	0.773	16
C2H2 zinc finger family	at5g43170	AZF3 (ARABIDOPSIS ZINC-FINGER PROTEIN 3)	0.730	37
MYB domain transcription factor family	at5g67300	AtMYB44	0.716	47
GRAS transcription factor family	at4g17230	SCL13 (SCARECROW-LIKE 13)	0.710	54
AP2/EREBP	at4g36900	RAP2.10	0.703	65
bZIP transcription factor family	at3g62420	basic region/leucine zipper motif 53 (BZIP53)	0.697	71
WRKY domain transcription factor family	at4g31550	WRKY11 (WRKY DNA-binding protein 11)	0.686	83
WRKY domain transcription factor family	at4g01250	AtWRKY22	0.676	93
putative transcription regulator	at5g05140	transcription elongation factor-related	0.675	94
WRKY domain transcription factor family	at2g23320	WRKY15	0.673	96
unclassified	at1g76590	zinc-binding family protein	0.671	98

Within the RNA category for Rap2.4d (Tab. 11), ZAT10 showed the strongest correlation (r_s = 0.9). The C2H2-type zinc finger transcription factor ZAT10 responds to the abiotic stresses like salt and osmosis (Hahn et al. 2013). Since no data are available about the function of Rap2.4d, a high co-response with ZAT10 endorsed putative involvement to similar abiotic stress in roots. Strong positive correlation was also present for Rap2.10 (r_s = 0.7), a member of the AP2/EREB family, which negatively controls cold stress responses under normal growth (Tsutsui et al. 2009). Strikingly, transcription factors belonging to the WRKY (3 genes) and bZIP family (2 genes) were found to be presented several times. WRKY transcription factors tightly regulate the plant defense transcriptome positively and negatively (Journot-Catalino et al. 2006). bZIP transcription factors enhance stress robustness in plants (Fujita et al. 2005) since they are able to bind ABA response elements (ABRE) and mediate ABA dependent signals (Zhang et al. 2008) upon drought or heat (Finkelstein et al. 2002). bZIP proteins can also bind to ABREs without the ACGT core element, the coupling element 3 (CE3) (ACGCGTGTC) (Choi et al. 2000). This motif shows high similarity to the CE3-like motif (CACGCGATTC) where Rap2.4a, a close relative within the ERFIb family, can bind (Shaikhali et al. 2008). The AtMYB44 protein (r_s = 0.71) is involved in ABA signaling and mediates stress responses upon drought, salt and cold (Jung et al. 2008).

The RNA category revealed that several transcription factors were correlated with Rap2.4b, whereas for Rap2.4d the bZIP, WRKY and C2H2 transcription factors are overrepresented. The co-responding transcription factors represent similar stress responses. Thus, overlapping in drought and salt stress involvement for both Rap2.4

genes is present. Difference exists in putative heat stress involvement for Rap2.4b and cold stress participation for Rap2.4d, highlighted through a strong positive correlation to transcription factors, which regulate such stress responses.

3.1.2.1.6 Evaluation of the positive top 100 co-response genes with Rap2.4b and Rap2.4d upon abiotic stress in roots

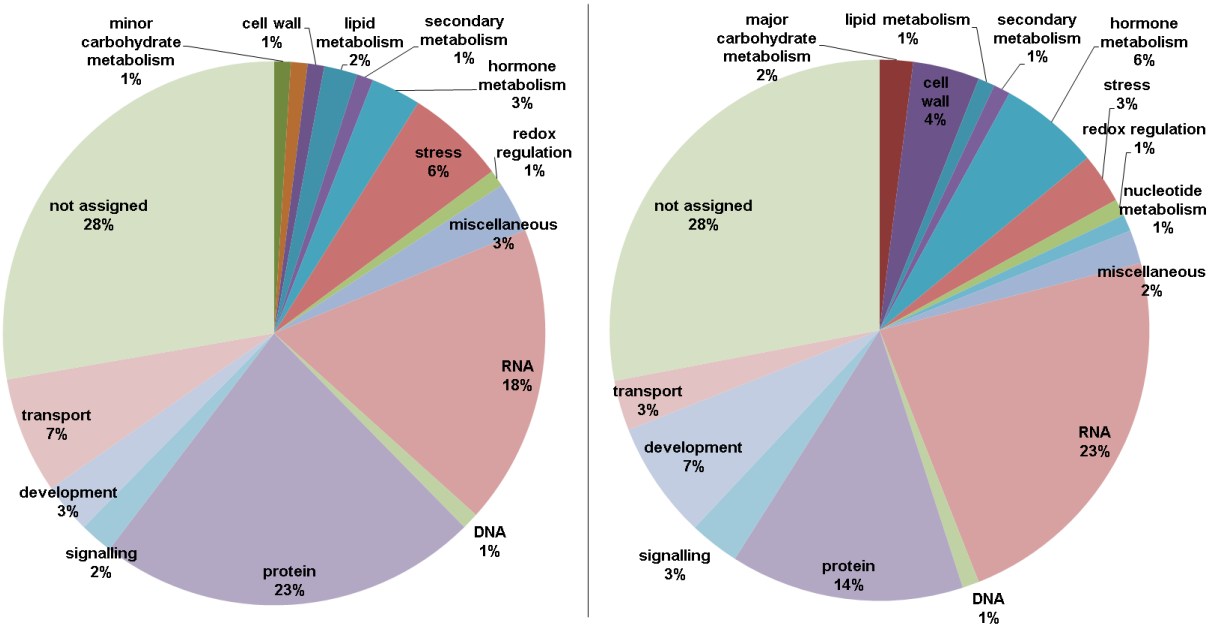

Fig. 12: Co-response for Rap2.4b (left) and Rap2.4d (right) with single gene query analysis and the matrix types abiotic stress in roots (atge0250); exposure according to MapMan.

The evaluation of top 100 co-responsive genes upon abiotic stress in roots (Fig. 12) showed similar values for the major category (> 10%) RNA (2.4b = 18%; 2.4d = 23%). Moreover, also an increase in this category for both Rap2.4 genes compared with developmental series is present (2.4b and 2.4d = 13%). The category protein was the second major one and showed higher value for Rap2.4b (23%); Rap2.4d, however, displayed a decrease with 14% compared to 19 % from developmental series (Fig. 11). A deeper look into the protein category for Rap2.4b, displayed an imbalance in favor of genes responsible for protein degradation (14 genes) towards genes involved in protein phosphorylation (3 genes). On the contrary, for Rap2.4d a similar number of genes belonging to these gene groups was found (5 genes associated to phosphorylation versus 6 genes associated to degradation). ERFIb genes are stress related transcription factors (Nakano et al. 2006). Under normal conditions many transcription factors are expressed at basal levels and are degraded via ubiquitin-proteasome pathway (Mizoi et al. 2012). Transcription is activated independently by a stress signal that could additionally stabilize

or activate the protein. This mechanism is proposed for the AP2/EREBP gene DREB2A (Mizoi et al. 2012). A similar process is feasible for Rap2.4b and Rap2.4d. Here, a protein-protein interaction with BPM proteins was shown in an Y1H approach (Weber et al. 2009). PBM proteins are involved in protein targeting prior to degradation via the 26S proteasome (Weber at al. 2009). BPM1 and BPM2 were the most favorable candidates for *in planta* assembly with Rap2.4b (Weber et al. 2009). Among strongly correlated genes with Rap2.4b from abiotic stress in roots, BPM2 (At3g06190; $r_s = 0.6$) was associated into the protein category. Thus, higher amount of genes involved in protein degradation hints towards a tight post-translational control of Rap2.4b. The stress signal triggers a high mRNA induction and enhanced protein translation; the higher protein amount would be, thus, balanced by a higher ubiquitination and degradation rate, respectively. For Rap2.4d neither genes involved in protein degradation were present, nor was a BPM family member present among co-responsive genes. This transcription factor is less likely controlled in similar manner in roots upon abiotic stress (complete list of genes assigned into the protein category is available in appendix table 19 and table 20).

The amount of co-responsive genes associated to the RNA category, showed the highest value for both Rap2.4 genes. Higher transcription rates point towards enhanced transcription factor activity is roots as response to abiotic stress by regulation of gene activity in a positive or negative manner.

3.1.2.1.7 Detailed analysis of the RNA category and associated genes for Rap2.4b and Rap2.4d upon abiotic stress in roots

The RNA BINs were analyzed in more detail to elucidate functional Rap2.4 characteristics via connection to other transcription factors, which display the predominant genes. In case of Rap2.4b, transcription factors from the AP2/EREBP, bZIP and zinc finger families were present, each with 2 genes (Tab. 12).

Tab. 12: Rap2.4b single gene query upon abiotic stress in roots; genes strongly correlated and assigned into the category RNA according to MapMan. As an indicator for correlation, the Spearman´s non-parametric measure of correlation was calculated. Small p-value indicates strong correlation; the p-value is < 0.001 for all correlations.

Function	Gene	Description	r_s	Rank
bZIP transcription factor family	at3g10800	putative bZIP transcription factor (bZIP28)	0.748	1
AP2/EREBP	at1g22190	Rap2.4d	0.695	6
C3H zinc finger family	at2g25900	AtTZF1; A. thaliana Cys3His zinc finger protein	0.681	9
G2-like transcription factor family, GARP	at4g37180	myb family transcription factor	0.670	12
Histone acetyltransferases	at1g79000	HAC1	0.645	15
putative transcription regulator	at5g60410	SIZ1; small ubiquitin-like modifier (SUMO) E3 ligase	0.644	16
bZIP transcription factor family	at2g46270	GBF3 (G-BOX BINDING FACTOR 3)	0.624	31
GRAS transcription factor family	at1g07530	scarecrow-like transcription factor 14 (SCL14)	0.619	36
regulation of transcription	at3g04740	SWP (STRUWWELPETER)	0.615	39
NAC domain transcription factor family	at3g15500	ANAC055	0.610	41
RNA binding	at1g29400	AML5 (ARABIDOPSIS MEI2-LIKE PROTEIN 5)	0.607	44
MYB-related transcription factor family	at5g04760	myb family transcription factor	0.585	58
bHLH,Basic Helix-Loop-Helix family	at3g47640	POPEYE (PYE), regulating response to iron deficiency	0.585	59
Vascular Plant One Zinc Finger TF (VOZ)	at2g42400	ATVOZ2 (vascular plant one zinc finger protein 2)	0.574	69
RNA binding	at1g58470	ATRBP1 (RNA-BINDING PROTEIN 1)	0.567	75
C2H2 zinc finger family	at3g19580	AZF2 (ARABIDOPSIS ZINC-FINGER PROTEIN 2)	0.566	77
HB,Homeobox transcription factor family	at2g01430	ATHB17	0.559	91
MYB domain transcription factor family	at2g03470	myb family transcription factor	0.557	94
AP2/EREBP	at4g16750	encodes a member of DREB subfamily A-4	0.551	99

The Rap2.4b transcription factor showed the strongest correlation to bZIP28 ($r_s = 0.75$). It was reported that bZIP28 triggers the unfolded protein response (UPR) in the endoplasmic reticulum upon stress (Srivastava et al. 2012). The zinc finger protein AtTZF1 also strongly correlated with Rap2.4b ($r_s = 0.68$). AfTZF1 is induced by ABA signals (Lin et al. 2011) as well as the NAC transcription factor ANAC055 ($r_s = 0.68$) (Tran et al. 2004). SIZ1 ($r_s = 0.64$) mediates drought responses independent from the ABA pathway (Catala et al. 2007) and inhibits polyubiquitination of ICE1, an upstream element in the cold stress regulon (Miura et al. 2007, Mozoi et al. 2011). The correlations support the report about Rap2.4b mediated drought stress regulation (Lin et al. 2008).

Besides drought-responsive genes, several genes involved in light responses were also positively co-expressed with Rap2.4b. The bZIP transcription factor GBF3 ($r_s = 0.62$) is light induced (Jakoby et al. 2002), SCL14 ($r_s = 0.62$) is down-regulated by FR-light (Ibarra et al. 2013). Rap2.4b was addressed as signal molecule in FR-light responses and was down-regulated by blue light as well as red/far-red light (Lin et al. 2008): Notably, there is evidence for overlapping functions of light and ethylene in plants (Knee et al. 2000). At first, it's surprising to find correlations of light related genes in roots. Nevertheless, Molas and co-workers (2006) reported about red and blue light pathways in roots of seedlings. Hence, RAP2.4b could interfere between light and ethylene signaling pathways in roots (Lin et al. 2008) through regulation of genes containing the ethylene-responsive G-box and drought responsive element (DRE) (Lin et al. 2008). Moreover, it was reported that light is an important additional factor upon cold- and drought-stress induced transcription mediated by DRE elements (Kim et al. 2002).

Rap2.4d, the closest family member of Rap2.4b (Nakano et al. 2006; Weber et al. 2009), was present among the co-responsive transcription factors (r_s = 0.7) upon abiotic stress in roots, demonstrating that Rap2.4b and Rap2.4d are positively co-regulated over a wide range of stress treatments. This analysis demonstrated that several of the highly co-responding genes with Rap2.4b are involved in drought response and / or ABA signaling but also mediate light signaling in roots. The RNA category for Rap2.4d displayed an overrepresentation of C2H, MYB and C2C2 transcription factors (Tab. 13).

Tab. 13: Rap2.4d single gene query upon abiotic stress in roots; genes strongly correlated and assigned into the category RNA according to MapMan. As an indicator for correlation, the Spearman´s non-parametric measure of correlation was calculated. Small p-value indicates strong correlation; the p-value is < 0.001 for all correlations.

Function	Gene	Description	r_s	Rank
HB,Homeobox transcription factor family	at3g61890	ATHB-12	0.737	12
bZIP transcription factor family	at2g46270	GBF3 (G-BOX BINDING FACTOR 3)	0.733	14
C2C2(Zn) DOF zinc finger family	at5g39660	CDF2 (CYCLING DOF FACTOR 2)	0.725	16
C2H2 zinc finger family	at5g59820	ZAT12	0.704	28
putative transcription regulator	at1g07590	pentatricopeptide (PPR) repeat-containing protein	0.699	31
C2C2(Zn) DOF zinc finger family	at5g62430	CDF1 (CYCLING DOF FACTOR 1)	0.696	34
AP2/EREBP	at1g78080	Rap2.4b	0.695	37
MYB domain transcription factor family	at5g17300	myb family transcription factor	0.694	38
C2H2 zinc finger family	at3g19580	AZF2 (ARABIDOPSIS ZINC-FINGER PROTEIN 2)	0.693	40
RNA.processing 3' end processing	at1g66500	Pre-mRNA cleavage complex II	0.680	47
unclassified	at5g37540	Eukaryotic aspartyl protease family protein	0.677	52
AP2/EREBP	at4g25480	DREB1A (DEHYDRATION RESPONSE ELEMENT B1A)	0.674	55
G2-like transcription factor family, GARP	at4g37180	myb family transcription factor	0.672	57
CCAAT box binding factor family, HAP2	at5g12840	ATHAP2A	0.672	58
C2H2 zinc finger family	at3g52800	zinc finger (AN1-like) family protein	0.670	61
C3H zinc finger family	at4g29190	zinc finger (CCCH-type) family protein	0.667	66
unclassified	at2g46670	pseudo-response regulator	0.663	69
C2H2 zinc finger family	at5g04340	ZAT6	0.661	73
MYB domain transcription factor family	at5g04760	heat shock protein, Hsp40	0.652	81
MYB domain transcription factor family	at5g54230	AtMYB49 (myb domain protein 49)	0.651	84
putative transcription regulator	at3g57540	remorin family protein	0.644	93
C2C2(Zn) DOF zinc finger family	at5g60850	OBP4	0.641	99
MYB domain transcription factor family	at5g62470	Encodes a R2R3 type Myb transcription factor	0.640	100

The ATHB-12 transcription factor showed the strongest correlation with Rap2.4d (r_s = 0.74). ATHB-12 enhances gene transcription upon drought stress by negative modulation of appropriate ABA pathways (Valdes et al. 2012).

ZAT12 was highly co-responsive (r_s = 0.7) and is needed for abiotic stress and reactive oxygen signaling (Davletova et al. 2005). Moreover, ZAT12 negatively controls the C-REPEAT/DRE BINDING FACTOR (CBF) regulon upon cold stress but also decimates heat stress responses (Vogel et al. 2005). ZAT6 is required for transcriptional response upon cold and osmotic stress (Ciftci-Yilmaz et al. 2008).

The C2H2-Dof genes CDF1 and CDF2 negatively control the CONSTANS (CO) promoter activity in a redundant manner and modulate its diurnal expression rhythm whereas CO induces flowering (Fornara et al. 2009). The expression of G-box binding factor 3 (GBF3)

was reported to be light dependent (Jakoby et al. 2002). Besides, the gene was also available among co-responsive genes with Rap2.4b (see table 8; $r_s = 0.62$).

Within the AP2/EREBP genes, Rap2.4b was positively correlated with Rap2.4d ($r_s = 0.7$). Moreover, DREB1A showed strong co-response with Rap2.4d ($r_s = 0.67$), DREB1A demonstrated high binding preference to the DRE core sequence A/GCCGAC (Sakuma et al. 2002). The gene is cold-induced and important for cold-stress handling (Mizoi et al. 2011). Rap2.4b, the closest relative of Rap2.4d (Nakano et al. 2006), binds also to DRE elements (Lin et al. 2008).

The analysis revealed positive correlation of transcription factors responsible for cold-stress response and genes involved in drought stress regulation with Rap2.4d. Thus, the strong correlation with Rap2.4b supports a common role in stress handling.

The starting point for the ERFIb investigation was the *in silico* analysis of expressional correlation between Rap2.4b and Rap2.4d. It revealed putative overlap upon abiotic stresses in roots. The data used for co-response analysis was generated from several different abiotic stress types like cold, heat or salt (Tab. 7).

Two questions were raised:

1) Is a correlation of Rap2.4b and Rap2.4d also present in leaf tissue and which particular abiotic stress could be responsible for the strong correlation?

2) Since the ERFIb family consists of in total 8 genes, is a similar correlation between the other genes feasible?

To address these questions, firstly an experiment with different temperatures was performed. The co-response analysis displayed for Rap2.4b and Rap2.4d correlations with genes involved in ABA pathways. Rap2.4d showed correlations with cold-stress related transcription factors, whereas Rap2.4b was co-expressed with heat-stress related genes. Temperature is a determinant trigger for AP2 transcription factors, for example, the DREB subfamily plays a major role in cold-stress response (Sakuma et al. 2002; Mizoi et al. 2011). Higher temperature can also adjust the AP2-gene activity like exemplarily shown for the AP2 transcription factor DREB2A (Sakuma et al. 2006). So far nothing is known about the consequence of temperature upon ERFIb transcript intensities.

Microarray signal detection can be problematic due to its low sensitivity towards transcriptional changes in low abundance genes like transcription factors (Holland 2002; Baginsky et al. 2009). This is particularly true for the ERFIb genes since the expressional

in silico analysis with data originating from microarray experiments could only be performed with a limited number of genes. However, the expression of all Rap2.4 genes is important to elucidate their function in abiotic stress. For this purpose, qPCR was used to monitor the expression profile for the whole ERFIb family.

3.2 ERFIb expression upon different abiotic stresses

3.2.1 Effect of different temperatures on Rap2.4 mRNA abundance after long term

Two week old Col-0 wild-type (wt) plants were grown under standard conditions and exposed for one week to 10 and 30°C under 120 µmol photons m^{-2} s^{-1} light intensity in a climate controlled chamber. Plants cultivated at 20°C served as controls. After RNA isolation and cDNA synthesis, the ERFIb expression was monitored by qPCR.

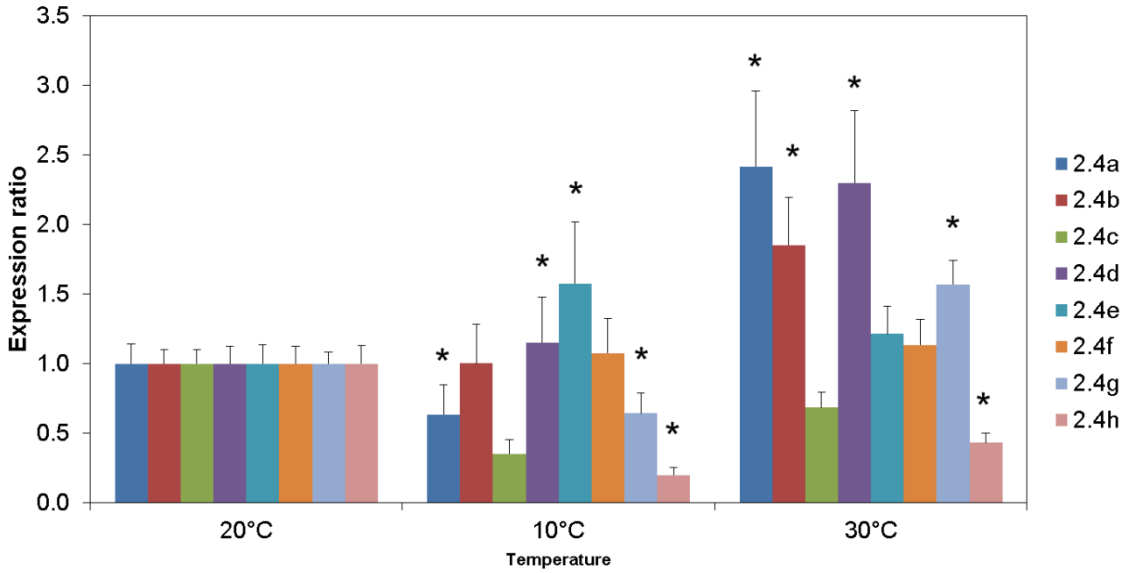

Fig. 13: Relative ERFIb transcript levels in Col-0 wt plants, exposed to different temperatures; plant material was pooled from three independent plants and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to Act7 transcript amount in Col-0 wt grown under 20°C. The relative transcript levels of Col-0 wt grown under 20°C were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from control treatment under 20°C.

The result displayed upon 10°C treatment a decreased expression for Rap2.4a (0.5-fold), Rap2.4c (0.3-fold), Rap2.4g (0.6-fold) and Rap2.4h (0.2-fold). Only the Rap2.4e transcript level was elevated (1.5-fold ± 0.4). Rap2.4b and Rap2.4d showed no difference in

transcript abundance relative to 20°C. Upon 30°C Rap2.4a, Rap2.4b and Rap2.4d genes showed the highest relative transcript levels (> 2-fold). The Rap2.4e transcript level was 1.5-fold increased. For Rap2.4g an increased relative mRNA level was present (1.6 ± 0.2; $p<0.05$). The Rap2.4c (0.7) and Rap2.4h (0.4) transcript levels were significantly decreased ($p<0.05$).

The analysis did not show putative cold-triggered changes regarding the Rap2.4d transcript level as presumed from the cold-response in the co-response analysis (Tab. 13). The applied temperatures were rather of moderate intensities. A transcriptional change of Rap2.4d, as response towards cold stress, could maybe take place upon lower temperatures. In general, Rap2.4a and Rap2.4g showed gradual reactions towards temperature shift with down-regulation upon cold and up-regulation in case of elevated temperatures. Rap2.4c and Rap2.4h were down-regulated at lower temperatures, whereas upon 10°C less than at 30°C. Hence, it depicts steady state responses.

3.2.2 Transcriptional changes of ERFIb genes upon short term cold-stress treatment

To elucidate more precisely a putative cold stress response, 4 week old Col-0 wild-type plants were grown under standard conditions and exposed for 24 hours to 4°C prior to mRNA isolation for cDNA synthesis and qPCR analysis.

