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DEPARTMENT OF ARCTIC AND MARINE BIOLOGY

Characterization of a Cytokinin Response Factor in *Arabidopsis thaliana*

Bernd Ketelsen

A dissertation for the degree of Philosophiae Doctor

Spring 2012

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I Acknowledgements

It is almost five years ago that I moved north of the Arctic Circle for doing my PhD studies at the University of Tromsø. It was a long way to this final point. I am happy that I did not have to walk it all alone. Many people have accompanied, supported and helped me throughout this time. Some people deserve a special thank:

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II Abbreviations

ABA	Abscisic Acid
ABI4	Abscisic Acid Insensitive 4
AHK	Arabidopsis Histidine Kinase
AHP	Arabidopsis Histidine Phosphotransfer Protein
AP2	Apetala 2
ARC, PARC	Accumulation And Replication Of Chloroplasts, Paralog Of ARC
ARR	Arabidopsis Response Regulator
ATP, ADP, AMP	Adenosine Tri-, Di-, Monophosphate
BA	N^6-Benzyladenine
CBF	C-Repeat Binding Factor
CDK	Cyclin-Dependent Protein Kinases
CK	Cytokinin
CKX	Cytokinin Oxidase/Dehydrogenase
CRF	Cytokinin Response Factor
DNA	Deoxyribonucleic Acid
DPE	Downstream Promoter Element
DRE	Drought Response Element
DREB	Drought Response Element Binding Protein
ERE	Ethylene Response Element
EREBP	Ethylene Response Element Binding Protein
ERF	Ethylene Response Factor
GFP	Green Fluorescent Protein
GTF	General Transcription Factor
HXK	Hexokinase
Inr	Initiator
IPT	Adenosine Phosphate Isopentenyltransferase
NLS	Nuclear Localisation Signal
PDV	Plastid Division Protein
PIC	Transcriptional Preinitiation Complex
RAV	Related To ABI3/VP1
RM	Root Meristem

RNA	Ribonucleic Acid
SAM	Shoot Apical Meristem
SEX1	Starch Excess 1
TAD	Transactivation Domain
TCS	Two Component System
TF	Transcription Factor
WHY	Whirly

III Explanatory Remarks

Gene names and gene abbreviations are written in *CAPITAL LETTERS* in italics, protein names and protein abbreviations are written in CAPITAL LETTERS.

IV Abstracts

Acclimation responses to environmental growth conditions in plants involve complex and fine-tuned signalling networks. Environmental signals, biotic and abiotic, are received and mediated, and lead eventually to transcriptional regulation. The plant hormones are vastly involved in these processes as intercellular mediators whereas the reception of such a hormone at the site of action involves intracellular protein signalling cascades. This thesis investigates the function of the transcription factor CRF5 of *Arabidopsis thaliana* which was so far known to be integrated in the course of cytokinin signalling and cotyledon development. AtCRF5 belongs to the ERF subfamily of the AP2/EREBP transcription family and is accordingly carrying an AP2 DNA binding domain which was shown to enable members of this family to bind to the *cis*-regulatory elements Ethylene Responsive Element (ERE) and the Drought Responsive Element (DRE).

By yeast transactivation assays it was possible to show that AtCRF5 acts not only as an activator of transcription but also that the responsible transactivation domain is located among the 98 final amino acids. A detailed sequence analysis of this so far uncharacterized region and a phylogenetic analysis revealed two conserved sites which could be found throughout the plant kingdom. These sites group the CRF subfamily into four clusters of which only three can be found in Arabidopsis. Promoter interaction studies revealed that AtCRF5 is able to interact with the *cis*-regulatory DRE element of *RD29A* and to induce gene expression. The regulation of gene expression of components of the cytokinin signalling pathway could be confirmed by expression analysis of transgenic Arabidopsis lines although it could not be proven if this is a direct or indirect effect. Also the crosstalk between cytokinins and other hormones might be facilitated through AtCRF5. Phenotypic studies on transgenic Arabidopsis seedling constitutively overexpressing *AtCRF5* showed pleiotropic effects like shorter roots, smaller shoots, and fewer chloroplasts per cell as well as a hexose/sucrose ratio shift towards the hexoses. Surprisingly, the shoot and the root phenotype could be reversed by adding sucrose to the growth medium which points towards an effect of AtCRF5 on sugar metabolism.

Summarized, it could be shown in this thesis that AtCRF5, as a component of the cytokinin signalling pathway, is able to connect this pathway with the regulatory action of AP2/EREBP transcription factors. AtCRF5 is involved in hormonal crosstalk and sugar metabolism in *Arabidopsis thaliana.*

Abstract in norwegian

Planter responderer på ulike miljøfaktorer gjennom et intrikat nettverk av signaloverføringer. Biotiske og abiotiske signaler som fanges opp fra omgivelsene bearbeides i planten og fører til reguleringer på transkripsjonsnivå. Denne prosessen involverer i stor grad plantehormoner, som binder til intercellulære reseptorer og setter i gang proteinsignalering i cellen. I denne avhandlingen presenteres en studie av funksjonen til transkripsjonsfaktoren CRF5 i *Arabidopsis thaliana* (AtCRF5), som er kjent for å være involvert i cytokininsignalering og utvikling av frøblader. AtCRF5 tilhører en undergruppe av transkripsjonsfamilien AP2/EREBP, og har et AP2 domene som medierer bindingen til enkelte cis-regulerende elementer.

Analyser har vist at domenet for transkripsjonsaktivering i AtCRF5 er lokalisert blant de siste 98 aminosyrene av proteinet. En detaljert sekvensanalyse av denne regionen har identifisert to områder som er konservert i planteriket. Disse områdene deler subfamilien CRF videre inn i fire grupper, hvorav bare tre er identifisert i Arabidopsis. Interaksjonsstudier med promotor viser at AtCRF5 kan interagere med det *cis*-regulerende DRE elementet hos *RD29A* og indusere genuttrykk. Reguleringen av genuttrykket i komponenter av cytokininsignaleringen kunne bekreftes ved å analysere uttrykket i transgene planter, men det har ikke blitt funnet beviser for hvorvidt dette er en direkte eller indirekte effekt. Også kommunikasjonen mellom cytokinin og andre hormoner kan være styrt gjennom AtCRF5.

Fenotypiske studier av transgene planter som overuttrykker *AtCRF5* viste flere pleiotropiske effekter, blant annet kortere røtter, mindre skudd og færre kloroplaster per celle. I tillegg ble det observert en endring i forholdet mellom heksose og sukrose. Fenotypen i skudd og røtter kunne reverseres ved tilsetning av sukrose til vekstmediet, noe som indikerer at AtCRF5 også har en effekt på plantenes sukkermetabolisme.

Sammenfattet kunne det vises at AtCRF5, som en komponent i signalering av plantehormonet cytokinin, forbinder denne signalveien med regulatoriske funksjoner av AP2/EREBP transkripsjonsfaktorer. AtCRF5 er involvert i hormoncrosstalk og sukkermetabolismen i *Arabidopsis thaliana*.

V List of Manuscripts

Manuscript 1

Bernd Ketelsen, Rainer Schwacke, Kirsten Krause, and Karsten Fischer

Transcriptional activation by Cytokinin Response Factor 5 is governed by an acidic C-terminus containing two conserved domains

Submitted to Plant Cell Reports

Manuscript 2

Bernd Ketelsen, Stian Olsen, Kirsten Krause, and Karsten Fischer

Cytokinin responsive factor 5 (CRF5) is involved in root development, hormonal crosstalk and sugar metabolism in Arabidopsis thaliana

Submitted to Planta

Manuscript 3

Bernd Ketelsen, Stian Olsen, Karsten Fischer, and Kirsten Krause

Correlation between 16S/18S rDNA ratio and chloroplast copy numbers in cotyledons of Arabidopsis thaliana*: use for assessment of the impact of cytokinin response factor 5 on chloroplast development*

Submitted to Endocytobiosis and Cell Research

Supplementary Manuscript

Rainer Schwacke, Karsten Fischer, Bernd Ketelsen, Karin Krupinska, and Kirsten Krause

Comparative survey of plastid and mitochondrial targeting properties of transcription factors in Arabidopsis and rice

Published in Molecular Genetics and Genomics 2007; 277: 631-46.

VI Introduction

Terrestrial plants face a great problem when environmental changes occur: They are unable of movement to search for better life conditions (nutrients, light) or to avoid stresses (drought, temperature, competition) like animals can (for illustration see Figure 1). Terrestrial plants cope with their situation by growing and developing new or reshaping existing organs or discarding them. The ability to react plastic, the phenotypic plasticity, is genomically encoded and believed to increase fitness (Schlichting, 1986; West-Eberhard, 1989; Gilroy and Trewavas, 2001). The regulatory network connecting signal transduction with gene regulation is the control room in plasticity processes (Pandey and Somssich, 2009; Gilroy and Trewavas, 2001). Plant hormones act as messengers by being produced in single cells or tissue and being transported to their target sites. There, the "message" is received and complex inner cellular signalling cascades eventually lead to specific modulations through modified gene expression based on the action of an enormous variety of transcriptional regulators, the transcription factors.

The study of transcription factors is important to get a deeper insight into the complex mechanisms which work at the molecular basis of the development of all organisms. Other regulatory processes downstream of transcriptional regulation, during gene expression in the broadest sense, are also involved in signalling. Alternative splicing, microRNA action, and post-translational regulations to mention but a few should not be disregarded (Chen and Rajewsky, 2007; Filipowicz et al., 2008; Chen and Manley, 2009; Schütze et al., 2008). This introduction, however, focuses mainly on the characteristics and regulatory functions of transcription factors in general, one transcription factor family (AP2/EREBP) in particular and its connection to cytokinin signalling.

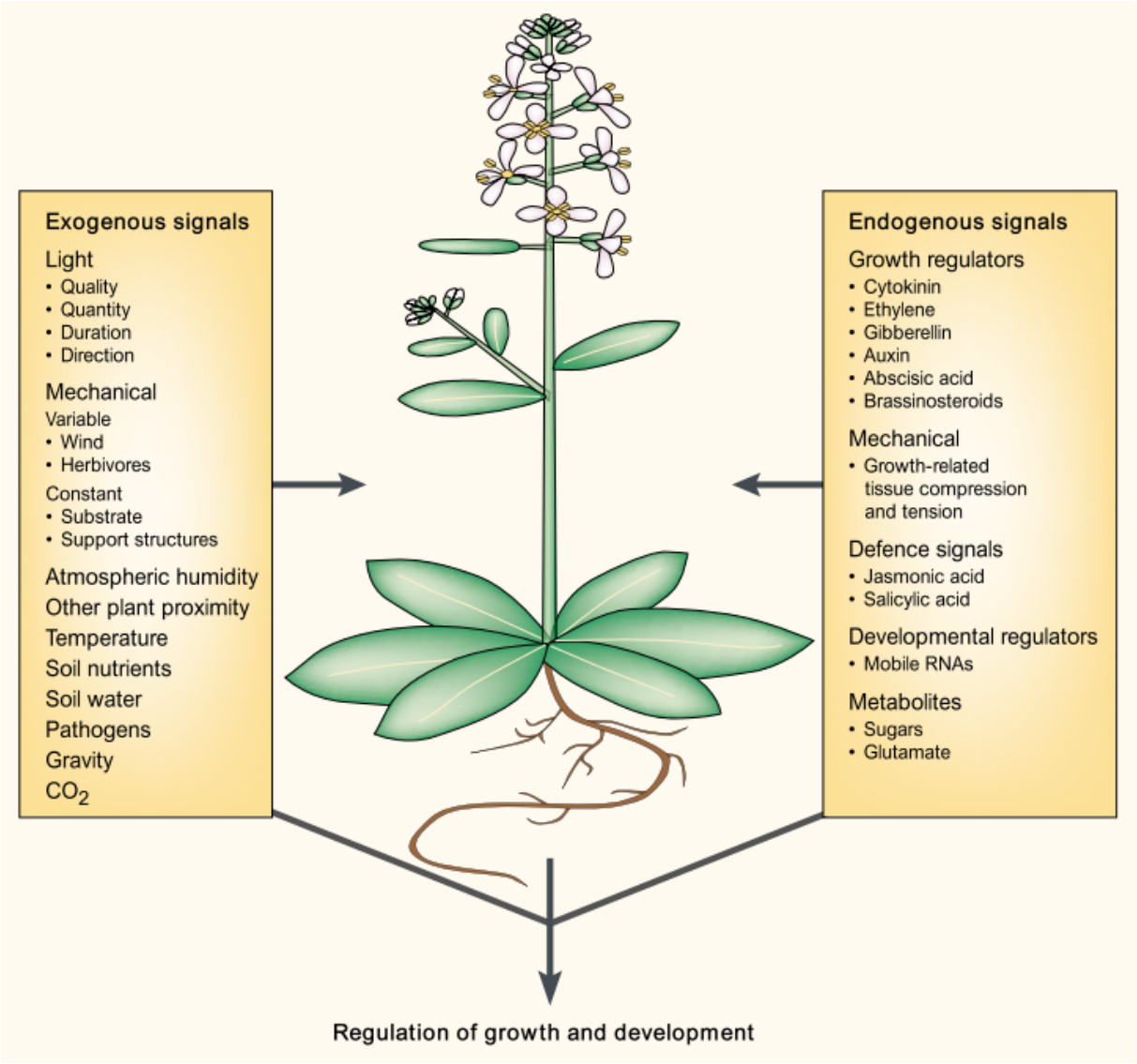


Figure 1 - Regulation of plant growth and development (Gilroy and Trewavas, 2001).

A Transcription Factors

It is estimated that around 5% of all genes in eukaryotes code for transcription factors (TFs). Transcription factors display a crucial component in differential gene expression (Riechmann and Ratcliffe, 2000). They specifically bind *cis*-regulatory elements in promoter sequences and accordingly they are able to alter the expression of their associated genes. Cell types, tissues, and plant developmental stages as well as adaptations to stresses are defined by a specific regulatory network which consists of a particular set of differentially expressed transcription factors and a thereby altered gene expression according to the effective conditions (Palaniswamy et al., 2006; Dietz et al., 2010).

The comparatively high number of TFs in regard to the total number of different proteins in plants is due to numerous gene duplication events that might have happened by segmental and tandem duplications as well as whole genome duplications. Especially duplicates of genes involved in transcription, signal transduction, and development are retained after duplication events (Maere et al., 2005; Rizzon et al., 2006). The thereby achieved gene redundancy might be a response to complexity of environments (Gilroy and Trewavas, 2001).

Figure 2 - The general transcription machinery in eukaryotes and its interactions with transcriptional activators.
Abbreviations: Activators: DBD, DNA binding domain; AD, activation domain; General Cofactors: TAFs, TATA box associated factors; USA, upstream stimulatory activity-derived cofactors; Transcription Pre-initiation Complex (PIC): GTFs, General Transcription Factors; DNA elements: TATA, TATA box; Inr, initiator; DPE, Downstream Promoter Element. After (Thomas and Chiang, 2006).

