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Subcellular localization and functional characterization of the *Arabidopsis thaliana* homologue of Cellular Repressor of E1A-stimulated genes (CREG)

Diplomarbeit

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eingereicht von

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Zusammenfassung

„Cellular repressor of E1A-stimulated genes“ (CREG) ist ein Phosphoglykoprotein in den Lysosomen tierischer Zellen. CREG wurde ursprünglich in *Drosophila melanogaster* (dCREG) identifiziert und mittlerweile wurden homologe Gene im menschlichen Genom (hCREG), in *Arabidopsis thaliana* (aCREG) und in der Maus (mCREG) gefunden. In Versuchen mit tierischer Zellkultur wurde gezeigt, dass CREG an Prozessen wie Zellteilung und Differenzierung teilnimmt. Überexpression von CREG inhibiert Zellwachstum und fördert Differenzierung. Obwohl die Sequenz des Gens und die tertiäre Struktur des Proteins vollständig geklärt wurden gibt es keinen Hinweis darauf durch welche Mechanismen es den Zellzyklus beeinflusst. Die Untersuchung der subzellulären Lokalisation des Proteins in tierischen Zellen ergab, dass es sich hierbei um ein lysosomales Protein handelt. Bislang wurde CREG nur in tierischen Systemen und in Drosophila untersucht.

Ziel dieser Arbeit war es daher, Einsicht in die subzelluläre Lokalisation und Funktion von CREG in pflanzlichen Zellen zu erlangen. Hierfür wurden zwei Konstrukte kreiert (aCREG-GFP und aCREG-mRFP), die durch konfokale Laser-Scanning Mikroskopie in den Zellen von *Nicotiana benthamiana* und *Arabidopsis thaliana* lokalisiert wurden. Die Analyse ergab, dass sich aCREG aller Wahrscheinlichkeit nach in den Vakuolen pflanzlicher Zellen befindet. Da pflanzliche Vakuolen homolog zu tierischen Lysosomen sind, stimmen diese Ergebnisse auch mit den Lokalisierungsstudien in tierischen Zellen überein.

Für die funktionelle Analyse von aCREG wurden *A. thaliana* Wildtyp Pflanzen mit zwei aCREG knock-down Linien unter abiotischen Stress-Bedingungen verglichen. Die Setzlinge wurden erhöhten Konzentrationen von Natriumchlorid und Saccharose ausgesetzt. Auch der Einfluss erhöhter Außentemperatur während des Wachstums oder das Fehlen von Saccharose im Wachstumsmedium wurden untersucht. Die Ergebnisse deuten darauf hin, dass aCREG keinen direkten Einfluss auf die Reaktion von Pflanzen auf abiotischen Stress ausübt.

Abstract

"Cellular repressor of E1A-stimulated genes" (CREG) is a phosphoglycoprotein found in the lysosomes of mammalian cells. CREG was first identified in the *Drosophila melanogaster* (dCREG) genome, but meanwhile CREG homologues have been also identified in the human (hCREG), *Arabidopsis thaliana* (aCREG) and the mouse (mCREG) genome. Experiments with mammalian cell culture have shown CREG to inhibit cell division and to promote cell differentiation. The mechanisms by which CREG influences cell growth are not clear yet, although the sequence and tertiary structure of the protein are known. Subcellular localization studies in mammalian cells have shown CREG to reside in lysosomes. Until now, only data on the mammalian and Drosophila CREG variants are available.

The aim of this work was to investigate the subcellular localization of CREG in plant cells and to get an insight into its functions there. For subcellular localization studies in *Nicotiana benthamiana* and *Arabidopsis thaliana*, two constructs (aCREG-GFP und aCREG-mRFP), that were analysed by confocal laser scanning microscopy, were created. The analysis shows, that aCREG is localized in the vacuoles of plant cells. This result is in accordance with the fact that vacuoles are the plant homologues of mammalian lysosomes and therefore agree with the localization studies in mammalian cells.

For functional analysis of aCREG in *A. thaliana*, wildtype seedlings were compared to two aCREG knock-down lines under abiotic stress conditions. The seedlings were subjected to elevated sodium chloride and sucrose levels in the growth medium. The effects of elevated temperature or sucrose depletion were also investigated. The results indicate that aCREG is dispensible for the abiotic stress response of plants.

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1 Introduction

1.1 Cellular Repressor of E1A-stimulated Genes (CREG)

1.1.1 Identification

CREG was first identified during a yeast-two-hybrid screen for TATA binding protein (TBP) interaction partners in *Drosophila melanogaster* (Veal *et al.*, 1998). Sequence analysis revealed amino acid similarity with two regions of the E1A protein, CR1 and CR2. Further analysis identified a CREG homologue in a human cDNA library. It displayed 31% amino acid identity to *Drosophila* CREG (dCREG). Other results showed that hCREG mRNA is widely expressed in adult human tissues and that hCREG mRNA levels increased during terminal differentiation of several cell lines (Veal *et al.*, 2000). hCREG displayed an anti-oncogenic activity (Veal *et al.,* 1998), but until today no enzymatic or other biochemical activity could be found. Recently CREG homologues in mice (mCREG) and *Arabidopsis thaliana* (aCREG) have been identified. The amino acid identity of hCREG to mCREG is 77%, to dCREG 31% and to aCREG 29% (Figure 1).

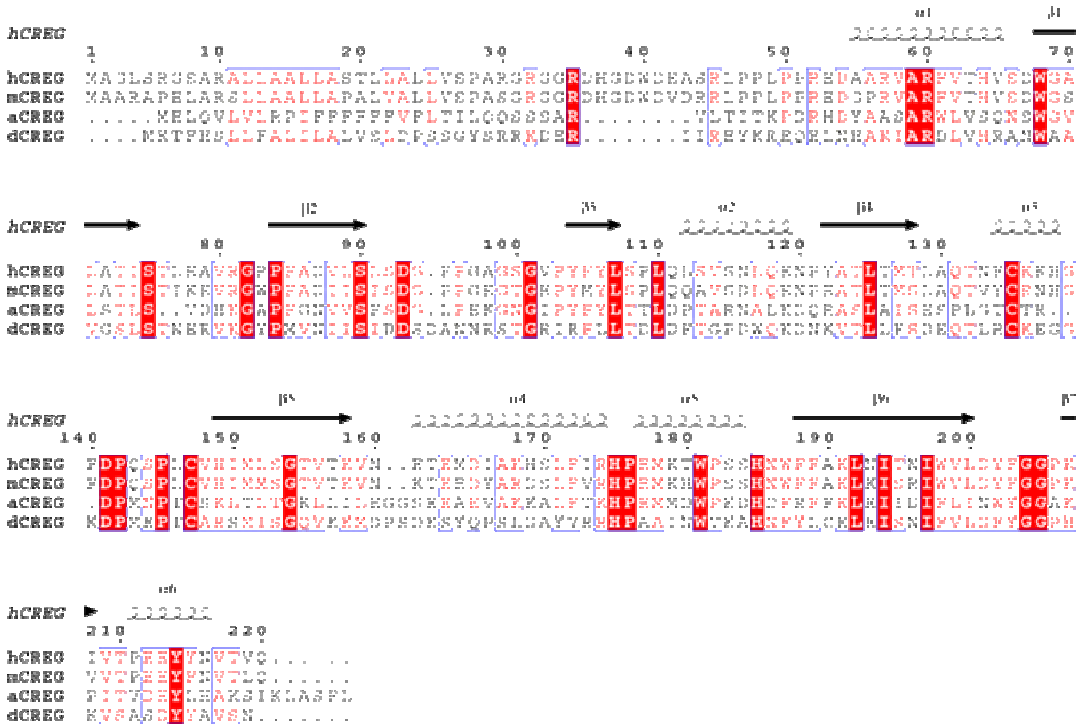

Figure 1: Amino Acid homology between hCREG, mCREG, aCREG and dCREG based on sequence alignment. Secondary structure elements based on the crystal structure of hCREG are indicated above the amino acid sequence. Boxes indicate conserved amino acid sequences, highly conserved residues are shown in red.