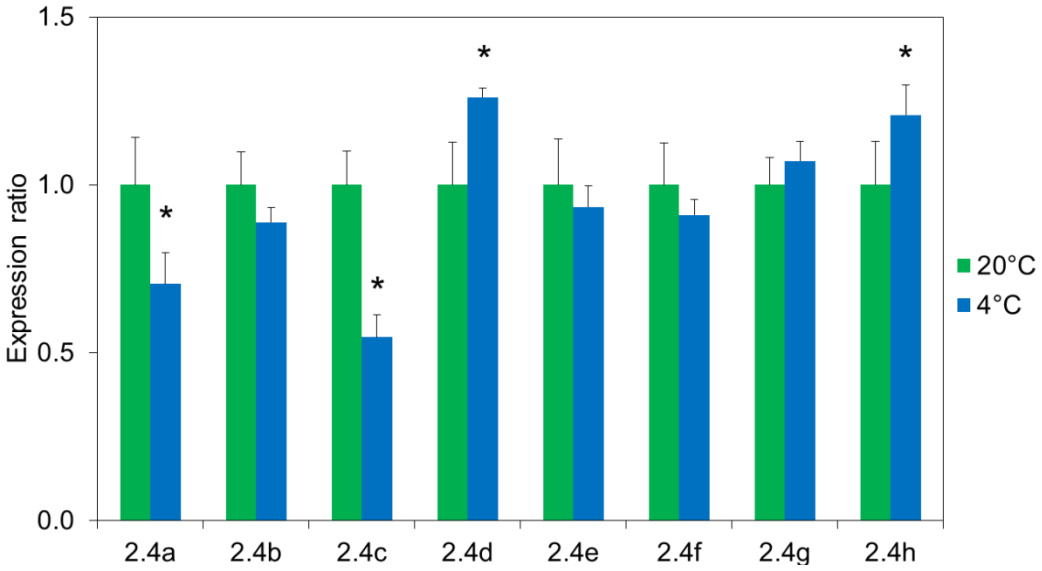

Fig. 14: Relative ERFIb transcript levels in Col-0 wt plants exposed to different temperatures; plant material was pooled from three independent plants and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to relative Act7 transcript amount in Col-0 wt plants grown under 20°C. The relative transcript levels of Col-0 wt grown under 20°C were set to 1.0. Data's are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from control treatment with 20°C temperature.

In general, the differences in gene expression levels were less after 24h cold treatment (Fig. 14) than after 7 days growth in the cold (Fig. 13). In relation to 20°C the regulatory thresholds were in the range of 0.5 – 1.3 only.

For Rap2.4a and Rap2.c similar trends were observed, whereas Rap2.4e and Rap2.4f did not respond to short term cold. Rap2.4d showed the strongest effect of all eight genes (1.3-fold; $p<0.05$), while Rap2.4b was slightly down-regulated (0.9-fold). Rap2.4c displayed the lowest transcript level (0.5 ± 0.1), comparable with values obtained from exposure at 10°C. Also the Rap2.4a transcript amount was down-regulated (0.7-fold), if compared to values from the 10°C experiment. The Rap2.4f regulation tendency was consistent with results obtained from the 10°C experiment, demonstrating that the transcription factor is probably not eminently triggered by the conditions used for cold stress treatment; maybe longer incubation at 4°C would induce a more intense

transcriptional change. The Rap2.4e mRNA level was 1.5-fold elevated upon 10°C treatment, probably longer incubation at low temperatures results in transcript accumulation. Rap2.4g was significantly down-regulated ($p<0.05$) in the 10°C treatment, most likely caused by the duration of cold exposure. Rap2.4h transcript level was increased upon 24 hours cold treatment (1.2 ± 0.08; $p<0.05$). Contrary, upon longer incubation at 10°C the Rap2.4h mRNA abundance was negatively impaired (0.2-fold).

According to these results, a distinction between a stress period (24h; 4°C) and a post-stress period (7 days; 10°C) with appropriate transcript level adjustment is feasible (Fig. 15). Thus, in the stress period after the beginning of stress stimulus the transcription was elevated and accompanied by transcript accumulation. In the transition from stress period to the post-stress period the transcript level was adjusted to a new steady state condition. Kreps and co-workers (2002) analyzed changes in the transcriptome after cold stress and observed similar changes for several genes, at which the time-scale differed (3h stress and 27h post-stress).

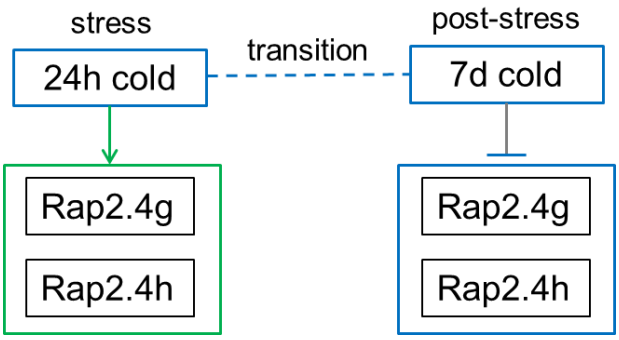

Fig. 15: Exposure of stress and post-stress effect on the Rap2.4g and Rap2.4h transcript level upon cold stress; based on significant qPCR results; green arrow indicates up-regulated gene expression, blue, broken line indicates transition from stress to post-stress period.

Results from cold stress treatment in two different experiments revealed transcriptional regulation of Rap2.4 genes in a positive and a negative manner. In general, Rap2.4a and Rap2.4c were down-regulated by cold stress, independent from duration of stress stimuli and without additive effect of a temperature below 10°C. Thus, 10°C could be seen as the threshold value for Rap2.4a and c.

None of the cold treatments had a significant effect on the Rap2.4f transcript amount. However, Rap2.4d was the only transcription factor positively affected in the transcript level upon short term 4°C treatment, at which the cold intensity and treatment length seemed to play an important role. An involvement of Rap2.4d in cold stress is supported by a strong and positive correlation with several transcription factors important for low

temperature response. Furthermore, in an advanced Y1H screen Rap2.4d was shown to bind a motif sequence in promoter region of KIN1, suggesting a regulatory function *in vivo* (Jote Bulcha, PhD thesis). Therefore, Rap2.4d represents a good candidate to putatively modulate the cold-tolerance in a genetic engineering approach. Rap2.4g and Rap2.4h showed diverse roles. On the one hand, upon long term cold-stress both genes were decreased in the expression ratio. On the other hand, after 24 h cold-treatment both genes displayed slight elevated mRNA levels. It is concluded that the treatment duration seems to be critical for both Rap2.4 genes. Additionally, a co-regulation for both genes is indicated.

3.2.3 Transcriptional changes of selected ERFIb genes upon heat stress treatment

The transcription factors Rap2.4a, Rap2.4b and Rap2.4d showed the highest induction upon heat stress treatment at 30°C (Fig. 13). Since so far no report exists concerning involvement or ERFIb transcription factors in heat stress response, a heat stress setup was applied with 10 day old Col-0 wild-type plants grown on solid MS medium in petri dishes. An advanced experimental setup was chosen to monitor a putative transcript increase due to elevated temperature combined with different periods of time. Since aerial heating resulted in not consistent temperature distribution, the whole experiment was performed in a water bath with submerged samples in darkness according to Ikeda et al. (2011). The experimental design was as follows: on the verge of stress treatment the appropriate petri dish with samples was packed in a freezer bag and closed air-tight. To avoid an emerging, the samples were weighted down with a weight plate.

Larkindale et al. (2005) distinguished between basal heat stress and acquired heat stress response: basal heat stress comprehends the exposure to temperatures above the optimal for growth. In this case the samples were exposed to 45°C for 90 min in darkness, following a recovery period of 5 d in light. Acquired heat stress consists of short period at moderately high temperatures prior to heat stress with otherwise lethal heat treatment (Larkindale et al. 2005). Here, the samples were incubated at 37°C for 150 min. After 120 min at room temperature the samples were exposed to 45°C for 30 and 60 min, respectively. The recovery lasted for 5 days prior to RNA isolation for cDNA synthesis and qPCR analysis. Expression ratios of Rap2.4a, Rap2.4b and Rap2.4d were monitored since these genes displayed strongest induction at 30°C (Fig. 13).

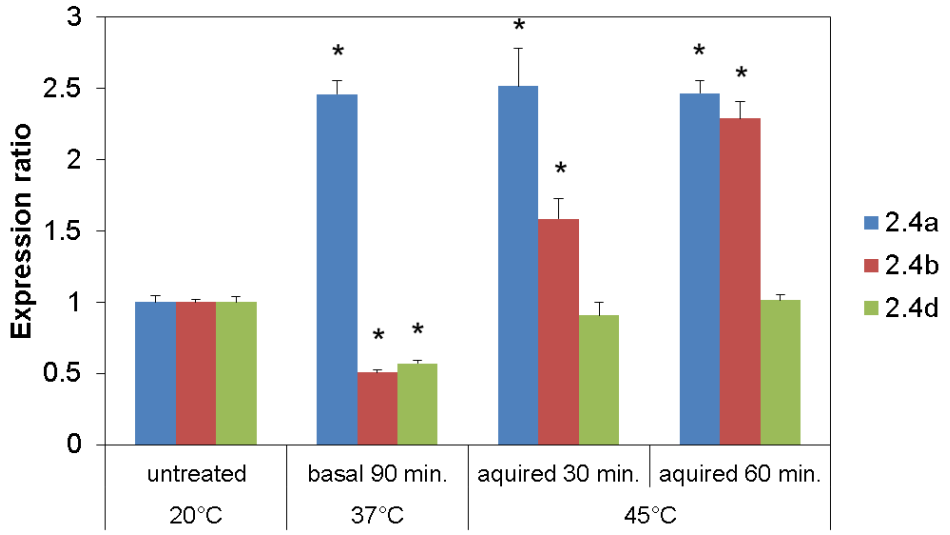

Fig. 16: ERFIb relative transcript levels in Col-0 wt plants, exposed to different temperatures; plant material was pooled from three independent plants and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to relative Act7 transcript amount in Col-0 wt plants grown under 20°C. The relative transcript levels of Col-0 wt grown under 20°C were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from untreated control.

The expression ratio for Rap2.4a was equally increased in all 3 treatments (2.5-fold). Strikingly, Rap2.4b and Rap2.4d were down-regulated after basal heat treatment. Contrary, the expression levels were elevated after 7 d at 30°C (Fig. 13). Hence, the relatively short exposure to 45°C had a repressive effect on the mRNA levels. The situation was changed when acquired heat stress was applied. Here, the Rap2.4b transcript levels increased gradually with the duration of heat stress exposure. Also, the Rap2.4d transcript level gradually increased with the duration of acquired heat stress but was not above wild-type expression ratio. In reference to results obtained in 3.2.1, Rap2.4a is generally triggered by elevated temperatures because already the treatment with 30°C induced its expression, this value can be assigned as threshold value. In terms of Rap2.4b, a longer duration of exposure is crucial since upon a pretreatment at 37°C and following second incubation at 45°C the transcript ratio was elevated (2.4-fold). The induction of Rap2.4d depends on the duration of appropriate treatment since only after one week exposure to 30°C; a significant mRNA increase was observed (Fig. 13).

The expressional data reflected the involvement of Rap2.4a and Rap2.4b transcription factors in heat stress response with differences in induction kinetics. The participation of AP2 domain containing transcription factors in responses upon high temperatures was successfully demonstrated. For example, overexpression of AtDREB1A improved heat stress tolerance in Chrysanthemum (Hong et al. 2009) and overexpressing the ERF/AP2

pepper transcription factor CaPF1 in Virginia pine enhanced the tolerability towards heat stress (Tang et al. 2005). Thus, Rap2.4a and Rap2.4b may be the right candidates for Arabidopsis tolerance engineering towards changed susceptibility to heat stresses.

3.2.4 Transcriptional changes of selected ERFIb genes upon salt stress treatment

The transcription factors Rap2.4b and Rap2.4d displayed strong correlation upon abiotic stress treatment. However, cold and heat stress experiments showed only a co-regulation upon higher temperatures but not upon cold stress. Salt stress was listed among the abiotic stresses concerning the co-response analysis (Tab. 7). To check this particular stress type, an experimental setup was performed with 4 week Col-0 wild-type plants grown on vertical plates with MS medium containing 50 mM NaCl. The cultivation in this setup enabled a separation between leaves and roots. Transcriptional changes for Rap2.4b and d were analyzed by qPCR in leaves and roots.

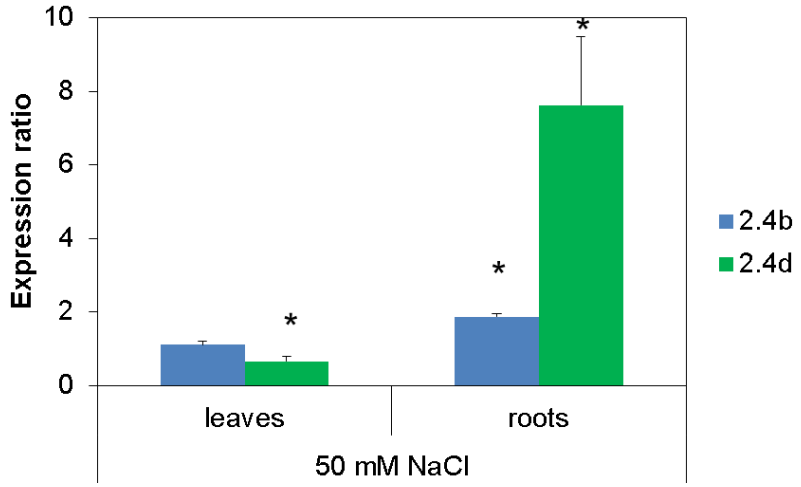

Fig. 17: Relative Rap2.4b and Rap2.4d transcript levels in Col-0 wt plants, grown on vertical MS plates with 50 mM NaCl; plant material was pooled from three independent plants and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to relative Act7 transcript in Col-0 wt grown on NaCl free MS medium plates. The relative transcript levels of Col-0 wt grown on NaCl free MS medium plates were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from control plants grown on NaCl free plates.

Upon salt stress treatment in leaves, the Rap2.4d transcript level was slightly decreased (0.6-fold) and Rap2.4b was slightly up-regulated (1.2 fold). In roots both Rap2.4 genes were expressed at high levels, at which Rap2.4d was 8-fold up-regulated and Rap2.4b 2-fold.

The comparison of transcriptional enhancement in leaves and roots demonstrated that Rap2.4b and d are co-regulated in roots upon salt stress, confirming results obtained from co-response analysis (Tab. 9). There, a strong positive correlation was exclusively available in roots. Salt and drought stress are often associated because both boost ABA signaling (Shinozaki et al. 2000). Rap2.4b binds to DRE elements and is independent from ABA signaling tracks (Lin et al. 2008). For Rap2.4d a putative induction due to ABA is feasible as well as a similar elevated transcript level upon drought stress. This is substantiated through a strong correlation of several important ABA related transcription factors in co-response analysis (Tab. 11). Moreover, Lin et al. (2008) reported that overexpression of Rap2.4b in Arabidopsis improved the drought tolerance. It can be supposed that Rap2.4d could also enhance the exposure to drought or salt stress and the overexpression of both TFs may combine these positive effects and result in plants with even greater tolerance to stress.

3.3 Dependency between ERFIb transcription factors

Rap2.4b and Rap2.4d displayed a strong correlation in co-response analysis (Tab. 9) and showed in qPCR experiments overlapping co-regulation, especially upon salt stress in roots (Fig. 17).

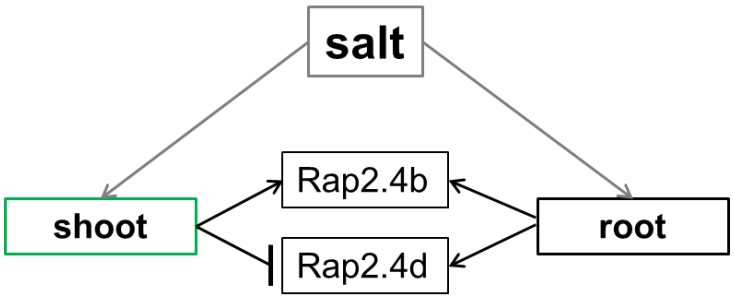

Fig. 18: Exposure of co-regulated Rap2.4b and Rap2.4d transcript level upon salt stress in roots, based on qPCR results; black arrow indicates up-regulated gene expression, black stop line down-regulated gene expression.

However, divergence mRNA ratios were measured upon cold and heat stress. Rap2.4a transcript ratios were elevated upon heat stress (Fig. 17) but down-regulated by cold treatment (Fig. 13). Rap2.4g and Rap2.4h were co-regulated upon cold stress treatment in dependence on stress duration (Fig. 14).

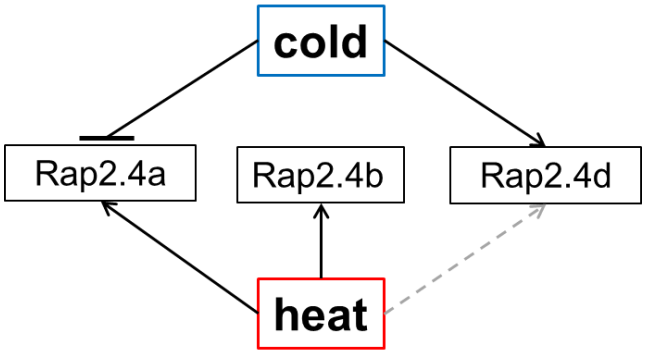

Fig. 19: Exposure of regulatory effects on the Rap2.4a, Rap2.4b and Rap2.4d transcript level upon cold and heat stress; based on qPCR results; black arrow indicates up-regulated gene expression, black stop line down-regulated gene expression. Broken line demonstrates no distinct change.

Previous work showed upon Rap2.4a knock-down an impairing impact on other ERFIb genes (Shaikhali et al. 2008). For example, the Rap2.4b transcript level was elevated, whereas the expression level of Rap2.4c was not affected. In summary the data suggests a possible dependency like competition or compensation between Rap2.4 genes.

Various reports describe dependency between TFs. For example, Zhang and co-workers (2003) reported about compensation by functional redundancy for stress responsive TGA transcription factors. Mu and colleagues (2013) demonstrated partially redundant roles in developmental processes for NF-Y transcription factors in Arabidopsis. A similar assumption was published for SHINE transcription factors (Shi et al. 2011). On the contrary, several WRKY transcription factors are supposed to act antagonistically in regulating pathogen responses (Xu et al. 2006). MADS transcription factors are supposed to antagonize for binding sites (Smaczniak et al. 2012); a competitive regulation was also shown between bZIP proteins (Schindler et al. 1992).

To investigate the possibility of similar regulation concerning compensation or competition between ERFIb genes, two different reverse genetics approaches were used and analyzed by qPCR:

- T-DNA insertion lines for all available Rap2.4 genes were selected and analyzed for homozygous T-DNA insertions. Subsequently the effect upon down-regulation on the remaining ERFIb transcripts was analyzed.

- Single AP2 transcription factors were transiently overexpressed to analyze the transcriptional reaction between the ERFIb genes.

3.3.1.1 Identification of homozygous T-DNA insertion lines using PCR-based genotyping

Using the online tool T-DNA express (http://signal.salk.edu/cgi-bin/tdnaexpress), T-DNA insertion lines were selected. For Rap2.4e no homozygous T-DNA insertion line was available. A homozygous deficiency in this gene possibly causes lethality.

Preference was set to lines with putative insertions in the exon region. Such an insertion has a high probability to affect the transcript level negatively and to inhibit or reduce the protein expression (Wang et al. 2008). Seeds from chosen and available ERFIb T-DNA insertion lines were received from the Nottingham Arabidopsis Stock Center (UK) and grown on soil. Genomic DNA from two week old plants was used to test the homozygosity of the T-DNA insertion. PCR based genotyping with primer pairs specific for the respective ERFIb gene (RP primer) and the left T-DNA border (LB primer) were used to verify if the T-DNA in localized at the predicted site. In a second PCR with LP and RP primer encasing the T-DNA insert position and specific for the appropriate gene the lines were tested for homozygosity as the PCR showed an amplicon of correct size only in wild-type plants. The positions of T-DNA insertions according to T-DNA express are indicated in figure 20, whereas all insertions are located in the only exon present.

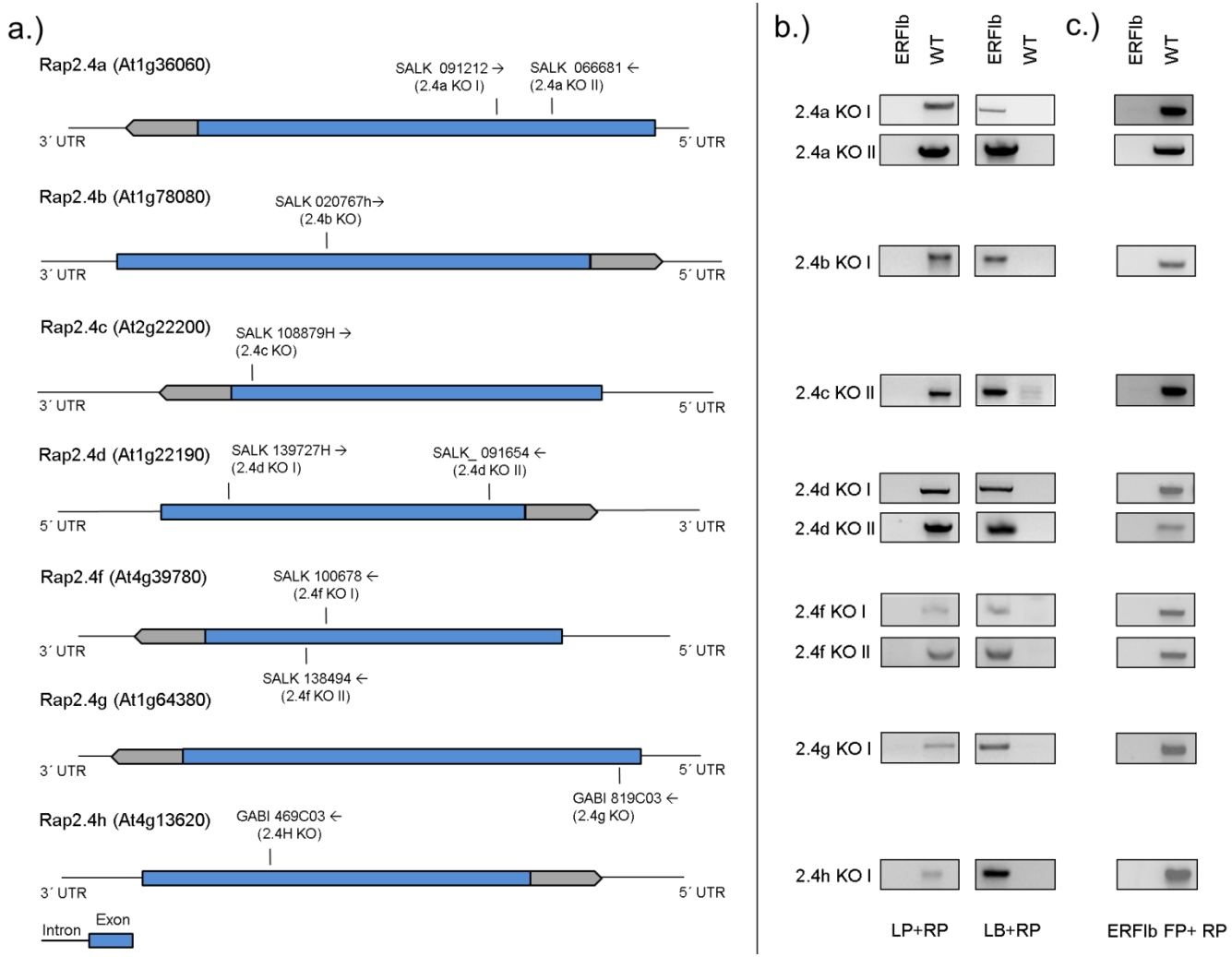

Fig. 20: a.) ERFIb T-DNA insertion lines with position and direction of each insert; genotyping confirmation for homozygous genotype in the analyzed Rap2.4 T-DNA lines b.) PCR with LP and RP primer, encasing the T-DNA insert position and specific for the appropriate gene, showed an amplicon of correct size only in wild type plants**;** PCR reaction with ERFIb gene (RP primer) and the left T-DNA border (LB primer) were used to verify the T-DNA insertion **c.)** PCR with the primer pair ERFIb FP and LP confirmed T-DNA insertion, since no amplification is present due to the T-DNA insert compared with Col-0 wt.