Interactions between TFs and the transcription machinery are mediated by general cofactors which interact with the transcription preinitiation complex (PIC). The PIC is successively assembled and starts with the binding of the General Transcription Factor TFIID to the TATA box, the initiator (Inr) or the downstream promoter element (DPE). The other General

Transcription Factors (GTFs) gradually join and together with the RNA Polymerase II they complete the PIC. The PIC alone is able to run basal transcription. But the interaction with the general cofactors and transcriptional activators or repressors is required for regulating gene expression (for illustration see Figure 2; reviewed in (Thomas and Chiang, 2006)).

A-1 The AP2/EREBP Transcription Factor Family

The core transcription machinery is highly conserved throughout the kingdoms. But on the regulatory level differences are huge between the animal kingdom and the plant kingdom. The independent evolution of both kingdoms with the last ancestor being a protist, is reflected on TF level as well. For instance, transcription factor families like the homeodomain containing TFs or the MADS-box proteins originated before divergence of the two kingdoms (Liu et al., 1999; Chen and Rajewsky, 2007). Their developmental functions, however, have evolved into different directions (Chen and Rajewsky, 2007). Other TF families can only be found in one kingdom. The AP2/EREBP transcription factor family (also known as ERF transcription factor family) was until recently thought to only be present in plants (Riechmann and Meyerowitz, 1998). Yet Magnani and co-workers have found homologs in a cyanobacterium, a ciliate, and in two viruses. In these organisms these proteins are predicted to be HNH-endonucleases. It was proposed that the AP2/EREBP TF family in plants originates in these organisms and was introduced into plants via lateral gene transfer (Magnani et al., 2004).

Currently 147 gene loci in the Arabidopsis genome (Nakano et al., 2006), 200 in poplar (Zhuang et al., 2008), 132 in grapevine (Zhuang et al., 2009), 131 in cucumber (Hu and Liu, 2011), and 163 loci in the rice genome (Sharoni et al., 2011) are considered to be coding for members of this family. The AP2/EREBP transcription factor family is classified into 5 subfamilies: DREB (57 members in Arabidopsis), ERF (65), AP2 (18), RAV (6), and others (1) (Zhuang et al., 2009). The two biggest subfamilies are divided in both cases into 6 groups (DREB: A-1 - A-6, ERF: B-1 - B-6) (for illustration see Figure 3; (Sakuma et al., 2002)).

AP2/EREBP transcription factors have been shown to be involved in growth and development (Rashotte et al., 2006; Kubo and Kakimoto, 2000; Okazaki et al., 2009; Dietz et al., 2010; Wellmer and Riechmann, 2005), hormone response (Solano et al., 1998; Lorenzo et al., 2003; Rashotte et al., 2006; Niu et al., 2002; Hu et al., 2004), and abiotic stress response (Liu et al., 1998; Sun et al., 2008; Licausi et al., 2011). In comparative studies it was shown that the expression of members of this family is regulated in different degrees to various hormones

and stresses (Krishnaswamy et al., 2011). Through analysis of coexpression datasets it was concluded that many AP2/EREBP transcription factors are interdependently regulated and are therefore building a complex network (Dietz et al., 2010). Developmental functions of selected AP2/EREBP TFs will be revisited in Chapter VI A-3.

A-2 Interaction of Members of the AP2/EREBP Transcription Factor Family with DNA and the Transcription Machinery

Typically, TFs contain a DNA binding domain, an oligomerization domain, a transcription regulation or transactivation domain, and a nuclear localization signal (NLS) (Liu et al., 1999). The DNA binding domains of TFs are binding to DNA bases of specific *cis*-regulatory elements. DNA binding domains usually have a basic character and are highly conserved (Liu et al., 1999). Because of this, TFs are often grouped into families according to sequence similarities of their DNA binding domain (Liu et al., 1999; Riechmann and Meyerowitz, 1998b). Other well described DNA binding domains besides the AP2 domain are e.g. the zinc finger domain, the bZIP domain or the homeodomain (reviewed in (Liu et al., 1999)).

Plant TFs usually contain one or two copies of the same DNA binding domain (Liu et al., 1999). In case of AtERF1 it was shown that the AP2 domain of the AP2/EREBP TFs comprises a three-stranded β-sheet and one α-helix. TF-DNA interaction is being established through the three-stranded β-sheet (for illustration see Figure 4; (Allen et al., 1998)).

For many TFs, the corresponding *cis*-regulatory element is known. For example, it has been shown for the AP2/EREBP TF family that Arabidopsis DREB1A, DREB2A and TINY are capable to specifically bind the drought response element (DRE) G/ACCGAC (Liu et al., 1998; Sakuma et al., 2002; Sun et al., 2008). This element has been found in the promoter of *rd29a* whose expression is responsive to cold, drought and ABA signalling (Jeon et al., 2010; Liu et al., 1998; Msanne et al., 2011). The second prominent *cis*-regulatory element of the AP2/EREBP TFs is the GCC-box or ethylene response element (ERE; AGCCGCC) which for instance can be bound by AtERF1 – 5 (Fujimoto et al., 2000; Allen et al., 1998) but also by TINY which means that this TF is able to bind both *cis*-regulatory elements which are characteristic for the AP2/EREBP TFs (Sun et al., 2008).

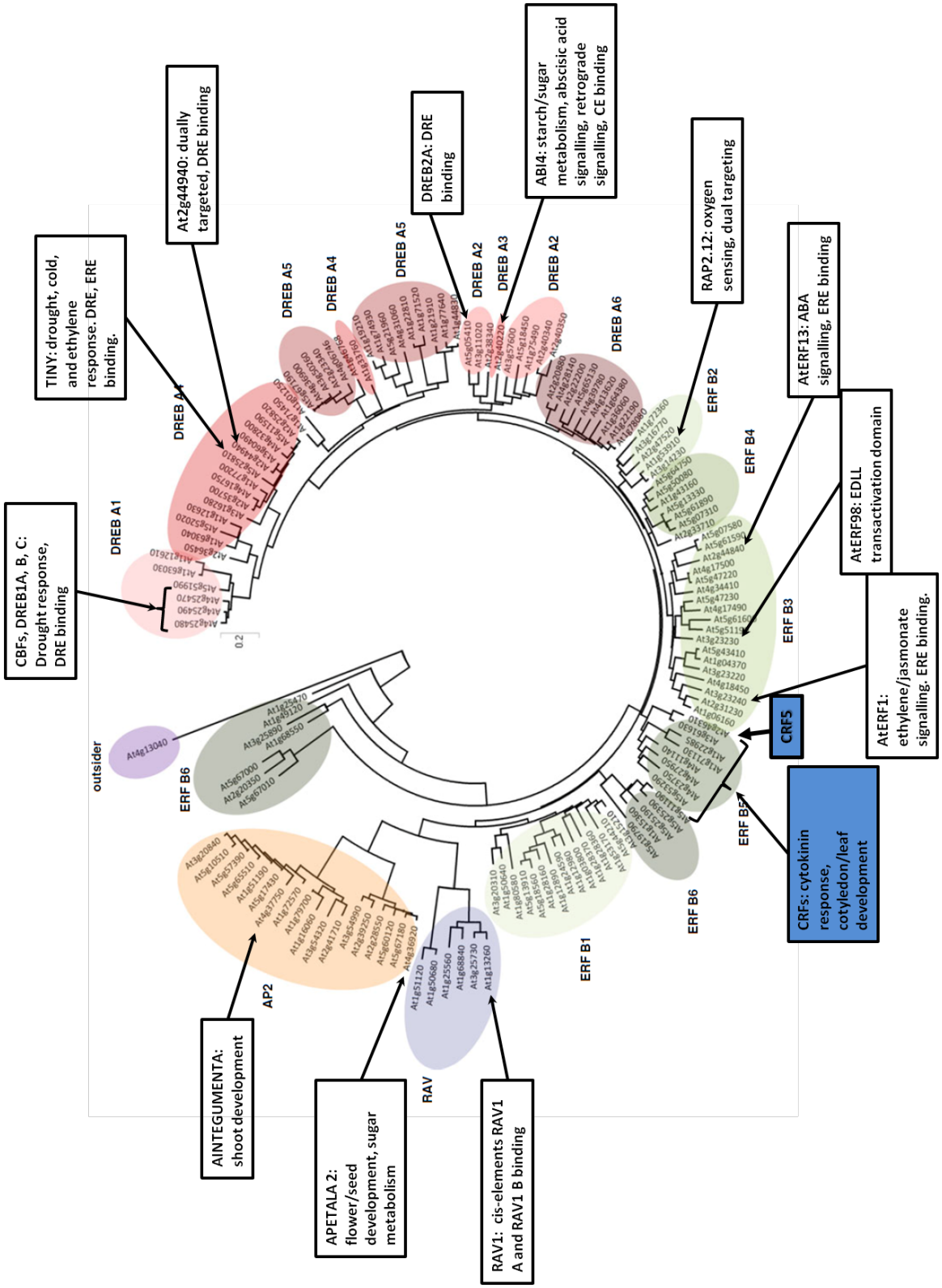


Figure 3 - The AP2/EREBP transcription factor family.
Phylogenetic tree of all AP2/EREBP transcription factors of *Arabidopsis thaliana*. Arrows with descriptions mark members of the AP2/EREBP TFs which are mentioned in this thesis. Subfamilies and subgroups are indicated after Sakuma et al., (2002). Figure modified after Dietz et al., (2010).

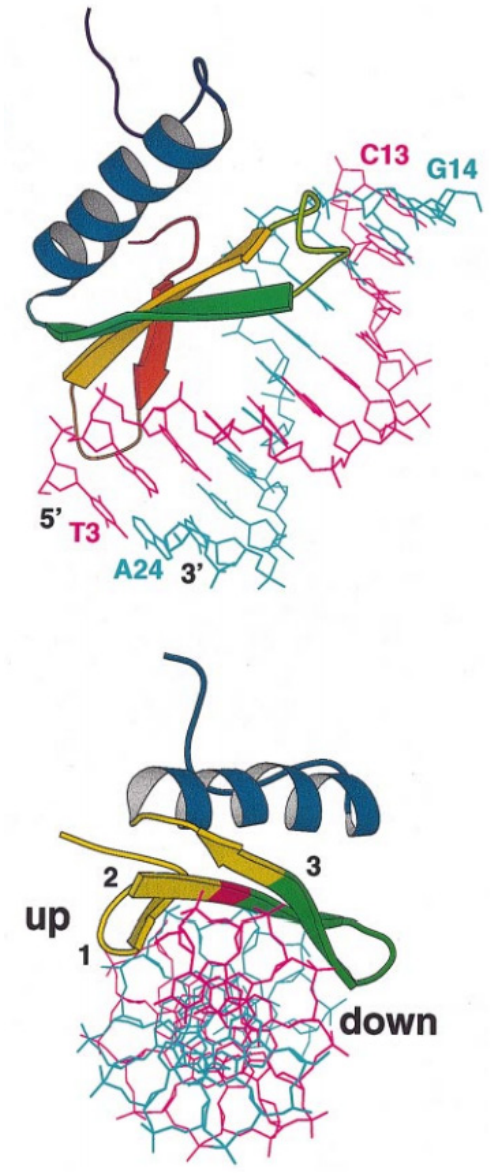


Figure 4 – Three dimensional structure of the GCC-Box binding AP2 domain of AtERF1 (Allen et al., 1998).

Maize ABI4, another AP2/EREBP TF, binds to the so called coupling element (CE1; CACCG), a binding element composed of a DNA sequence very similar to the DRE element (Niu et al., 2002).

In parallel, it seems as if these *cis*-elements and the ability to be bound by AP2 proteins are conserved throughout the plant kingdom. This could be observed in the case of one wheat AP2/EREBP protein, namely CBF1, which is the homolog to DREB1A in Arabidopsis and binds preferentially to the base sequence TTGCCGACAT which contains the DRE (Xue, 2002). Also, Arabidopsis ERF13 is able to bind the CE1 element which was bound by ABI4 from Maize (Lee et al., 2010).

But there is also evidence that there are not only DRE and ERE that work as *cis*-regulatory elements for AP2/EREBP TFs (Reeves et al., 2011). RAP2.12 binds the hypoxia responsive element ATCTA (Licausi et al., 2011). And RAV1 is able to bind a bipartite *cis*-regulatory element (CAACA and CACCTG) but RAV1 is in that respect special that it belongs to the RAV subfamily and contains in addition to the AP2 domain also the B3 DNA binding domain (Kagaya et al., 1999). (For an overview over known cis-regulatory elements connected to the AP2/EREBP TF family see Table 1.)

Through binding assays it was shown that most exchanges of single bases of the two major *cis*-regulatory elements, DRE and ERE, only lead to a reduction of interaction strength between TF and the *cis*-regulatory element. However, exchanges of the core CCG-sequence led to drastic reductions. Resulting from these and other studies a core base composition of CCG plus flexible flanking sequences for AP2/EREBP TFs emerged (Xue, 2002; Fujimoto et al., 2000; Niu et al., 2002; Liu et al., 1998; Sakuma et al., 2002). This flexibility is supported by studies which have shown that *cis*-regulatory elements are in general very low conserved (Chen and Rajewsky, 2007).

Table 1 - Known *cis*-regulatory elements bound by members of the AP2/EREBP transcription factor family

***Cis*-regulatory element or TF**	**Sequence**	**References**
DRE, HvCBF1	(T)G/ACCGAC(AT)*	(Stockinger et al., 1997; Liu et al., 1998; Sakuma et al., 2002; Xue, 2002)
CE1	CACCG; TGCCACCGG	(Niu et al., 2002; Lee et al., 2010)
GCC box, ERE	AGCCGCC	(Fujimoto et al., 2000; Allen et al., 1998; Ohme-Takagi and Shinshi, 1995)
RAP2.12	ATCTA	(Licausi et al., 2011)
RAV1-A	CAACA	(Kagaya et al., 1999)
RAV1-B	CACCTG	(Kagaya et al., 1999)

*** and variations of this (Sakuma et al., 2002)**

Repressive transcription factors can act by competitive binding to *cis*-regulatory elements and thereby inhibit binding of transcriptional activators. Dimerization with a transcriptional repressor can also disable a transcriptional activator to bind *cis*-regulatory elements. Ohta and co-workers (2001) showed that a specific motif is carried by transcriptional repressors of the AP2/EREBP TF family. This so called EAR motif (ERF-associated amphiphilic repression; (L/F)DLN(L/F)(x)P) which is carried by the ERF3 homolog in *Nicotiana tabacum* also disabled transcriptional activation ability of transcriptional activators when fused to the activation domain (Ohta et al., 2001).

To act as a proper activating transcription factor a transcription activation domain (transactivation domain; TAD) is necessary for the interaction with the previously mentioned general cofactors (see Figure 2). Transactivation domains are often characterized by a high number of acidic amino acids, prolines, and glutamines (Schwechheimer et al., 1998; Liu et al., 1999; Sainz et al., 1997; Ptashne, 1988). Just recently an acidic TAD in AP2/EREBP TFs was discovered by Tiwari and co-workers (2012). They located the responsible region at the C-terminal end of AtERF98 which is conserved in ortholog protein sequences throughout the plant kingdom (EDLL motif) (Tiwari et al., 2012). Also in CBF1 an acidic TAD was located at the C-terminus. Although conserved acidic amino acids were found in the TAD, conserved hydrophobic amino acids seem to play a more important role in transcriptional activation (Wang et al., 2005). Amphipathic helices might play a role in transcription activation, as well (Liu et al., 1999; Ptashne, 1988).