Recently, two novel members of the CREG family have been identified: human CREG2 (hCREG2) and mouse Creg2 (mCreg2). Expression of hCREG2 and mCreg2 has been observed only in brain tissue, whereas hCREG is ubiquitously expressed in most adult tissues (Kunita *et al.*, 2002).

CREG has also been identified in several proteomic approaches with the aim to fully characterize the lysosomal proteome. *N*-glycosidically linked mannose-6-phosphate (M6P) residues specifically label soluble lysosomal proteins. Therefore proteomic approaches conventionally rely on immobilized M6P receptors to capture them (Journet *et al.*, 2000). Using a combination of one- and two-dimensional gel electrophoresis and protein identification by *N*-terminal sequencing Journet *et al*. identified 15 proteins, of which 12 were well known lysosomal hydrolases, one was a putative lysosomal protein and two (leukocystatin and human cellular repressor of E1A-stimulated genes (hCREG)) were

described for the first time as M6P containing proteins. The same group used this approach two years later for the comparison of the lysosomal proteome in undifferentiated and differentiated monocytic cells as well as in a breast cancer cell line (Journet *et al.*, 2002). Among the 22 identified proteins hCREG was found again, and the results also show that hCREG undergoes proteolytic processing (removal of a 14 residue *N*-terminal extension). hCREG was also identified in a screen of the human brain lysosomal proteome (Sleat *et al.*, 2005) and in a mass spectrometric approach to determine the positions of M6P-modified *N*-glycans in lysosomal proteins (Sleat *et al.*, 2006). These findings are in contradiction to previous theories that CREG is a transcriptional regulator, because as such it should reside in the nucleus or cytoplasm. Therefore further studies are required to understand the function of hCREG in the lysosomal compartment.

1.1.2 Putative functions of CREG

Being the transforming factor of adenoviruses, E1A intervenes with the control mechanisms for transcription in the host cell in order to propagate cell division and proliferation, thereby inhibiting differentiation. E1A cooperates with oncogenes, such as *ras*, to reprogram cellular gene expression and transform primary cells. E1A was shown to interact with several transcriptional regulators of cell proliferation, including the tumor suppressor retinoblastoma protein, pRb, and the coactivators p300 and CBP, to activate and repress expression of particular genes (Veal *et al.*, 1998). CR1 and CR2, two conserved regions of E1A that mediate binding to pRb and other cellular transcription factors, such as TATA-binding protein (TBP), have been shown to be required for E1A mediated transcriptional regulation and cellular transformation. E1A has been observed to activate transcription through several response elements, including the binding site for E2F, a cell-cycle regulated transcription factor, in the adenoviral E2 promoter.

Since dCREG was initially identified as a TBP-binding protein, the ability of hCREG to bind TBP was investigated in vitro and showed that hCREG can bind not only TBP but also pRB and the related p107 and p130 proteins. Expression of hCREG was shown to reduce the efficiency with which E1A and the oncogene *ras* cooperate to transform cells. In transfection assays, it has also been demonstrated that hCREG represses E1A mediated activation of adenovirus E2 and cellular hsp70 promoters.

These results suggest that CREG might be involved in the transcriptional control of cell growth, inhibiting proliferation and promoting differentiation.

In 2000 Veal *et al*. confirmed a function of CREG during cell differentiation. They found that expression of CREG is enhanced upon in vitro differentiation of NTERA-2 cells induced by retinoic acid or hexamethylene bisacetamide. They also found that constitutive overexpression of CREG enhances neuronal differentiation of NTERA-2 cells and also that NTERA-2 cells grown in CREG-enriched media showed enhanced differentiation upon stimulation with retinoic acid. CREG is not expressed at significant levels in pluripotent mouse embryonic stem cells but expression levels rise during differentiation. CREG has repeatedly been implicated in cell differentiation processes (Di Bacco and Gill, 2003, Han *et al.*, 2008) by keeping cells or tissue in a mature state and thus appears to counteract pathological de-differentiation and overgrowth. Other putative functions of CREG include regulation of the ERK1/2 pathway in cardiac hypertrophy (Bian *et al.*, 2009, Xu *et al.*, 2004) and an involvement in the turnover of the M6P/IGF2R during IGF-mediated hypertrophy in ileal epithelial cells (Gordon *et al.*, 2005).

As mentioned above, CREG was identified as a novel M6P-modified protein. Such proteins carry mannose-6-phosphate moieties on their glycan structures and are recognized by M6P receptors. In mammalian cells these are the M6P/IGF2R and MPR 46. Both receptors bind M6P-modified proteins and target them to the lysosomes after protein synthesis. The M6P/IGF2R is a multifunctional transmembrane glycoprotein of about 300 kDa that is built of a large extracellular domain containing 15 repeat regions and a small cytoplasmic domain. The receptor has two regions for binding of M6P residues and one region for IFG2 (Insulin like growth factor 2) binding. It is not only located in the Golgi membrane but also on the plasma membrane where it is responsible for binding of extracellular IGF2 and its delivery into lysosomes for degradation. The receptor can also bind other growth factors and cytokines like the latent form of transforming growth factor-β (Dennis and Rifkin, 1991), leukaemia inhibitory factor (Blanchard *et al.*, 1999), and proliferin (Dahms and Hancock, 2002), which are all M6P modified. The receptor plays a critical role as a negative regulator of cell growth as it influences signalling of growth factors across the cell membrane. The M6P/IGF2R has been demonstrated to be required for CREG-induced growth inhibition (Di

Bacco and Gill, 2003). How CREG cooperates with the M6P/IGF2R to suppress cell proliferation is not clear yet. It is possible that secreted CREG is internalized upon binding to the M6P/IGF2R and then transferred to its site of action, or that CREG influences M6P/IGF2R interactions with other ligands, like IGF2 (Han *et al.*, 2009).

In conclusion it can be said that CREG has putative functions in cell growth control by facilitating tissue hypertrophy in some tissues whereas it permits maturation in others, depending on the stage of development.

1.1.3 Structural analysis of CREG

In 2005 the 3D-crystal structure of hCREG (*aa* 49-220) was solved (Sacher *et al.*, Figure 2). It showed that hCREG monomers display a β-barrel fold and that hCREG appears to form a tight homodimeric complex. Gel filtration studies performed on recombinant mCREG revealed that this homologue also forms dimers. Three potential glycosylation sites (Asn 160, Asn 193 and Asn 216) map to a confined patch opposite the dimer interface, and thus upon dimerization CREG presents a multivalent ligand for the M6P/IGF2R. Structural homologues of hCREG are proteins that bind flavin mononucleotide (FMN), but the putative FMN-binding pocket of the hCREG homodimer is sterically blocked by a small loop. This loop was shown to be required for CREG function in growth control, but how this function is carried out remains unknown. The first 31 amino acids of murine and human CREG and the first 23 *aa* in dCREG target the protein to the secretory pathway of the cell (signal peptides estimated by SignalP 3.0, Technical University of Denmark). hCREG and mCREG undergo proteolytic maturation, as the first 14 *aa* (propeptide) are cleaved off by proteases in the lysosome (Schahs *et al.*, 2008). mCREG shares two of the three glycosylation sites of hCREG: Asn 160 and 216. dCREG is also modiefied by *N*-glycosylation on Asn 87. *A. thaliana* CREG has no identified *N*-glycosylation site.

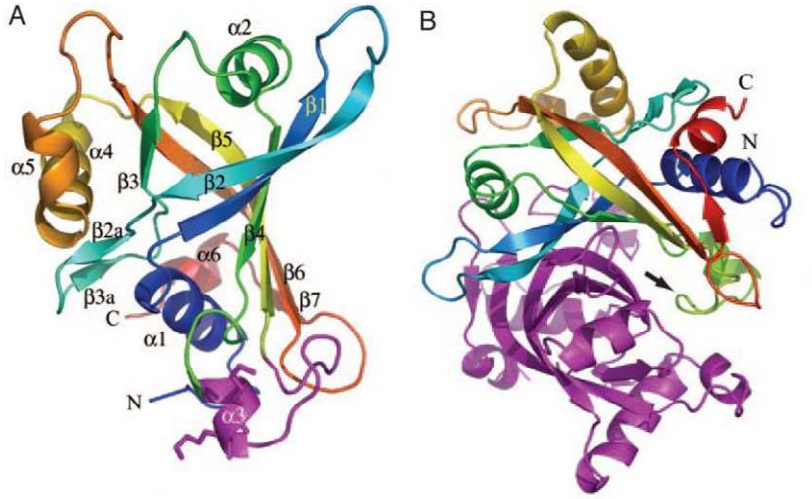

Figure 2: The crystal structure of human CREG.
A: Monomeric subunit;
B: Homodimeric complex. The arrow indicates the loop region (Sacher *et al.*, 2005).