3.3.1.2 Determination of T-DNA copy number

The integration of T-DNA into the genome results not necessary in a single copy (Alonso et al. 2003; Ülker et al. 2008). Before further analysis, the number of T-DNA inserts was analyzed, which should be optimally single ones for the T-DNA line of interest. In case of several T-DNA insertions, an additional confirmation by a second T-DNA insertion line for the same gene was performed to affirm the effects observed and to exclude that possible results are not caused by a nearby mutation (Radhamony et al. 2005). For copy-number determination a qPCR approach (Yang et al. 2012) was performed with genomic DNA from homozygous ERFIb T-DNA insertion plants. Since the T-DNA insertion lines for Rap2.4g (2.4g KO) and for Rap2.4h (2.4h KO) are confirmed to have single T-DNA insertions according to the line specific information (http://www.gabi-kat.de/), the T-DNA insertion line 2.4h KO served as single copy control.

Tab. 14: C_T values based on the T-DNA control gene 35S and the endogenous control PrxQ; Estimates of T-DNA copy number in T-DNA insertion lines.

T-DNA line	T-DNA control C_T value	$(E_T)\ \Delta\ C_T\ T$	Endogenous control C_T value	$(E_C)\ \Delta\ C_T\ C$	$2 * (E_T)\ \Delta\ C_T\ T / (E_C)\ \Delta\ C_T\ C$	Copy number
2.4a KO I	15.24 ± 0.09	1.71	19.45 ± 0.15	7.67	9.00	9
2.4a KO II	20.03 ± 0.49	0.07	24.06 ± 0.64	0.28	7.94	8
2.4b KO	22.1 ± 0.08	0.24	22.99 ± 0.76	0.07	0.55	1
2.4c KO	17.79 ± 0.33	1.31	19.02 ± 0.39	2.30	1.14	1
2.4d KO I	15.25 ± 0.09	1.67	19.48 ± 0.19	7.62	9.13	9
2.4d KO II	15.81 ± 0.61	5.17	20.13 ± 0.34	1.06	9.71	10
2.4f KO I	20.29 ± 0.46	0.23	22.78 ± 0.02	0.17	2.71	3
2.4f KO II	21.53 ± 0.13	0.10	22.23 ± 0.03	0.25	0.49	1
Negative control	-	0.00	20.16 ± 0.25	1.04	0.00	0
Single-copy control	18.18 ±0.08	-	19.77 ± 0.32	-	-	1

The results showed for Rap2.4a, that line 2.4a KO I includes more than 5 T-DNA insertions, consequently the second T-DNA insertion line 2.4a KO II was also used for additional analysis. For Rap2.4b and Rap2.4c single T-DNA insertions were determined. For Rap 2.4d the line 2.4d KO I showed more than 5 T-DNA copies, the second line 2.4d KO II was included for analysis. For Rap2.4e no homozygous T-DNA insertion line could be selected. Hence, this gene could not be tested in this analysis procedure. The line 2.4f KO I displays 3 T-DNA copies, therefore the second line 2.4f KO II was also considered for analysis.

For the lines 2.4a KO I and 2.4d KO I additional Southern-blot hybridizations (Fig. 21) with probes against the T-DNA were performed to verify the results obtained with the qPCR method. For both lines approximately 6-7 T-DNA insertions could be counted. Southern blotting may not exactly estimate the copy number due to the integration in tandem repeats with variance regarding the orientation of the T-DNA (Kim et al. 2003; Makarevitch et al. 2003; Zhang et al. 2008; Yang et al. 2012). Therefore, qPCR results can be regarded as more precise in case of copy number determination (Yang et al. 2012).

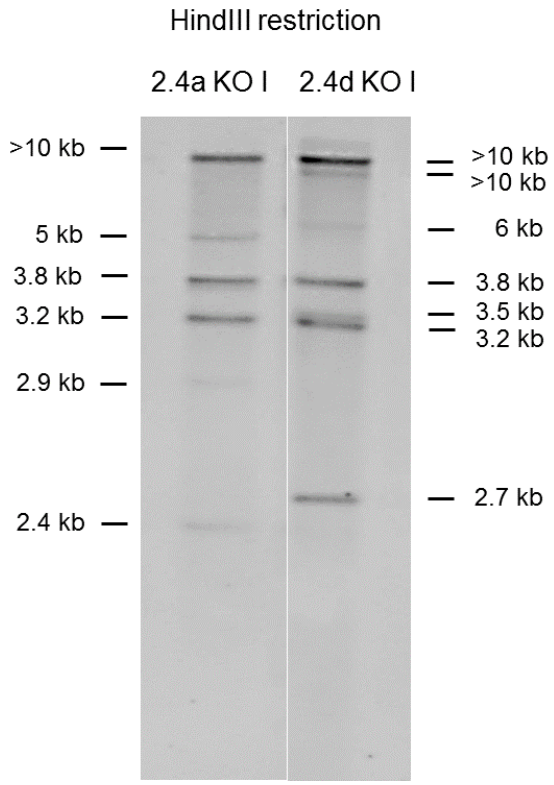

Fig. 21: Estimation of T-DNA insertions for the Rap2.4a KO I and Rap2.4d KO I T-DNA insertion lines with Southern-blot after HindIII restriction; the resulting fragments showed a putative amount of 6 copies for Rap2.4a KO I line and 7 copies for Rap2.4d KO I line.

3.3.2 Effect on plant growth due to ERFIb deficiency in T-DNA insertion lines

To check whether the ERFIb knock-downs affect the plant growth, the rosette diameters and the leaf numbers were quantified in 4 week old plants from each ERFIb KO line.

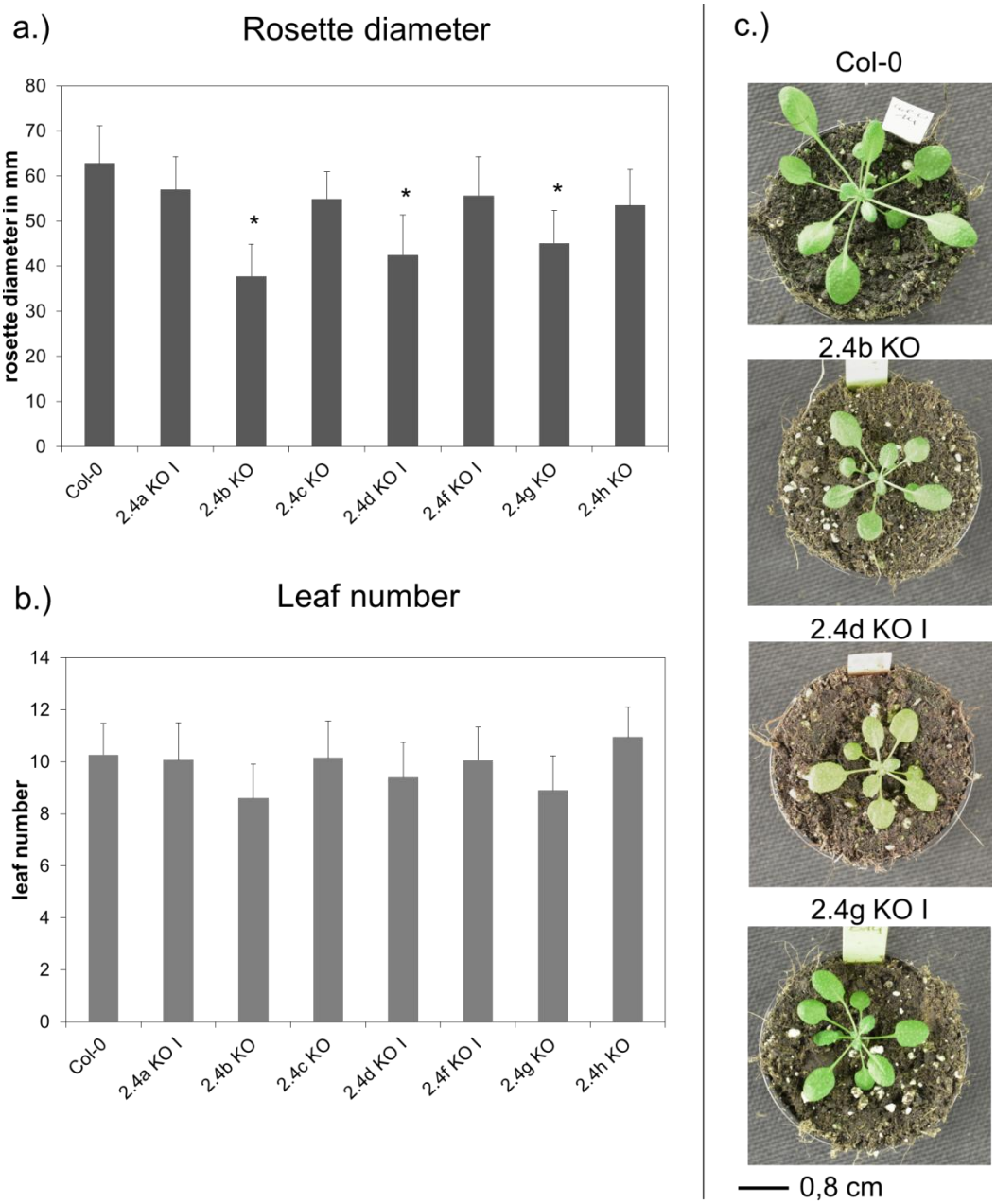

Fig. 22: a.) Rosette diameter and b.) leaf number of Col-0 wt and homozygous ERFIb KO plants grown under standard conditions for 4 weeks; 15-20 plants for each T-DNA insertion line were used. * indicates significant differences from the control sample Col-0 wt (Student *t*-test, *p<0.05) **c.) Pictures show exemplarily 4 week old homozygous plants from Rap2.4b, Rap2.4d and Rap2.4g KO I lines with decreased rosette diameter**.

Taken the rosette diameter as a parameter for plant growth (Fig. 22a), a significant decrease was demonstrated for the lines 2.4b KO, 2.4d KO I and 2.4g KO. The remaining T-DNA insertion lines displayed no difference towards Col-0 wild-type. The data's suggest an involvement of ERFIb genes in plant growth or development regulation, especially for Rap2.4b and Rap2.4d. Noteworthy, a similar decrease was also available in the Rap2.4d KO II line. Referring to developmental expression data obtained from Genevestigator (Fig. 10), Rap2.4b was expressed at high levels in the rosette stage that is comparable to the 4 week old plants used (Fig. 20b). Thus, the Genevestigator data would support at least in case of Rap2.4b a putative relevance in development. Genome wide analysis of transcriptomic changes during different developmental steps in Arabidopsis revealed that important genes were highly expressed at the corresponding time point (Ma et al. 2005).

3.3.3 Effect on photochemical efficiency of Photosystem II due to ERFIb deficiency in T-DNA insertion lines

To check if an impaired ERFIb expression affects functionality of the photosynthetic machinery, the photochemical efficiency of Photosystem II (PSII) was measured, after 20 min dark incubation, with a PAM fluorometer. The resulting value F_v/F_m determines the maximum quantum efficiency of PSII and derives from the ratio between measured maximal (F_{max}), and initial (F_o) fluorescence (Sperdouli et al. 2012).

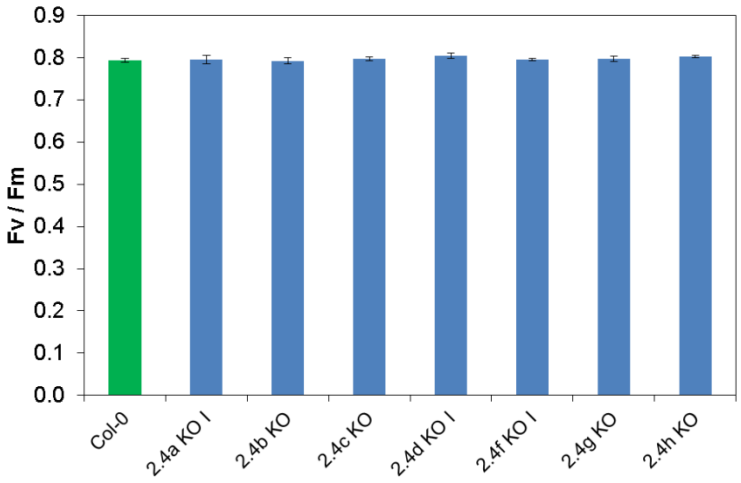

Fig. 23: The maximum photochemical efficiency of PSII reaction (F_v/F_m) in Col-0 wt and homozygous ERFIb KO I plants grown under standard conditions for 4 weeks; 10 plants for Col-0 wt and each T-DNA insertion line were used prior 20 min of dark adaption.

Decreasing changes in the F_v/F_m ratio would reflect decreasing PSII activity as well as an impaired integrity of the D1 protein in the PSII reaction center (Armbruster et al. 2010).

The results revealed no significant difference in PSII integrity and functionality between Col-0 wt and the T-DNA insertion lines, demonstrating that ERFIb genes do not affect PSII formation under standard light conditions.

The LHC availability correlates with the chlorophyll content (Hemelrijk et al. 1992). Hence a visible change in leaf color could deliver crude information about differences in the sizes or numbers of the light-harvesting antenna. Nevertheless, no visible change in leaf color was present among the analyzed Rap2.4 T-DNA insertion lines.

It should be mentioned that upon Rap2.4a knock-down several LHC associated genes displayed decreased transcript amounts (Shaikhali et al. 2008). They encode light-harvesting chlorophyll a/b-binding proteins and are part of PSI or II (Jansson 1999).

According to the results obtained (Fig. 23) Rap2.4b knock-down does not impair the maximum photochemical efficiency of PSII reaction efficiency under standard light conditions. However, seedlings over-expressing Rap2.4b and grown under continuous far-red, red and blue light conditions displayed decreased mRNA levels for CAB3 (Lin et al. 2008). CAB3 is important for chlorophyll accumulation (Sun et al. 2011). Nevertheless, no effect on chlorophyll content could be detected upon Rap2.4b knock-down or overexpression (Lin et al. 2008).

For Rap2.4d no PSII or photosynthesis related genes were available in the co-expression analysis (Tab. 11; Tab. 13). Nonetheless, the advanced Y1H analysis for Rap2.4d binding targets revealed several photosynthesis involved genes (PhD thesis, Jote Bulcha).

Yet several authors report about general enhanced photosynthetic capability related to AP2 transcription factors but without special reference to PSII or associated parts of photosynthesis. For example, an improvement in photosynthesis was demonstrated after overexpression of AP2 transcription factors like AtCBF1 or AtNFXL1 in Arabidopsis (Hsieh et al. 2002b; Lisso et al. 2006) or GhDREB1 in cotton (Shan et al. 2007). The enhancement is explained as a positive side effect since these transcription factors act as upstream regulators and are mainly responsible for systemic abiotic stress responses after drought or cold, whereas photosynthesis is mostly also negatively affected (Saibo et al. 2009).

3.3.4 ERFIb genes in homozygous T-DNA insertion lines

To investigate the possibility of similar regulation concerning compensation or competition between ERFIb genes, like reported exemplarily for TGA or WRKY transcription factors (Zhang et al. 2003; Xu et al. 2006), T-DNA insertion lines for all available Rap2.4 genes were selected and analyzed for homozygous T-DNA insertions. To analyze the effect upon down-regulation on the other ERFIb transcripts, mRNA from homozygous T-DNA insertion plants was isolated for qPCR analysis. In case of several insertions, a second T-DNA insertion line was included for the appropriate genes.

3.3.4.1 Transcriptional changes upon ERFIb genes in Rap2.4a deficient plants

Since the T-DNA copy number for 2.4a KO I line showed more than 5 insertions (Tab. 14), the additional line 2.4a KO II, which has 8 insertions, was also used for mRNA level analysis.

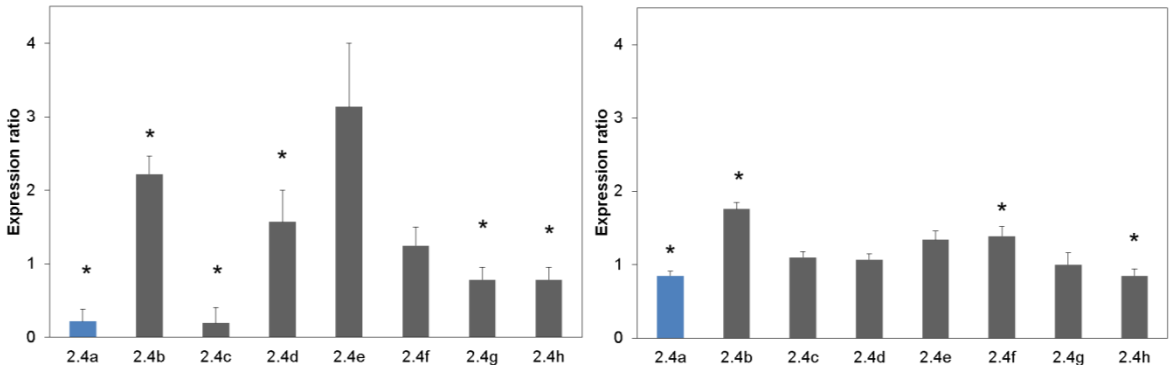

Fig. 24: Relative ERFIb transcript levels in the homozygous lines Rap2.4a KO I (left) and Rap2.4a KO II (right); rosette leaves from 3 independent plants were harvested and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to relative Act7 transcript amount in Col-0 wt plants. The relative transcript levels of Col-0 wt were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from control Col-0 wt plants.

The strongest analogy in expressional changes between both T-DNA insertion lines was demonstrated through the up-regulation of Rap2.4b and the down-regulation of Rap2.4h. The elevated Rap2.4b transcript level in Rap2.4a deficient plants was also reported by Shaikhali and co-workers (2008).

In the KO I line the Rap2.4a transcript level was down-regulated (0.2 ± 0.08). Rap2.4b, Rap2.4c as well as Rap2.4e and Rap2.4f showed up-regulated expression values. In case

of Rap2.4b the mRNA level was 2-fold higher, for Rap2.4e even 3-fold higher (3.1 ± 0.86) compared with Col-0 wild-type. Rap2.4d, Rap2.4g and Rap2.4h were negatively impaired. Notably, Rap2.4c displayed the strongest down-regulation (0.2 ± 0.1).

The KO II line displayed weak down-regulation of the target gene Rap2.4a (0.8 ± 0.02). The genes Rap2.4b and Rap2.4c were elevated in the expression, whereas Rap2.4b showed the highest up-regulation (1.8 ± 0.04).

It can be presumed that Rap2.4c, e, f and g changed the transcript level in correlation with the level of Rap2.4a knock-down. For example, the Rap2.4c mRNA ratio was decreased to a lower transcript level upon strong reduction of Rap2.4a transcript abundance in the Rap2.4a KO I line. However, in the Rap2.4a KO II line the Rap2.4c mRNA level was not significantly down-regulated (1.1 ± 0.04). In case of Rap2.4e an opposite effect was monitored: a 3-fold higher Rap2.4e transcript amount was available upon appropriate high Rap2.4a down-regulation in the KO I line. For comparison in the KO II line the Rap2.4e expression level was slightly elevated (1.3 ± 0.06) upon Rap2.4a knock-down (0.8-fold). Thus, for Rap2.4d and Rap2.4e a loop-feedback mechanism on mRNA level could be feasible.

3.3.4.2 Transcriptional changes upon ERFIb genes in Rap2.4b deficient plants

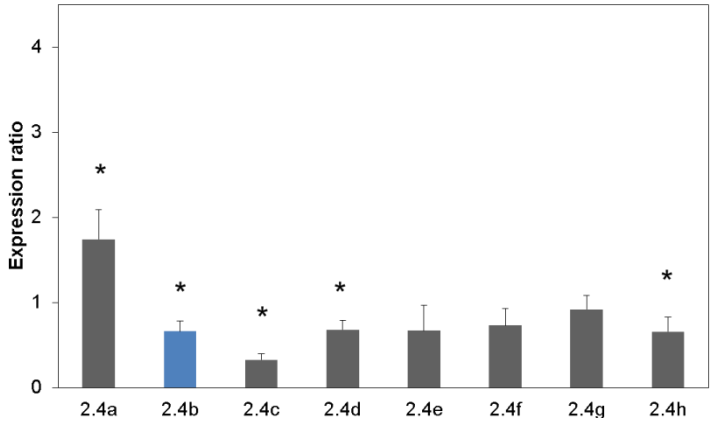

Fig. 25: Relative ERFIb transcript levels in the homozygous Rap2.4b T-DNA insertion line; rosette leaves from 3 independent plants were harvested and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to relative Act7 transcript amount in Col-0 wt plants. The relative transcript levels of Col-0 wt were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post-hoc analysis; Bars with * are significantly different ($p<0.05$) from control Col-0 wt plants.

The knock-down of Rap2.4b resulted in an increased Rap2.4a mRNA level (1.7 ± 0.5). All other ERFIb genes were down-regulated in their transcript abundance. Especially Rap2.4c was strongly down-regulated (0.3 ± 0.07). Rap2.4d, e and f showed similar low relative transcript levels (0.66 - 0.73). Rap2.4g was slightly decreased (0.9 ± 0.16); the Rap2.4h mRNA level was significantly down-regulated (0.6 ± 0.2).

3.3.4.3 Transcriptional changes upon ERFIb genes in Rap2.4c deficient plants

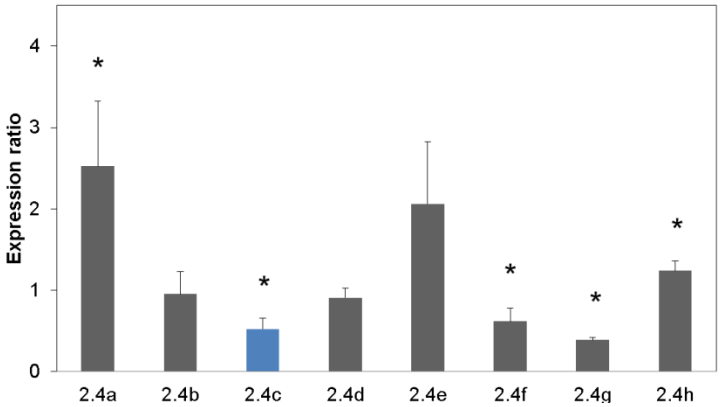

Fig. 26: Relative ERFIb transcript levels in the homozygous Rap2.4c T-DNA insertion line; rosette leaves from 3 independent plants were harvested and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to relative Act7 transcript amount in Col-0 wt plants. The relative transcript levels of Col-0 wt were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post-hoc analysis; Bars with * are significantly different ($p<0.05$) from control Col-0 wt plants.

Upon Rap2.4c knock-down (0.5 ± 0.1), Rap2.4a (2.5 ± 0.8) and Rap2.4e (2 ± 0.7) displayed increased transcript levels. The expression ratios for Rap2.4b, c, f and h showed no significant change. Rap2.4g (0.4 ± 0.03) was down-regulated.

3.3.4.4 Transcriptional changes upon ERFIb genes in Rap2.4d deficient plants

The copy number estimation for the 2.4d KO I line resulted in more than 5 insertions (Tab. 14). Therefore, the second line 2.4d KO II was also used for transcriptional analysis.

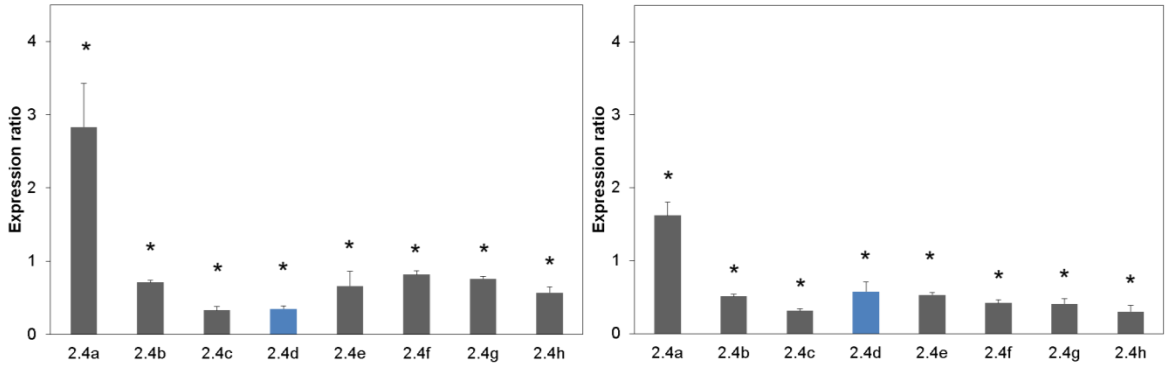

Fig. 27: Relative ERFIb transcript levels in the homozygous lines Rap2.4d KO I (left) and Rap2.4d KO II (right); rosette leaves from 3 independent plants were harvested and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to relative Act7 transcript amount in Col-0 wt grown under 20°C. The relative transcript levels of Col-0 wt were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post-hoc analysis; Bars with * are significantly different ($p<0.05$) from control Col-0 wt plants.