A-3 Functions of AP2/EREBP Transcription Factors

As mentioned earlier, many TFs of the AP2/EREBP protein family are involved in stress and hormone response (or in stress response mediated by hormonal signalling).This has been extensively studied by expression analyses and phenotypic analyses of loss-of-function mutants or constitutively overexpressing plant lines. Very often TFs of the AP2/EREBP family play a role in plant development. The overexpression of AP2/EREBP TFs leads in many cases to a retarded growth phenotype (for illustration see Figure 5 and Table 2) whereas the loss-of-function mutants do not show any obvious phenotypic change possibly due to gene redundancies as mentioned earlier. Along with this phenotype in transgenic Arabidopsis lines overexpressing DREB1A, an improved cold resistance was observed (Maruyama et al., 2009). The connection between AP2/EREBP TFs and cold acclimation is the cold responsive DRE *cis*-regulatory element as mentioned in the previous chapter (Stitt and Hurry, 2002).

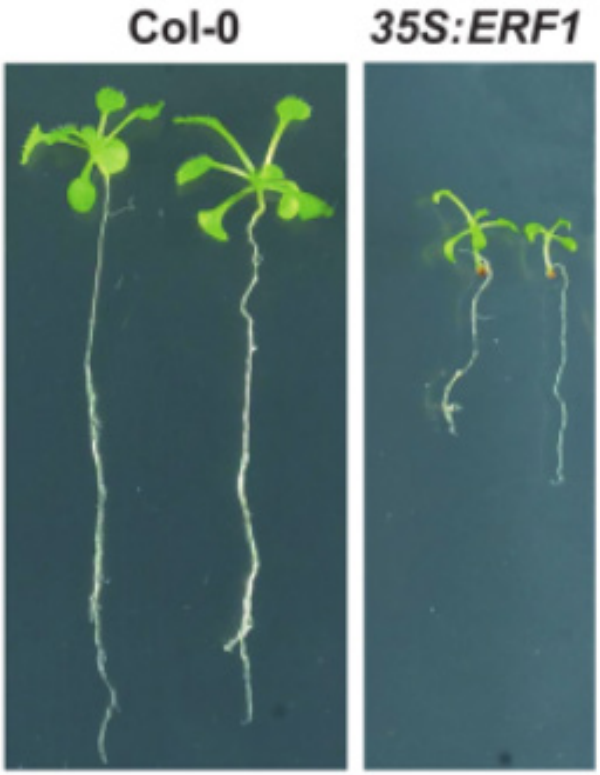


Figure 5 - Retarded growth in transgenic AP2/EREBP TF *ERF1* overexpressing Arabidopsis seedlings.
Plants were grown for 2 weeks on agar plates. *ERF1* is constitutively expressed controlled by CaMV 35S promoter. Modified after (Lorenzo et al., 2003).

Originally the AP2/EREBP TF family was discovered by being able to bind the ethylene responsive element ERE (Ohme-Takagi and Shinshi, 1995; Allen et al., 1998) and through that interaction, ethylene response was directly regulated (Hass et al., 2004; Fujimoto et al., 2000). In the meantime other members of this TF family have been shown to be involved in various other hormone signalling pathways: In abscisic acid signalling (ABI4; (Finkelstein et al., 2002; Reeves et al., 2011)), jasmonate signalling (ERF1; (Lorenzo et al., 2003)), or in cytokinin response (CRFs; (Rashotte et al., 2006, 2003)).

A-4 Dual Targeting of Transcription Factors

The import of transcription factors into the nucleus occurs in the same manner as other proteins are imported. They either contain an NLS (Boulikas, 1993, 1994), they bind a protein which subsequently gets imported, or they simply do not exceed a size of 40 kDa which enables them to enter the nucleus in a transport independent way (Görlich and Mattaj, 1996).

In most cases, TFs are located only to the nucleus after they have been synthesized in the cytoplasm. However, in some cases it has been shown that transcription factors can be located in different compartments of the cell. The ability of proteins to localize to more than one compartment of the cell is called dual targeting and can be regarded as post-translational regulatory mechanism. Mechanisms leading to dual targeting of proteins are numerous: protein folding, post-translational modification and protein–protein interaction are essentially involved (Karniely and Pines, 2005).

Dually targeted TFs are part of the retrograde communication of the organelles and other cellular compartments with the nucleus (Silva-Filho, 2003; Small et al., 1998; Schwacke et al., 2007; Krause and Krupinska, 2009). Especially dually targeted transcription factors might enable cellular compartments, which are unable or limited to produce their own proteins, to directly "ask" for supplemental proteins.

A coordinated release of a dually targeted transcription factor from the plastids has been shown in case of WHY1. WHY1 is imported first into the chloroplast. The processed version of WHY1 then is able to translocate to the nucleus where it regulates expression of genes such as *PR1* (Grabowski et al., 2008; Isemer et al., 2012). Yet another example for dually targeted TFs is the AP2/EREBP TF RAP2.12. This TF is involved in oxygen sensing. It binds under aerobic conditions to the plasma membrane associated acetyl-CoA-binding proteins ACBP1 and 2. But under anaerobic conditions as simulated by submergence, the TF disassociated from its binding partner and entered the nucleus to regulate expression of genes involved hypoxia acclimation (Licausi et al., 2011). Through combination of several localization prediction programs many other transcription factors have been found which are potentially dually targeted. One of the candidates with the highest score is the AP2/EREBP transcription factor AT2G44940 which was shown to be able to enter the nucleus and the plastids at the same time (Schwacke et al., 2007).

This study also led to the discovery that two cytokinin response factors CRF5 and CRF6, both AP2/EREBP TFs, might be capable of locating to two compartments, namely to the mitochondria as well as to the nucleus (Schwacke et al., 2007).

A-5 The Cytokinin Response Factors

The cytokinin response factors (CRFs) belong to the B-5 subgroup of the ERF subfamily of the AP2/EREBP transcription factor family (Sakuma et al., 2002). In Arabidopsis this subgroup counts 8 members (AtCRF1-8). As mentioned in the previous chapters CRFs are involved in cytokinin signalling and two members are *in silico* predicted to be potentially dually targeted. Phylogenetic analyses have revealed that the CRFs underwent numerous duplication events and that they are present in all so far sequenced plant genomes including dicots and monocots (Rashotte and Goertzen, 2010).

The peptide sequence of CRFs is composed of three major regions: The central AP2 DNA binding domain is flanked by the well conserved N-terminal part, which contains highly conserved motifs (the TEH and the CRF domain), and the relatively low conserved C-terminal part containing a putative phosphorylation site (SP(T/V)SVL motif) (Rashotte and Goertzen, 2010). The CRF domain was shown to be necessary and sufficient for protein-protein interaction amongst all CRFs. In addition this domain enables CRFs to interact with Arabidopsis Histidine Phosphotransfer Proteins (AHPs) of the cytokinin TCS (Cutcliffe et al., 2011). So far the C-terminal region of CRFs has not been very well described (for illustration see Figure 6).

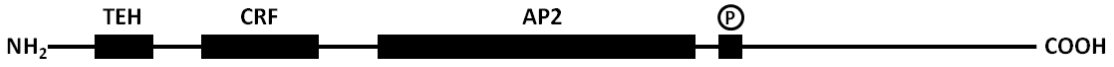


Figure 6 - Domain composition in Cytokinin Response Factors 1 – 6.
CRF7 and CRF8 lack the C-terminal region including Ⓟ. The AP2 domain is responsible for DNA binding, the CRF domain for protein-protein interactions. The TEH domain, as well as the CRF domain , the AP2 domain, and the putative phosphorylation site Ⓟ are highly conserved in related sequences throughout the plant kingdom (Rashotte and Goertzen, 2010).

It was shown that *AtCRFs* rapidly respond to cytokinin (CK) treatment. Not only the transcript level rapidly increased after application of cytokinins but also the location of CRF1-6 changed from cytoplasm to the nucleus (Rashotte et al., 2006), an effect that was shown for AHPs as well (Hwang and Sheen, 2001). In tomato it was shown that the transcriptional response to cytokinin is not restricted to CRFs of *Arabidopsis thaliana* (Rashotte and Goertzen, 2010; Shi et al., 2011). But some CRF genes in tomato (*SlCRFs*) were also responding to other abiotic factors such as NaCl, methyl jasmonate, ethylene, and salicylic acid (Shi et al., 2011). The fact, that *CRF2* and *CRF5* belong to the group of 20 genes which are most frequently appearing in gene expression studies connected to cytokinin signalling further emphasize the importance of the CRFs during cytokinin signalling (Brenner et al., 2012). Through microarray expression studies it was found that transcription of *CRF2* is also induced by cold whereas the transcription level of *CRF5* in roots is strongly induced during salt stress (Brenner et al., 2012). It was shown that ARRs at least indirectly regulate the expression of *CRF2* and *CRF5* (Taniguchi et al., 2007).

The CRF loss-of-function Arabidopsis mutants showed changes in gene expression of known cytokinin affected genes which are partly also regulated by type-B ARRs (for illustration see Figure 7). For instance, upon cytokinin treatment the gene expression of *CELL WALL INVERTASE 1* was less up-regulated as well as many response regulators of the cytokinin pathway and a receptor histidine kinase (*AHK4*) (Rashotte et al., 2006). However, only triple loss-of-function mutants were studied in this respect.

Phenotypic effects in embryo, cotyledon and leaf development were also observed in CRF loss-of-function mutants of Arabidopsis. These effects became only visible the more CRF genes were knocked out which might indicate genetic redundancy among the CRFs (Rashotte et al., 2006).

CRF2 is regarded to play a role in the signal transduction of cytokinin to induce chloroplast division (Okazaki, 2009). But this is the only function so far known which can be connected to one single member of the whole CRF subgroup. This study will be revisited in more detail in Chapter VI D-2.

B Cytokinins

Cytokinins are one of the six major classes of plant hormones besides abscisic acid, auxin, gibberellins, ethylene, and brassinosteroids. They are involved in many different processes

during plant development, e.g. development of meristematic tissue (Werner et al., 2003), differentiation of cells and cell cycle control (reviewed in (De Veylder et al., 2007; Dewitte and Murray, 2003; Francis, 2007)), apical dominance (reviewed in (Ongaro and Leyser, 2008; Shimizu-Sato et al., 2009)), plastid development (Lochmanová et al., 2008; Vandenbussche et al., 2007; Okazaki et al., 2009), source-sink relations (reviewed in (Roitsch and González, 2004)), and senescence (Swartzberg et al., 2011; Köllmer et al., 2011; Balibrea Lara et al., 2004).

Naturally occurring cytokinins are adenine derivatives. They are classified by the configuration of their N^6-side chain either being isoprenoid (e.g. *trans*-Zeatin) or aromatic (e.g. Benzyladenine (BA)) cytokinins. The first limiting step of cytokinin synthesis is catalyzed by adenosine phosphate isopentenyltransferases (IPTs) which utilize preferably ATP or ADP (but also AMP). Degradation and inactivation of cytokinins is conducted through irreversible cleavage of the N^6-side chain by cytokinin oxidase/dehydrogenases (CKXs) (Mok and Mok, 2001; Sakakibara, 2006).

Cytokinin synthesis is dependent on the availability of IPTs, CKX, and Cyp735A (a cytochrome P450 monooxygenase). Gene expression of these cytokinin synthesis components is, amongst others, controlled by auxin, abscisic acid and by cytokinin itself (Sakakibara, 2006; Brenner et al., 2005, 2012; Werner et al., 2006). Cytokinins are preferentially synthesized in tissues that are rich in dividing cells such as root tips and young leaves (Nordström et al., 2004) and act as local or long distance signals (Hirose et al., 2008).

C The Cytokinin Signalling Pathway

Cytokinin recognition is mediated by a phosphotransfer cascade which is similar to the two-component signal pathways (TCS) found in most bacteria and yeast. In these organisms the TCS functions as a sensor of environmental changes (Argueso et al., 2010; To and Kieber, 2008; Ferreira and Kieber, 2005; Romir et al., 2010; Brenner et al., 2012). The simplest composition of such a TCS comprises a sensor histidine kinase as the signal receiver and a response regulator which becomes activated after phosphorylation through the sensor histidine kinase. Subsequently, activated response regulators act as transcriptional regulators of gene expression.

In Arabidopsis three sensor histidine kinases responsible for CK perception are known: CRE1 (AHK4, WOL), AHK2 and AHK3 (Yamada et al., 2001; Nishimura et al., 2004). After signal

perception, the Arabidopsis sensor histidine kinases transfer a phosphate group not directly to a response regulator but autophosphorylate an aspartate before the phosphate group is transferred to Histidine Phosphotransfer Proteins (AHPs) (Lohrmann and Harter, 2002; Mähönen et al., 2006). The AHPs act as signal mediators in form of a phosphate shuttle between the receptor histidine kinases and response regulators (Arabidopsis response regulators (ARRs)) (Hwang and Sheen, 2001; Punwani and Kieber, 2010). Two types of ARRs are known in Arabidopsis. Type-A ARRs contain only a receiver domain whereas type-B ARRs consist of a receiver domain and a transcription factor domain. Cytokinin treatment rapidly raises the expression levels of genes encoding type-A ARRs but does not change the expression of type-B ARRs (Hwang and Sheen, 2001; Ferreira and Kieber, 2005; D'Agostino et al., 2000). Type-A ARRs are negative regulators of cytokinin action while type-B ARRs are activators of the expression of CK response genes but also genes encoding type-A ARRs. This leads in turn to a dampening of the CK effect; a negative feedback loop (To et al., 2004). Just recently it was found that components of the CK signalling pathway like several type-A ARRs and AHK2 and AHK3 are also involved in the cold response of *Arabidopsis thaliana* (Jeon et al., 2010) and type-B ARR ARR2 seems to also interfere with ethylene signalling through interaction with a *cis*-element found in the promoter of ERF1 (Hass et al., 2004).

One important transcription factor family up-regulated after CK application is the Cytokinin Response Factor family (CRF) as mentioned in the previous chapter (Rashotte et al., 2006). All CRFs are like ARRs able to interact with AHPs (Cutcliffe et al., 2011) and they are regulating partly the same targets as Type-B ARRs (Rashotte et al., 2006) (for illustration see Figure 7).