1.1.4 Subcellular localization of CREG

When CREG was first identified, the question was raised where in the cell the protein resides. In 2000 Veal *et al.* used immunofluorescent staining to show that hCREG is a secreted glycoprotein. CREG was found in the perinuclear region of the cell, which is typical for the ER (Endoplasmic Reticulum) and the Golgi apparatus. They found secreted hCREG in tissue culture media and concluded that the *N*-terminal signal sequence is necessary for secretion. The notion of CREG being a secreted protein is now widely accepted (Di Bacco and Gill, 2003, Han *et al.*, 2008, Kunita *et al.*, 2002, Sacher *et al.*, 2005, Xu *et al.*, 2004).

However, recent results point out that CREG is a lysosomal protein (Journet *et al.*, 2000, Journet *et al.*, 2002, Sleat *et al.*, 2005, Sleat *et al.*, 2006) that co-localizes with lysosomal marker proteins (cathepsin D and LAMP-1), and has putative lysosomal functions (Schahs *et al.*, 2008).

How CREG influences cell growth while residing in the lysosome remains to be elucidated. It is possible that CREG is being activated in the lysosome and then in turn activates a signalling cascade leading to changes in transcription in the nucleus.

1.1.5 The *Arabidopsis thaliana* homologue of CREG

So far, all studies on CREG have been conducted with the mammalian or *D. melanogaster* homologues. The biological processes in which *A. thaliana* CREG is involved are unknown. aCREG has a *N*-terminal 29 *aa* signal peptide targeting it to the secretory pathway. The predicted tertiary structure of the protein resembles that of hCREG, and it also contains a domain related to the FMN-binding split barrel. It is located on the At2g04690 locus of the *A. thaliana* genome. The gene is interspersed by 5 introns, and two estimated splice variants exist: At2g04690.1 and At2g04690.2 (www.arabidopsis.org) (Figure 3). The difference between the two splice variants is, that At2g04690.2 is 21 bp (or 7 *aa*) shorter than At2g04690.1. Thus, the two splice variants not only differ in the length of the gene product but also in the coding sequence.

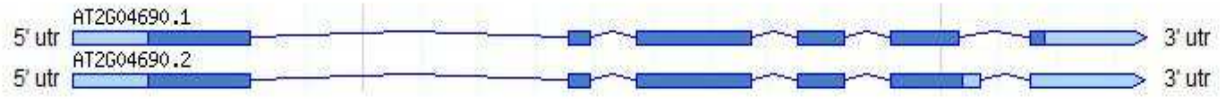

Figure 3: The splice variants of At2g04690. 5' and 3' utr (untranslated regions) sequences are shown in light blue, gene coding sequences in dark blue. Introns are shown as connecting lines.

The subcellular localization of *A. thaliana* CREG remains to be investigated. Two reports have identified aCREG in plant tissues: once in the vacuolar proteome (Carter *et al.*, 2004) and once in the plant cell wall (Irshad *et al.*, 2008).

1.2 Aims of the project

In this diploma thesis, the subcellular localization of aCREG was investigated, as well as its biological role.

- Subcellular localization studies were performed with C-terminal fusion constructs of aCREG with GFP (green fluorescent protein) or mRFP (monomeric red fluorescent protein). The fusion constructs were used for agroinfiltration and transient

expression in *Nicotiana benthamiana* epidermal leaf cells and analysed by confocal laser scanning microscopy.

- Also, the fusion proteins were stably expressed in *A. thaliana* for subcellular localization by confocal laser scanning microscopy and sucrose density gradient fractionation.

- The biological function of aCREG was investigated by subjecting aCREG RNAi knock-down lines to abiotic stress. The stress conditions investigated include salt stress, sucrose stress and heat stress, with the aim to elucidate whether aCREG participates in stress responses.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and Solvents

Fluka

Acetosyringone

Acrylamide/ Bisacrylamide solution 40% (29:1)

Agar

APS (ammonium peroxisulfate)

D(+)-glucose monohydrate

Dithioerythritol (DTE)

DMSO (dimethylsulfoxide)

Gentamycin-sulfate

Potassium chloride

Potassium dihydrogenphosphate

Silver nitrate

Sodium acetate

Sodium acide

Sodium hydrogenphosphate

Sodium thiosulfate pentahydrate

TEMED (*N,N,N',N'*-tetramethyl-ethane-1,2-diamine)

Gibco BRL

Phenylmethanesulphonylfluoride (PMSF)

Helena Chemical Company

Silwet L-77

PeqLab

Agarose

Riedel-de Haen

Sodium carbonate

Rethmann

Developer

Fixing Solution

Roth

Acetic Acid (100%)

Bovine Serum Albumin

Citric acid monohydrate

Disodium hydrogen phosphate

Ethylenediaminetetraacetic acid (EDTA)

Ethanol (100%)

Chloroform (100%)

Glycerol (100%)

Glycine

Hydrochloric acid (37%)

Isopropanol (2-Propanol)

Methanol

SDS (sodium dodecyl sulphate)

Sodium chloride

Sodium hypochlorite (12% in water)

Tris base

Tryptone

Yeast extract

Sigma-Aldrich

Ampicillin sodium salt

Brefeldin A

Ethidiumbromide

Formaldehyde

Glutardialdehyde

Kanamycin-sulfate

D-Mannitol

Mercaptoethanol

MES hydrate

Murashige and Skoog basal medium

P-nitrophenyl *N*-acetylglucosamine (PNP-GlcNAc)

Phytagel

Polyvinylpolypyrrolidone (PVPP)

Ponceau S

Protease-inhibitor cocktail for plant cell and tissue extracts

Sodium phosphate dodecahydrate

Triton X-100

Tween 20

Wiener Zucker

Sucrose

2.1.2 Buffers & Solutions

Acetosyringone

stock solution: 1M; 10 µL aliquots stored at -20° C

Ethidiumbromide

stock solution: 10 mg/ml (in ddH_2O)

working solution: 600 ng/µL; stored at +4° C

Extraction buffer

10 mM Tris

1% SDS

1 mM EDTA

pH 8.0

Infiltration buffer (for agroinfiltration; freshly prepared and stored at room temperature for no longer than one day)

50 mM MES, pH 5.6

2 mM $Na_3PO_4*12H_2O$

0.5 % (w/v) D(+)-glucose

200 µM Acetosyringone

ddH_20

Infiltration buffer (for vacuum infiltration), pH 7.5

100 mM Tris-HCl

2 mM EDTA

10 mM $MgCl_2$

Stored at 4°C

PBS (phosphate buffered saline), pH 7.4

137mM NaCl;

2.7 mM KCl;

8.1 mM $Na_2HPO_4.2H_2O$;

1.9 mM KH_2PO_4

ddH_2O

PBST

PBS, 0.1% Tween 20

PCR buffer (homemade)

10 x: 0.1 M Tris-HCl, pH 8.8
0.5 M KCl
15 mM $MgCl_2$
1 % Triton X-100

Ponceau S stain

working solution: 0.1 % Ponceau S in 5% acetic acid

Running Buffer (for SDS-PAGE)

10 mM Tris
80 mM glycine
1.4 mM SDS
ddH_2O

Sample Buffer (Laemmli; for SDS-PAGE)

4 x: 250 mM Tris-HCl, pH 6.8
40 % glycerol
8 % SDS
20 % mercaptoethanol
0.2 % bromophenol blue
dH_2O