The T-DNA insertion strongly decreased the Rap2.4d mRNA level in both analyzed Rap2.4d KO lines (2.4d KO I= 0.3 ± 0.02; 2.4d KO II = 0.5 ± 0.01). Moreover, in both T-DNA insertion lines an elevated Rap2.4a transcript ratio was observed, at which the expression was highly elevated in the Rap2.4d KO I line (2.8 ± 0.6). All other ERFIb genes showed down-regulated mRNA abundances. Especially Rap2.4h was comparably negatively affected in both T-DNA insertion lines and displayed the lowest values (2.4d KO I= 0.6 ± 0.1; 2.4d KO II = 0.3 ± 0.01).

3.3.4.5 Transcriptional changes upon ERFIb genes in Rap2.4f deficient plants

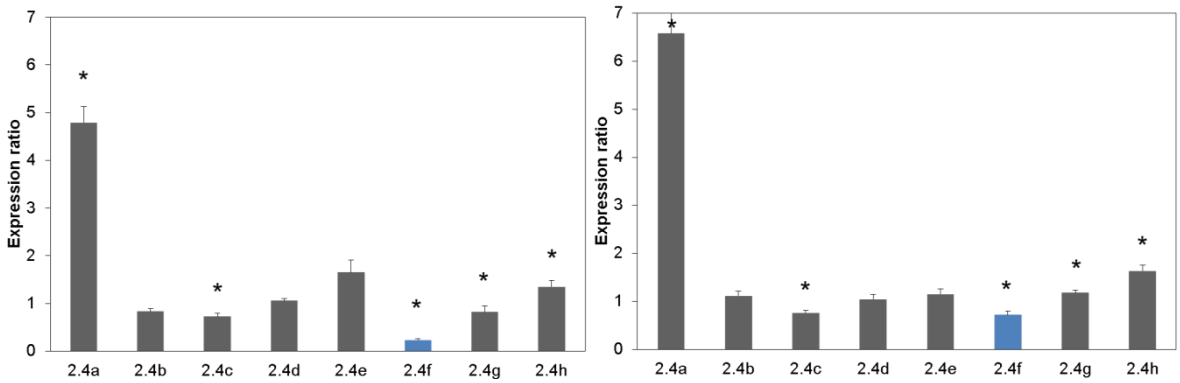

Fig. 28: Relative ERFIb transcript levels in the homozygous Rap2.4f T-DNA insertion lines KO I (left) and KO II (right); rosette leaves from 3 independent plants were harvested and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to Act7 transcript amount in Col-0 wt plants. The relative transcript levels of Col-0 wt were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from control Col-0 wt plants.

The Rap2.4f transcript amount was in both lines due to the T-DNA insertion decreased, whereas in the KO I line the mRNA level was stronger affected (Rap2.4f KO I = 0.2 ± 0.01; Rap2.4f KO II = 0.7 ± 0.03). The Rap2.4a transcript level was highly induced in both lines (Rap2.4f KO I = 4.8 ± 0.3; Rap2.4f KO II = 6.6 ± 0.7). Also Rap2.4h mRNA was up-regulated in both lines (Rap2.4f KO I = 1.3 ± 0.13; Rap2.4f KO II = 1.6 ± 0.06). The remaining ERFIb genes demonstrated expressional changes with dependency towards the Rap2.4f mRNA level. For instance, the Rap2.4e transcript level was higher (1.7 ± 0.12) in case of stronger Rap2.4f down-regulation in the KO I line. In contrast, the Rap2.4e transcript level was only slightly elevated in the KO II line (1.2 ± 0.05).

3.3.4.6 Transcriptional changes upon ERFIb genes in Rap2.4g deficient plants

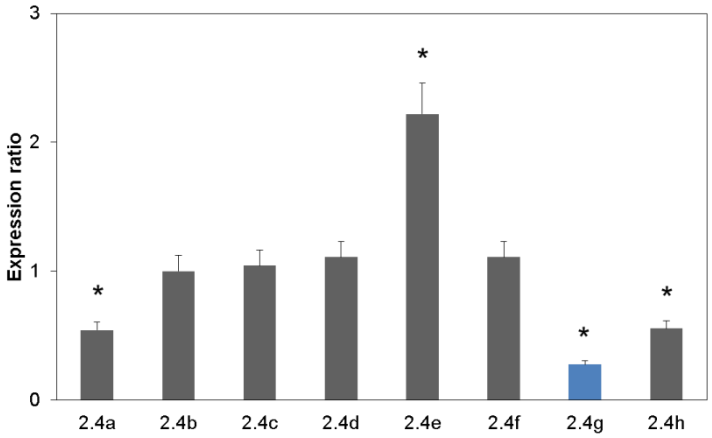

Fig. 29: Relative ERFIb transcript levels in the homozygous Rap2.4g T-DNA insertion line; rosette leaves from 3 independent plants were harvested and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to relative Act7 transcript amount in Col-0 wt plants. The relative transcript levels of Col-0 wt were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from control Col-0 wt plants.

Upon decreased Rap2.4g transcript abundance, Rap2.4a and Rap2.4h were down-regulated. Rap2.4e showed up-regulated transcript level (2.2 ± 0.24). Rap2.4b, c, d and f were not especially affected in the relative transcript amount.

3.3.4.7 Transcriptional changes upon ERFIb genes in Rap2.4h deficient plants

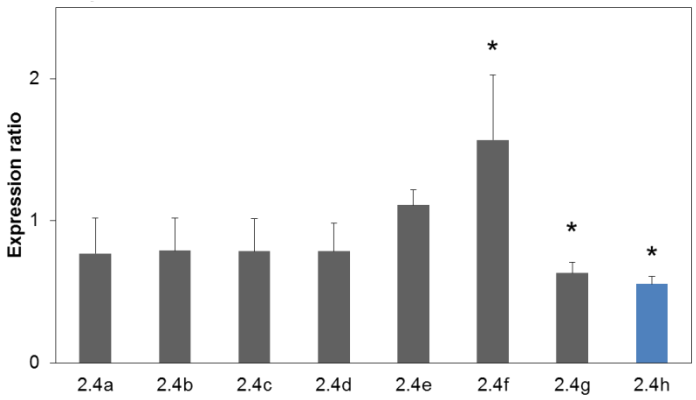

Fig. 30: Relative ERFIb transcript levels in the homozygous Rap2.4h T-DNA insertion line; rosette leaves from 3 independent plants were harvested and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to Act7 transcript amount in Col-0 wt. The relative transcript levels of Col-0 wt were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from control Col-0 wt plants.

In case of Rap2.4h knock-down, the Rap2.4f mRNA ratio was elevated (1.6 ± 0.45), the Rap2.4e transcript amount displayed slight elevated ratio (1.1 ± 0.1). The expression of Rap2.4a, b, c and d was down-regulated to similar value of 0.8, however, without significance. Moreover, only Rap2.4g demonstrated significant down-regulation (0.6 ± 0.07).

3.3.4.8 General view about ERFIb dependency upon particular Rap2.4 knock-down

The knock-down analysis of individual ERFIb genes demonstrated major influences between ERFIb genes. The results were summarized in a matrix (Fig. 31) to study putative interplays on the transcriptional level within the ERFIb family.

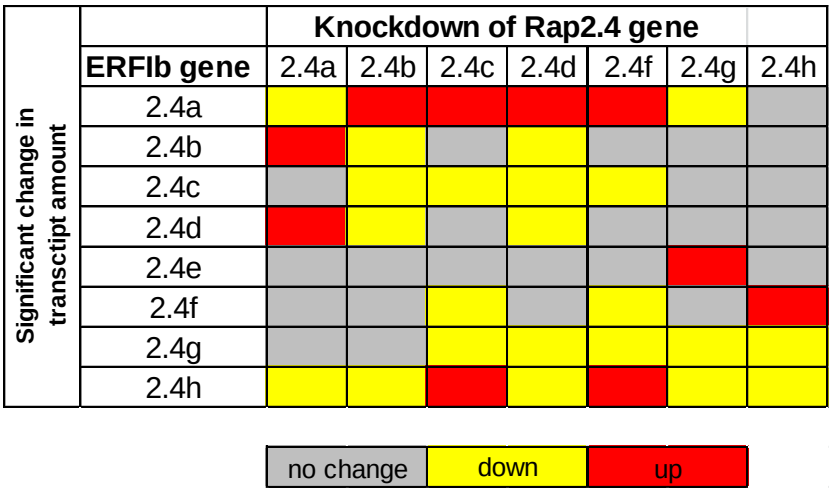

Fig. 31: Interplay between ERFIb after individual Rap2.4 knock-down according to qPCR data; appropriate colors display no change; down-regulation or up-regulation of relative transcript amount; only significant values are indicated, in case of data originating from two T-DNA insertion lines for a particular gene, only distinct and significant changes in transcript abundance present in both lines are indicated.

The overview about the leverage of individual ERFIb knock-down and transcriptional modulation, respectively, displays several features:

- Rap2.4a is up-regulated in various ERFIb deficient plants. Outstanding is the inverse expressional pattern between Rap2.4a and Rap2.4b as well as Rap2.4d. Furthermore, nearly the same expressional profile of ERFIb is present after knock-down of Rap2.4b or d.
- Rap2.4h is down-regulated in most T-DNA insertion lines, except upon Rap2.4c and Rap2.4f knock-down. Moreover, lack of Rap2.4h affects the transcript level of remaining ERFIb genes with the weakest effect. Rap2.4g is often negatively co-regulated with Rap2.4h, suggesting inverse transcription control.
- Another determinant is the common decrease in transcript amount of ERFIb genes after the knock-down of Rap2.4b as well as Rap2.4d. On the opposite, these transcription factors are not exceptionally regulated after the knock-down of other ERFIb genes.
- Rap2.4e depicts an exceptional case because its transcript abundance is exclusively up-regulated after Rap2.4g knock-down; the knock-down of other ERFIb genes remains ineffective.

3.3.5 Exemplarily compensation between Rap2.4b and Rap2.4d expression upon salt stress in Rap2.4b T-DNA insertion line

The initial analysis of Rap2.4b and Rap2.4d demonstrated a tissue dependent co-expression upon salt-stress treatment (Fig. 17). A potential transcriptional dependency between these transcription factors could be demonstrated upon Rap2.4b knock-down, where Rap2.4d transcript amount was negatively impaired. To further investigate a putative compensation, a salt stress experiment was performed with homozygous Rap2.4b KO line. The relative Rap2.4b and Rap2.4d expression ratio was analyzed by qPCR.

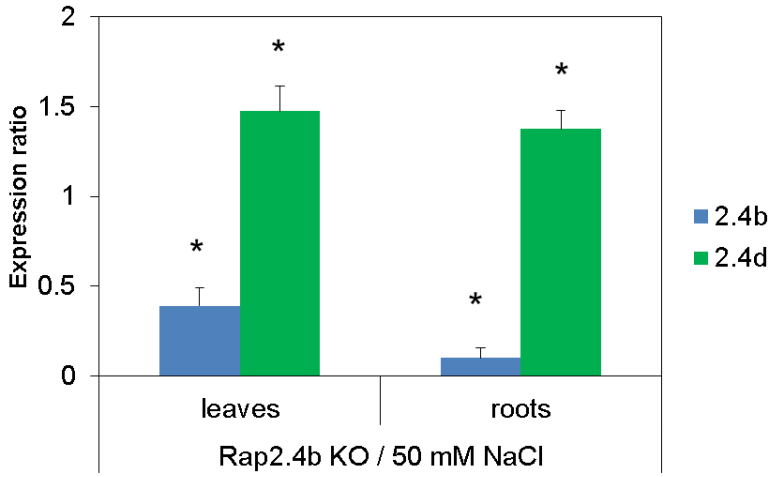

Fig. 32: Relative transcript level of Rap2.4b and Rap2.4d in Rap2.4b KO line in leaves and roots, grown on vertical MS plates with 50 mM NaCl; plant material was pooled from three independent plants and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to Act7 transcript in Col-0 wt grown on NaCl free MS medium plates. The relative transcript levels of Col-0 wt grown on NaCl free MS medium plates were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from control plants grown on NaCl free plates.

Under standard conditions and Rap2.4b knock-down the relative Rap2.4d transcript level was also suppressed (Fig. 25). The situation changed when plants, deficient in Rap2.4b expression, were exposed to 50 mM NaCl. Under these conditions, the lack of Rap2.4b transcript in leaves was balanced by increased Rap2.4d expression (Fig. 32 left). This is contrary to the situation in Col-0 wild-type, where upon 50 mM salt-stress treatment the Rap2.4d transcript abundance was decreased in leaf tissue and Rap2.4b expression level was slightly up-regulated (Fig. 17).

The experiment demonstrated a contingent compensatory regulation on transcript level between Rap2.4b and Rap2.4d in leaf tissue (Fig. 33) and refines the *in silico* result of co-regulation upon abiotic stress with the possibility of compensation.

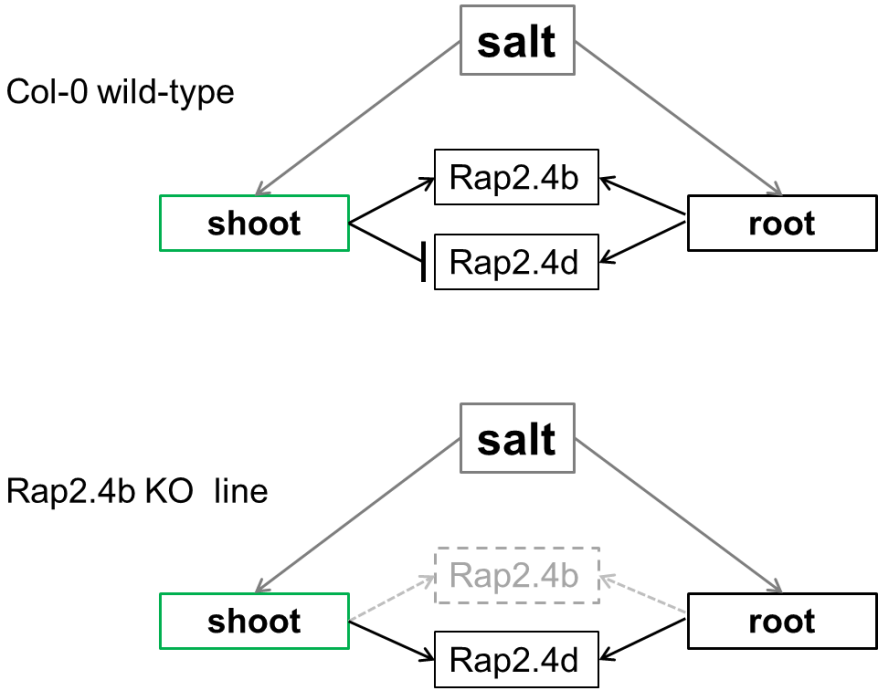

Fig. 33: Exposure of compensatory effects between Rap2.4b and Rap2.4d transcript level upon salt stress treatment in comparison to Col-0 wt; based on qPCR results; black arrow indicates up-regulated gene expression, black stop line down-regulated gene expression. Grey, broken line demonstrates decreased Rap2.4b transcript amount due to T-DNA insertion.

3.3.6 Effect on ERFIb transcript levels upon transient Rap2.4 overexpression

The evaluation of ERFIb T-DNA insertion lines suggested putative interplay within the ERFIb family. The knockdown of single ERFIb transcription factor specifically impacted on the remaining ERFIb members. Down-regulation indicates dependency between transcription factors, i.e. Rap2.4g and Rap2.4h. Up-regulation is feasible in case of compensation between Rap2.4b and Rap2.4d, Rap2.4a and Rap2.4b are inversely regulated in case of one-sided knock-down (Fig. 31).

To further test a putative interplay between ERFIb genes, the controlled overexpression of single ERFIb transcription factors was analyzed. For this approach an estradiol inducible system was established, using the pMDC7 plasmid (Zuo et al. 2000; Curtis et al. 2003).

The estradiol system does not harmfully influence the plants and can be precisely controlled due to its very low basal expression level (Zuo et al. 2000).

12 day old Arabidopsis plantlets were transiently transformed by vacuum infiltration with Agrobacteria containing the appropriate pMDC7 plasmid. After transfection the plantlets were transferred on freshly prepared petri dishes with solid MS medium containing estradiol and cefotaxime.

Estradiol induces the expression (Zuo et al. 2000; Curtis et al. 2003) and cefotaxime prevents further growing of present Agrobacteria on transfected plantlets. Several publications confirm the usability of cefotaxime in case of Agrobacteria related transformations, without secondary effects for the plants (Yang et al. 2008; Ahmed et al. 2011).

After two days incubation under standard conditions, all plantlets from one petri dish were collected for RNA isolation and additional cDNA synthesis for qPCR mediated transcript control. Col-0 wild-type plantlets transformed with empty pMDC7 plasmid served as mock-control.

3.3.6.1 Estimation of optimal estradiol concentration and correlation with transcript amounts

Initial experiments were performed to verify the functionality of estradiol induction and correlation between estradiol concentrations versus the resulting transcript amount. For this, Rap2.4c introduced in pMDC7 plasmid was used for transient transformation. A good relation between high induction levels and an estradiol concentration from 5 µM was reported (Zuo et al. 2000). Therefore, the concentrations of 5 µM and 10 µM estradiol were tested.

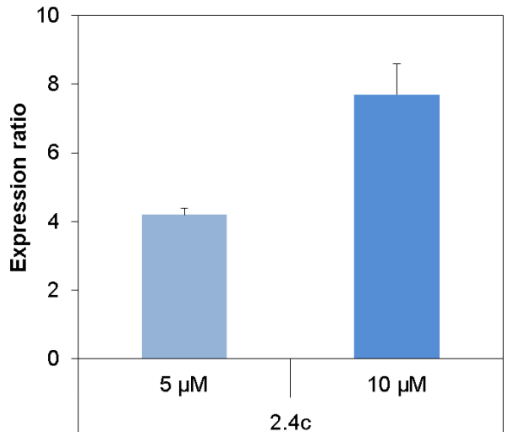

Fig. 34: Relative transcript level of Rap2.4c upon estradiol induced transient overexpression (5 and 10 µM); 16 plantlets were pooled from 2 replicates and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to the Act7 transcript level in Col-0 wt plants which were transiently transformed with empty plasmids. The relative transcript level of Col-0 wt, transiently transformed with empty plasmids, was set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM).

The result displayed a good dependency between estradiol concentration and relative transcript amount. Here, ideally upon doubled estradiol concentration the transcript level displayed nearly twice the number (4-fold versus 7.7-fold).

3.3.6.2 The effect of transient Rap2.4a overexpression on the ERFIb transcript levels

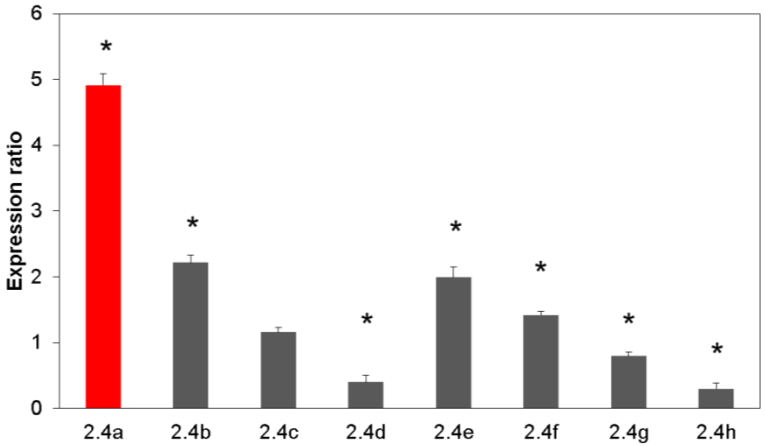

Fig. 35: Relative transcript level of Rap2.4a (red bar) upon estradiol induced transient overexpression (10 µM); 16 plantlets were pooled from 2 replicates and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to the relative Act7 transcript level in Col-0 plants transiently transformed with empty plasmids. The relative transcript levels of Col-0 wt transiently transformed with empty plasmids were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). Results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from Col-0 wt plants transiently transformed with empty plasmids.

The overexpression of Rap2.4a affected all other ERFIb genes except Rap2.4c. Rap2.4b and Rap2.4e were 2-fold up-regulated. Rap2.4d and h were strongly decreased in expression level. For Rap2.4f, slightly increased transcript abundance was displayed.

3.3.6.3 The effect of transient Rap2.4b overexpression on the ERFIb transcript levels

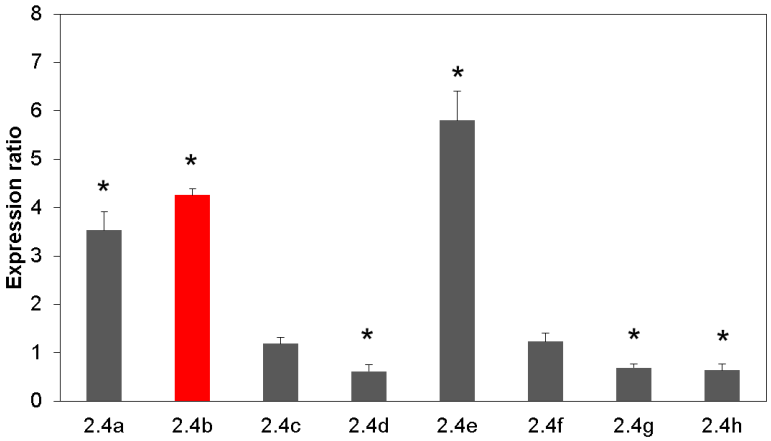

Fig. 36: Relative transcript level of Rap2.4b (red bar) upon estradiol induced transient overexpression (10 µM); 16 plantlets were pooled from 2 replicates and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to the relative Act7 transcript level in Col-0 plants transiently transformed with empty plasmids. The relative transcript levels of Col-0 wt transiently transformed with empty plasmids were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). Results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from Col-0 wt plants transiently transformed with empty plasmids.

After the overexpression of Rap2.4b, Rap2.4a (3.5-fold) and Rap2.4e (5.8-fold) transcript levels were up-regulated. Contrary, Rap2.4d, g and h were decreased (0.5-fold) in relative mRNA level. Rap2.4c and Rap2.4f showed no change in transcript content.

3.3.6.4 The effect of transient Rap2.4c overexpression on the ERFIb transcript levels

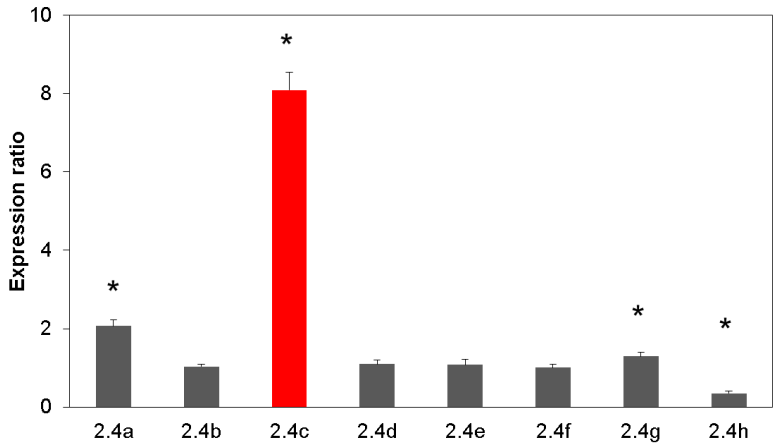

Fig. 37: Relative transcript level of Rap2.4c (red bar) upon estradiol induced transient overexpression (10 µM); 16 plantlets were pooled from 2 replicates and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to the relative Act7 transcript level in Col-0 plants transiently transformed with empty plasmids. The relative transcript levels of Col-0 wt transiently transformed with empty plasmids were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). Results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from Col-0 wt plants transiently transformed with empty plasmids.

The induced Rap2.4c overexpression elevated the transcript level of Rap2.4a (2-fold) and Rap2.4g (1.5-fold). Only Rap2.4h was decimated (0.4-fold). Rap2.4d, e and f were not affected in relative expression ratio.