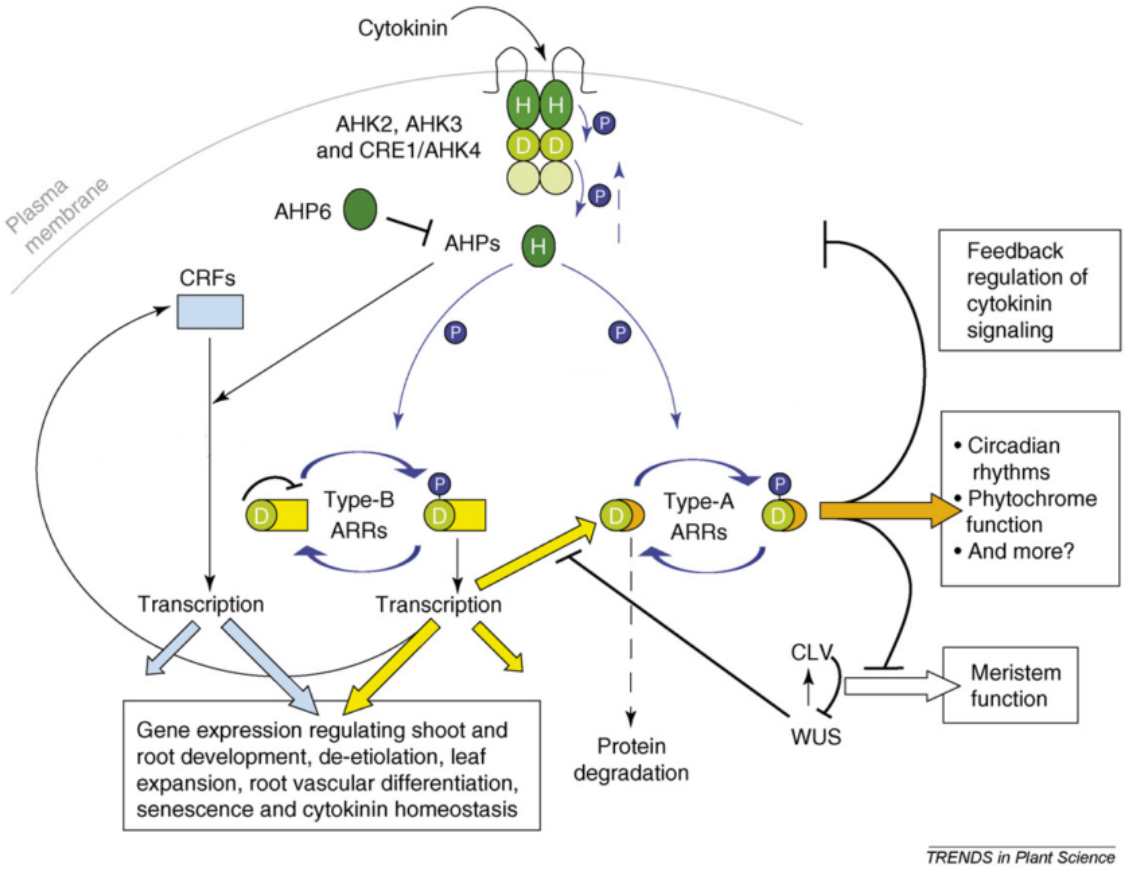


Figure 7 - Model of cytokinin signalling.
Modified after (To and Kieber, 2008). Nuclear membrane removed due to revised and unresolved mechanisms of AHP (and CRF?) import into the nucleus (Punwani et al., 2010).

D The Action of Cytokinins during Plant Development

Cytokinins are influencing many aspects of plant development and so do AP2/EREBP TFs. Both actors, CK and AP2/EREBP TFs, are connected by the small cytokinin responsive subgroup Cytokinin Response Factors. In the following chapter only cytokinin effects on plant development which are directly relevant for this thesis will be described. Many more effects are known as mentioned in Chapter VI B. The cell-cycle will be addressed as the commonly accepted principal site of action of cytokinins.

D-1 Differentiation of Cells and Cell Cycle Control

Meristematic tissue consists of undifferentiated, undetermined cells which can develop into organs and tissues. Various meristem types are providing the basis for plant development. Meristem behaviour, size, shape and cell differentiation requires a complex succession of

differential gene expression. Phytohormones such as auxin and cytokinins play a significant role in the regulation of meristems (Rupp et al., 1999; Hamant et al., 2002; Dello Ioio et al., 2007; Müller and Sheen, 2008; Moubayidin et al., 2009). The best studied meristems in plants are the Shoot Apical Meristem (SAM) and the Root Meristem (RM).

The plant cell cycle comprises mitosis (M), cytokinesis, postmitotic interphase (G_1), DNA synthetic phase (S), and postsynthetic interphase (G_2). The transitions from G_1- to S-phase and the transition from G_2- to M-phase are controlled by so called 'principal control points' (Francis, 2007). Cyclin-dependent protein kinases (CDKs) and cyclins are essential regulators of the cell cycle judging especially over these 'principal control points'.

Cytokinins, auxin and sucrose are concertedly regulating the cell cycle by inducing the expressions of cyclins and cyclin-dependent protein kinases which are regulating the crucial passage from the G_1-phase to the S-phase (Inzé and De Veylder, 2006; De Veylder et al., 2007; Francis, 2007; Menges et al., 2006; Sieberer et al., 2003). It was also shown that cytokinins together with auxin regulate the second transition point from G_2- to M-phase (Dewitte and Murray, 2003; Werner et al., 2008). Cytokinins and sucrose induce the expression of the cyclin *CycD3* and thereby enhance the transition from G_1-phase to the S-phase (Dewitte et al., 2007; Riou-Khamlichi et al., 2000). Sucrose is also able to induce the expression of *CycD2*. Auxin on the other hand induces the expression of the cyclin-dependent protein kinase A (*CDKA*). CycDs and CDKA form phosphorylatable complexes which subsequently induce the entrance into the S-phase in which the genetic material of the cell gets replicated (Francis, 2007; Inzé and De Veylder, 2006). Differentiating plant cells arrest in the G_1-phase of the cell cycle (Riou-Khamlichi et al., 1999) (for illustration see Figure 10).

D-1-1 Regulation of the Shoot Apical Meristem

The outermost tip of the shoot, the shoot apex, is represented by the shoot apical meristem (SAM). Leaves and branches are generated of precursors originating from these undifferentiated cells.

After the discovery of cytokinins it was shown that this class of phytohormones was able to induce shoot formation in growing callus tissue (Werner et al., 2003; Skoog and Miller, 1957). Werner and co-workers were able to prove that cytokinin deficient Arabidopsis plants exhibited reduced activity of apical and floral shoot meristems. The SAM in *CKX* overexpressing plants contained significantly fewer cells than observed in wild type plants. But not only cell proliferation is controlled by cytokinins. It is suggested that the transition

from undifferentiated cells to differentiated primordia is mediated by cytokinins as well. Cytokinins are necessary for cell division and cytokinin deficiency causes cell differentiation. It is speculated that due to a connection between homeobox genes and cytokinins a concentration gradient of cytokinin and its antagonist auxin might judge over cell fate like homeobox genes in Drosophila development (Werner et al., 2003). The interplay of cytokinins and auxin is also responsible for the regulation of apical dominance. Auxin inhibits axillary bud outgrowth whereas cytokinin induces shoot branching (Shimizu-Sato et al., 2009; Ongaro and Leyser, 2008).

One important factor that is playing a significant role in transmitting a cytokinin signal to changes in meristematic development is *STIMPY* (*STP, WOX9*). Its expression is regulated by cytokinins and, in addition, the overexpression of this homeobox gene is able to partially compensate for growth defects in Arabidopsis mutants which are unable to sense cytokinins (Wu et al., 2005; Skylar et al., 2010).

Furthermore, an analogy between the phenotypes of cytokinin deficient plants and the effects of an AP2 transcription factor, *AINTEGUMENTA*, on plant development was emphasized due to its involvement in the control of plant organ cell number and organ size (Werner et al., 2003; Mizukami and Fischer, 2000). The ectopic expression of *AINTEGUMENTA* in Arabidopsis resulted in enlarged shoot organs and increased cell numbers. However, most reported ectopical expressions of AP2/EREBP TFs resulted in a retarded shoot growth (see Table 2).

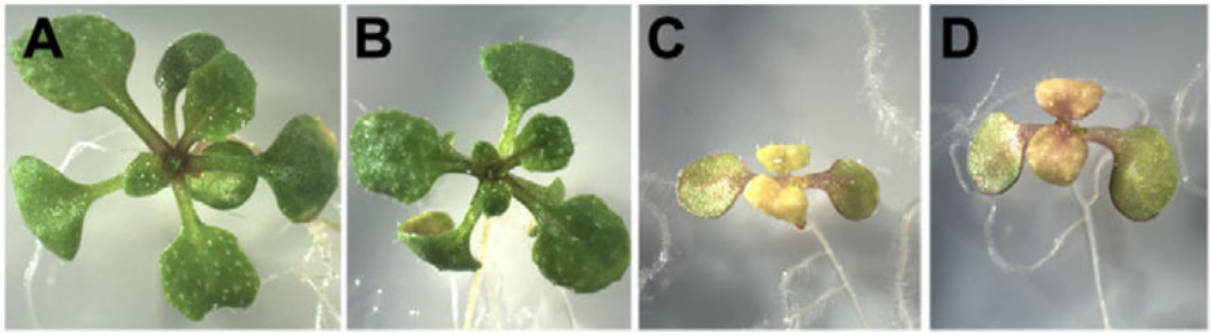


Figure 8 - Reduced growth of *Arabidopsis thaliana* treated with cytokinins.
Seedlings were grown for seven days on MS medium containing (A) no cytokinins, (B) 10 nM, (C) 100 nM, or (D) 1 µM *trans*-zeatin. Modified after Skylar et al., 2010.

Although cytokinins lead to a higher proliferation rate in SAM, shoot growth on media containing cytokinins is inhibited. An aspect which will be revisited in Chapter VI D-3 (Skylar et al., 2010) (for illustration see Figure 8).

D-1-2 Regulation of the Root Meristem

In the root tip the root meristem (RM) is the source for undifferentiated cells. Root apical meristem size is controlled by the "key regulators" CKs and auxin (Dello Ioio et al., 2007; Blilou et al., 2005; Kuderová et al., 2008). In these RMs CKs are determining the meristem size in an opposed manner to their action in the SAM. Exogenous application of CKs as well as *IPT* overexpression leads to a decrease in meristem size. However, growth of the primary root in cytokinin deficient *CKX* overexpressing plants and *IPT* mutants is accelerated due to an increased size of the RM (for illustration see ; (Werner et al., 2003; Miyawaki et al., 2006; Werner et al., 2001)). Additionally, the length of lateral roots in cytokinin deficient plants is drastically increased (Miyawaki et al., 2006).

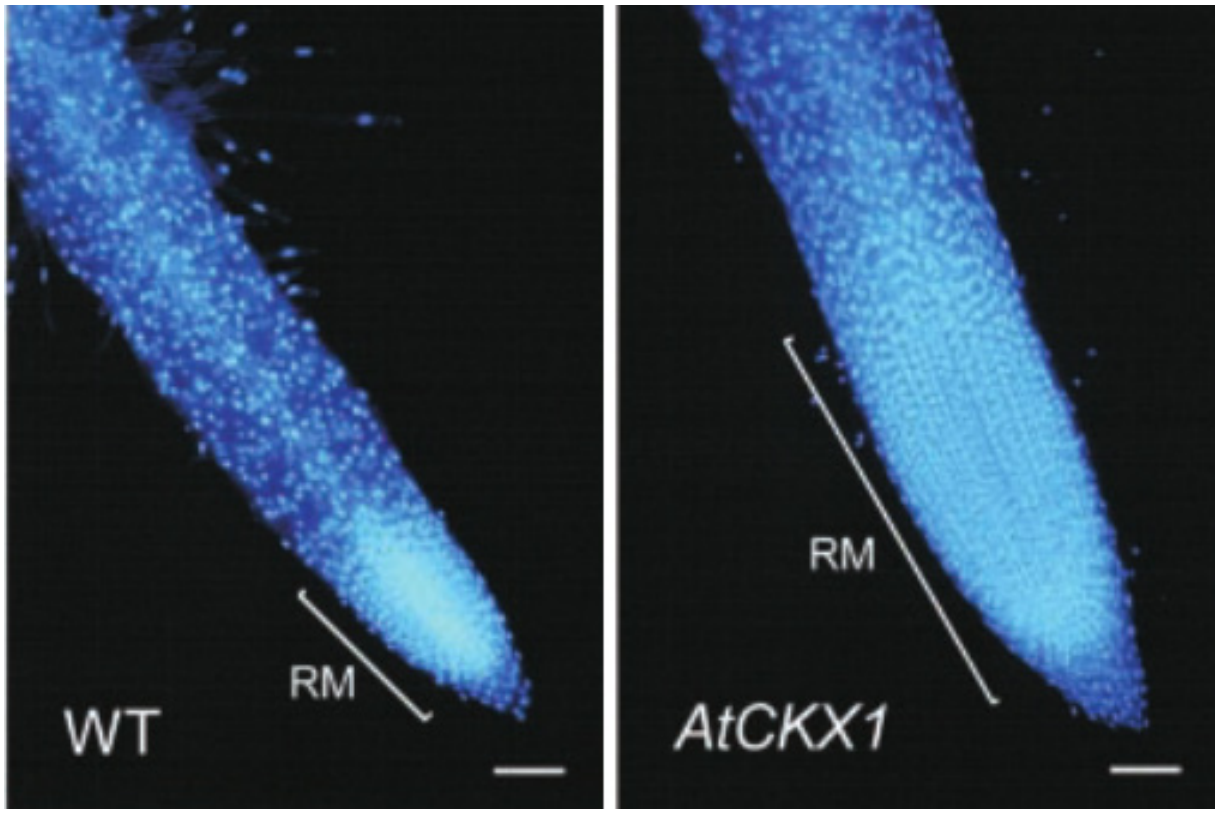


Figure 9 - Increased cell number in roots of cytokinin deficient Tobacco plants.
Cytokinin deficiency was achieved by overexpression of the cytokinin degradating enzyme CYTOKININ OXIDASE/DEHYDROGENASE 1 of *Arabidopsis thaliana* (AtCKX1). Nuclei are stained with the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI). RM, root meristem. Bar: 100 µm. Modified after (Werner et al., 2001).

The auxin/cytokinin crosstalk in root development is indicated by the auxin-dependent induction of certain *IPTs* in roots (*AtIPT5* and *AtIPT7*; (Miyawaki et al., 2004)).

Root length is also drastically reduced in *ERF1* overexpressing Arabidopsis seedlings (Lorenzo et al., 2003). This study, however, aimed for proving a connection between this TF and jasmonate and ethylene signalling. A reduced root phenotype has been observed in plant lines which constitutively overexpress components of the TCA which influence cytokinin signalling like response regulator *ARR22* or *ARR2* (Kiba et al., 2004; Hwang and Sheen, 2001).

D-2 Chloroplast Development and Photomorphogenesis

The only so far known function of a CRF has been found in connection with chloroplast development as mentioned in Chapter VI A-5. In transgenic Arabidopsis which constitutively overexpress *CRF2* it has been found that these plants exhibit more and smaller chloroplasts per cell than wild-type plants. In addition, transcription of plastid division proteins (PDVs) is up-regulated. Both effects are mimicking the effect of CKs. Due to the fact that *CRF2* expression is also induced by cytokinins (see Chapter VI A-5) it was concluded that *PDVs* are not only under cytokinin control but in particular regulated by CRF2 (Okazaki et al., 2009). Plastid division proteins (PDVs) have an important function in the chloroplast division machinery. Plastid division proteins (PDV 1 and 2) have been shown to be crucial for the recruitment of dynamin to the division site (Miyagishima et al., 2006; Okazaki et al., 2009, 2010). A higher expression of PDV1 or PDV2 led to an increase in number of chloroplasts per cell as well as a decrease in size. Accordingly, a lower expression showed the opposite effect (Okazaki et al., 2009).