Stripping Solution

0.2 M Glycine-HCl, pH 2.5
0.05% Tween-20
100 mM 2-Mercaptoethanol

TAE (Tris-acetate-EDTA), pH 8.2

40 mM Tris-acetate;
1 mM EDTA, pH 8.2

ddH_2O

TE buffer (Tris-EDTA), pH 7.5

10 mM Tris-HCl

1 mM EDTA

ddH_2O

Transfer Buffer (for Western Blot); stored at +4° C

for 1 L: 3 g Tris base

14.4 g glycine

200 mL methanol

ddH_2O

2.1.3 Enzymes

Fermentas

BamHI (10 U/µL), XbaI (10 U/µL)

(including appropriate 10x buffers)

Pfu (*Pyrococcus furiosus*) Polymerase(2.5 U/µL)

(including 10x Pfu Buffer)

T4-DNA ligase

(including 10x ligase buffer)

Promega

GoTaq® Polymerase (500 U/µL)

(including 5x Green GoTaq® Buffer and 5x Colorless GoTaq® Buffer)

2.1.4 DNA markers & loading dyes

Fermentas

6x DNA Loading Dye

GeneRuler™ 1 kb DNA Ladder

GeneRuler™ 100 bp Plus DNA Ladder

GeneRuler™ 100 bp DNA Ladder

Lambda DNA/ EcoRI+HindIII Marker

PageRuler™ Prestained Protein Ladder

Prestained Protein Molecular Weight Marker

2.1.5 Media

All media were autoclaved after preperation and stored at +4° C.

LB (Luria-Bertani) liquid

0.5 % (w/v) yeast extract

1 % (w/v) bacto-tryptone/peptone

0.5 % (w/v) sodium chloride

LB agar

0.5 % (w/v) yeast extract

1 % (w/v) bacto-tryptone/peptone

0.5 % (w/v) sodium chloride

1.5 % agar

MSS (Murashige and Skoog Sucrose medium), pH 5.7-5.8 (adjusted with 1N KOH)

0.43 % (w/v) MS-Basal medium (Sigma, M5519)

2 % (w/v) sucrose

1 % (w/v) agar

MSS for sucrose stress experiment, pH 5.7-5.8 (adjusted with 1N KOH)

0.43 % (w/v) MS-Basal medium (Sigma, M5519)

0%; 1%; 4.5% (w/v) sucrose

1 % (w/v) agar

MSS for salt stress experiment, pH 5.7-5.8 (adjusted with 1N KOH)

0.43 % (w/v) MS-Basal medium (Sigma, M5519)

0.5g/l MES hydrate

1% (w/v) sucrose

1 % (w/v) agar

+/- 140 mM NaCl

H+ medium for heat stress experiment, pH 5.7-5.8 (adjusted with 1N KOH)

1.6g/l Hoaglands Nr.2 Basal Salt Mixture (Sigma)

1% (w/v) sucrose

1 % (w/v) agar

SOC (Super Optimal broth for Catabolite repression), pH 7.0

2 % (w/v) bacto-tryptone

0.5 % (w/v) yeast extract

10 mM NaCl

2.5 mM KCl

10 mM $MgCl_2$

10 mM $MgSO_4$

20 mM D(+)-glucose monohydrate

2.1.6 Antibiotics

Ampicillin

stock solution: 100 mg/mL (in dH_2O)

working solution: 100 µg/mL

Gentamycin

stock solution: 50 mg/mL (in dH_2O)

working solution: 25 µg/mL

Kanamycin

stock solution: 50 mg/mL (in dH_2O)

working solution: 50 µg/mL

2.1.7 Antibodies

Primary antibodies:

Mouse anti athCREG affipur (homemade 24.01.08)

working solution: 1:1000 in PBST/ 3% BSA/ 0.02% NaN_3

Mouse anti-GFP [Roche Applied Science]

working solution: 1:2000 in PBST/ 3% BSA/ 0.02% NaN_3

Rabbit anti-mRFP [US biological]

working solution: 1:1000 in PBST/ 3% BSA/ 0.02% NaN_3

Rabbit anti-TIP (homemade)

working solution: 1:4000 in PBST/ 0.5% BSA/ 0.02% NaN_3

Secondary antibodies:

Goat anti-rabbit IgG (peroxidase conjugate) [Sigma-Aldrich]

working solution: 1:50 000 in PBST/ 0.5% BSA

Goat anti-mouse IgG (peroxidase conjugate) [Jackson Immuno Research Laboratories]

working solution: 1:10 000 in PBST/ 0.5% BSA

2.1.8 Molecular Biology Kits

MSB® Spin PCRapace [Invitek]

Wizard® *Plus* Minipreps DNA Purification System [Promega]

NucleoSpin® Extract II [Macherey-Nagel]

BigDye® Terminator v3.1 Cycle Sequencing Kits [Applied Biosystems]

BCA™ Protein Assay Kit [Pierce]

Supersignal West Pico Horseradish Peroxidase (HRP) Detection Kit [Pierce]

2.1.9 Bacterial Strains

Agrobacterium tumefaciens UIA143

genotype: *autC58 recA::Ery140(pAtC58)+pmp90(Gent^R)*

E. coli DH5α, electrocompentent [homemade]

genotype: *F$^-$ recA1 endA1 gyrA96 supE44 relA1 deoR Δ(lacZ-argF)U169 hsdR1 thi-1 1$^-$ j89Δlac Δ(lacZ)M15*

2.1.10 Plants

In this work *Nicotiana benthamiana* wild type plants were used for *Agrobacterium tumefaciens* leaf infiltration studies. *Arabidopsis thaliana* wild type plants (ecotype Columbia-0) were used for floral dip transformation with *A. tumefaciens*. Two *A. thaliana* aCREG-knockdown lines (line T4/1 and T4/2) as well as two *A. thaliana* lines carrying the p20F aCREG-GFP vector (p20F aCREG T4/1 and p20F aCREG T4/2) had been obtained earlier at our institute (Christiane Veit, Institute of Applied Genetics and Cell Biology, BOKU, Vienna). The knockdown was achieved by *A. thaliana* transformation with a hairpin construct harbouring the sequence for siRNA complementary to the aCREG gene. Screening and confirmation of the knock down effect and the aCREG-GFP expression was done before this work started. For CLSM (confocal laser scanning microscopy) localization studies two *A. thaliana* lines expressing different marker constructs were used. The first one expressed a PIP2A-GFP fusion protein, marking the plasma membrane. PIP2A (plasma membrane intrinsic protein 2A) is an aquaporin localizing in the plasma membrane. This *A. thaliana* line had initially been obtained from the European Arabidopsis Stock Centre (NASC; http://arabidopsis.info) and represents a T-DNA insertion line in a Col-2 background. The

second line was harbouring a ST-mRFP fusion construct, where ST stands for sialyl transferase, marking the Golgi apparatus. The seeds for this line were kindly provided by Prof. Chris Hawes (Oxford Brookes University, UK).

2.1.11 Vectors

For CLSM localization studies in *N. benthamiana* epidermal leaf cells two different constructs were used. In the p20F vector the aCREG protein was fused C-terminally to GFP (green fluorescent protein) whereas in the p31 vector it was fused to mRFP (monomeric red fluorescent protein). The p20F, as well as the p31 vector, is derived from pPT2 (Strasser *et al.*, 2005). The aCREG gene lacking its stop codon is inserted in between the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter and the GFP or mRFP gene lacking the start codon.

For the p20F-aCREG construct (made by Christiane Veit, Institute of Applied Genetics and Cell Biology, BOKU, Vienna) the aCREG sequence was amplified through PCR from aCREG cDNA from Col-0 and cloned into the PCR4-TOPO vector (described in section 3.1). The gene was then subcloned into the p20F vector. In this work, the aCREG gene from the PCR4-TOPO vector was subcloned into the p31 vector (described in section 3.2). This vector (p31-aCREG-mRFP) was then first introduced into *E. coli* DH5α and then into *A. tumefaciens* UIA 143 for *N. benthamiana* argoinfiltration and *A. thaliana* transformation.

Upon agroinfiltration of *N. benthamiana* leaves the fusion protein is transiently expressed and the localization can be monitored by CLSM. Upon floral dip agroinfiltraion of *A. thaliana* the fusion protein is stably expressed.