3.3.6.5 The effect of transient Rap2.4d overexpression on the ERFIb transcript levels

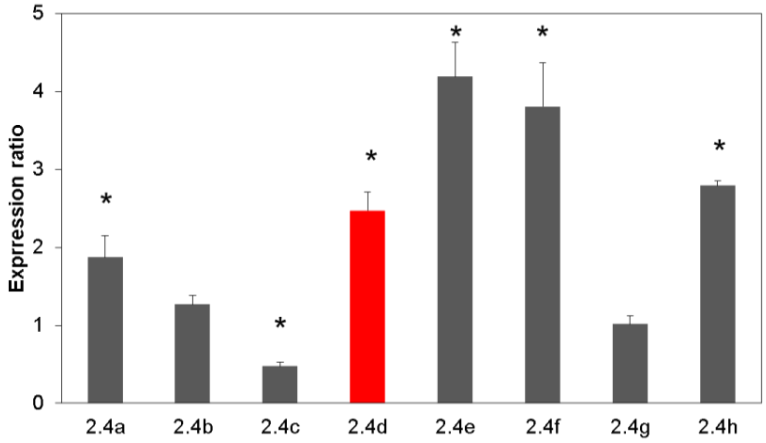

Fig. 38: Relative transcript level of Rap2.4d (red bar) upon estradiol induced transient overexpression (10 µM); 16 plantlets were pooled from 2 replicates and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to Act7 transcript level in Col-0 plants transiently transformed with empty plasmid. The relative transcript levels of Col-0 wt transiently transformed with empty plasmids were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). Results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from Col-0 wt plants transiently transformed with empty plasmids.

The overexpression of Rap2.4d affected the expression level of Rap2.4a (1.9-fold), Rap2.4e (4.2-fold), Rap2.4f (3.8-fold) and h (2.8-fold) positively and Rap2.4c negatively (0.5-fold). Rap2.4b and g were not changed in expression levels.

3.3.6.6 The effect of transient Rap2.4e overexpression on the ERFIb transcript levels

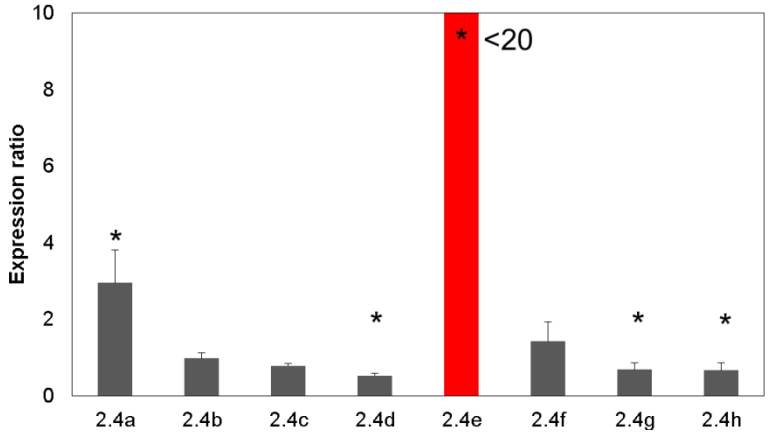

Fig. 39: Relative transcript level of Rap2.4e (red bar) upon estradiol induced transient overexpression (10 µM); 16 plantlets were pooled from 2 replicates and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to the relative Act7 transcript level in Col-0 plants transiently transformed with empty plasmids. The relative transcript levels of Col-0 wt transiently transformed with empty plasmids were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). Results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from Col-0 wt plants transiently transformed with empty plasmids.

The overexpression of Rap2.4e resulted in 3-fold up-regulated Rap2.4a mRNA level. In contrast, Rap2.d (0.5-fold), Rap2.4g (0.7-fold) and Rap2.4h (0.7-fold) were down-regulated. No change in transcript amount was monitored for Rap2.4b, Rap2.4c and Rap2.4f.

3.3.6.7 The effect of transient Rap2.4f overexpression on the ERFIb transcript levels

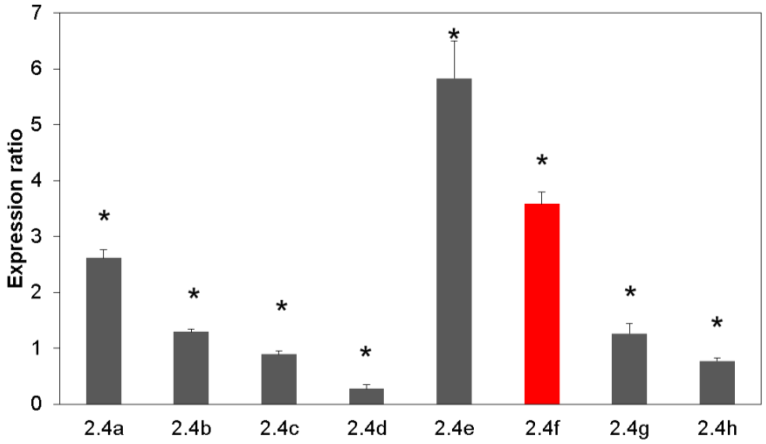

Fig. 40: Relative transcript level of Rap2.4f (red bar) upon estradiol induced transient overexpression (10 µM); 16 plantlets were pooled from 2 replicates and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to the relative Act7 transcript level in Col-0 plants transiently transformed with empty plasmids. The relative transcript levels of Col-0 wt transiently transformed with empty plasmids were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). Results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from Col-0 wt plants transiently transformed with empty plasmids.

Upon Rap2.4f overexpression, Rap2.4b (1.3-fold), Rap2.4e (5.8-fold) and Rap2.4g (1.3-fold) showed elevated expression levels. Rap2.4c (0.9-fold) and Rap2.4h (0.8-fold) were slightly decreased; the Rap2.4d mRNA level was strongly decreased (0.3-fold).

3.3.6.8 The effect of transient Rap2.4g overexpression on the ERFIb transcript levels

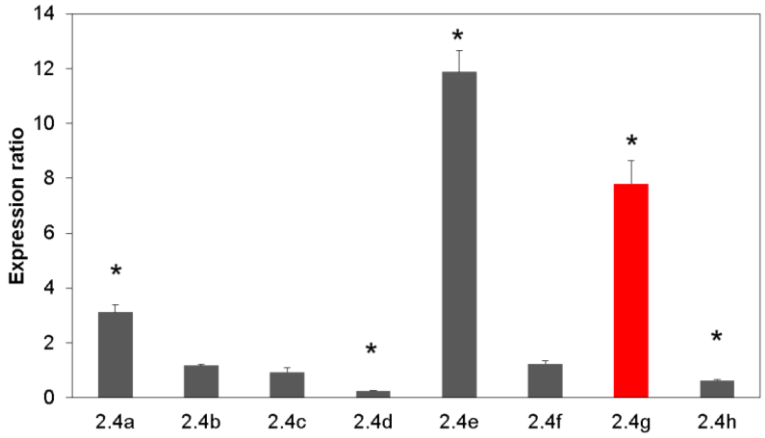

Fig. 41: Relative transcript level of Rap2.4g (red bar) upon 10 µM estradiol induced transient overexpression; 16 plantlets were pooled from 2 replicates and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to the relative Act7 transcript level in Col-0 plants transiently transformed with empty plasmids. The relative transcript levels of Col-0 wt transiently transformed with empty plasmids were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). Results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from Col-0 wt plants transiently transformed with empty plasmids.

The triggered Rap2.4g overexpression caused a decrease in Rap2.4d (0.3-fold) and Rap2.4h (0.6-fold) transcript abundance. Contrary, Rap2.4e (11-fold) as well as Rap2.4g (8-fold) were up-regulated.

3.3.6.9 The effect of transient Rap2.4h overexpression on the ERFIb transcript levels

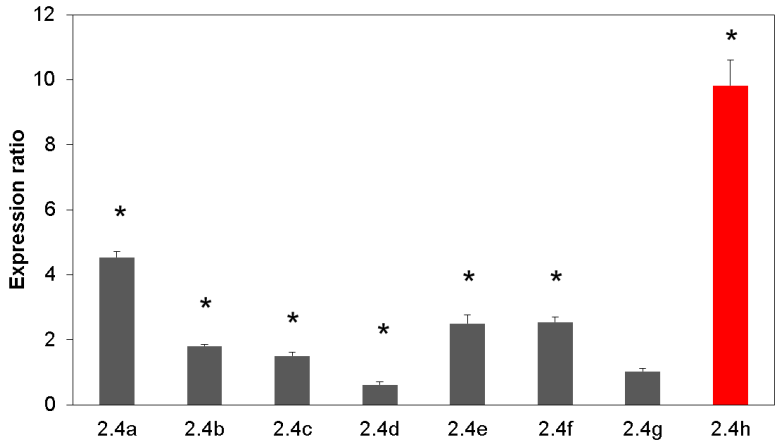

Fig. 42: Relative transcript level of Rap2.4h (red bar) upon 10 µM estradiol induced transient overexpression; 16 plantlets were pooled from 2 replicates and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to the relative Act7 transcript level in Col-0 plants transiently transformed with empty plasmids. The relative transcript levels of Col-0 wt transiently transformed with empty plasmids were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from Col-0 wt plants transiently transformed with empty plasmids.

Chemically induced Rap2.4h overexpression led to up-regulation of mRNA abundances present for Rap2.4a, b, c, e and f. Only Rap2.4d was shown to be down-regulated.

3.3.6.10 The comparison between knock-down conditioned and overexpression induced changes in the ERFIb transcript profile reveals regulatory patters

The initial analysis of transcriptional changes in the ERFIb expression pattern due to T-DNA knock-down, endorsed putative dependency between Rap2.4 genes.

However, primarily the arrangement of results from knock-down analysis and overexpression demonstrated regulatory relations between individual Rap2.4 transcription factors and the definition of certain regulatory features, respectively.

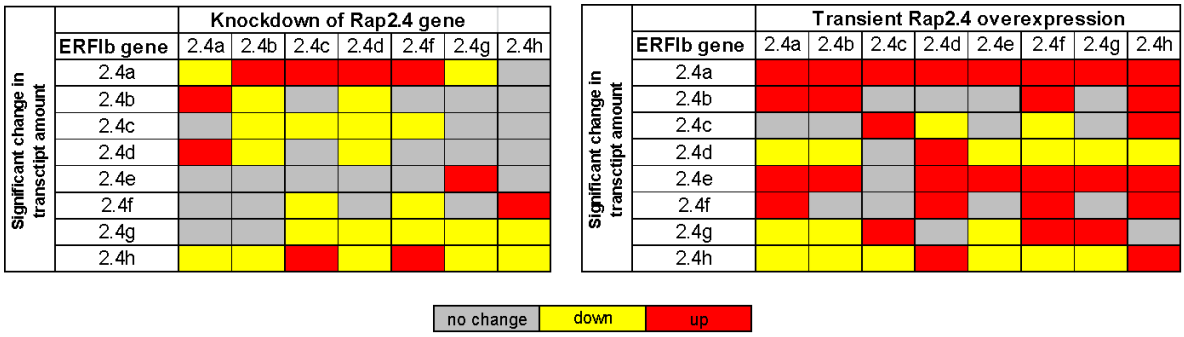

Fig. 43: Interplay between ERFIb genes after individual Rap2.4 knock-down and transient overexpression according to qPCR data; appropriate colors display no change; down-regulation or up-regulation of relative transcript amount; only significant values are indicated.

Notably, Rap2.4a and Rap2.4e were up-regulated in response of all Rap2.4 TFs. In contrast, Rap2.4h was predominantly down-regulated in both data sets. The Rap2.d transcript was decreased after overexpressing of all TFs, expect Rap2.4c.

3.3.6.11 Rap2.4a preferably negatively regulates other ERFIb genes

The reaction of Rap2.4a partly reflects the already present up-regulation in the knock-down lines for Rap2.4b, Rap2.4c, Rap2.4d and Rap2.4f. For these cases an overall up-regulation of Rap2.4a can be presumed. Here, an “out-of-balance” situation is the driving force towards general up-regulation (Fig. 44), without distinction between more or less transcript availability of the mentioned Rap2.4 genes.

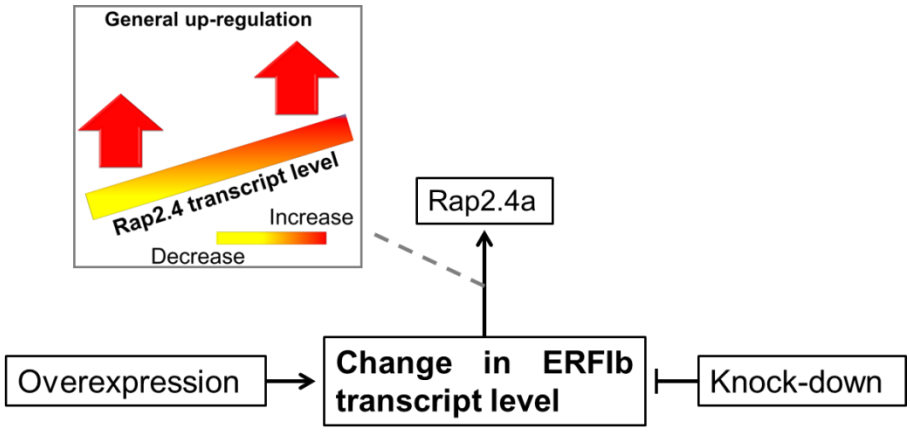

Fig. 44: Theoretical regulatory mechanism in case of “out-of-balance” disturbance of transcript level leading to a general up-regulation of Rap2.4a transcript level; black arrow indicates up-regulated gene expression, black stop line down-regulated gene expression.

3.3.6.12 Interplay between Rap2.4a and Rap2.4g

Upon Rap2.4g knock-down, Rap2.4a was negatively regulated, whereas after Rap2.4g overexpression a positive up-regulation for Rap2.4a was available (Fig. 43). This parallelism indicates that Rap2.4a is co-regulated with Rap2.4g.

The Rap2.4g mRNA abundance changed only after strong induction of Rap2.4a expression level and was not impaired after Rap2.4a knock-down. Thus, the activating stimulus for positive down-regulation (Fig. 45) is the increased amount of Rap2.4a transcript. Noteworthy, Rap.2.4g is the only transcription factor co-regulating Rap2.4a mRNA abundance two-directionally (Fig. 45).

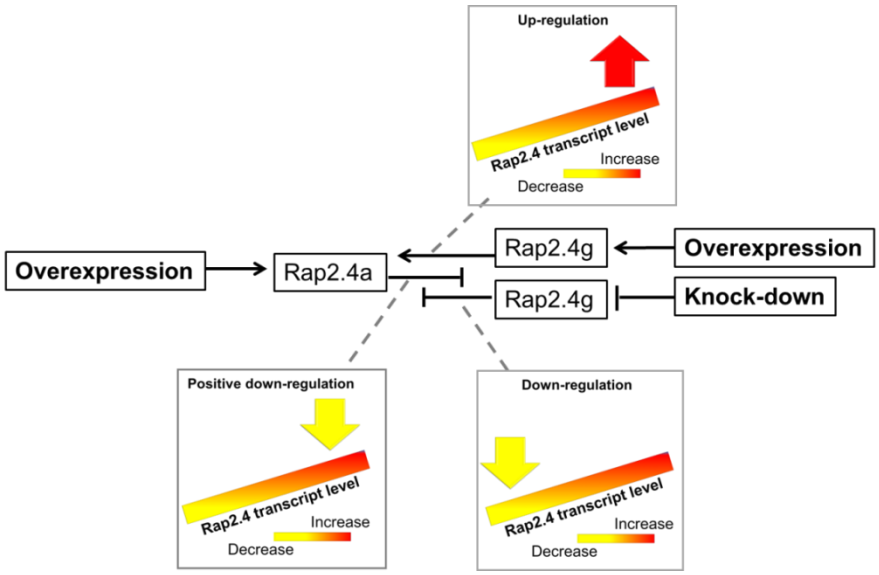

Fig. 45: Putative dependency between Rap2.4a and Rap2.4g; black arrow indicates up-regulated gene expression, black stop line down-regulated gene expression.

3.3.6.13 Interplay of Rap2.4a towards Rap2.4b and Rap2.4d

Rap2.4a inversely co-regulated Rap2.4b and Rap2.4d transcript levels regarding knock-down and overexpression (Fig. 46). Upon Rap2.4a knock-down, Rap2.4b and Rap2.4d were increased in mRNA amount and vice versa. On the contrary, positive or negative changes in Rap2.4b or Rap2.4d transcript levels, up-regulate Rap2.4a mRNA abundance. This kind of regulation among the ERFIb genes is unique. Moreover, it indicates that Rap2.4a expression is regulated by imbalances in Rap2.4b and Rap2.4d expression.

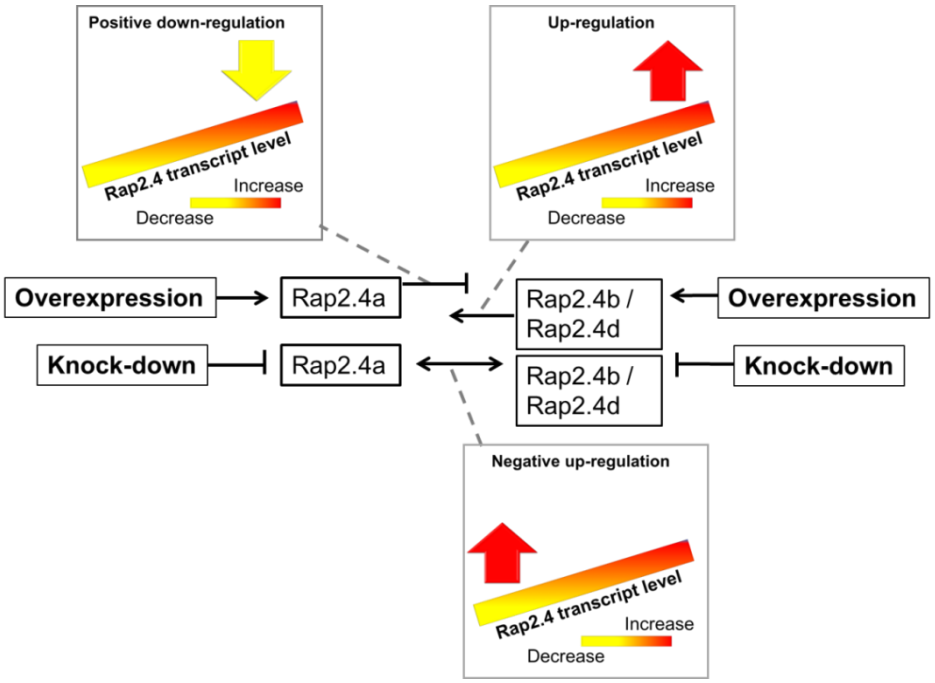

Fig. 46: Putative dependency between Rap2.4a and Rap2.4b/Rap2.4d. Elevated Rap2.4a transcript down-regulates Rap2.4b and Rap2.4d, enhanced Rap2.4b and d expression level up-regulated Rap2.4a mRNA amount; Rap2.4a knock-down increases Rap2.4b and Rap2.4d transcript level and vice versa; black arrow indicates up-regulated gene expression, black stop line down-regulated gene expression.

3.3.6.14 Rap2.4h up-regulates other ERFIb genes but is mostly down-regulated

The Rap2.4h mRNA level is mainly negatively regulated. Additionally, the Rap2.4h regulation is also uncoupled from a strict tendency of positive or negative mRNA level of another appropriate ERFIb transcription factor. The determinant factor must be an unbalanced transcript level. Further, a similar but reversed regulation, namely general up-regulation, is present for Rap2.4a (Fig. 44). Moreover, this general Rap2.4h down-regulation (Fig. 47) was also present upon changes in Rap2.4b, Rap2.4c and Rap2.4g transcriptional level.

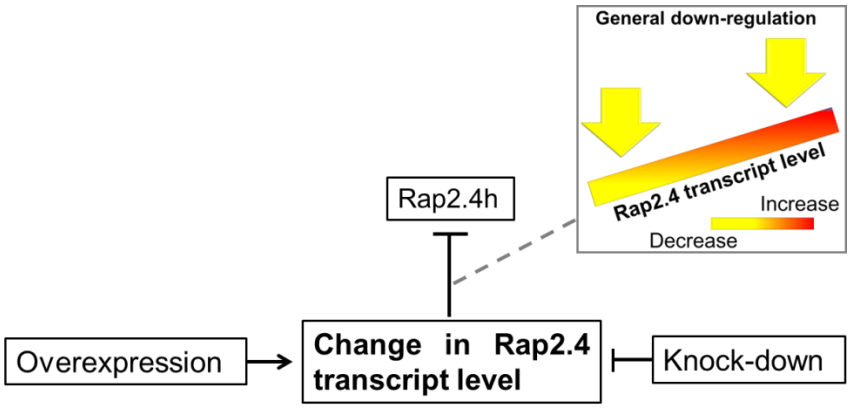

Fig. 47: Theoretical regulatory mechanism in case of "out-of-balance" disturbance of transcript level leading to a general down-regulation of Rap2.4h transcript level; black arrow indicates up-regulated gene expression, black stop line down-regulated gene expression.

The relationship between Rap2.4a and Rap2.4h revealed to be more complex. One the one hand, only after the Rap2.4h knock-down Rap2.4a is not regulated on the transcript level (Fig. 43 left). On the other hand, only after strong Rap2.4h overexpression the Rap2.4a transcript level was highly increased. Consequently, the trigger for positive Rap2.4a up-regulation (Fig. 48) might be linked to the elevated Rap2.4h mRNA amount. A Rap2.4a dependent, one-sided loop-feedback regulation of Rap2.4h transcription seems possible.

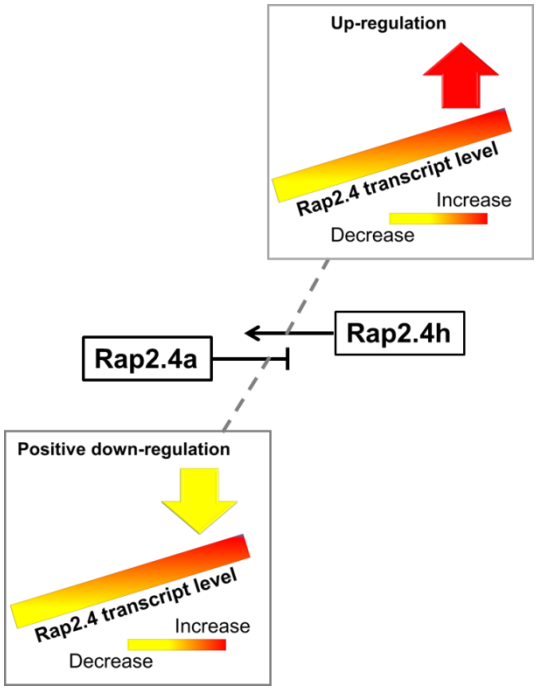

Fig. 48: Putative dependency between Rap2.4a and Rap2.4h for putative one-sided loop-feedback regulation of Rap2.4h transcript. Elevated Rap2.4a transcript down-regulates Rap2.4h, enhanced Rap2.4h expression level up-regulated Rap2.4a mRNA amount; black arrow indicates up-regulated gene expression, black stop line down-regulated gene expression.

However, several exceptions were observed: Rap2.4h was reversely regulated relative to the transcript levels of Rap2.4c and Rap2.4f (Fig. 43). The down-regulation of Rap2.4c and f triggered directly or indirectly the expression of Rap2.4h transcript abundance and the overexpression of Rap2.4c and Rap2.4f decreased the Rap2.4h expression level.

Rap2.4h regulated Rap2.4c positively upon overexpression, the Rap2.4f transcript ratio was generally increased upon changes in Rap2.4h mRNA level. Rap2.4d co-regulated Rap2.4h and determined the type of transcriptional regulation. Thus, up-regulation and down-regulation are the working mechanisms (Fig. 49). In this relationship, the regulatory potential of Rap2.4h was demonstrated by positive Rap2.4d down-regulation (Fig. 42) upon strong overexpression. Notably, this regulation partially corresponds to the interplay between Rap2.4a and Rap2.4h (Fig. 48).