Photomorphogenesis is the light dependent development of organs in plants. In cotyledons, photomorphogenesis is observable in the development of chloroplasts after germination out of proplastids and etioplasts. Later in cotyledon development they also undergo chloroplast fission as usual in true leaves (Pogson and Albrecht, 2011). Exogenously applied CKs are able to partly substitute light signals and induce de-etiolation of dark grown seedlings (Chory et al., 1994; Riefler et al., 2006). In tobacco the overexpression of cytokinin degrading enzymes such as *CYTOKININ OXIDASE/DEHYDROGENASE* (*CKX*) led to an earlier differentiation of plastids and later in development to a partial disorganization of thylakoids (Werner et al., 2008).

Another interesting link between CKs and photomorphogenesis has been found in connection with cryptochromes. Cryptochromes are light receptors and one of the first components of photomorphogenesis induction in blue light. They interact in their activated form with COP1 (CONSTITUTIVELY PHOTOMORPHOGENIC 1) which is a negative regulator of photomorphogenesis in etiolated seedlings. COP1 is able to ubiquitinate HY5 which is a bZIP transcription factor that induces the expression of light response genes, in particular anthocyanin biosynthesis genes (Wang et al., 2001). In absence of COP1 or in presence of CKs, HY5 protein accumulates without increasing the transcription rate of *HY5*. It was concluded that CKs in this process have the function of preventing HY5 from ubiquitination by COP1 and from subsequent degradation at the so called photobodies in the nucleus (Van Buskirk et al., 2012; Vandenbussche et al., 2007).

In contrast to the effect of cytokinins on photomorphogenesis and chloroplast development, it was shown that levels of proteins of the photosynthetic apparatus were not drastically different in cytokinin deficient tobacco plants compared with plants with an elevated cytokinin level (Cortleven et al., 2011).

D-3 Sugar and Starch Metabolism and Source/Sink Regulation

Physiologically, a plant can be divided into photosynthetically active source tissue and photosynthetically less active or inactive sink tissue. Source tissue is characterized by carbohydrate export due to higher production than consumption whereas sink tissue imports carbohydrates (Roitsch and Ehneß, 2000; Leopold and Kawase, 1964). Source tissues are for example mature leaves whereas sink tissues are flowers, roots, developing seeds, or young leaves.

Plants successfully adapt to changing sugar availability by changing their developmental programmes. Many sugar response pathways are intertwined with abiotic and biotic stress signalling pathways (Gibson, 2004). The role of abscisic acid (ABA) is opposing the role of glucose regarding cell cycle activity in cotyledon development. However, sucrose and ABA both promote nutrient accumulation during plant development (Finkelstein and Gibson, 2002). Werner and co-workers found in cytokinin deficient *CKX1* and *CKX2* tobacco mutants that soluble sugar content in sink leaves was drastically reduced while the starch content stayed on a normal level. In source leaves however the starch content was drastically reduced while soluble sugars were found to be on a normal level (Werner et al., 2008). Already in

1978 Longo and co-workers found that the CK benzyladenine is able to change the sugar and starch composition of cotyledons of watermelon (Longo et al., 1978).

Cytokinins are able to induce gene expression of cell wall invertases and thereby they are influencing senescence or source/sink relations (Roitsch and Ehneß, 2000; Roitsch and González, 2004; Balibrea Lara et al., 2004) (for illustration see Figure 10). Cell wall invertases are part of the apoplastic unloading pathway. They are bound to the cell walls and irreversibly hydrolyze sucrose to fructose and glucose which can subsequently be transported into the cells. These hexoses represent the carbohydrate supply for the sink tissue (Roitsch and Ehneß, 2000; Roitsch and Tanner, 1996; Werner et al., 2008). In addition to this effect, CKs are strengthening the sink by accelerating the cell cycle together with glucose.

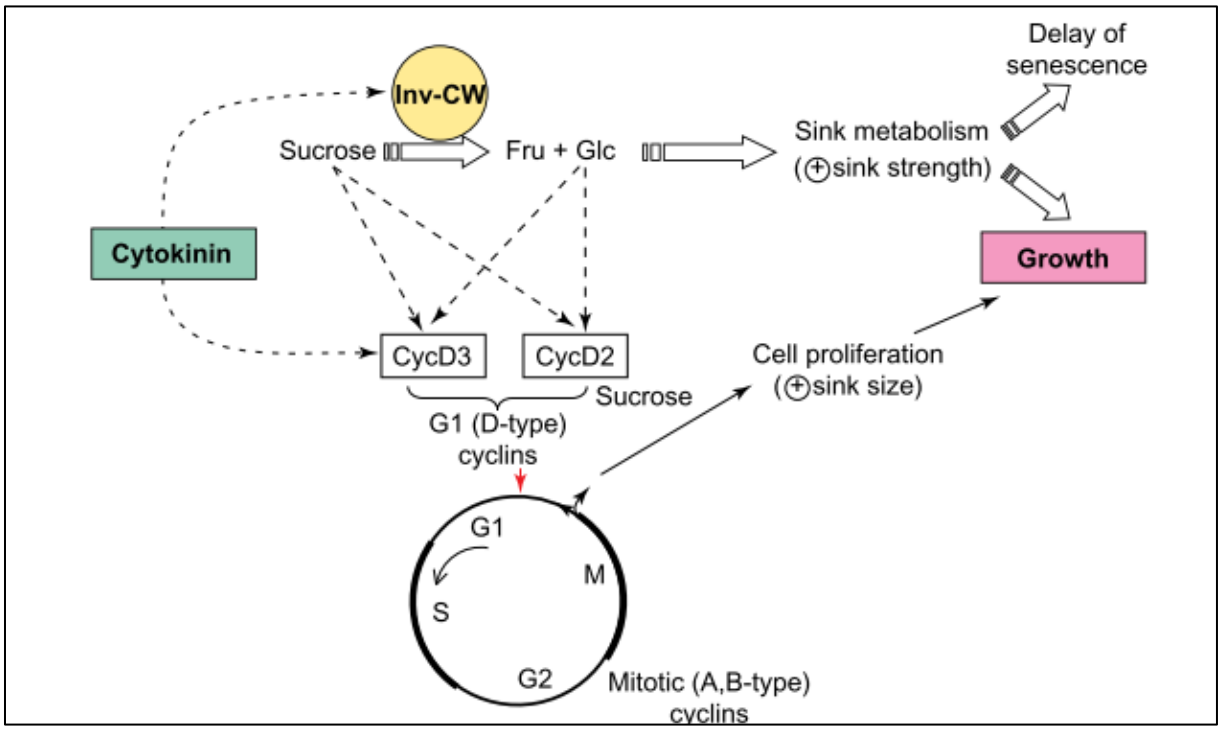


Figure 10 - Cell cycle control by cytokinins.
Inv-CW, cell wall invertase; Fru, fructose; Glc, glucose. Modified after (Roitsch and González, 2004).

Yet another link connecting a cytokinin mediator, sucrose signalling and cell-cycle control has been found in form of *STIMPY*. Loss-of-function mutants exhibited retarded shoot growth which was explained by cell cycle arrest. Due to a missing sucrose signal, seedlings stopped growing. The addition of sucrose to the medium, however, led to a completely normal shoot development (Skylar et al., 2010; Wu et al., 2005).

VII Aims of this Study

Recent studies have in depth illuminated the effect of cytokinins on plant development on the one hand. Components of the cytokinin signalling process have been discovered and characterized. On the other hand many members of the AP2/EREBP transcription factor family have been characterized in regards to their transcription factor properties and they have shown to be involved in various developmental processes as well.

The aim of this study was to further characterize the AP2/EREBP transcription factor CRF5 of *Arabidopsis thaliana* which in the beginning of this study was only known to be involved in cytokinin signalling and leaf development. The paramount question of this thesis was: Is CRF5 as a component of the cytokinin signalling cascade functioning as regulator of related downstream processes? To shed light on this, this thesis tried to tackle the following questions: What is the cellular location of CRF5 and is it possibly dually targeted? Is it possible to identify transactivation elements of CRF5? What effect does constitutive overexpression of *CRF5* have on plant development? Does this phenotype resemble formerly described cytokinin induced phenotypes or is it rather comparable with known phenotypes of AP2/EREBP TF gain-of-function or loss-of-function lines? Is the gene expression of selected genes affected? Which interaction partners does CRF5 have? Does CRF5 bind known AP2 domain associated *cis*-regulatory elements?

VIII Summaries of Manuscripts

This thesis is a cumulative collection of three manuscripts and one supplemental manuscript. The supplemental manuscript preceded my PhD studies but provided experimental data on which parts of this thesis base.

Manuscript 1

Bernd Ketelsen, Rainer Schwacke, Kirsten Krause, and Karsten Fischer

Transcriptional activation by Cytokinin Response Factor 5 is governed by an acidic C-terminus containing two conserved domains

Submitted to Plant Cell Reports

Cytokinin response factors (CRFs) are transcription factors involved in hormone signal transduction. The CRFs consists of an N-terminal dimerization domain (CRF domain), a DNA-binding AP2 domain and a C-terminal part of unknown function. Using a combination of sequential deletions and yeast-two-hybrid assays, we provide evidence that the C-terminus can confer transactivation activity to the protein CRF5. Two conserved motives and several conserved acidic and aromatic amino acid residues were identified in the otherwise heterogeneous C-terminus of most CRFs. These conserved regions contributed cooperatively to the activation of target gene transcription, suggesting a modular structure of the transactivation domain.

Manuscript 2

Bernd Ketelsen, Stian Olsen, Kirsten Krause, Karsten Fischer

Cytokinin responsive factor 5 (CRF5) is involved in root development, hormonal crosstalk and sugar metabolism in Arabidopsis thaliana

Submitted to Planta

Cytokinin response factors (CRFs) are transcription factors involved in cytokinin signalling. They have been previously shown to play a role in embryo and cotyledon development. To get further insights into the physiological functions of CRFs Arabidopsis thaliana plants overexpressing CRF5 (CRF5-8^{OE}) were analyzed. The transgenic plants showed an inhibition of primary root growth and a reduction of the lateral root system. This phenotype resembles that of cytokinin treated wild-type plants and of transgenic plants overexpressing other components of cytokinin signal transduction. The expression of several genes known to be involved in cytokinin signalling was induced in the CRF5-8^{OE} plants, such as CRF6, the type-A response regulator ARR5, the cytokinin receptor AHK4 and the cell wall invertase 1. In addition, two genes known to be activated by auxin and ABA are also upregulated in the CRF5 overexpressing plants. One of these genes is directly activated by binding of CRF5 to a DRE promoter element. These results are discussed with respect to the role of CRF5 in cytokinin signal transduction and hormonal crosstalk.

Manuscript 3

Bernd Ketelsen, Stian Olsen, Karsten Fischer, and Kirsten Krause

Correlation between 16S/18S rDNA ratio and chloroplast copy numbers in cotyledons of Arabidopsis thaliana*: use for assessment of the impact of cytokinin response factor 5 on chloroplast development*

Submitted to Endocytobiosis and Cell Research

The regulation of plastid density and size per cell by phytohormone-induced signaling cascades has been a focus of research many decades ago but has recently experienced a revival. Evidence for a connection between cytokinin levels, a transcription factor belonging to the cytokinin response factor group and the expression level of two plastid division proteins are likely just the beginning of a new field of endosymbiosis research that will require the screening of potential candidate genes under a variety of conditions in order to map the effects on chloroplast numbers per cell.

We report here on a comparison of two methods for the determination of chloroplast copy numbers per cell. The direct counting of chloroplasts in 3-D models of cells reconstructed from optical sections using a fluorescence microscope equipped with an ApoTome is a highly accurate method but is time-consuming and tedious. In contrast, the determination of plastid to nuclear DNA ratio using the 16S and 18S rDNA genes, respectively, is a very rapid method suitable to screen large numbers of tissues, mutant seedlings or seedlings grown under different conditions. Although it targets the DNA instead of the plastids, it correlates rather well with the counting method and can be recommended for initial investigations or for large experimental set-ups.

Supplemental Manuscript

Rainer Schwacke, Karsten Fischer, Bernd Ketelsen, Karin Krupinska, and Kirsten Krause

Comparative survey of plastid and mitochondrial targeting properties of transcription factors in Arabidopsis and rice

Published in Molecular genetics and genomics 2007; 277: 631-46.

A group of nuclear transcription factors, the Whirly proteins, were recently shown to be targeted also to chloroplasts and mitochondria. In order to find out whether other proteins might share this feature, an in silico-based screening of transcription factors from Arabidopsis and rice was carried out with the aim of identifying putative N-terminal chloroplast and mitochondrial targeting sequences. For this, the individual predictions of several independent programs were combined to a consensus prediction using a naïve Bayes method. This consensus prediction shows a higher specificity at a given sensitivity value than each of the single programs. In both species, transcription factors from a variety of protein families that possess putative N-terminal plastid or mitochondrial target peptides as well as nuclear localization sequences, were found. A search for homologues within members of the AP2/EREBP protein family revealed that target peptide-containing proteins are conserved among monocotyledonous and dicotyledonous species. Fusion of one of these proteins to GFP revealed, indeed, a dual targeting activity of this protein. We propose that dually targeted transcription factors might be involved in the communication between the nucleus and the organelles in plant cells. We further discuss how recent results on the physical interaction between the organelles and the nucleus could have significance for the regulation of the localization of these proteins.

IX Work in Progress

Some results which did not find their way into one of the attached manuscripts due to thematic reasons or incompleteness are described and discussed in the following section.

A Interaction Partners of CRF5

After having determined and deactivated the transactivation ability of CRF5 (see Manuscript 1) a yeast-two-hybrid screen against an Arabidopsis cDNA library was conducted. A recent study of Cutcliffe and co-workers (2011) shows the ability of all members of the CRF subgroup to interact with each other and, in addition, to interact with AHPs and in some extend with ARRs. This study, however, directly examined interactions with known components of the cytokinin TCS one-by-one without a full scale Yeast-Two-Hybrid screening against a complete cDNA library. We tried to find interaction partners outside this commonly known signalling machinery to improve our knowledge about which proteins CRF5 might be associated to, first, in case it is located in the cytoplasm, and second, when it is acting as a transcriptional regulator in the nucleus.

A-1 Interaction Partners of CRF5 - Material and Methods

A-1-1 Cloning

The complete coding sequence of *HGL1* was PCR amplified based on cDNA clone U25165 (ABRC) with B-site containing primers (HGL1 CDS B1 for: GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGACTTTAAGTGGGGATAG, HGL1 CDS B2 rev: GGGGACCACTTTGTACAAGAAAGCTGGGTATGGGAAACTTGTGACTTCATATTC). The PCR product was used as template for a BP-clonation first into pDONR207 which then subsequently was used in recombinational cloning to fuse the coding sequences in frame to the coding sequences of the binding and activation domains into Gateway cassette (Invitrogen) containing pGBKT7 and pGADT7, respectively.