The pVKH18-En6-ST-mRFP vector (Saint-Jore-Dupas *et al.*, 2006), a construct of sialyl transferase fused to mRFP, was used to mark the Golgi apparatus.

2.2 General Methods

2.2.1 Agarose gel electrophoresis

For separation of DNA fragments by electrophoresis, 1-1.5% (w/v) (depending on the size of the DNA fragment) agarose gels were prepared. The appropriate amount of agarose was mixed with 1x TAE buffer and heated up in the microwave. After the agarose was dissolved it was left to cool. After cooling, 100µl of ethidium bromide working solution were added to 100 ml agarose solution and the gel was poured into the gel casting chamber with sample combs inserted, and left to solidify. The gels could be stored for up to one month at 4°C when soaked in 1x TAE buffer. Samples for electrophoresis were prepared by mixing them with 6x loading buffer.
Electrophoresis was performed at 100V until the DNA bands were sufficiently separated. Visualization of the DNA bands was carried out in the UV transilluminator (Gel Doc, BioRad).

2.2.2 Photometric measurement of DNA concentration and bacterial suspension cultures

DNA concentration was determined by measuring the purified DNA at a 1:50 dilution with ddH_2O at 260 nm in a quartz cuvette (Quartz spectrometer cell micro, BIO-RAD). The spectrophotometer (BIO-RAD smart Spec 300) was blanked with ddH_2O.
Cell concentration in bacterial suspension cultures was determined for agroinfiltration. The OD (optical density) of a 1:10 dilution in infiltration medium was measured at 600 nm in a plastic cuvette (Greiner). Infiltration medium was used as blank.

2.2.3 Preparation of LB-agar and MSS plates

The required amount of LB agar or MSS medium was molten in the microwave and left to cool down, before the appropriate antibiotic stock was added. The agar was then poured into plastic petri dishes and left to solidify at room temperature. Plates were stored at 4°C and could be used up to two months.

2.2.4 Confocal laser scanning microscopy (CLSM)

Subcellular localization of aCREG-GFP/ mRFP of marker constructs was analysed in a Leica TCS SP2 confocal laser scanning microscope (Leica). Images were obtained using a 40 x 1.25 numerical aperture or 63 x 1.4-numerical aperture oil immersion objective. GFP was excited with a 488 nm argon laser and its emission was recorded from 505 to 535 nm, whereas mRFP was excited with a 543 nm helium-neon laser and its emission was recorded from 605 to 635 nm.

N. benthamiana leaves were analysed 72 h after infiltration. A small area of the infiltrated *N. benthamiana* leaf or a whole *A. thaliana* seedling (day 4 – day 8) where analysed placed face down on a glass slide.

2.3 Molecularbiological methods

2.3.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was used for screening *E. coli* colonies for positive transformants and for sequencing DNA.

The technique is used to amplify a copy of a piece of DNA. This is accomplished by several cycles of repeated heating and cooling, allowing DNA melting and subsequent enzymatic replication. A PCR reaction mix contains the template DNA, forward and reverse primer (in the case of sequencing only one primer is used), a thermostable DNA polymerase, deoxynucleotide triphosphates (dNTPs) (in the case of sequencing also dideoxynucleotide triphosphates (ddNTPs) as chain terminators) and a buffer, providing the optimum conditions for the polymerase chain reaction.

The reaction mix for screening of *E. coli* colonies for positive transformants contained 5 µl bacterial DNA, 12 pmol of the forward primer 35S-7 (5'- ATTGATGTGATATCTCCACTGAC-3') and the reverse primer aCREG rev2 (5'- AAGGGTTAGTTTAGAGCAAGTAGGATTCA-3'), 10 mM dNTP's, 0.5 µl Taq-Polymerase, 2 µl of 10 x PCR buffer and the appropriate amount of ddH_2O to reach the final volume of 20 µl. The PCR program used is listed in Table 1.

The sequence of the aCREG-mRFP cDNA construct was determined using the chain terminator method (Sanger, 2004). The sequencing PCR reaction mix (10 µl) contained 200 ng DNA (miniprep) and 12 pmol/ of the forward primer 35S-7 (5'-ATTGATGTGATATCTCCACTGAC-3') or the reverse primer mRFP-4 (5' GAGCCCTCCATGCGCACCTTGAA-3'). All other components were included in the BigDye mix and 5 x Sequencing buffer (Applied Biosystems). The PCR program used is listed in Table 1.

Table 1: Thermocycler programs for sequencing PCR and screening PCR

	Sequencing PCR		Screening PCR	
Step	Duration [min]	Temperature[°C]	Duration [min]	Temperature[°C]
Initial Denaturation	1	95	2	95
Denaturation	0,3	95	0,5	95
Primer annealing	0,3	50	0,3	50
Primer extension	1,5	60	1,5	72
Cycles	27		44	
Final extension			2	72

2.3.2 DNA sequencing

After amplification of the DNA fragment by sequencing PCR (see section 2.2.1) the DNA was precipitated with sodium-acetate-ethanol. For this, the samples were transferred into clean 1.5 ml Eppendorf tubes and filled up to 50 µl with ddH_2O. Then 5 µl 3M NaAc pH 5.2 (1/10 of volume) and 125 µl Ethanol abs. (-20°C) (2.5 x of the volume) were added and the mixture was incubated for 20 min. on ice. The samples were then centrifuged for 20 min. at 14 000 rpm/RT and the supernatant was discarded. The pellet was washed with 250 µl 70% EtOH and centrifuged for 5 min. at 14 000 rpm/RT. After the supernatant was discarded the pellet was dried and kept at 4°C until sequencing.

For sequencing by capillary electrophoresis the ABI Prism^{TM} 3100 Genetic Analyser was used and analysis of the obtained chromatograms was performed with the computer program SeqMan (DNAStar).

2.3.3 Bacterial cultures

For liquid cultures of *E. coli* and *A. tumefaciens* a single colony was picked and resuspended in 5 ml LB medium containing the appropriate antibiotic. The glass tubes were incubated at

37°C (*E. coli*) or 30°C (*A. tumefaciens*) for 18 h at 180 rpm. The same incubation temperatures and periods applied to LB agar plates, except for *A. tumefaciens* LB agar plates, which were incubated for 48 h.

2.3.4 DNA extraction for PCR screening

For screening of *E. coli* DH5α colonies for positive p31-aCREG-mRFP transformants a single colony was picked from the LB plate, suspended in 50 µl TE buffer and the rest of the colony streaked out on a masterplate. The masterplate was incubated at 37°C. The bacterial suspension was vortexed, denatured for 5 min at 95°C and centrifuged for 1 min at max. speed. 5 µl of the supernatant were used as PCR template. The PCR product was loaded on an agarose gel. If a colony was positive for the p31-aCREG-mRFP construct, the masterplate was used to propagate the clone.

2.3.5 Plasmid purification

5ml of DH5α overnight culture grown in LB medium were pelleted in a microcentrifuge (Eppendorf) for 5 min at 10 000 rpm/RT and the plasmid-DNA was isolated with the Wizard *Plus* Minipreps DNA Purification System (Promega) according to the manufacturer's instructions. The plasmid DNA was eluted in 100µl of the supplied Nuclease Free Water and stored at -20°C.

2.3.6 Restriction enzyme digest

Two different digestion preparations were used. For cloning, a 100 µl preparation was made, whereas only 20 µl reaction mixes were used for test digestions to check for the presence of a desired insert, when screening for positive transformants. All enzymes and buffers were obtained from Fermentas. The digestion mixes were incubated from 2-4 h at 37°C. The following reaction mixes were set up:

Test digestion (20 µl):

x µl plasmid DNA (approx. 500 ng)
2 µl 10 x buffer (as recommended by the supplier)
2 µl restriction enzyme (10 U/µl)
x µl ddH_2O

20 µl total volume

Preparative digestion (100 µl)

x µl plasmid DNA (approx. 3500 ng)
10 µl 10 x buffer (as recommended by the supplier)
5 µl restriction enzyme (10 U/µl)
x µl ddH_2O

100 µl total volume

For cutting with two different enzymes (double digest) the DNA was precipitated with the MSB® Spin PCRapace [Invitek] purification kit after the first digestion reaction and digested with the second enzyme after elution in the appropriate amount of ddH_2O.