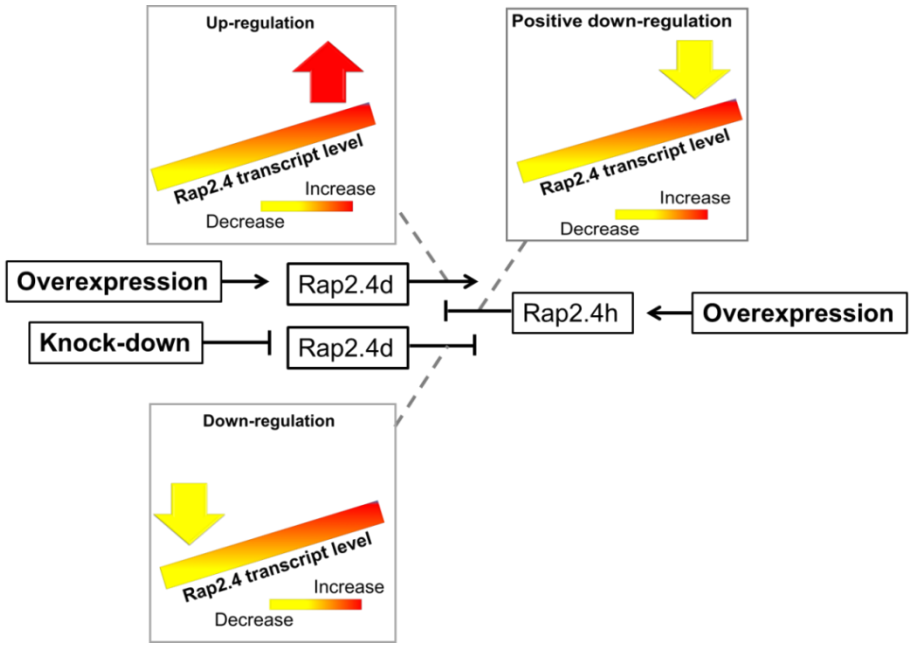

Fig. 49: Interplay between Rap2.4d and Rap2.4h; black arrow indicates up-regulated gene expression, black stop line down-regulated gene expression.

In general, the Rap2.4h knock-down poorly affected other ERFIb genes. However, overexpression demonstrated a negative impulse on Rap2.4b, Rap2.4c and Rap2.4d mRNA levels.

3.3.6.15 Rap2.4e transcript level is mainly affected by overexpression of other ERFIb genes

No data is available about the effect of Rap2.4e knock-down on the remaining ERFIb genes. According to the qPCR results from T-DNA insertion lines (Fig. 43 left) one can conclude that the Rap2.4e mRNA abundance was not affected after the knock-down of nearly all ERFIb genes.

The situation changed upon overexpression (Fig. 43 right) in which the Rap2.4e transcript level was increased and the determinant factor must be the strong up-regulation of the overexpressed gene. A similar situation of highly induced ERFIb gene was not present in any analyzed knock-down line. This supports the presumption for the mechanism of up-regulation (Fig. 50).

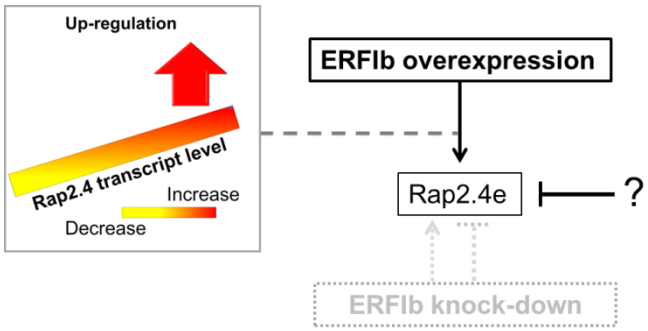

Fig. 50: Theoretical regulatory mechanism: Rap2.4e is up-regulated if the transcript level of another Rap2.4 gene is strongly increased; black arrow indicates up-regulated gene expression, black stop line down-regulated gene expression. Grey dotted line demonstrates no regulatory influence of ERFIb knock-down on Rap2.4e expression level.

Nevertheless, the intensity of transcript up-regulation could be an important modulator. Furthermore, Rap2.4e is in no case decreased by changing ERFIb transcript levels. It remains unclear how the aspect of negative Rap2.4e regulation could be maintained; either an upstream regulator could negatively impact on the transcription or maybe a certain abiotic or biotic stress. However, it also be can suggested that Rap2.4e is very sensitive towards any misbalances in ERFIb genes and could act as a sensor to detect and pinpoint such changes and trigger compensatory mechanisms.

3.4 The role of ERFIb transcription factors in chloroplast antioxidant system

Besides the regulatory effect of ERFIb gene expression on other ERFIb genes, the impact on putative target genes was investigated. It was described that a 35S-promoter mediated transient Rap2.4a overexpression increased the signal of a 2CPA-promoter-YFP fusion construct in Arabidopsis mesophyll protoplasts (Shaikhali et al. 2008). Moreover, down-regulated Rap2.4a transcript amount resulted in decreased level of 2CPA expression (Shaikhali et al. 2008).

2CPA is encoded in the nucleus (Baier and Dietz 1997) and participates in the antioxidant system within the chloroplast (Baier et al. 2000). There, the enzyme ensures protection from oxidative damages originating from reactive oxygen species like hydrogen peroxide (H_2O_2), for example, produced during photosynthesis (Baier et al. 2004).

ERFIb genes share the same DNA binding domain and the particular Rap2.4 knock-down or overexpression points towards interplay within the gene family (Fig. 36). Thus, in can be presumed that an involvement in regulation of antioxidant enzymes like 2CPA is not exclusively present for Rap2.4a but could also be possible for the remaining ERFIb genes. However, a direct regulatory function is so far only known for Rap2.4a and is based on interaction with the 2CPA promoter in Y1H experiments (Shaikhali et al. 2008).

Supporting, results from Y1H assay demonstrated strong interaction of Rap2.4h with the 2CPA promoter (Fig. 51) (Collaboration with Jote Bulcha). All other ERFIb genes, namely Rap2.4b-g, showed no interaction (Jote Bulcha; PhD thesis).

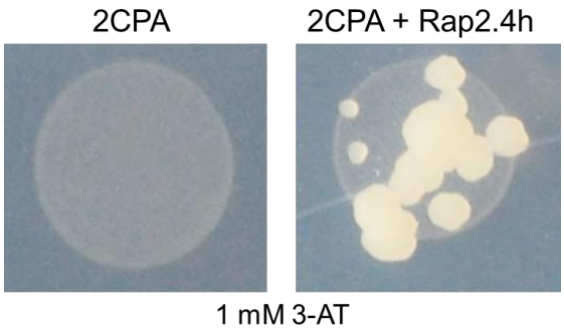

Fig. 51: Yeast-One-Hybrid hybrid assay demonstrating the interaction of 2CPA promoter with Rap2.4h transcription factor. The negative control is empty pACT2 vector co-transformed with reporter plasmid containing 2CPA. The interaction was assayed on media lacking Lys, Trp and His with 1 mM 3-Amino-1,2,4-triazole (3-AT).

3.4.1 Influence of Rap2.4a and Rap2.4h knock-down on transcript abundance of the antioxidant enzymes 2CPA and 2CPB

A direct positive regulation of 2CPA promoter activity, triggered through slight redox disturbances was shown for Rap2.4a (Shaikhali et al. 2008). Furthermore, the abundance of Rap2.4a transcript can affect the 2CPA mRNA status (Shaikhali et al. 2008). It is not known whether Rap2.4h is able to influence the 2CPA transcript level. To investigate this assumption, the relative expression levels of 2CPA and 2CPB were analyzed in homozygous Rap2.4a and Rap2.4h T-DNA insertion lines by qPCR.

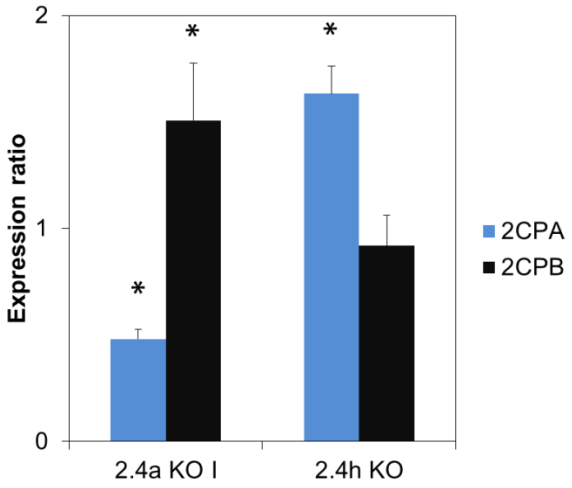

Fig. 52: Relative 2CPA and 2CPB transcript levels in homozygous Rap2.4a KO I and Rap2.4h KO T-DNA insertion lines; rosette leaves from 3 independent plants, grown under standard conditions for 4 weeks, were harvested and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to Act7 transcript amount in Col-0 wt plants. The relative transcript levels of Col-0 wt were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from control Col-0 wt plants.

The effect of Rap2.4a and Rap2.4h knock-down affects the relative transcript level of both antioxidant enzymes with different impact intensities (Fig. 52). Strikingly, the 2CPA mRNA level was highly down-regulated in case of decreased Rap2.4a transcript abundance (0.5 ± 0.04); this effect is in agreement with data from Shaikhali and colleagues (2008). In parallel the 2CPB transcript level was increased, indicating a compensational effect among the antioxidant enzymes. Previous experiments demonstrated that the knock-down of Rap2.4a also decreased the Rap2.4h transcript level, but not vice versa. Thus, it can be suggested that the displayed negative 2CPA regulation is mainly accomplished through deficiency in Rap2.4a transcript amount.

In contrast, the Rap2.4h knock-down did not affect Rap2.4a mRNA but increased the 2CPA transcript level (1.6-fold); the 2CPB mRNA level was not changed. Thus, the increased 2CPA transcript level could be mainly caused by stronger Rap2.4a effects in the absence of Rap2.4h.

3.4.2 Transient overexpression of Rap2.4a and Rap2.4h Influence the 2CPA and 2CPB transcript level

If the knock-down of Rap2.4a decreased the 2CPA transcript level and the knock-down of Rap2.4h up-regulated the 2CPA expression level, the transient overexpression of these two TFs should subsequently have up-regulated or down-regulated the 2CPA mRNA amount, respectively.

To test this presumption, 12 day old plantlets were transiently transformed with appropriate constructs to overexpress Rap2.4a and Rap2.4h as described in 3.3.6.2.

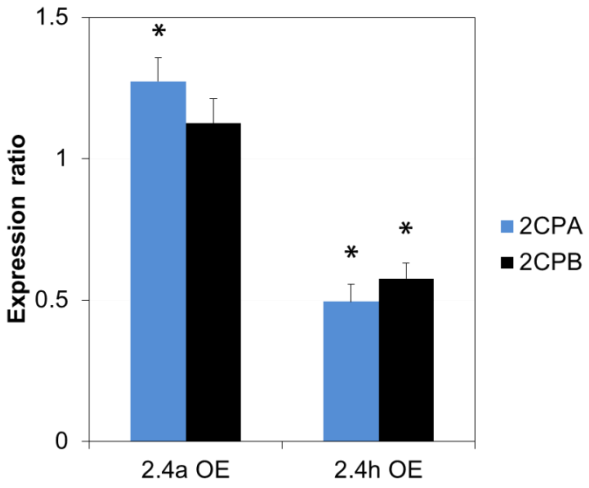

Fig. 53: Relative transcript level of 2CPA and 2CPB upon 10 µM estradiol induced transient overexpression of Rap2.4a and Rap2.4h; 16 plantlets were pooled from 2 replicates and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to Act7 transcript level in Col-0 plants transiently transformed with empty plasmid. The relative transcript levels of Col-0 wt were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from Col-0 wt plants transiently transformed with empty plasmids.

The transient overexpression of Rap2.4a results in an up-regulated 2CPA transcript level and confirms a positive regulatory function like demonstrated by Shaikhali and colleagues (2008). On the contrary, the overexpression of Rap2.4h decreased the relative 2CPA transcript level (0.5-fold) and endorses the assumed ability to negatively regulate the

2CPA transcript amount. Noteworthy, the additional negative regulation of 2CPB (0.6-fold) suggests indirect or direct co-regulation with 2CPA. The relative 2CPA transcript level is only little affected upon Rap2.4a overexpression (1.3-fold). It should be noted that according to developmental expression data from Genevestigator (Hruz et al. 2008), during the seedling stage (comparable to the age of used plantlets) 2CPA and 2CPB are highly expressed (Fig. 54). Referring to this, Baier and co-workers (2004), reported about high 2CPA promoter activity, preferably in young tissue parts. This implicates that despite of the already high 2CPA activity, Rap2.4a may not further activate 2CPA since it is already at its maximum level.

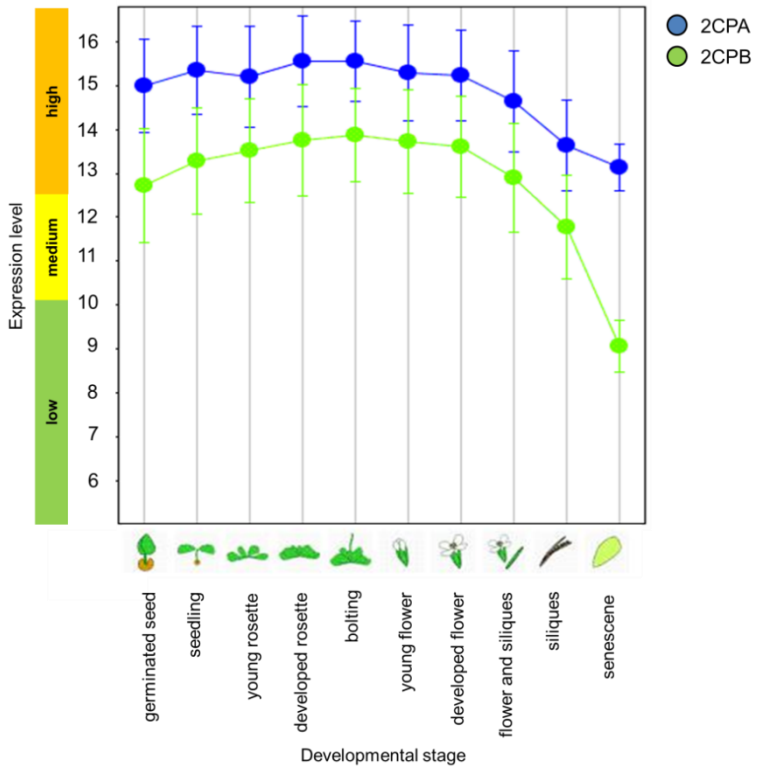

Fig. 54: log2 expression of ERFIb genes during developmental stages, signal intensity on a 22k array; modified according to Genevestigator Version 4; Development analysis dataset.

3.4.3 Transient Rap2.4a and Rap2.4h overexpression regulates 2CPA promoter activity

The question rises, if the increased Rap2.4a or Rap2.4h transcript level could affect the 2CPA promoter activity, impartial of post-transcriptional 2CPA regulation (Baier et al. 2004). Therefore, 12 day old homozygous plantlets, harboring a GUS-reporter gene fused to a 2CPA promoter fragment (Baier et al. 2004), were transiently transformed with appropriate constructs to overexpress Rap.24a and Rap2.4h like described in 3.3.6.2.

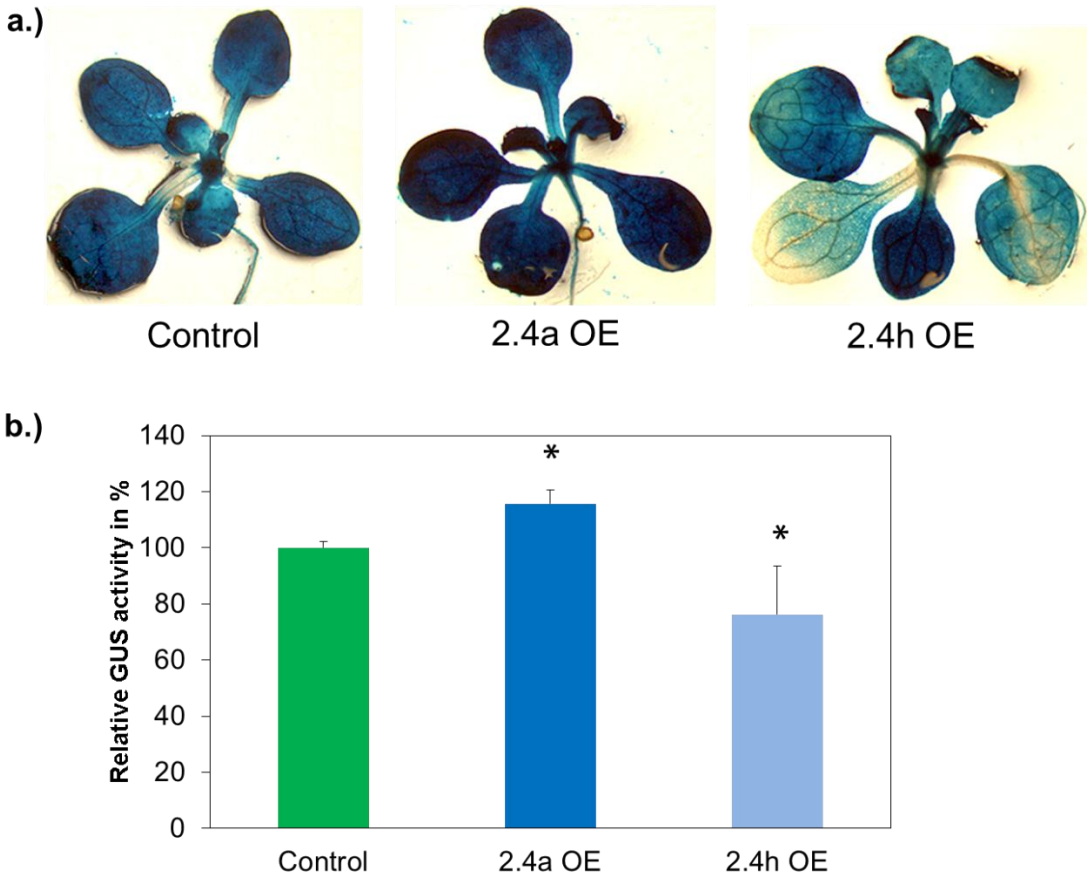

Fig. 55: a.) GUS staining pattern due to 2CPA promoter activity upon estradiol induced transient overexpression (10 µM) of Rap2.4a and Rap2.4h in comparison to control plantlets transiently transformed with empty plasmids. Pictures are representative, experiment was performed once with 16 plantlets for each construct (n=16) **b.) Measured GUS activity due to 2CPA promoter activity after estradiol (10 µM) induced transient overexpression of Rap2.4a and Rap2.4h** in comparison to control plantlets transiently transformed with empty plasmids. Data are means of 2 biological and 12 technical replicates each (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from Col-0 wt plants transiently transformed with empty plasmids.

The transient overexpression of Rap2.4a (Fig. 55) demonstrated up-regulated 2CPA promoter activity. The transformed plantlets displayed a general more intense GUS

staining pattern in all tissues (Fig. 55a left) as well as a 16% higher total GUS activity compared with control (Fig. 55b). In contrast, the transient overexpression of Rap2.4h resulted in less stained leaves with even almost unstained leaf tip (Fig. 55a right). The measured total GUS activity was decreased to 77% as compared with control plantlets (Fig. 55b). Noteworthy, leaf tissue with high photosynthesis activity, displayed less stained areas. This data confirms that Rap2.4a and Rap2.4h are able to regulate the 2CPA promoter activity in opposite manner.

Notably, Bondino and co-workers (2009) reported about GUS expression in 10 day old Arabidopsis plantlets, derived from high and low 2CPB promoter activity after heat and cold stress treatment. The resulting strong GUS staining after heat stress was comparable to the 2CPA promoter driven GUS expression, after transient Rap2.4a overexpression. In contrast, the GUS staining intensity after cold stress was similarly less strong as described for 2CPA promoter activity after transient Rap2.4h overexpression (Fig. 55a right). The authors concluded that the differences in GUS expression and consequently in 2CPB promoter activity relied on the appropriate stress type (Bondino et al. 2009).

The 2CPA protein was inactivated upon cold stress (König et al. 2003). The Rap2.4a transcript level was down-regulated and Rap2.4h mRNA abundance was up-regulated after 4°C treatment (Fig. 14). Therefore, it can be suggested that Rap2.4a and Rap2.4h regulate 2CPA promoter under different prevailing environmental premises like diverse abiotic stresses and are good candidates for improvement of, for instance, tolerances towards abiotic stresses in a genetic engineering approach like already demonstrated for various other AP2 TFs (reviewed in Xu et al. 2011)

The relationship between Rap2.4a and Rap2.4h is of importance in terms of 2CPA regulation. The analysis of potential interplay between Rap2.4 genes, according to data generated by qPCR from ERFIb T-DNA insertion lines and transient overexpression studies, demonstrated a decrease of Rap2.4h mRNA abundance upon Rap2.4a knock-down and overexpression (Fig. 24; Fig. 35). Additionally, Rap2.4a was up-regulated upon overexpression of Rap2.4h (Fig. 42), whereas its expression level did not exceed the Rap2.4h transcript level and vice versa. The proposed one-sided feedback loop regulation of Rap2.4h transcription through Rap2.4a could be adaptive (Fig. 56) in terms of 2CPA promoter regulation, since is controls permissiveness of the activating transcription factor Rap2.4a upon stress.

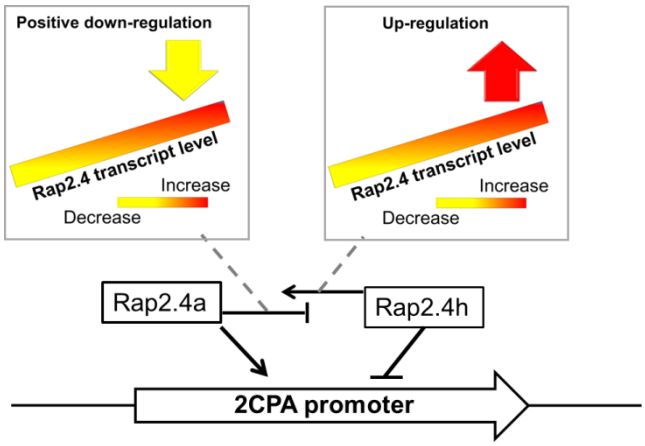

Fig. 56: Proposal for 2CPA promoter regulation with Rap2.4a as positive and Rap2.4h as negative factor, **respectively**. Regulation of Rap2.4h transcription might be regulated through putative Rap2.4a dependent feedback loop. Black arrow indicates up-regulated gene expression, black stop line down-regulated gene expression.

3.4.3.1 The effect of combined Rap2.4a and Rap2.4h knock-down on 2CPA transcription

The effort to determine the mechanism of the interplay between Rap2.4a and Rap2.4h in regulation of 2CPA expression, revealed the assumption of a one-sided negative feedback-loop (Fig. 56). This presumption originates on qPCR results from knock-down and overexpression experiments.

To further elucidate the putative dependency between Rap2.4a and Rap2.4h, four week old homozygous double knock-down plants, originating from a cross between homozygous plants from the Rap2.4a KO I line and the Rap2.h KO line, were compared by qPCR analysis.

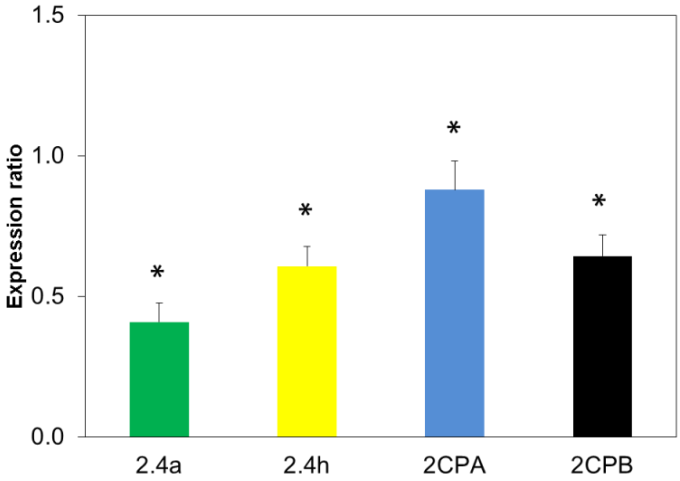

Fig. 57: Relative Rap2.4a, Rap2.4h, 2CPA and 2CPB transcript level in homozygous Rap2.4a/Rap2.4h double knock-down mutant plants grown for 4 weeks under standard conditions; rosette leaves from 3 independent plants were harvested and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to Act7 transcript amount in Col-0 wt. The relative transcript levels of Col-0 wt were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from control Col-0 wt plants.