The *CRF5* full length genes were amplified on Arabidopsis cDNA with the primer pair: TOPO-CRF5-for: CACCATGAAAAGCCGAGTGAGAAAATCC and CRF5-rev: CTTATCCAACAAATGATCTTGG. The product was inserted into the TOPO cloning vector

pENTR/SD/D-TOPO (Invitrogen) before it was recombined into pGADT7 (equipped with Gateway cassette for Gateway cloning).

A-1-2 Yeast-Two-Hybrid Analysis

For the yeast-two-hybrid screen, yeast cells (strain Y187, clontech) containing the non-autoactivating bait coding vectors pGBKT7-CRF5ΔV (lacking bp 601-750) and pGBKT7-CRF5ΔVI (lacking bp 751-882), respectively, were mated with the yeast strain AH109 containing the *A. thaliana* cDNA library CD4-30 (in the vector pAD-GAL4-2.1 (Stratagene), obtained from the Arabidopsis Biological Resource Center (ABRC)) until an OD_{600} of ~0.6 was reached. Cells were precipitated and resuspended according to the instructions in the User Manual of the Matchmaker GAL4 Two-Hybrid System 3 (Clontech). The cell suspension was plated on SD plates lacking the amino acids Ade, His, Leu, Trp (Quadruple Drop Out medium (QDO) for high-stringency selection. Diluted cell suspensions were spread on plates containing SD/-Leu/-Trp for estimation of transformation efficiency. The plates were incubated for 7 days at 30° C and growing colonies were transferred to new plates. Colony PCRs were performed with library specific primers (SP/pGADT7 GAAAGGTCGAATTGGGTACC, ASP/pGADT7: AACCTTGATTGGAGACTTGACC) and resulting PCR fragments were sequenced. DNA sequencing results yielding coding sequences which were not in frame with the coding sequence of the activation domain of the library vector disqualified the candidate. Remaining sequences were subjected to BLAST search. Library plasmids containing interesting candidates which passed the first filter were isolated, amplified in *E. coli* OneShot TOP10 cells (Invitrogen), and transferred back into yeast strain AH109. Confirmative small scale mating was performed with both bait vectors as well as pGBKT7 as negative control. The selection was carried out on maximum stringency plates and positive colonies were transferred to fresh QDO plates.

To test identified interacting proteins for auto-activation activity, candidate cDNAs were cloned into pGBKT7, introduced into yeast strain Y187. The transformants were subjected to filter lift assays as described in Cao et al. (1997).

A-1-3 GST-pull down Assays

GST-tagged CRF5 was expressed in *E.coli* (BL21 Star; Invitrogen) after cloning the full length genes into pDEST15 (Invitrogen) by Gateway cloning (Invitrogen). Overnight cultures in Luria Broth (LB) with Kanamycin (50µg/ml) were used for inoculation of fresh LB/Kan medium to an OD_{600} of 0.05. After approximately 2-3 hours of incubation at 37°C to an OD_{600} of 0.4, protein expression was induced by adding IPTG to 0.1 mM end concentration. The

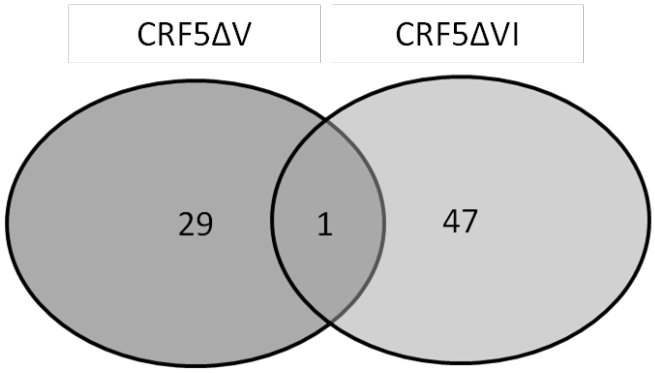
cells were harvested after 2 hours of incubation, pelleted and frozen at -80°C. Ice cold NETN+ buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.2 % Nonidet P-40, 6 mM EDTA, 6 mM EGTA, protease inhibitors (Roche)) was added and the re-suspended cell suspension was treated with 3 rounds of 15 sonication pulses. After brief centrifugation the supernatant was analyzed by western blotting with an anti-GST antibody (Sigma) for expression of GST-tagged proteins.

100 µl of glutathione-linked agarose beads (50 % suspension in PBS buffer) were incubated in a rotation shaker for 1 hour at 4 °C with 1 ml of GST-tagged protein or only the GST-tag containing supernatant. After repeated washing of the beads with PBS buffer (0.1 M sodium phosphate pH 7.2, 0.7 % NaCl (w/v)) the bead suspension could be stored for maximum one week at 4 °C as a 50 % suspension in PBS buffer containing protease inhibitors.

^{35}S-Methionine labeled proteins were produced by using the TNT T7 Quick Coupled Transcription/Translation system (Promega) with the vector pET161/GW/D-Topo (Invitrogen) containing the coding region of *CRF5* and the 3'-terminal 501 bp of *HGL1* under the control of a T7 promoter. In vitro translated proteins and protein coupled agarose beads were incubated together in NETN+ buffer for 2 h at 4°C. After repeated washing (5 times) of the beads in cold NETN buffer (NETN+ without protease inhibitors) bound proteins were released by adding SDS containing protein sample buffer and separated by SDS PAGE. Radioactively labeled proteins were detected by autoradiography using the Personal Molecular Imager FX system (BioRad).

A-2 Interaction Partners of CRF5 - Results and Discussion

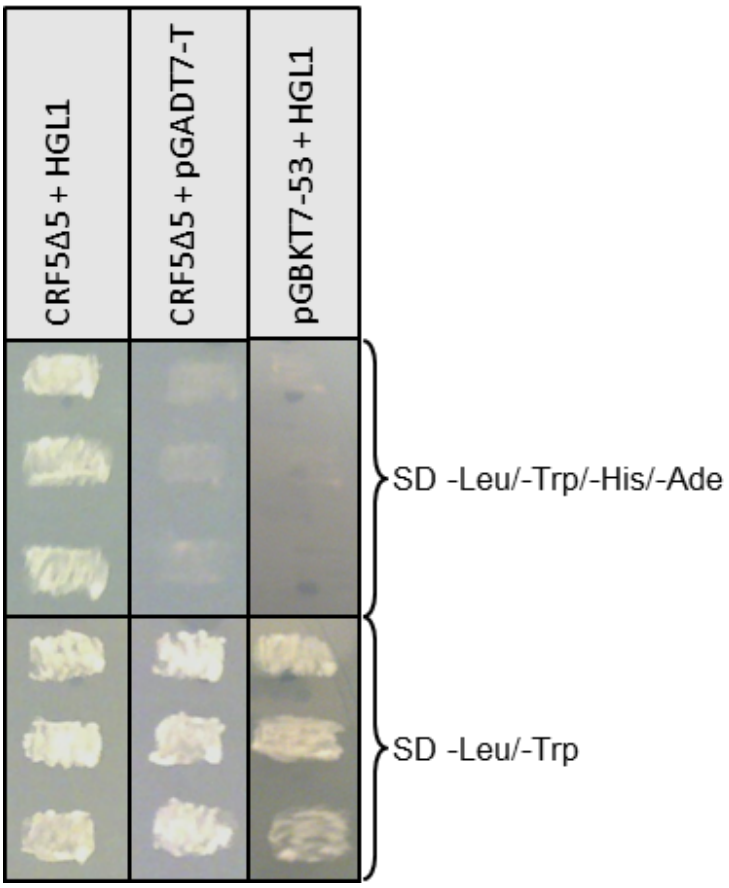
A yeast two hybrid screen against an Arabidopsis cDNA library produced more than 300 colonies on highest stringency medium. However, after sequencing and sorting of potential false positives a group of 77 interaction candidates passed this process. Only one candidate was found with both baits (CRF5ΔV and CRF5ΔVI) (see Supplemental Figure 1). The cDNA sequence of this candidate matched the final 501 base pairs of *HGL1*(*At3g23640*). HGL1 is considered to be involved in the degradation of cytosolic heteroglycan to glucose. A process associated to starch degradation (Lu and Sharkey, 2006). Interaction studies of CRF5ΔV and the full-length HGL1 confirmed this interaction. No interaction was detectable either with the bait negative control pGADT7-T or the prey negative control pGBKT7-53 which are included in the used Matchmaker Kit (see Supplemental Figure 2).



Supplemental Figure 1 – Venn diagram of different potential interacting candidates of CRF5.
Candidates were discovered after conducting a yeast-two-hybrid approach with the non-auto-activating crf5 deletion constructs CRF5ΔV and CRF5ΔVI. Shown are the total numbers of sequences in frame with the activation domain of the library vector pAD-GAL4 2.1. Library vector DNA of colonies growing on quadruple drop-out medium was isolated, specifically amplified in *E.coli* and sequenced.

Preliminary interaction studies with switched bait-prey vectors and mutated versions of CRF5 with successive deletions of around 50 amino acids (CRF5ΔI – CRF5ΔVI; described in detail in Manuscript 1) and the final 166 amino acids of HGL1 showed an interaction with CRF5ΔIII, ΔIV, ΔV, and ΔVI but a much weaker interaction with CRF5ΔI and CRF5ΔII (data not shown). CRF5ΔI and CRF5ΔII are both lacking parts of the CRF domain described by Cutcliffe and co-workers which was shown to be necessary and sufficient for protein-protein interactions within the CRFs and with AHPs of the TCS (Cutcliffe et al., 2011). It

should be noted that these interactions with swapped bait-prey vectors could only be shown on less stringent medium lacking only Leu, Trp, and His. No growth was detectable on highest stringency medium like in Supplemental Figure 2. In addition, the full-length version of HGL1 fused to the activation domain in the bait vector exhibited transactivation activity and hence was not usable for interaction verification without further adjustments.

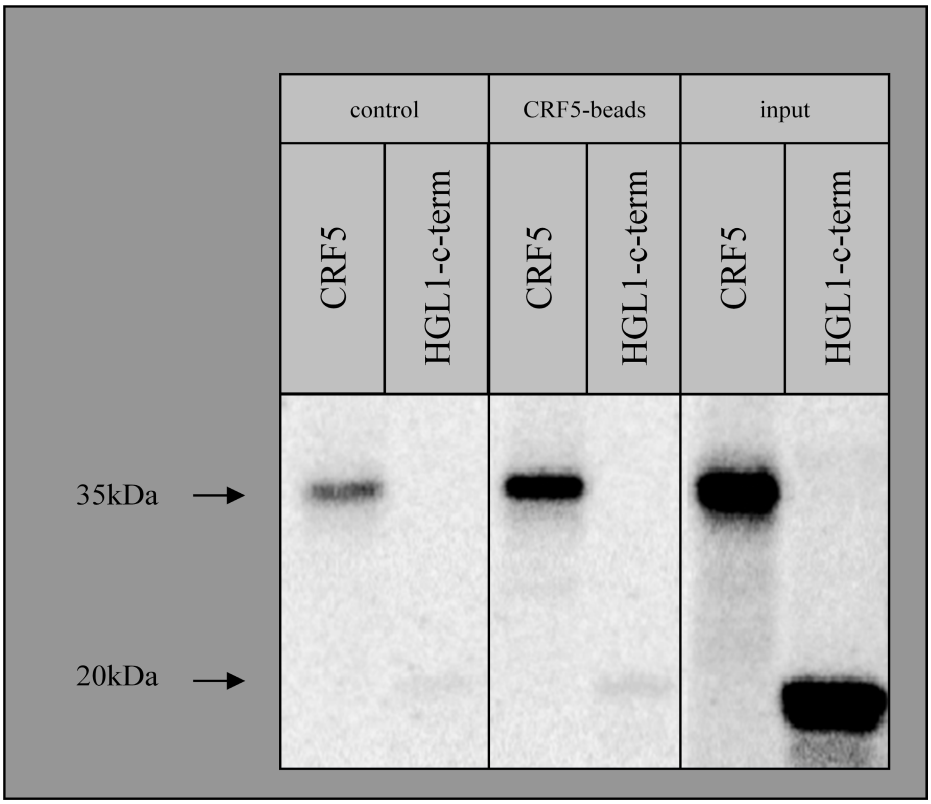


Supplemental Figure 2 – Interaction of CRF5 and HGL1 in yeast.
Growth of diploid Yeast strains on double (-Leu/-Trp) or quadruple (-Leu/-Trp/-His/-Ade) drop-out media. CRF5Δ5 was fused to the GAL4 binding domain (BD) and HGL1 was fused to the GAL4 activation domain (AD). Negative controls: pGBKT7-53 (53-BD) and pGADT7-T (T-AD). Shown are the results of 3 independent diploid yeast strains.

Approaches to verify the interactions between CRF5 and HGL1 by means of coimmunoprecipitation and GST-pulldown did not succeed so far. We did not achieve to synthesize full length HGL1 protein *in vitro* probably due to the high molecular weight of about 111 kDa. Therefore we decided to use the C-terminal end of HGL1 (HGL1-C-term) with a molecular weight of 20 kDa for GST-pulldown assays. In these, no obvious difference

of the amounts of precipitated HGL1-C-term from GST-tag-coupled (control) or GST::CRF5-coupled beads was detectable. A very little amount was precipated by both types of beads and therefore considered as background binding.

The homodimerization ability of CRF5 (Cutcliffe et al., 2011), however, could be verified by these GST-pulldown assays. Much more ^{35}S-Methionine labelled CRF5 could be precipitated by GST::CRF5-coupled glutathione agarose beads than with only GST-tag-coupled agarose beads (see Supplemental Figure 3).



Supplemental Figure 3 – *In vitro* interaction of CRF5 and HGL1.
Autoradiogram of a GST-pulldown of ^{35}S-Methionine labelled CRF5 and the C-terminal end of HGL1, respectively, with agarose beads coupled with GST::CRF5 or GST only. Input: raw *in vitro* translated 35S-methionine labelled protein, control: GST-coupled agarose beads.

The interaction of a transcription factor and HGL1 is to say at least atypical. To our knowledge there has been shown no interaction between enzymes of the sugar or starch metabolism and transcription factors yet. Transcription factors interact with other TFs or with activating phosphatases (e.g. (Cutcliffe et al., 2011; Desveaux et al., 2004; To and Kieber, 2008)). Kinases have been found in our raw list of candidates after sequencing but none of

which past the filtering process. It has been shown that transcriptional activators interact with the transcription apparatus through general cofactors (for illustration see Figure 2; reviewed in (Roberts, 2000; Thomas and Chiang, 2006)). Nevertheless, such a candidate was not found amongst all putative interaction partners.