To verify that the digestion products were of the expected size, the digestion mix and molecular weight markers (Fermentas) were loaded on an agarose gel and electrophoresed for 30 min. at 100 V.

2.3.7 Purification of DNA fragments from agarose gel

Purification was performed with the NucleoSpin® Extract II [Macherey-Nagel]. After electrophoresis the agarose gel band containing the desired DNA fragment was cut out with a clean scalpel and put into a pre-weighted 1.5 ml Eppendorf tube. The weight of the agarose gel band was determined and 200 µl NT buffer were added for every 100 mg of gel. According to the manufacturer's manual the sample was incubated at 60°C until the agarose gel was completely dissolved (approx. 15 min) and the sample was then loaded onto a

NucleoSpin column. The sample was centrifuged (1 min, 11 000 rpm, RT), followed by a washing step (adding 600 µl NT3 buffer and centrifuging for 1 min at 11 000 rpm/RT) and dry-centrifugation (2 min, 11 000 rpm, RT) to remove residual ethanol. To recover the purified DNA, 25 µl NE buffer were added and the DNA was eluted by centrifugation (1 min, 11 000 rpm, RT). The DNA was stored at -20°C.

2.3.8 DNA ligation

DNA ligation was performed with T4 ligase from Fermentas, and the corresponding 10 x ligase buffer. The molar ratio of vector:insert (both restricted and purified) was usually 1:3, as calculated below.

$$\text{ng insert} = \frac{\text{ng vector x kb insert}}{\text{kb vector}} \times \frac{3}{1}$$

The following reaction mixture was prepared and incubated overnight in a waterbath at 14°C.

Ligation mix:

x µl vector DNA (approx. 100 ng)
x µl insert DNA (calculated as above)
1 µl T4 DNA ligase
1 µl 10 x ligase buffer
x µl ddH_2O

10 µl total volume

2 µl of the reaction mix were then directly used to transform *E. coli* DH5α by electroporation.

2.3.9 Transformation by electroporation

For transformation, 100 µl of electrocompetent DH5α were thawed on ice and then mixed with 2 µl ligation mix. Electroporation was carried out in pre-cooled electroporation cuvettes. Immediately after transformation, 900 µl prewarmed (37°C) SOC medium was added and the cells were incubated for 1 h at 37°C with gentle shaking. After incubation, 50 µl of the cell suspension were plated on LB agar plates containing kanamycin and incubated over night at 37°C.

The same technique applied for UIA143 transformation, except that approx. 400 ng purified plasmid (miniprep) was used for transformation instead of ligation mix and that the cells were kept at 30°C instead of 37°C. Also the incubation of LB agar plates (containing kanamycin and gentamycin) took up to 48 h due to the slower growth rate of *A. tumefaciens*.

2.3.10 Cryostocks

For cryostocks, 750 µl of a 5 ml *E. coli* or *A. tumefaciens* overnight liquid culture were trarferred into a cryostock tube and 500 µl sterile glycerol (87%) was added. The cryostocks were stored at -80°C.

2.4 Protein methods

2.4.1 Total protein extraction

Total protein was extracted from *N. benthamiana* leaves and *A. thaliana* roots, leaves or seedlings for subsequent SDS-PAGE and Western blot analysis. 40-50 mg plant material were placed into 2 ml round Eppendorf tubes together with two sterile metal beads and put into liquid nitrogen. The frozen samples were ground in a mixermill for 1 min at amplitude 60. After the sample was pulverised 1.25 µl Extraction buffer were added to 1 mg of plant material, vortexed and left on room temperature for 10 min. The samples were transferred to clean 1 ml Eppendorf tubes and centrifuged for 10 min at 10 000 rpm on 4°C. The supernatant was transferred into a clean tube and again centrifuged (5 min, 10 000 rpm and

4°C). The supernatant was either directly used for measurement of protein concentration and SDS-PAGE or stored at -20°C.

2.4.2 Determination of protein concentration

Protein concentration was determined with the BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions. The technique is based on a colour change proportional to the protein concentration in the sample. When reacting with the peptide bonds of proteins, Cu^{++} is reduced to Cu^{+}, and subsequent formation of a chelate complex with bicinchoninic acid causes a colour change from green to purple. The absorbance of the intense purple complex can be monitored at around 550 nm (Smith *et al.*, 1985).

Reagent A was mixed with reagent B 50:1 to give the reagent mastermix. To 200 µl mastermix in a 96-well-plate were added either 20 µl of a BSA standard (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.063 mg/ml, 0.031 mg/ml, 0.016 mg/ml or 0 mg/ml; dilution in PBS) or the appropriate amount of sample (from 1-5 µl). The plate was incubated at 37°C for 30 min and the absorbance was measured at 550 nm on a plate reader (Victor 1420 multilabel counter, Perkin Elmer Wallac). Protein concentration was calculated based on the BSA standard curve (Microsoft Excel).

2.4.3 SDS-PAGE

SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) was performed according to established protocols (Laemmli, 1970). A 15% separation gel and a 6% stacking gel were used (Table 2)

Table 2: Components necessary to prepare 20 ml 15% separation gel and 8 ml 6% stacking gel.

	15% separation gel	6% stacking gel
Aqua dest. (ml)	7,1	5,84
40% AA/Bis (ml)	7,5	1
Tris (1.5 M, pH 8.8) (ml)	5	-
Tris (0.5 M pH 6.8) (ml)	-	1
10% SDS (µl)	200	80
10% APS (µl)	200	80
TEMED (µl)	20	8

Protein samples were mixed with 3 x sample buffer, denatured at 95°C for 5 min and cooled on ice. The polymerised gels were inserted into the electrophoresis apparatus filled with running buffer and the samples were loaded onto the gels. Electrophoresis was first performed at 100 V for 15 min (stacking of proteins) and then at 200 V (protein separation) until the loading dye was running out of the gel. After SDS-PAGE the gels were either used for silver staining or for Western blot analysis.

2.4.4 Protein detection in gels by silver staining

Protein bands in gels were visualized through silver staining (Switzer *et al.*, 1979). The technique is based on the reduction of silver ions by the amino acid side chains to metallic silver. Silver staining provides a very sensitive tool for protein visualization with a detection level down to the 0.3-10 ng level. The required solutions are listed below (indicated for 100 ml total volume, filled up with dest. water), as well as the incubation times of the gel in the respective solutions.

1. Fixation solution

40 ml ethanol

10 ml acetic acid

50 ml ddH_2O

2. Sensitizing solution

30 ml ethanol

250 µl glutardialdehyde

314 mg sodium thiosulfate pentahydrate

6.8 g sodium acetate

3. Staining solution

Destilled water was mixed with 40 µl formaldehyde, then 250 mg silvernitrate were dissolved. The solution was prepared shortly before use and kept in the dark.

4. Developing solution

2.5 g sodium carbonate

20 µl formaldehyde

5. Terminating solution

1.46g EDTA-Na_2 (50mM EDTA) pH 8.0

Procedure:

Table 3: Incubation times for Silver staining.

	Incubation [min]
1. Fixation solution	min. 30
2. Sensitizing solution	30
wash (dest. water)	3 x 5
3. Staining solution	20
wash (dest. water)	2 x 1
4. Developing solution	2-5
5. Terminating solution	10

2.4.5 Western blot

Western blotting allows the detection of specific proteins in a sample. The procedure involves the electrophoretic transfer of proteins from the SDS-gel to sheets of nitrocellulose.

The immobilised proteins can then be visualized by the reaction with specific antibodies (Burnette, 1981).