The decrease in both Rap2.4 transcription factors in parallel resulted in slightly down-regulated 2CPA expression level (0.9-fold). The additionally measured 2CPB mRNA amount was strongly decreased (0.6-fold).

Upon Rap2.4a or Rap2.4h knock-down the 2CPA transcript was down-regulated (0.48-fold) and up-regulated (1.6-fold), respectively. Upon knock-down of both Rap2.4 genes, the 2CPA transcript level was nearly not affected (Fig. 57). Since a preeminent majority for, at least, one putatively regulating TF is no more available it can be presumed that the lack of both putative regulators mimics the situation of 2CPA transcript regulation when both TFs are in balance (Fig. 58 right).

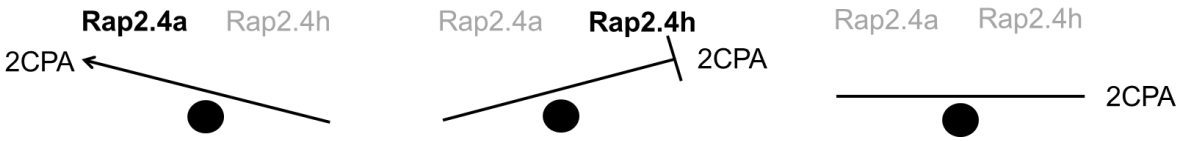

Fig. 58: Changes in 2CPA transcript abundance in dependence on Rap2.4a or Rap2.4h deficiency; left: knock-down of Rap2.4h enhances the positive 2CPA regulation by Rap2.4a; centre: the knock-down of Rap2.4a facilitates the negative effect of Rap2.4h on 2CPA regulation; right: the knock-down of both TFs mimics a balanced Rap2.4a and Rap2.4h transcript level where upon the 2CPA transcript level is not affected.

3.4.3.2 Effect on Rap2.4a and Rap2.4h transcript level upon deficiency in 2CP genes

The overexpression of Rap2.4h decreased the 2CPA and 2CPB expression. The knockdown of Rap2.4a decreased 2CPA mRNA abundance, whereupon the 2CPB expression level was up-regulated, potentially to compensate the lowered amount of 2CPA transcript. This observation raises the question raises if a disturbance in 2CPA and / or 2CPB transcript levels could affect the mRNA levels of Rap2.4a and Rap2.4h, in a kind of loop-feedback regulation.

To investigate this possibility, homozygous Arabidopsis knock-down plants, deficient either in 2CPA, 2CPB or both genes, were cultivated for 4 weeks under standard conditions. Plant material was harvested from each mutant line and used for RNA isolation prior to cDNA synthesis. qPCR enabled the monitoring of changes in the relative expression levels of Rap2.4a and Rap2.4h.

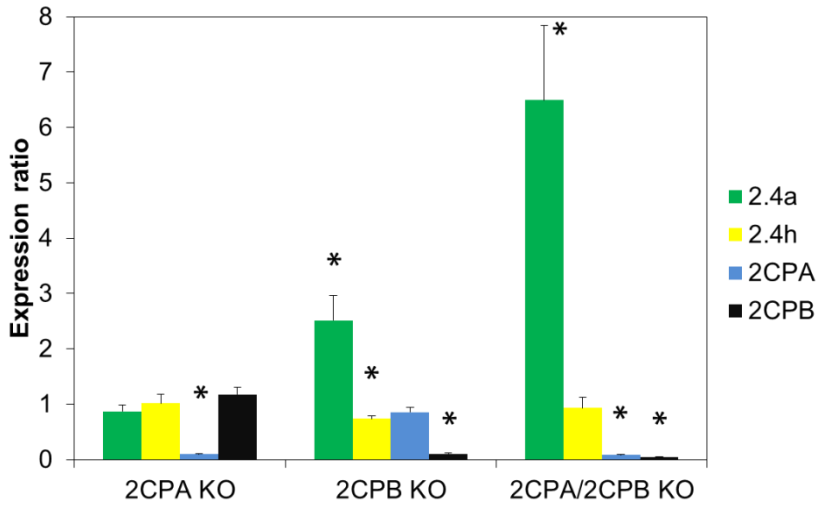

Fig. 59: Relative ERFIb transcript levels in homozygous 2CPA, 2CPB, and double knock-out mutant plants; rosette leaves from 3 independent plants, grown for 4 weeks under standard conditions, were harvested and further used for RNA isolation. The relative expression ratio was measured by qPCR and normalized to Act7 transcript amount in Col-0 wt. The relative transcript levels of Col-0 wt were set to 1.0. Data's are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from control Col-0 wt plants.

The qPCR results from different 2CP KO lines (Fig. 59) revealed no up-regulation of the Rap2.4a transcript amount in 2CPA knock-down plants. The Rap2.4a transcript amount displayed even a slight decrease. Rap2.4a was up-regulated upon lack of 2CPB (2.5-fold) and even stronger (6.4-fold) if both 2CP genes were knocked-out. This leads to the assumption that Rap2.4a transcript is triggered upon 2CPB breakdown and highly induced

after complete outfall in 2CP transcript. This suggests very probably no negative 2CPA feedback loop effect.

The knock-down of Rap2.4h enhances the 2CPA mRNA level most likely because the positive regulatory effect of Rap2.4a is strengthened. Results from induced Rap2.4a overexpression demonstrated an exceptionally strong decreasing effect on the Rap2.4h transcript level (Fig. 35). Regarding this regulatory dependency, one could presume that Rap2.4h mRNA abundance was not down-regulated in case of 2CPA knock-out because Rap2.4a expression was not up-regulated. On the contrary, upon 2CPB knock-out and 2CPA/2CPB double knock-out Rap2.4h was down-regulated, most likely due to a strong increase in Rap2.4a expression. Hence, also for Rap2.4h a positive or negative loop-feedback regulation caused by impaired 2CPA transcript level is not available. More prominent is the Rap2.4a mediated regulation of Rap2.4h like demonstrated upon overexpression of Rap2.4a (Fig. 35).

3.4.3.3 Global impact on the ROS status upon knock-down of Rap2.4a and Rap2.4h

Rap2.4a transcript level was positively regulated with the increased levels of chloroplastic H_2O_2 (Maruta et al. 2012). After Rap2.4a knock-down, besides 2CPA also the transcript levels of other antioxidant enzymes, namely stromal ascorbate peroxidase (sAPx), thylakoid bound peroxidase (tAPx) as well as superoxide dismutase 2 (Csd2), were down-regulated (Shaikhali et al. 2008). All these enzymes contribute to ROS detoxification (Rizhsky et al. 2003; Maruta et al. 2010). At the same time, transcript abundances of the general ROS marker gene ZAT10 (Mehterov et al. 2012) and ascorbate peroxidase 2 (APx2), as indicator for changes in cytosolic H_2O_2 level (Karpinski et al. 1997), were up-regulated. This implicates that Rap2.4a directly or indirectly supports ROS regulated pathways operating all over the cell. The elevated ROS levels in chloroplasts, caused by less enzymatic antioxidant activity promote triggering of ROS related genes for cytosolic antioxidant enzymes like APx2 (Karpinski et al. 1997).

To investigate this assumption, the overall ROS level was analyzed in 4 week old homozygous Rap2.4a KO I plants. Since Rap2.4h seems to be the counterpart or Rap2.4a in 2CPA regulation and its transcript amount was not remarkably affected upon 2CPA knock-down, Rap2.4h KO plants were also incorporated to analyze the putative effect on ROS abundance.

Leaves were stained with nitro blue tetrazolium (NBT) (Kawai-Yamada et al. 2004). It allows histochemical detection of endogenous superoxide anions ($O_2^{-\bullet}$) (Jabs et al. 1996), a precursor from hydrogen peroxide. Under non-stress conditions, the major amount of superoxide anions derives from chloroplasts and photosynthesis related processes (Asada 1999; Apel and Hirt 2004; Scarpeci et al. 2008).

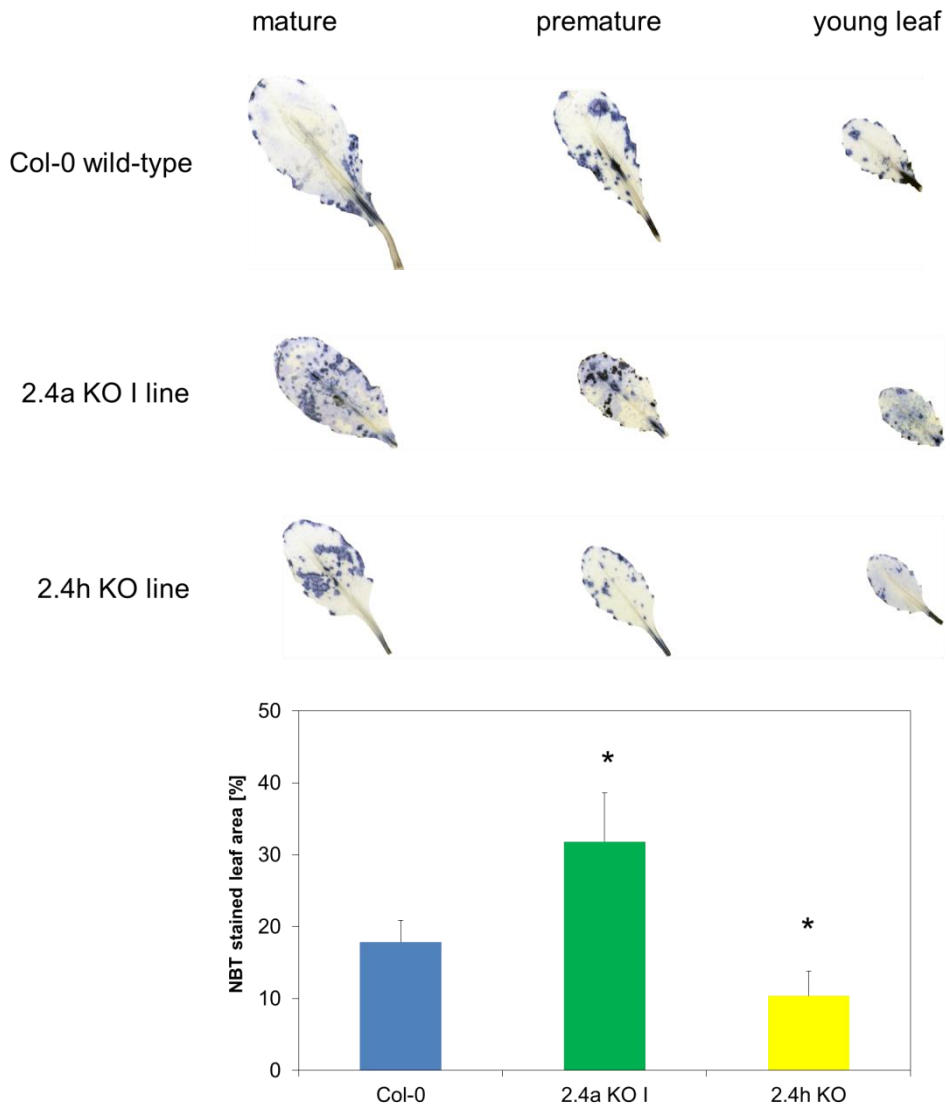

Fig. 60: NBT staining of superoxide anions ($O_2^{-\bullet}$) in rosette leaves of 4 week old homozygous Rap2.4a KO I and Rap2.4h KO plants showed differences in ROS accumulation. Mature, premature and young leaves were stained to gain an overall ROS status; Col-0 wt served as control. Pictures are representative; 4-5 plants from each genotype were used for staining. Blue colored area point towards accumulation of ROS molecules. The percentage of stained area was evaluated with ImageJ. The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from control Col-0 wt plants.

The knock-down of Rap2.4a resulted in a higher accumulation of superoxide anions as well as higher portion of stained area in comparison towards the control (Fig. 60). This observation is consistent with up-regulation of ROS sensitive genes like ZAT10 and APx2 in Rap2.4a KO plants (Shaikhali et al. 2008). By contrast, leaves from plants lacking Rap2.4h transcripts were less stained. Besides 2CPA, the transient Rap2.4h overexpression additionally decreased the 2CPB mRNA level (Fig. 53). In this case one could assume that Rap2.4h potentially negatively regulates other antioxidant enzymes or pathways responsible for ROS detoxification. Thus, the absence could result in a higher ROS detoxification.

3.4.3.4 The impact of ROS on Rap2.4a, Rap2.4h and 2CPA transcript level

Pulido and co-workers (2010) confirmed an elevated content of H_2O_2 relative to the fresh weight (+15 %) in 2CP deficient plants; at once Rap2.4a transcripts were highly up-regulated in plants lacking both peroxiredoxins (Fig. 59 right). Thus, an elevated H_2O_2 level, caused by a disturbed antioxidant system in chloroplasts, could directly or indirectly affect the Rap2.4a transcript abundance.

Moreover, it could be demonstrated that the application of H_2O_2 (< 3 mM) in protoplasts overexpressing Rap2.4a, resulted in higher 2CPA-driven reporter gene activity (> 50 %) (Shaikhali et al. 2008). Moreover, the accumulation of chloroplastic H_2O_2 through estradiol inducible RNAi silencing of tAPx resulted in increased Rap2.4a transcript level (2.2-fold) (Maruta et al. 2012). However, the 2CPA promoter activity decreased after application of high H_2O_2 concentrations (Shaikhali et al. 2010).

Rap2.4h is most likely less affected by an increased H_2O_2 level since it´s transcript abundance was slightly down-regulated in 2CPA/2CPB double knock-out plants (Fig. 59). Thus, Rap2.4a is participating in several regulatory pathways. Together with the antagonist Rap2.4h, Rap2.4a can fine tune the 2CPA promoter activity in chloroplasts when minor changes in the redox poise occur. Upon oxidative stress the TF forfeits this ability (Shaikhali et al. 2008 and 2010) and assumedly gains significance in other ROS associated pathways. To this end, no data exist showing changes in Rap2.4a, Rap2.4h and 2CPA transcripts at the same time in case of an elevated ROS level.

The usage of external detergents is widely approved to experimentally investigate oxidative stress responses. For example, the herbicide methyl viologen (MV) is a redox-active substance that is photochemically reduced in chloroplasts and triggers

photooxidative stress responses. MV acts as alternative electron acceptor taking electrons from the iron-sulfur cluster Fe-S_A/Fe-S_B of PSI (Fujii et al. 1990). Further, PQ is reoxidized by transferring electrons to O_2 and generating $O_2^{-\bullet}$ (Kim and Lee 2003). Subsequently, superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{-\bullet}$ into O_2 and H_2O_2 (Kim and Lee 2003).

MV was successfully used in many different studies handling with genetic approaches or antioxidant system responses (op den Camp et al. 2003; Melchiorre et al. 2009). However, the application of MV or H_2O_2 does not necessarily reflect a comparable stress response *in planta*. Especially the usage of H_2O_2 makes it difficult to credit a certain change in transcript level to a specific organelle or cell compartment as the presence of H_2O_2 is not spatially limited (op den Camp et al. 2003).

A more elegant and advanced way to study ROS conditioned gene expression *in vivo*, is to manipulate antioxidant enzymes (Apel and Hirt 2004) like tAPx (Maruta et al. 2012) or genetically control ROS generation like singlet oxygen ($^1O_2$) (op den Camp et al. 2003; Laloi et al. 2007; Kim et al. 2012) in the conditional fluorescent in blue light (*flu)*-mutant (Meskauskiene et al. 2001).

3.4.3.5 The conditional *flu*-mutant enables induced generation of singlet oxygen in plastids

The nuclear encoded FLU protein negatively regulates the glutamyl-tRNA reductase in the tetrapyrrole pathway of the chlorophyll biosynthesis (Meskauskiene et al. 2001; Meskauskiene and Apel 2002; Terry et al. 2013). Due to the mutational defect, the level of the chlorophyll precursor protochlorophyllide can no longer be controlled in the *flu*-mutant in darkness and increases disproportionally (Meskauskiene et al. 2001). Upon a shift from dark to light, the photo-sensitive protochlorophyllide (Matringe et al. 1989) produces $^1O_2$ in plastids (op den Camp et al. 2003), which accumulates in the chloroplasts and finally causes general tissue damage (Fig. 61) and cell death after long term exposure (op den Camp et al. 2003; Kim et al 2012). Growing under continuous light rescues the mutant (Meskauskiene et al. 2001).

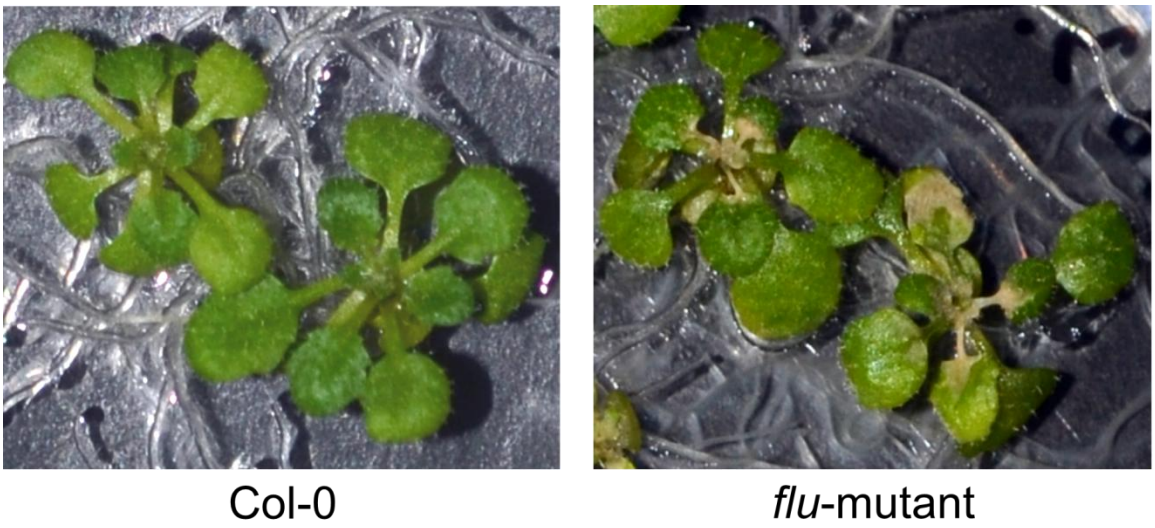

Fig. 61: Comparison of 2 week old Col-0 wt plants (left) and *flu*-mutant plants (right) 2 days after dark/light shift; pictures are exemplary**;** the *flu*-mutants display less green leaves as well as bleached leaves caused by long term singlet oxygen accumulation in chloroplasts.

3.4.3.5.1 Short term expressional changes after ROS induction in the conditional *flu* mutant

To investigate whether Rap2.4a and 2CPA transcript levels could be induced through signals that originate from ROS generated inside chloroplasts, the *flu*-mutant was used. Rap2.4h was also considered in the analysis to check its presumed weak susceptibility to ROS level perturbations.

ROS marker genes were included to gain more insight which ROS species were predominantly available. For example, direct H_2O_2 quantification in plants can be influenced by various factors, like ascorbate, polyphenols or sample handling (Veljovic-Jovanovic et al. 2002; Queval et al. 2008). Various reports demonstrated dissimilar H_2O_2 concentrations in plants, even if the same measuring procedure was applied (Queval et al. 2008). Thus, the usage of ROS marker genes omits such problems and provides indirect but more accurate information about sensed ROS levels (Mehterov et al. 2012). ROS marker genes were considered according to table 15.

Tab. 15: Selection of ROS marker genes for qPCR analysis in *flu*-mutant plants.

ROS-repsonse	Gene	Description	Reference
general	ZAT10 (at1g27730)	C2H2-type zinc finger transcription factor	Mehterov et al. 2012
chloroplastic H_2O_2	ROS M1 (At5g59845)	gibberellin-regulated family protein	Maruta et al. 2012
chloroplastic $^1O_2$	BAP1 (At3g61190)	Bonzai1-associated protein	op den Camp et al. 2003
chloroplastic $^1O_2$	ROS M2 (At5g57560)	xyloglucan endotransglucosylase	op den Camp et al. 2003

Seeds from homozygous *flu*-mutant plants and Col-0 wt plants were germinated on MS medium in perti dishes for 2 weeks under continuous light and 100 µmol photons m^{-2} s^{-1} light intensity in a climate controlled chamber. The further experimental setup referred to op den Camp et al. (2003). Mutant and Col-0 plantlets were incubated in darkness for 8 h and transferred back to light to induce $^1O_2$ generation in plastids. 30 and 60 min after onset of irradiation, 8 plantlets from 2 different petri dishes from Col-0 and the *flu*-mutant plantlets, respectively, were pooled for RNA isolation. After cDNA synthesis, the transcript levels were monitored by qPCR.

Rap2.4a mRNA levels were up-regulated (Fig. 62) (30 min: 2.4-fold; 60 min: 2.55-fold), in response to chloroplast $^1O_2$ accumulation. Contrary, the 2CPA transcript abundance was down-regulated (30 min: 0.68-fold; 60 min: 0.5-fold). This may be explained with previous reports, whereupon the 2CPA activity decreased upon high H_2O_2 concentrations (König et al. 2002; Shaikhali et al. 2010).

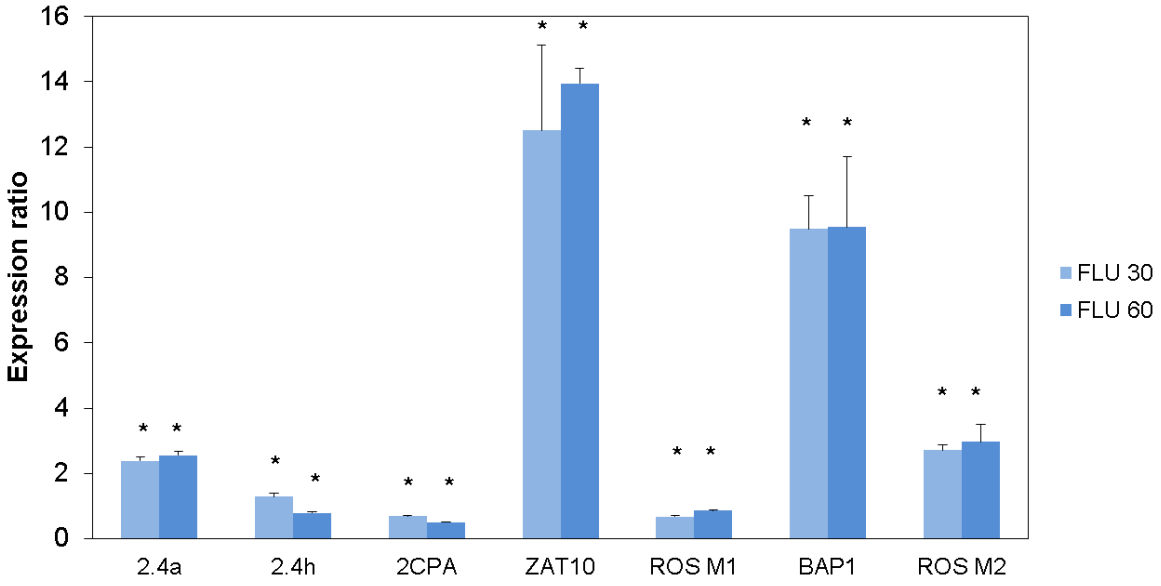

Fig. 62: Relative transcript levels in homozygous *flu*-mutant plants; plants were grown on solid MS medium in perti dishes for 2 weeks under continuous light and 100 µmol photons m^{-2} s^{-1} light intensity in a climate controlled chamber**.** 8 plantlets from 2 different petri dishes were pooled for RNA isolation from Col-0 and the *flu*-mutant, respectively, 30 and 60 min after reillumination. The relative expression ratio was measured by qPCR and normalized to Act7 transcript amount in Col-0 wt. The relative transcript levels of Col-0 wt reilluminated after 30 and 60 min, respectively, were set to 1.0. Data are means of 2 biological and 3 technical replicates (± SEM). The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis; Bars with * are significantly different ($p<0.05$) from control Col-0 wt plants.