One very interesting interaction between a TF and a protein apart from the usual interactions was described by Licausi and co-workers. RAP2.12, a member of the AP2/EREBP transcription factor family, was found to bind to the membrane associated proteins ACPB1 and ACPB2 until hypoxia was sensed by the plant. The release of RAP2.12 led to an induction of expression of hypoxia regulated genes (Licausi et al., 2011). A similar function can be the reason for an interaction of CRF5 with HGL1. HGL1 is putatively located to the cytoplasm (Lu and Sharkey, 2006) and CRF5 (GFP::CRF5) can be located to the cytoplasm as well until application of cytokinin which leads to a location change to the nucleus (Rashotte et al., 2006). A cytokinin signal might trigger the release of CRF5 from HGL1 whereupon the TF might enter the nucleus and specifically regulate gene expression. We were able to show that CRF5 in overexpressing plants is able to induce cytokinin response on phenotypic as well as on regulatory level including the transcriptional induction of *CELL WALL INVERTASE 1* an enzyme hydrolyzing sucrose to glucose and fructose (see Manuscript 2; (Roitsch and González, 2004). A connection of sucrose metabolism and *CRF5* overexpression could also be shown by a sucrose/hexose shift towards the hexoses in CRF5-8^{OE} plants. A reversion of cytokinin related effects on plant development, e.g. shorter roots and retarded shoot growth, which was also observed in these transgenic plants, could be obtained by supplementing the growth medium with sucrose. The expression of *HGL1* however was altered neither by cytokinins nor by ectopical expression of *CRF5* which means that there would be no direct link between CRF5-HGL1 interaction and expression regulation of *HGL1*. Nevertheless, this interaction has only been shown in yeast so far. Further analyses are inevitable.

It has been shown that CRF5 is exhibiting a strong auto-transactivation activity in yeast (Manuscript 2). This means, that there must be an interaction with components of the transcription machinery. It might be a possibility to in depth analyze with which general cofactors in yeast CRF5 interacts and to use this knowledge to find its counterpart in Arabidopsis.

B Cellular Localization of GFP-tagged CRF5

In the Supplemental Manuscript (Schwacke et al., 2007) several *in silico* target prediction programs were combined in order to increase the accuracy and significance for target predictions of transcription factors. The main focus was put on the discovery of dually targeted transcription factors, which could play a role in retrograde signalling between the organelles and the nucleus.

The prediction score for CRF5 to be targeted to the mitochondria and the nucleus was with 11.0 relatively high compared to the already published dually targeted transcription factor WHY2 which has a recently updated score of 15.2 (ARAMEMNON).

However, in another study, CRF5 was shown to be located in the cytoplasm of Arabidopsis protoplasts until the phytohormone cytokinin was added to the protoplast suspension (Rashotte et al., 2006). This was leading to a localization change of CRF5 into the nucleus. No localization was detected in either the chloroplasts or the mitochondria. In this study, a GFP-tagged version of CRF5 was used with the GFP fused to the N-terminus of CRF5 (GFP::CRF5). Organelle import is mostly facilitated by presequences or transit peptides functioning as an import address. These are located at the N-termini of proteins (reviewed in (Kovács-Bogdán et al., 2010)). A fusion to GFP at this end would essentially inhibit its function as an import signal. That is why we decided to conduct localization approaches with a CRF5-GFP hybrid in which the GFP-tag was located at the C-terminal end of CRF5 to prove the ability of CRF5 to be dually targeted.

B-1 Cellular Localization of GFP-tagged CRF5 - Material and Methods

B-1-1 Cloning

The *CRF5* full length gene in the TOPO cloning vector pENTR/SD/D-TOPO (see Chapter IX A-1-1) was used in Gateway recombinational cloning with the binary plant vector pEG103 (Earley et al., 2006).

B-1-2 Transient Transformation of Arabidopsis Protoplasts and Onion Epidermal Cells

A light grown cell suspension culture of Arabidopsis mesophyll cells was grown for 7 days before it was diluted 1:1 with murashige and skoog (MS) medium containing sucrose (30 g/L), B5 vitamins (4 ml/L, Sigma), naphthalene acetic acid (0.5 mg/L) and kinetin (0.1 mg/L)

(pH 5.5). The culture was grown for another 3 days at + 22 °C. Then the cell suspension was sedimented for 5 minutes at 1,500 rpm before it was incubated shaking (dark, 50 rpm) on glass petri dishes with 0.25 % cellulase and 0.05 % mazerozyme (Rio Yakult) in MS containing 0.34 M glucose and 0.34 M mannitol (pH 5.5). After 2.5 hours the protoplast suspension was transferred into centrifuge tubes and sedimented for 5 minutes at 800 rpm. The pellet was washed once with MS containing 0.34 M glucose and 0.34 M mannitol (pH 5.5) and resuspended in MS containing 0.28 M sucrose (pH 5.5). After centrifugation for 5 minutes at 800 rpm the upper band containing intact protoplasts were transferred into microcentrifuge tubes and kept dark on ice until further use.

The protoplast transformation followed largely the protocol of (Lazzeri et al., 1991). 50 µL of the protoplast suspension were mixed with 15 µL DNA (5 µg - 15 µg) and 150 µL PEG solution (30 % PEG 8000, 0.45 M mannitol, 0.1 M $Ca(NO_3)_2$; pH 9). After incubation at room temperature the mixture was pelleted and washed twice with MS containing 0.34 M glucose and 0.34 M mannitol (pH 5.5).

After at least 16 hours of incubation in the dark at room temperature, transformed protoplasts were observed by fluorescence microscopy.

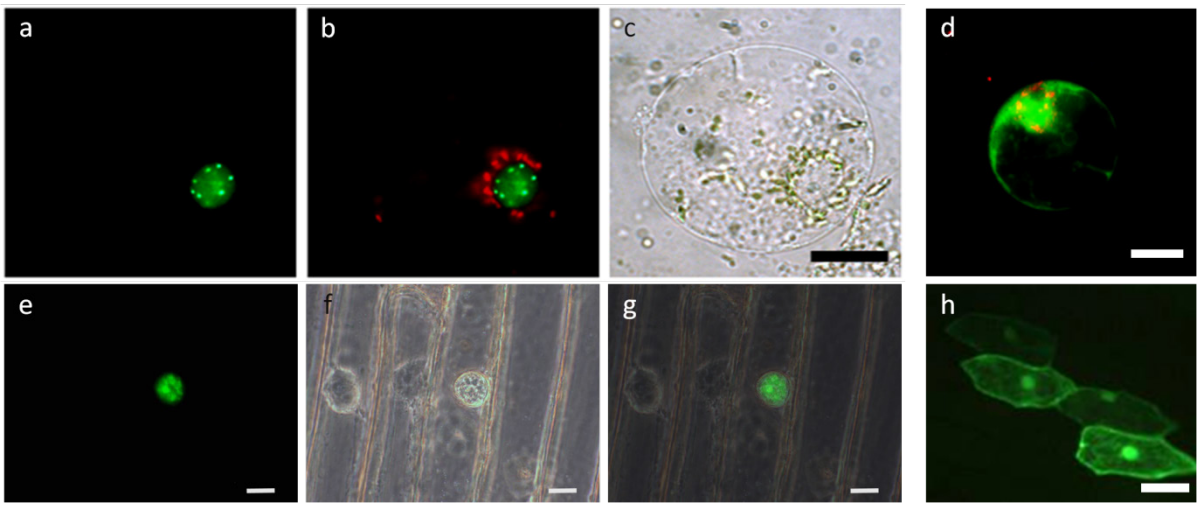
Onion epidermal cells were transformed using the biolistical transformation method modified after (Krause et al., 2005). Inner onion peels were placed on agar plates (1.2% w/v) containing full strength MS medium (pH 5.8) (Sigma). Biolistic bombardments were performed with a PDS-1000/He instrument (BioRad). Acceleration of gold particles (1.5–3.0 µm) coated with 1-10 µg of recombinant plasmid DNA was used to transform onion epidermal cells under a vacuum of around 27 inches Hg and a helium pressure of 1350 psi. The plates containing the bombarded tissue were incubated for at least 16 h in the dark at 20–24 °C.

B-2 Cellular Localization of GFP-tagged CRF5 - Results and Discussion

To prove the ability of CRF5 to be dually targeted to the mitochondria and the nuclei transient transformations of Arabidopsis protoplasts and onion epidermal cells with a CRF5::GFP hybrid protein were conducted.

The basic biological property of transcription factors to be located to the nuclei could be shown (see Supplemental Figure 4). But the results were partly contrary to predictions about the cellular localization which have been performed with the help of the database

ARAMEMNON (http://aramemnon.botanik.uni-koeln.de/). This database uses data generated after procedures described in the supplemental Manuscript (Schwacke et al., 2007). Predicted were localizations in the nucleus but also in the mitochondria with a relatively high score of 11.0. This location could not be shown in the tested systems. Furthermore, we were unable to confirm the reported movement from the cytoplasm to the nucleus after cytokinin application (Rashotte et al., 2006). Weak cytoplasmatic localization was sometimes observable but never without a strong GFP signal in the nucleus. One reason for this opposed behaviour of our GFP-tagged CRF5 might be the C-terminal fusion to GFP. We decided to fuse GFP to the C-terminal end of CRF5 due to the fact that we suspected an N-terminal mitochondrial presequence whereas Rashotte and co-workers constructed a CRF5-GFP fusion protein with the tag located at the N-terminus (GFP::CRF5). Fusing GFP to the C-terminus might prevent CRF5 to locate naturally or *vice versa*.



Supplemental Figure 4 - Location of GFP-tagged CRF5 (CRF5::GFP) in Arabidopsis protoplasts (a-c) and onion epidermal cells (d-f).
Protoplasts derived from an Arabidopsis cell suspension culture. Control GFP fluorescence is shown in Arabidopsis protoplast (d) and onion epidermal cells (h). (a) and (e) show GFP fluorescence only, (b) shows GFP and chlorophyl auto-fluorescence, (c) and (f) show bright field pictures and (g) shows the merged picture of (e) and (f). The scale bars in (c-d) equate 5 µm, the scale bars in (e-g) equate 20 µm, and the scale bar in (h) equates 40 µm.

Nevertheless, the GFP-signal distribution did not appear to be evenly spread like in the case of GFP alone (see Figure 5 d and h). In Arabidopsis protoplasts and also in onion epidermal cells the fluorescence signal of CRF5::GFP formed nuclear body-like structures which varied

in our experiments in number and size. The molecular functions of nuclear bodies, Cajal bodies or speckles has been described in diverse studies and reach from transcriptionally active centres to protein degradation complexes, called photobodies, in connection with phyA (reviewed in (Van Buskirk et al., 2012; Mao et al., 2011; Reddy et al., 2012; Shaw and Brown, 2004)).

In Medicago ERN1 a transcription factor of the AP2/EREBP family with strong homology to RAP2.11 was also shown to localize to nuclear body-like structures (Andriankaja et al., 2007). And, in addition, it was found that plant hormones can also modify the appearance of nuclear speckles (Li et al., 2002). If CRF5 can be assigned to one of the above mentioned complexes has to be further elucidated.

Dual targeting of CRF5 between the mitochondria and the nucleus can be ruled out. This is also supported by detailed sequence analysis of all members of the CRF subgroup. Very high sequence similarities even conserved domains within the N-terminus of the proteins could be detected (Rashotte and Goertzen, 2010). Normally, presequences responsible for the import into mitochondria (or into chloroplasts) show high sequence diversity and completely lack conserved sequence motifs (Huang et al., 2009). The high prediction score for CRF5 and also CRF6 is possibly due to a high proportion of positively charged amino acids and, at the same time, low proportion of negatively charged amino acids at the N-terminus which is a normal feature of mitochondrial transit peptides as well (Huang et al., 2009). Abundance of positively charged and underrepresentation of negatively charged amino acids at the N-terminus is one criterion which is incorporated in localization prediction programs (e.g. (Emanuelsson et al., 2000)). This leads to false predictions when such an amino acid composition is incorporated in conserved N-terminal domains. Integrating motif analyses into target prediction programs as criterion for exclusion might be a way to reduce the probability of false positives.

X General Discussion

In this work, CRF5, a transcription factor of *Arabidopsis thaliana* known to be involved in cytokinin signalling was further analysed with respect to its cellular localization, interaction partners, regulatory targets, function, phylogeny, and domain composition. Most results are discussed extensively in Manuscripts 1 – 3 and the Supplemental Manuscript but some selected topics will be put into context with additional results of the previous section.

A CRF5 is not Dually Targeted

In the Supplemental Manuscript several *in silico* target prediction programs were combined in order to increase the accuracy and significance of target predictions of transcription factors. The main focus was put on the discovery of dually targeted transcription factors. The dual localization of TFs plays a role in retrograde signalling between the organelles and the nucleus. A coordinated release of TFs displays the simplest way to directly change gene regulation in the nucleus under control of the organelles.

CRF5 was proposed to be targeted to the mitochondria and the nucleus was with a prediction score of 11.0 which is above the threshold set by the prediction program but lower than the verified transcription factor WHY2 which has a recently updated score of 15.2 (ARAMEMNON). However, first localization experiments in Arabidopsis protoplasts and onion epidermal cells with C-terminally GFP-tagged CRF5 (CRF5::GFP) exhibited nothing but a nuclear localization in addition to a weak cytoplasmatic localization. These data contradict not only the prediction results but also earlier published results which stated a cytoplasmatic localization of CRF5 until application of cytokinins which then led to a localization change into the nucleus (see Supplemental Figure 4). One big difference, however, is that those localization experiments were conducted with an N-terminally tagged CRF5 and C-terminal tagging possibly interferes with the ability of CRF5 to change location (Rashotte et al., 2006).

Detailed sequence analyses of members of the CRF subgroup revealed very high sequence similarities within the N-terminus of the proteins which suggests that the CRFs do not possess a mitochondrial target signal. Presequences are normally not very well conserved, and do not contain any domains, whereas CRFs are characterized by the well conserved N-terminal CRF

domain (Kovács-Bogdán et al., 2010; Rashotte and Goertzen, 2010; Emanuelsson et al., 2000).

B Characterization of Functional Domains of CRF 5

As mentioned above, the N-terminal part of the CRFs have been shown to consist of the well conserved CRF domain (Rashotte and Goertzen, 2010; Cutcliffe et al., 2011). It turned out that the CRF domain is necessary and sufficient for the formation of homo- and heterodimers (Cutcliffe et al., 2011). We could confirm the homodimerization ability of CRF5 by GST-pulldown assays (Supplemental Figure 3). The DNA binding AP2 domain was already well described in several studies beginning with Ohme-Takagi and Shinshi in 1995 who found this conserved domain to be responsible for binding the *cis*-element AGCCGCC. We decided to focus on the hitherto undescribed C-terminal 98 amino acids of CRF5 which we found to be responsible for auto-transactivation in yeast two hybrid studies.

Two conserved motives were identified as described in Manuscript 1, each responsible for transactivation in combination with the other. Several conserved acidic and hydrophobic amino acids were identified in the transactivation domain but the actual function of these residues has to be determined in the future.

C The Phylogeny of the CRFs

The recently finished genome sequencing projects of several plant species provided the opportunity to gather sequences of ortholog proteins CRF proteins throughout genomes of dicots and monocots. Phylogenetic analyses described in Manuscript 1 revealed that the CRFs can be grouped into four clusters. Only three of which could be found in *Arabidopsis thaliana.* Each of these three clusters is represented by two Arabidopsis CRFs indicating that these pairs derived from the most recent whole genome duplication during evolution of the Brassicacea.