After assembly of the Western blot the blotting apparatus (BioRad) was filled with transfer buffer and ice and blotting was performed for 1 h at 100 V. After the transfer was completed the membrane was incubated in blocking solution (3% BSA in PBS/0.02% NaN_3) for one hour at room temperature. The membrane was then washed 3 x 5 min with PBST and incubated with the primary antibody overnight. After incubation with the first antibody the membrane was again washed (5 x 5 min with PBST) and incubated with the secondary antibody for 1.5 h (appropriate dilutions for all antibodies are listed in section 2.1.7). Finally, the membrane was washed 5 x 5 min with PBST, rinsed twice with PBS before detection was carried out. For detection of horseradish peroxidase activity, the membrane was incubated for 2 minutes in a 1:1 mixture of the chemiluminescent detection reagents (Supersignal West Pico Horseradish Peroxidase (HRP) Detection Kit [Pierce]). The excess reagent was drained off and the blot was put into a film cassette after sealing in a plastic bag. The blot was overlaid with an autoradiographic film (Amersham Hyperfilm ECL [GE Healthcare]) in a dark room and exposed for 10 seconds up to 20 minutes. After the required exposure time, the film was placed for two minutes in developer solution, rinsed in water and fixed in fixing solution (Rethmann).

After exposure the membranes could be stained with Ponceau-S to verify protein transfer and to compare total protein concentrations between the samples.

2.4.6 Stripping of nitrocellulose membranes

Stripping was required when the same blot had to be probed with a different antibody. The membrane was incubated in stripping solution at 60°C for 60 min under shaking. After stripping, the blot was washed 2 x 5 min in ddH_2O, then 4 x 5 min in PBST, and could then be reblocked again for 1 h at room temperature.

2.4.7 Sucrose density gradient fractionation

Sucrose density gradient (SDG) centrifugation was used to separate the different cellular compartments. The sucrose gradient was constructed by overlaying lower sucrose

concentrations onto higher concentrations (prepared by diluting 64,5% sucrose solution (40 g sucrose + 22 g H_20) with water, covering a range from 20%-52%, as shown in Table 3) in a centrifuge tube. The prepared sample was then poured on top of the lowest sucrose concentration and ultracentrifuged for 5 h at 33 000 rpm (L8-80M Ultracentrifuge, Beckman; SW41 Ti-rotor, swinging bucket). During this time the compartments in the sample travel through the gradient until their density (mass/volume) matches that of the surrounding sucrose. After centrifugation the different fractions were removed and subjected to further analysis.

Table 4: Overview of sucrose densities for SDG fractionation and the volumes of 64,5% sucrose solution and H_20 required for their preparation.

density [g/ ml]	sucrose w/w	total volume to make	wgt of 64,5% sol.	approx vol. (density=1.33 g/ml or 64,5%)	H_20
1.0810	20 %	10 ml	3.352	2.52 ml	6.98 ml
1.1036	25 %	12	5.133	3.86	7.54
1.1151	27.5 %	12	5.705	4.29	7.11
1.1270	30 %	12	6.290	4.73	6.67
1.1463	34 %	12	7.250	5.45	5.95
1.1612	37 %	12	7.990	6.01	5.39
1.1764	40 %	12	8.750	6.58	4.82
1.1920	43 %	12	9.536	7.17	4.23
1.2132	47 %	12	10.610	7.98	3.42
1.2186	52 %	6	6.000	4.51	1.19

Protein extration for SDG centrifugation:

Total protein extracts from *A. thaliana* roots were prepared for SDG centrifugation. For this, approx. 350 mg roots material was harvested (Col-0: 350 mg; aCREG RNAi T5/1: 377 mg; p20F aCREG-GFP: 265 mg), placed into a 2 ml round-bottom Eppendorff tube together with 3 metal beads and ground in a mixermill 4 x 1 min with amplitude 70. To 1 mg of plant material 1.2 µl 1 x complete extraction buffer was added.

2 x Extraction buffer:

1 ml 1 M Tris pH 7.5

25 µl 2 M KCl

2,6 ml 64,5% sucrose

400 µl 0,5 M EDTA

300 µl 0,5 M EGTA

200 µl 1 M β-glycerophosphate

475 µl H_2O

5 ml total volume

1 x Complete extraction buffer:

2 ml 2 x Extraction buffer

16 µl 1 M DTE

100 µl protease inhibitor cocktail [Sigma]

1 mM PMSF (solubilized in DMSO)

1,88 ml H_2O

4 ml total volume

For each sample 15 mg PVPP were left to soak overnight in 300 µl H_2O. The water was then removed and 50 µl 2 x extraction buffer were added. The PVPP mix was again left overnight and was used the next day. The obtained crude root extract was transferred into a new Eppendorf tube containing 50 µl PVPP solution. The samples were then subjected to several rounds of centrifugation (5 min, 1000 g; 1 min 1000 g; 4 min 2000 g; 5 min, 1000 g; 3 min 2000 g; always on 4°C), during which the supernatant was removed every time to give a total volume of approx. 400 µl. The remaining pellet was reextracted with 600 µl 1 M Tris / 20 mM EDTA, pH 7.5 (5 min, 3000 g, 4°C), and both supernatants of the sample were pooled. The sample was then again centrifuged and the clear supernatant was transferred into a clean tube. If the total volume was less then 1.2 ml, 1 M Tris / 20 mM EDTA (pH 7.5) was added. To

each sample 1 µl PMSF (1mM) was added before the samples were loaded onto the sucrose gradient. Before loading 40 µl of the sample was removed for analysis.

2.4.8 *β-N*-Acetylhexosaminidase assay

This enzyme assay was used to determine the *β-N*-acetylhexosaminidase content in the recovered fractions after SDG centrifugation. The assay was performed in 96-well-plates.

Sodium citrate buffer:

0,1 M citric acid, pH 4.6

0,04% NaN_3

0,2% BSA

1% Triton X-100

In each well 10 µl protein extract were mixed with 10 µl sodium citrate buffer and 20 µl substrate (10 mM PNP-GlcNAc) and left for 1 h at room temperature. The reaction was stopped with 80 µl 0,4 M glycine/ NaOH, pH 10,4 and absorbance was measured at 405 nm on a plate reader (Victor 1420 multilabel counter, Perkin Elmer Wallac). As a blank, 10 µl of heat-inactivated protein extract was used for the reaction.

2.4.9 Methanol / chloroform protein precipitation

Samples obtained by SDG centrifugation contain high levels of sucrose, and therefore protein precipitation was carried out as a purification step prior to Western blot analysis (Wessel and Flugge, 1984). For every 100 µl of sample 400 µl methanol were added and the sample was vortexed. Then 100 µl chloroform and 300 µl ddH_2O were added and the sample was centrifuged for 5 min at 8000 g. After centrifugation the precipitated protein of the sample accumulated in the interphase. The upper phase consisting of methanol and water was removed and 300 µl methanol were added. The protein was centrifuged for 10 min at full speed and the pellet was air-dried on ice before addition of 150 µl 1 x sample buffer (diluted in PBS).

2.5 Plant methods

2.5.1 Sowing of seeds

Seeds were surface sterilized with a 12% hypochlorite solution for 10 min on a shaker to avoid contamination with fungi and other plant pathogens. If the seeds were to be sowed on MSS plates then further washing with water and sowing was performed under sterile conditions in the laminar flow hood. The seeds were washed four times with 1 ml sterile ddH_2O, then mixed with 1 ml 0.2% agar and transferred onto appropriate agar plates. For abiotic stress experiments the seeds were first sowed onto control plates providing normal growth conditions. The MSS plates were covered with aluminium foil and kept in the dark for 48 h at 4°C before being transferred to the growth chamber (22°C). If the seeds were to be sowed on soil then washing was performed with ddH_2O and the seeds were placed in moistened soil. The pots were covered in cling film and kept in the dark for 48 h (4°C) to facilitate germination before putting them into the growth chamber (22°C).

2.5.2 Transfer of seedlings

Seedlings were either transferred from MSS plates onto soil or onto fresh MSS plates. Seedling transfer onto fresh MSS plates was carried out with the help of forceps under sterile conditions in a laminar flow hood. Seedling transfer onto soil did not require sterile working conditions.

2.5.3 Growing of plants

Before being potted into plastic pots (7 x 7 x 8 cm for *A. thaliana* and 9 x 9 x 9 cm for *N. benthamiana*), the soil was mixed with 1/3 vermiculite and sterilized by two cycles of freezing (-20°C, 16 h) and thawing (RT, 16 h). The plants were cultured in growth chambers, where the temperature was constantly 22°C under long-day conditions (16 h light per 24 h) and watered once every two days with tap water.