The Rap2.4h transcript level was transiently induced. It was quickly up-regulated upon the early increase in the $^1O_2$ level. 30 min after the dark/light shift and subsequent onset of

ROS accumulation, the expression level was comparable to Col-0. After additional 30 min, the mRNA amount level was decreased (0.8-fold).

The induction of ROS marker genes provided information about the respective ROS type (Mehterov et al. 2012). ZAT10 and BAP1 were highly increased; ROS M2 mRNA levels were also elevated. ZAT10 is generally induced by ROS occurrence (Mehterov et al. 2012), whereas BAP1 and ROM M2 specifically react upon $^1O_2$ accumulation inside chloroplasts in *flu*-mutant plants (op den Camp et al. 2003; Kim et al 2012). MV treatment did not change the BAP1 transcript abundance in *flu*-mutants (op den Camp et al. 2003), demonstrating that this gene is less sensitive towards chloroplast H_2O_2 signals. The transcript level of ROS M1, which indicates chloroplast H_2O_2 (Maruta et al. 2012), was slightly decreased (30 min: 0.7-fold; 60 min: 0.86-fold).

The results revealed a transcriptional dependency for Rap2.4a, Rap2.4h and 2CPA towards signals derived from ROS generated in chloroplasts. The specific induction of ROS marker genes allows the presumption that the accumulation of $^1O_2$ in chloroplasts is directly or indirectly triggering appropriate signals to mediate transcriptional changes in nucleus. At least in case of Rap2.4a and 2CPA the modulating effects of ROS were consistent with available reports where Rap2.4a was up-regulated upon chloroplastic H_2O_2 generation (Maruta et al. 2012) and the 2CPA activity was down-regulated after application of high amounts of H_2O_2 (König et al. 2003). Hence, one could presume that H_2O_2 and $^1O_2$ affect similar signaling cascades which modulate the transcription of both genes. Regarding Rap2.4h, this TF seems to be down-regulated very fast towards disturbances in ROS status. Accounting its role as putative negative regulator of 2CPA and maybe other antioxidant enzymes, it would make sense to decrease its regulating ability in chloroplasts to boost the ROS abolishment.

The proposed 2CPA regulation (Fig. 63) considers ROS derived changes on the transcript levels (Fig. 62). The two options allow either the fine tuning of 2CPA activity and / or reaction upon tough conditions reflected through increment in ROS, which could modulate retrograde signaling cascades (Nott et al. 2006; Foyer and Shigeoka 2011). Here, for instance, redox sensitive MAPK kinases could transduce the signals and affect TF activity like demonstrated for Rap2.3. This AP2 TF is negatively redox regulated by several MAPK cascade components (Pitzschke et al. 2009).

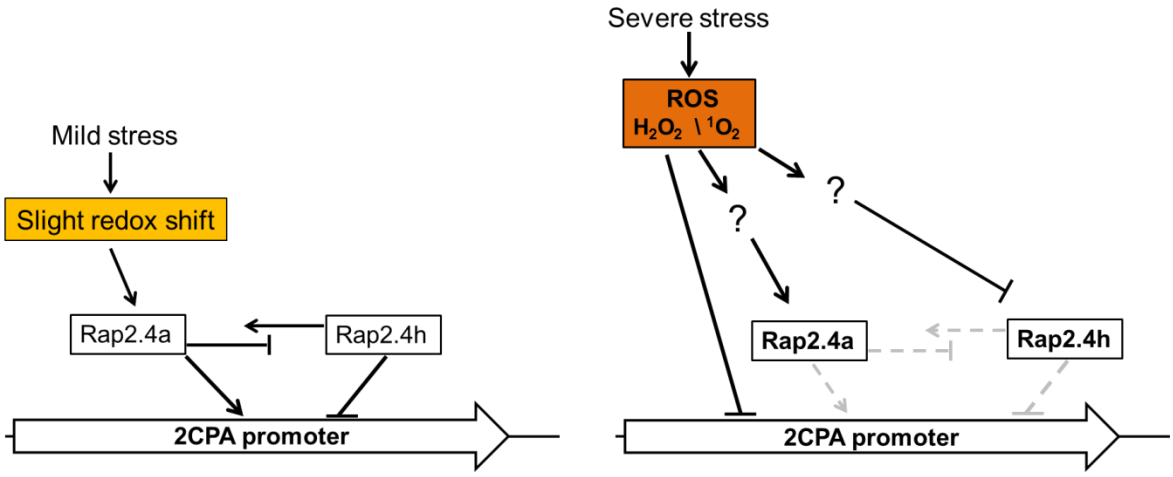

Fig. 63: Proposal for 2CPA promoter regulation with Rap2.4a as positive and Rap2.4h as negative factor upon mild stress conditions (left). Changes in ROS level could modulate signals important to regulate the transcription factors and down regulate 2CPA transcript amounts in terms of severe stress conditions (right). The direct transducer of the ROS signal could be a redox sensitive MAPK kinase.

3.4.3.5.2 Singlet oxygen could modulate signaling pathways involved in retrograde communication

The dark/light shift initiates $^1O_2$ accumulation in chloroplasts of *flu*-mutant plants (op den Camp et al. 2003; Foyer and Shigeoka 2011). Rap2.4a (Shaikhali et al. 2008), Rap2.4h as well as 2CPA (Baier et al. 1997) are nuclear encoded and further targeted to chloroplasts (Baier et al. 2000). Thus, the appropriate signaling pathway between both organelles that controls gene transcription could be retrograde signaling (Nott et al. 2006).

The question appears, if especially $^1O_2$ could be mainly responsible for the transcript regulation as signal provider. For example, there is evidence that H_2O_2 acts as signal transducer (Maurino and Flügge 2008; Balazadeh et al. 2012; Maruta et al. 2012). One reason could be the comparatively long half-life time (Bienert et al. 2007; Mubarakshina et al. 2010) allowing to act as signal outside of chloroplasts (Balazadeh et al. 2012). However, it is known that the life-time of $^1O_2$ is quite short (Hatz et al. 2007) but can be extended depending on available conditions (Foyer and Harbinson 1994).

It was demonstrated that $^1O_2$ molecules can disperse over wide ranges (200 nm/6 µs) (Skovsen et al. 2005). Notably, the prerequisite for this wide diffusion was a high amount of $^1O_2$ (Skovsen et al. 2005). The ongoing production of $^1O_2$ by protochlorophyllide upon reillumination (Meskauskiene et al. 2001) could maintain a stable level of $^1O_2$ in *flu*-mutants (op den Camp et al. 2003). Proof for the highly elevated $^1O_2$ level in the *flu*-mutant after dark/light shift comes from op den Camp and co-workers (2003). The high

accumulation could be demonstrated as relative fluorescence quenching of the dye DanePy (Hideg et al. 1998; op den Camp et al. 2003) and by increased fluorescence of the singlet oxygen sensor green (Flors et al. 2006). However, it is less likely that $^1O_2$ itself could be a direct transducer of information since the ROS abolishment in chloroplasts is highly efficient and $^1O_2$ is quenched by various electron donors (Foyer and Harbinson 1994). Hence, the diffusion of chloroplast-generated ROS species over long distances is limited (Mubarakshina et al. 2010). Therefore, the possibility of chloroplast $^1O_2$ triggered, far reaching signaling pathways is more likely.

Nevertheless, the *flu*-mutant system benefits from inducible and noninvasive $^1O_2$ production in plastids (op den Camp et al. 2003; Wagner et al. 2004; Kim et al 2012). Thus, the obtained results could display the possibility to regulate the nuclear transcription of Rap2.4a, Rap2.4h and 2CPA through $^1O_2$ mediated retrograde signaling.

3.5 Conclusions and perspectives

ERFIb transcription factors share common regulatory pathways in stress handling but also demonstrate individual stress responses. This variety and overlap facilitate manifold regulatory possibilities to react upon a wide range of abiotic stresses like cold or heat in a positive or negative manner. Up to now genetic engineering with AP2 TFs could improve the stress tolerance and subsequent the yield of various crop plants (reviewed in Xu et al. 2011). Experiments with abiotic stresses revealed that Rap2.4d can be proposed for testing its capability to improve the tolerance towards cold and salt stress (Fig. 14; Fig. 17). Rap2.4a and Rap2.4b should be considered to enhance heat stress permissiveness (Fig. 16). Stably transformed Arabidopsis plants overexpressing Rap2.4a and Rap2.4d, respectively, will provide more information about the usability to enhance stress tolerance.

The high number of 8 genes is a premise for co-regulation, compensation or competition between the ERFIb genes like demonstrated for other AP2 TFs (Eini et al. 2013). Bioinformatic analyses suggested co-expression between Rap2.4b and Rap2.4d during developmental stages and abiotic stress handling. Compensation was demonstrated upon salt stress where the knock-down of Rap2.4b was balanced by increased Rap2.4d mRNA level. A reverse genetic approach with knock-down and overexpression of individual ERFIb genes revealed a regulatory interplay between the TFs. Notably, Rap2.4e was the only TF where no homozygous T-DNA insertion line is available. Hence, an RNAi approach should be considered to investigate the relevance of Rap2.4e. Regulatory

competition is present between Rap2.4a and Rap2.4h. Both genes are antagonistically regulating the chloroplast antioxidant enzyme 2CPA. The analysis of particular regulatory mechanism altering the Rap2.4a and Rap2.4h expression revealed a modulation through $^1O_2$ mediated retrograde signaling pathways. NBT staining demonstrated that the lack of both genes directly or indirectly influences the overall ROS status and subsequently the antioxidant system. A direct regulation of other antioxidant enzymes like 2CPB should be investigated by Y1H approach.

4 References

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5 Appendix

Tab. 16: Primers used for quantification by qPCR.

Gene	gene code	Sequence Forward primer / reverse primer (5´ - 3´)
Rap2.4a	At1g36060	CGTCAGCGCCACAACAACATTC AACATCCGAAGTCGGTGAACCC
Rap2.4b	At1g78080	ATTTCGCCCGGCTTAACTTCCC AATCGCCTCCGATGTGAGATCC
Rap2.4c	At1g22190	TCCAACGATTCATCCGCGTT AGTGGAGATCCGACCCGTAT
Rap2.4d	At2g22200	TGAGTCACCGAGAAGCGATGAG CGATGAATATTCCGCCTGCGACTC
Rap2.4e	At5g65130	GTTCGAAACGGCTCAAGAAGCTG TTGAGACGAGCGTTGTCTCCTC
Rap2.4f	At4g39780	CACAGTTCAGACACGAGGATGG GCTTTGCGTCGACAGAGGAATG
Rap2.4g	At1g64380	GCTTTGCGTCGACAGAGGAATG ACCGCAGTAGTAGTTGTCGTCAC
Rap2.4h	At4g13620	AGGAACAGAACCCGTGTTTGGC CGTAAGCCATTGCTGCTTGCTC
Actin7	At5g09810	GTTGCCATTCAGGCCGTTCTTTC CAGAATCGAGCACAATACCGGTTG
2CPA	At3g11630	CCCAACAGAGATTACTGCCT ATAGTTCAGATCACCAAGCCC
2CPB	At5g06290	TCATACCCTCTTCCTCGGCATC ACCGACCAGTGGTAAATCATCAGC
ZAT10	At1g27730	TCACAAGGCAAGCCACCGTAAG TTGTCGCCGACGAGGTTGAATG
BAP1	At3g62290	ATCGGATCCCACCAGAGATTACGG AATCTCGGCCTCCACAAACCAG
ROS M1	At5g59845	TACTCTCAACCGCGGATTCGTC TCCTGCCTTTGAACATCTCACG
ROS M2	At5g57560	ACTACTGGCTCGTGGTTGTCAC TTCCTCTGCACCCATCTCATCC

Tab. 17: List of genes included in the protein category from top 100 co-expressing genes with Rap2.4b in developmental series.

protein category from top 100 co-expressing genes wirh Rap2.4b according to MapMan hierarchical categorization		
protein.targeting.secretory pathway.vacuole	at3g52850	ATELP1 (VACUOLAR SORTING RECEPTOR HOMOLOG)
protein.postranslational modification	at5g35980	protein kinase family protein
protein.postranslational modification	at3g51370	protein phosphatase 2C. putative / PP2C
protein.postranslational modification	at3g62260	protein phosphatase 2C. putative / PP2C
protein.postranslational modification	at5g04540	phosphatase/ protein tyrosine phosphatase
protein.postranslational modification	at4g29380	protein kinase family protein / WD-40 repeat family protein
protein.postranslational modification	at3g53930	protein kinase family protein
protein.postranslational modification	at4g19110	Protein kinase superfamily protein19740
protein.postranslational modification	at3g62260	protein phosphatase 2C. putative / PP2C. putative
protein.postranslational modification	at5g35980	Symbols: YAK1
protein.postranslational modification	at4g29380	protein kinase family protein / WD-40 repeat family protein
protein.degradation.autophagy	at1g54710	Symbols: ATATG18H
protein.degradation.AAA type	at1g02890	AAA-type ATPase family protein
protein.degradation.ubiquitin.E3.HECT	at5g02880	Symbols: UPL4 | UPL4
protein.degradation.ubiquitin.E3.RING	at5g63970	copine-related
protein.degradation.ubiquitin.E3.RING	at3g15070	zinc finger (C3HC4-type RING finger) family protein
protein.degradation.ubiquitin.E3.RING	at1g14200	RING/U-box superfamily protein
protein.degradation.ubiquitin.E3.RING	at5g63970	Copine (Calcium-dependent phospholipid-binding protein) family
protein.degradation.ubiquitin.E3.RING	at3g15070	RING/U-box superfamily protein
protein.degradation.ubiquitin.E3.SCF.FBOX	at2g02870	kelch repeat-containing F-box family protein
protein.degradation.ubiquitin.E3.SCF.FBOX	at1g23780	F-box family protein

Tab. 18: List of genes included in the protein category from top 100 co-expressing genes with Rap2.4d in developmental series.

protein category from top 100 co-expressing genes wirh Rap2.4d according to MapMan hierarchical categorization		
protein.synthesis.ribosomal protein	at5g16200	50S ribosomal protein-related
protein.synthesis.initiation	at1g69410	Encodes eIF5A-2
protein.synthesis.initiation	at5g54940	eukaryotic translation initiation factor SUI1
protein.targeting.secretory pathway.unspecified	at1g22180	SEC14 cytosolic factor family protein
protein.postranslational modification	at5g44290	protein kinase family protein
protein.postranslational modification	at1g30640	protein kinase. putative
protein.postranslational modification	at3g27560	encodes a protein with kinase domains
protein.postranslational modification	at5g44290	Protein kinase superfamily protein
protein.postranslational modification	at4g32300	S-domain-2 5 (SD2-5)
protein.postranslational modification	at4g33920	protein phosphatase 2C family protein
protein.postranslational modification	at1g30640	protein kinase. Putative
protein.postranslational modification.kinase.receptor like cytoplasmatic kinase III	at1g67470	protein kinase family protein
protein.postranslational modification.kinase.receptor like cytoplasmatic kinase VIII	at3g59350	serine/threonine protein kinase
protein.degradation.cysteine protease	at3g02070	Cysteine proteinases superfamily protein
protein.degradation.serine protease	at1g63120	ATRBL2 (ARABIDOPSIS THALIANA RHOMBOID-LIKE 2)
protein.degradation.ubiquitin.E2	at4g36800	RUB1
protein.degradation.ubiquitin.E3.RING	at3g07360	armadillo/beta-catenin repeat family protein
protein.degradation.ubiquitin.E3.RING	at3g05200	ATL6
protein.degradation.ubiquitin.E3.RING	at3g16720	RING-H2
protein.degradation.ubiquitin.E3.RING	at3g07360	PUB9 (PLANT U-BOX 9)
protein.degradation.ubiquitin.E3.RING	at3g16720	ATL2; protein binding / zinc ion binding
protein.degradation.ubiquitin.E3.RING	at1g63900	E3 Ubiquitin ligase family protein
protein.degradation.ubiquitin.E3.RING	at3g05200	Encodes a putative RING-H2 zinc finger protein ATL6
protein.degradation.ubiquitin.E3.RING	at3g07360	plant U-box 9 (PUB9)
protein.degradation.ubiquitin.ubiquitin protease	at1g04860	Encodes a ubiquitin-specific protease

Tab. 19: List of genes included in the protein category from top 100 co-expressing genes with Rap2.4b in abiotic stress in roots.

protein category from top 100 co-expressing genes wirh Rap2.4b according to MapMan hierarchical categorization		
protein.synthesis.elongation	at4g22780	Member of a family of ACT domain containing proteins
protein.targeting.peroxisomes	at3g04460	APM4/ATPEX12/PEX12 (PEROXIN-12)
protein.postranslational modification	at1g67580	protein kinase family protein
protein.postranslational modification	at3g55270	MKP1 (MKP1); MAP kinase phosphatase
protein.postranslational modification	at5g01820	Symbols: ATSR1, SnRK3.15, CIPK14, ATCIPK14
protein.degradation.cysteine protease	at1g09730	Cysteine proteinases superfamily protein
protein.degradation.ubiquitin.E3.RING	at3g12920	protein binding / zinc ion binding
protein.degradation.ubiquitin.E3.RING	at4g03510	RMA1 (Ring finger protein with Membrane Anchor 1)
protein.degradation.ubiquitin.E3.RING	at3g60220	Symbols: ATL4 | ATL4
protein.degradation.ubiquitin.E3.RING	at3g15070	zinc finger (C3HC4-type RING finger) family protein
protein.degradation.ubiquitin.E3.RING	at1g55530	zinc finger (C3HC4-type RING finger) family protein
protein.degradation.ubiquitin.E3.RING	at5g22000	encodes a RING-type E3 ubiquitin ligase
protein.degradation.ubiquitin.E3.RING	at1g14200	zinc finger (C3HC4-type RING finger) family protein
protein.degradation.ubiquitin.E3.RING	at2g22680	zinc finger (C3HC4-type RING finger) family protein
protein.degradation.ubiquitin.E3.RING	at4g33940	zinc finger (C3HC4-type RING finger) family protein
protein.degradation.ubiquitin.E3.RING	at3g12920	SBP (S-ribonuclease binding protein) family protein
protein.degradation.ubiquitin.E3.RING	at4g34100	RING/U-box superfamily protein
protein.degradation.ubiquitin.E3.RING	at3g47550	zinc finger (C3HC4-type RING finger) family protein
protein.degradation.ubiquitin.E3.RING	at3g43430	RING/U-box superfamily protein
protein.degradation.ubiquitin.E3.RING	at4g03510	RMA1 encodes a novel 28 kDa protein with a RING finger motif
protein.degradation.ubiquitin.E3.RING	at1g55530	zinc finger (C3HC4-type RING finger) family protein
protein.degradation.ubiquitin.E3.RING	at5g48655	zinc finger (C3HC4-type RING finger) family protein
protein.degradation.ubiquitin.E3.SCF.FBOX	at3g59940	kelch repeat-containing F-box family protein
protein.degradation.ubiquitin.E3.SCF.FBOX	at1g77000	AtSKP2;2 is a homolog of human SKP2
protein.degradation.ubiquitin.E3.BTB/POZ Cullin3.BTB/POZ	at3g06190	ATBPM2; protein binding

Tab. 20: List of genes included in the protein category from top 100 co-expressing genes with Rap2.4d in abiotic stress in roots.

protein category from top 100 co-expressing genes wirh Rap2.4d according to MapMan hierarchical categorization		
protein.targeting.secretory pathway.vacuole	at3g52850	ATELP1 (VACUOLAR SORTING RECEPTOR HOMOLOG)
protein.postranslational modification	at4g19110	Protein kinase superfamily protein
protein.postranslational modification	at3g51370	Protein phosphatase 2C family protein
protein.postranslational modification	at3g53930	Protein kinase superfamily protein
protein.postranslational modification	at5g35980	YAK1. protein kinase family protein
protein.postranslational modification	at3g62260	Protein phosphatase 2C family protein
protein.postranslational modification	at4g29380	protein kinase family protein
protein.postranslational modification	at5g04540	Myotubularin-like phosphatases II superfamily
protein.degradation.autophagy	at1g54710	ATATG18H
protein.degradation.AAA type	at1g02890	AAA-type ATPase family protein
protein.degradation.ubiquitin.E3.HECT	at5g02880	UPL4
protein.degradation.ubiquitin.E3.RING	at1g14200	zinc finger (C3HC4-type RING finger) family protein
protein.degradation.ubiquitin.E3.RING	at5g63970	Copine (Calcium-dependent phospholipid-binding protein) family
protein.degradation.ubiquitin.E3.RING	at3g15070	RING/U-box superfamily protein
protein.degradation.ubiquitin.E3.SCF.FBOX	at2g02870	Galactose oxidase/kelch repeat superfamily protein
protein.degradation.ubiquitin.E3.SCF.FBOX	at1g23780	F-box family protein

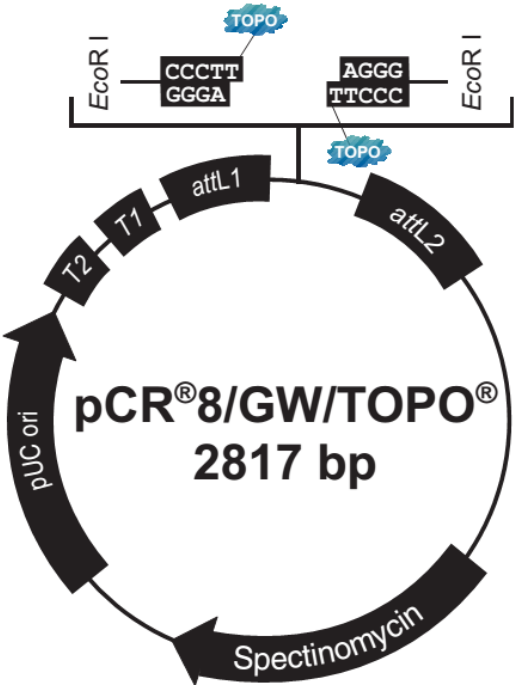

Fig. 64: Vector map of pCR8/GW/TOPO plasmid used for TOPO cloning mediated generation of entry clones; Spectinomycin resistance gene (SpnR); pUC ori: pUC origin of replication; T2 / T1: rrnB T2 and T1 transcription termination sequences to prevent basal transcription of the inserted PCR product in *E. coli*; attL1 / attL2: sites for recombinational transfer of appropriate gene into destination plasmid (Invitrogen, Carlsbad, USA).

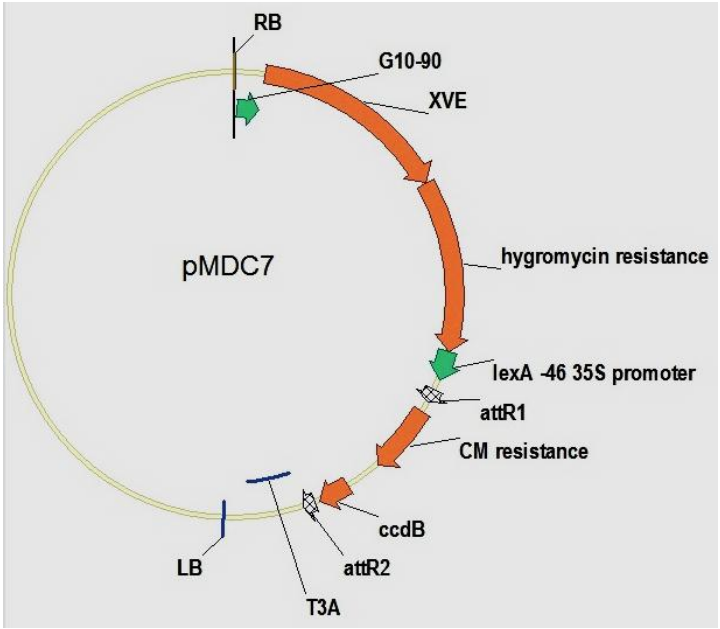

Fig. 65: Vector map of pMDC7 plasmid which served as destination vector in TOPO cloning procedures; G10-90: strong constitutive promoter; XVE: chimeric transcription activator; CM resistance: Chloramphenicol resistance gene; minimal promoter: lexA -46 35S; ccdB: positive-selection marker, lethal gene that targets DNA gyrase; attL1 / attL2: sites for recombinational transfer of appropriate gene (Curtis et al. 2003).

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I hereby declare that I have prepared and written the presented doctoral thesis myself using only the presented methods and sources. All sources from literature are marked as such and are properly cited.

Berlin, July 2013

Radoslaw Rudnik