These analyses also surfaced a motif conserved in all four clusters throughout all CRF orthologs. This motif is located at the outermost C-terminus of all CRFs. The function, however, could not be resolved.

D Protein-Protein Interaction of CRF5 and HGL1

After having located and deactivated the transactivation ability of CRF5, a yeast two hybrid screen against an Arabidopsis cDNA library was conducted (see Manuscript 2 and Work in

Progress). A heteroglycan glucosidase (HGL1) turned out to be the most reliable interacting candidate (see Supplemental Figure 2). Unfortunately, coimmunopreciptation or GST-pulldown experiments could not verify this interaction so far.

Neither the actual function nor the cellular localization of HGL1 is known. HGL1 is considered to be involved in the degradation of cytosolic heteroglycan to glucose. A process associated to starch degradation (Lu and Sharkey, 2006). However, the experimental proof of this theory is still missing. The interaction of a transcription factor and an enzyme involved in starch degradation is very interesting for two reasons. First, it would be the first evidence for an interaction of a TF with an enzyme of the starch metabolism. And second, the interaction with HGL1 would be the second link of CRF5 to starch metabolism, besides the upregulation of *SEX1*, which will be revisited in Chapter X F.

E The Ability of CRF5 to bind the *cis*-Acting Element DRE

Besides these indications of a possible integration of CRF5 in starch or sugar metabolism we were able to find more links between cytokinin, CRF5, and sugar metabolism. Especially the drought responsive element (DRE) located in the promoter region of *RD29A* seemed to be a good candidate for a link between CRF5 and sugar metabolism due to its connection to cold acclimation which has the documented effect of changing sugar levels in plants (e.g. (Stitt and Hurry, 2002)). The interaction of AP2/EREBP transcription factors with this element has been shown for some members of the DREB subfamily (Liu et al., 1998; Sun et al., 2008).

The direct interaction of CRF5 with the DRE could be shown by yeast one hybrid analysis. An indication of a direct interaction with the DRE element *in planta* has been found in the up-regulation of *RD29A* expression in *CRF5* overexpressors compared with wild type expression (see Manuscript 2). DREB1A, which is also a member of the AP2/EREBP transcription factor family, induces expression of *RD29A* through binding of the DRE, too (Maruyama et al., 2009; Liu et al., 1998). In addition to the up-regulation of *RD29A, AUR3* (*AUXIN UPREGULATED 3*) was also up-regulated. By promoter analyses, a DRE-like element was found in the promoter region of *AUR3* indicating a direct regulation of this gene, too.

F Comparing the Effect of Cytokinin Treatment with *CRF5* Overexpression

Gene expression analyses of the crf5 overexpressors (see Manuscript 2) also indicated that constitutive *CRF5* overexpression led to an up-regulation of known cytokinin responsive transcripts, e.g. *AHK4* and *ARR5*. *CELL WALL INVERTASES 1* (*CWINV1*) which is also

known to respond to cytokinins was up-regulated to almost double of its normal expression in wild type. Apart from the earlier mentioned abscisic acid marker *RD29A* and the auxin responsive marker *AUR3* other hormonal markers were not differentially regulated either in treated wild type plants or in *CRF5* overexpressing plants. But this indicates an involvement of CRF5 in the auxin/cytokinin and abscisic acid/cytokinin crosstalk.

Of the genes which products are involved in sugar and starch metabolism only *CWINV1* and *SEX1* were noticeably differentially expressed. Transcript levels were up-regulated in both *CRF5* overexpressors as well as in cytokinin treated wild type Arabidopsis seedlings. However, *SEX1* transcript level in *CRF5* overexpressors was about 12 times higher than in wild type compared to about 2.5 times in cytokinin treated wild type seedlings. The above mentioned genes which are differentially regulated in *CRF5* overexpressing plants are encoding proteins which in turn have an effect on gene regulation as well or can, *vice versa*, already be the product of an indirect regulation. Therefore, the primary CRF5 impact on the eventual gene regulation is difficult to be traced back. A direct interaction with *cis*-regulatory elements like shown for the DRE element can be a proof. Another possibility would be, to generate transgenic plant lines in which *CRF5* is put under the control of an inducible promoter. Short term changes in gene expression could better be accounted for and more directly be linked to CRF5.

G Phenotypic Analysis of *CRF5* Overexpressors

Phenotypical, *CRF5* overexpression mimicked the action of cytokinins on seedling growth. Pleiotropic effects like shorter roots, smaller shoots, and fewer chloroplasts per cell in cotyledons have been observed in Manuscript 2 and 3. In the case of chloroplasts per cell the effect of CRF5 seems to be contradictory to the effect of CRF2 in transgenic plants overexpressing these genes (Okazaki et al., 2009).

With respect to shoot and root growth the untreated wild type phenotype could be re-established by addition of sucrose. This could be shown in cytokinin treated seedlings as well. That sucrose depletion could be the reason for this effect was supported by sugar measurements conducted on four week old plants which showed a shifted sucrose/hexose ratio towards the hexoses without changing the total sugar amount.

One similar example could be found in another component of cytokinin signalling. The loss-of-function mutant of the homeobox protein STIMPY exhibits a similar phenotype as *CRF5*

overexpression and this phenotype could also be completely rescued by the addition of sucrose (Wu et al., 2005). This effect was explained by the need of a sucrose signal in these mutants in order to run the cell-cycle properly, a connection to sucrose depletion was not drawn (Skylar et al., 2010).

Table 2 - AP2/EREBP transcription factors which lead in case of constitutive overexpression to a dwarf-like phenotype

Name	Locus	Subfamily and subgroup	References
DREB1A (CBF3)	AT4G25480	DREB A-1	(Liu et al., 1998; Gilmour et al., 2004)
DREB2A	AT5G05410	DREB A-2	(Liu et al., 1998)
CBF1	AT4G25490	DREB A-1	(Gilmour et al., 2004)
CBF2	AT4G25470	DREB A-1	(Gilmour et al., 2004)
TINY	AT5G25810	DREB A-4	(Sun et al., 2008)
ERF1	AT3G23240	ERF B-3	(Solano et al., 1998; Lorenzo et al., 2003)
RAP2.6	AT1G43160	ERF B-4	(Krishnaswamy et al., 2011)
RAV1	AT1G13260	RAV	(Hu et al., 2004)

All these phenotypic effects of ectopic expression of *CRF5* mimicking cytokinin effects cannot directly be assigned to CRF5 so far. CRF5 is possibly inducing cytokinin response in the same manner as other positive regulators of the TCS are able to do (Kiba et al., 2004; Hwang and Sheen, 2001). The CRFs were shown to regulate several targets which are also regulated by type-B ARRs including components of the TCS (Rashotte et al., 2006). The up-regulation of *AHK4* and *ARR5* by *CRF5* overexpression could be confirmed in Manuscript 2. On the other hand, phenotypic responses to *CRF5* overexpression might also be due to the common AP2 domain, i.e. that it shares a set of common targets with other members of the AP2/EREBP TF family. For many AP2/EREBP TFs it has been shown that an ectopic expression can, for instance, lead to retarded shoot growth (see Table 2). Sugar levels changes have been observed in AP2/EREBP TF overexpression plants (Gilmour et al., 2004; Maruyama et al., 2009) and seeds of these (Ohto et al., 2005). In addition, a regulatory role of an AP2/EREBP TF in starch synthesis has been found in rice (Fu and Xue, 2010). The schematic depiction in Figure 11 tries to put recent data and data acquired in this thesis into context with special respect to the connection between the cytokinin signalling pathway and the AP2/EREBP TFs.

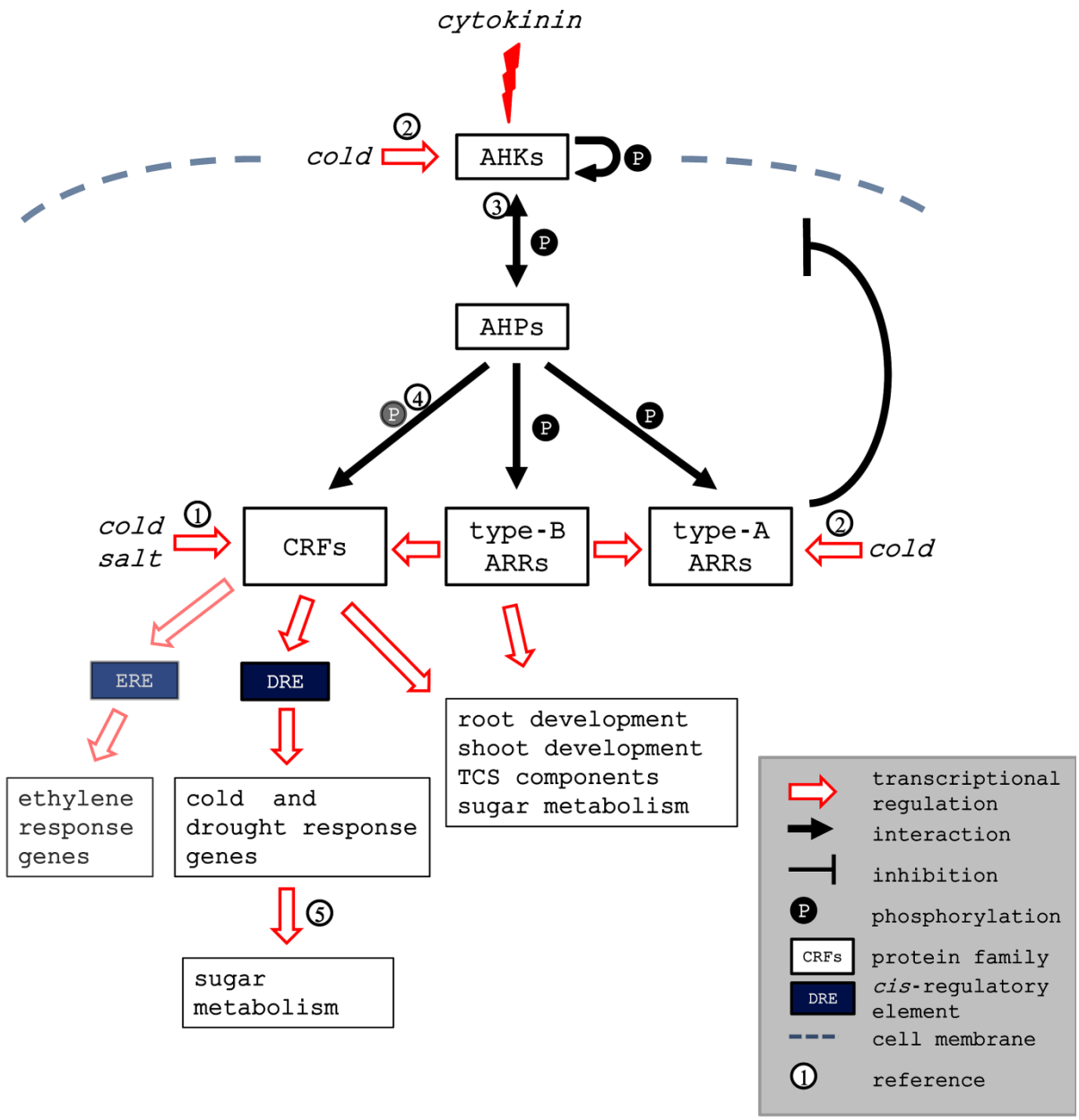


Figure 11 – Updated model of the cytokinin signalling pathway.
Integration of recent data and data of this thesis into the model based on To and Kieber 2008 References: (1) Dietz et al., 2010, (2) Jeon et al., 2010, (3) Mähönen et al., 2006, (4) Cutcliffe et al., 2011, (5) Maruyama et al., 2009.

XI Conclusions and Future Directions

Taken together, CRF5 is a positive regulator of cytokinin signalling in *Arabidopsis thaliana*. The transactivation domain is C-terminally located and comprises modules which are conserved throughout the plant kingdom. Overexpression of *CRF5* leads to effects known from exogenous application of cytokinin on plants in regards to shorter root growth and smaller shoot growth. Ectopic expression of *CRF5* and cytokinin application affects the expression of known cytokinin responsive genes in a similar manner. Contrary to cytokinin application, *CRF5* overexpression increases the expression of *RD29A* which is normally responding to drought, cold, or salt stress. The well described promoter region of *RD29A* harbours the drought responsive element (DRE) bound by some members of the AP2/EREBP subfamily DREB. This element could be shown to be bound by CRF5 as well which might therefore be a candidate to link cytokinin action to cold and ABA signalling. Whether this link or a direct regulation of genes coding for elements of the sugar metabolism like the *CELL WALL INVERTASE 1* is responsible for an observed sucrose/hexose shift has to be further investigated.

A lot of questions remain. CRF5 is only one of approximately 1,600 transcription factors in *Arabidopsis thaliana*. Nine percent of which are members of the AP2/EREBP family. Many are regulated by different abiotic and biotic stresses sometimes not only by a single one. Furthermore it seems as if *cis*-acting elements of one kind can be bound by many transcription factors of the same TF family though with different strengths. Transcription factors might compete for the same *cis*-acting element. It has been shown that transcription factors multimerize and interact with phosphatases in addition to the interaction with *cis*-regulatory elements and general cofactors. Furthermore, transcription factors have been shown to regulate other transcription factors including closely related family members. These interdependencies in many ways picture an extremely complex regulatory network which is yet only partially understood.

Redundancy in function of many transcription factors let it seem impossible to only study single loss-of-function mutants. Effects on plant development might be impossible to detect due to disguise by the natural variance in plant growth. Multiple loss-of-function mutants, however, increase the possibility of pleiotropic effects.

But now that the first hints of functions of CRF5 and other CRFs became apparent it might make sense to look more into their relation with cold and drought stress. This in connection with sucrose metabolism can be important with respect to solving the problem of cold sweetening in potatoes. The confirmation of the HGL1-CRF5 interaction would lead into the same direction. Down-regulation instead of complete loss-of-function of both, *CRF5* and *CRF6*, at the same time might also bear clues towards the function of these two closely related transcription factors. A double loss-of-function is reported to be lethal.

The ability of *CRF5* to bind the DRE element, a connection which has yet been shown only for members of the DREB subfamily and one member of the ERF subfamily (*TINY*), implies that *CRF5* is also able to bind the GCC-box (ERE) which is bound by closer related members of the ERF subfamily. Point mutational analyses of both *cis*-acting elements might yield a better match for CRF5. The resulting element can subsequently be searched for in promoters of all Arabidopsis genes to find further regulated genes. Other completely different *cis*-regulatory elements might also be bound and lead for example to the up-regulation of cytokinin related gene expression which cannot be explained by DRE or ERE elements absent in the promoters of *AHK4* and *ARR5*.

Post-translational regulations of the CRFs, the possibility of being phosphorylated and the controversial results of cellular CRF5 localization for instance, have to be studied as well.

XII References

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XIII Manuscripts

MANUSCRIPT 1

MANUSCRIPT 2

MANUSCRIPT 3

SUPPLEMENTAL MANUSCRIPT

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