2.5.4 Harvesting of *A. thaliana* seeds

After 2 months, the siliques of A. thaliana plants started to become brown. From this time on the plants were no longer watered until the whole plant had become dry. The siliques could then be crushed open to release the seeds, which were sieved to clear them of plant debris and put into Eppendorf tubes. The seeds were stored at 4°C.

2.5.5 Experimental procedure of abiotic stress experiments

A. thaliana seeds were sowed onto control plates providing standard growth conditions (1% agar, 2% sucrose) and kept at 22°C in the growth chamber. After 7 days growth in control conditions the seedlings were transferred onto fresh plates for stress experiments (MSS plates for sucrose stress containing 0%; 1%; or 4.5% sucrose, MSS plates for salt stress experiment containing 140 mM NaCl, H+ plates providing optimum conditions for growth at elevated temperatures). The plates for sucrose and salt stress experiments were again placed on 22°C, whereas the H+ plates were put into a growth chamber providing 30°C. After 12 days the roots of the seedlings were measured and the root length recorded. Images of the plates were then recorded using a conventional scanning device.

2.5.6 *A. thaliana* floral-dip

For stable transformation of *A. thaliana* plants the floral-dip method was used (Clough and Bent, 1998). A 500 ml overnight culture of *A. tumefaciens* containing the desired plasmid (carrying a kan-resistance gene) for transformation was prepared and the OD of the culture was measured until it reached 1,2. Then the *Agrobacteria* were harvested by centrifugation (600 rpm, 30 min, 4°C) and the pellet was resuspended in 100 ml 5% sucrose solution. The OD of the *Agrobacteria* suspension was again measured and the suspension was diluted with 0.5% sucrose solution until it reached an OD of 0,8. 300 µl/ l Silwet L-77 were added prior to floral dipping.

A. thaliana plants with numerous floral buds and a few siliques were dipped upside-down into the *A. tumefaciens* suspension for 3 min before being laid down in a tray and covered in cling film. The plants were kept in the dark at 18°C for 48 h before being transferred to the

growth chamber. The transformed progeny was selected on MSS plates containing kanamycin.

2.5.7 *A. tumefaciens* leaf infiltration

For transient expression in *N. benthamiana*, leaves were infiltrated with *Agrobacteria* (Batoko *et al.*, 2000). A 5 ml overnight culture of an *A. tumefaciens* UIA143 strain containing the desired plasmid was grown in the presence of kanamycin and gentamycin until it reached the stationary phase. 1 ml of the culture was then centrifuged at 5000 rpm for 5 min and the pellet was resuspended in infiltration buffer. The pellet was again centrifuged for 5 min and the supernatant was removed. After washing, the pellet was again resuspended in infiltration buffer and the OD600 was measured. The required volume of *A. tumefaciens* suspension was calculated for the desired OD600 in V_2 = 500 µl with the formula: $OD_1 * V_1 = OD_2 * V_2$. For *N. benthamiana* infiltration OD600 values of 0.03, 0.1 and 0.3 were used. The required volume was diluted to 1 ml with infiltration buffer and the bacterial suspension was delivered with the help of a 1 ml syringe without a needle to the lamina tissues of the leaves through the stomata of the lower epidermis. The infiltrated leaf area was marked with a pen and the plants were put back into the climate chamber. Expression was observed after 48 h.

2.5.8 Vacuum leaf infiltration

N. benthamiana leaves were subjected to vacuum infiltration to recover secreted proteins from the interstitial fluid (IF) (McCormick *et al.*, 1999). 48 h after *A. tumefaciens* infiltration, the infiltrated leaves were cut off and weighted. The leaves were placed in 500 ml infiltration buffer (100 mM Tris-HCl, 2 mM EDTA, 10 mM $MgCl_2$, pH 7.5) and subjected to a 80 mbar vacuum (MZ 2C vacuum pump, Vacuubrand) for 5 min. The IF was recovered by mild centrifugation (800 g, 15 min, 4°C) and the protein concentration was determined.

2.5.9 Plasmolysis

When subjected to hypertonic stress conditions the plant cell looses water and the plasmalemma pulls away from the cell wall, rendering the protoplasts visible under the

microscope. For this, whole *A. thaliana* seedlings (4 or 8 days old) were kept for 10 min in aqueous 0.5 M mannitol solution. The seedlings were then analysed under the microscope.

2.5.10 Perturbation of intracellular protein transport

Brefeldin A (BFA) is a lactone antibiotic that interferes with intracellular protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus, so that proteins become trapped in the ER. *N. benthamiana* leaves were *Agro*-infiltrated with the desired construct, expressing a fluorescing fusion protein. Leaves were harvested after 48 h and leaf pieces (0.5 x 0.5 cm) were placed into a 100 µM BFA solution. The pieces were incubated from 30 min to 1 h, during which they were constantly observed under the microscope.

2.5.11 Protease inhibitor treatment

N. benthamiana leaves infiltrated with the desired construct were subjected to a protease inhibitor (PI) treatment to counteract cleavage of the produced fusion protein. For PI treatment, *N. benthamiana* leaves were infiltrated with an *Agrobacterium* suspension, containing 100 µM E-64d ((2S,3S)-*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester) in the infiltration medium. E-64d is a membrane-permeable synthetic analog of E-64, a cysteine protease inhibitor. The 10 mM stock solution in DMSO was diluted 1:100 in infiltration medium prior to use. As a control, leaves were also infiltrated with 1% DMSO in the infiltration medium and with the *Agrobacterium* suspension in infiltration medium alone. 48 h post infiltration the leafs were observed under the confocal laser scanning microscope.

3 Results

3.1 Summary of previous results

Generation of the Arabidopsis thaliana aCREG RNAi lines

For stable silencing of CREG in *Arabidopsis thaliana* an aCREG RNAi hairpin construct was created (work done by Christiane Veit, Department of Applied Genetics and Cell Biology, BOKU, Vienna) using the puc18XTI2 plasmid (Figure 4).

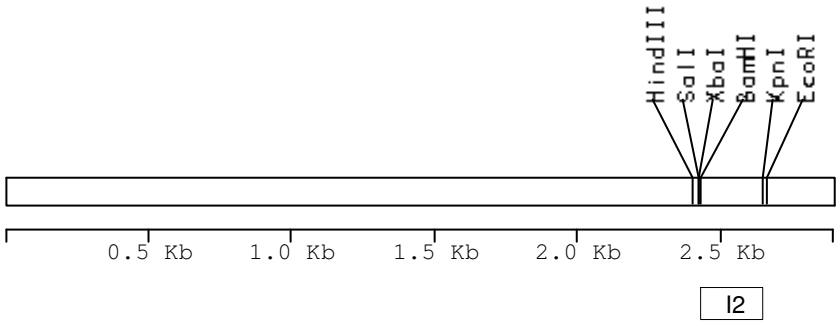

Figure 4: The puc18XTI2 plasmid. I2, Intron 2 from the *A. thaliana* XylT gene.

The puc18XTI2 plasmid contains the short intron 2 (=I2, 210 bp) from the *A. thaliana* XylT gene in between two multiple cloning sites. For the hairpin construct, the 193 bp sense fragment was created by means of PCR from the TOPO aCREG_19 template vector using fwd primer RCREG_1F (XbaI) 5'-ATATCTAGACGGTATACCTTACTTTTACTTAACAAC-3' and rev primer RCREG_2R (BglII) 5'-TATAAGATCTGCTTCCTCAGATCCTCCTTCC-3' (restriction sites underlined) and digested with XbaI and BglII before being cloned into the puc18XTI2 plasmid previously digested with XbaI and BamHI, giving the pCREGs plasmid. The 193 bp PCR antisense fragment was created with fwd primer RCREG_3F (KpnI) 5'-TATAGGTACCTGCTTCCTCAGATCCTCCTTCC-3' and rev primer RCREG_4R (EcoRI/BamHI) 5'-TATAGAATTCGGATCCGGTATACCTTACTTTTACTTAACAAC-3' from the TOPO aCREG_19 template vector. The PCR fragment was digested with KpnI and EcoRI and cloned into the pRCREGs plasmid, previously digested with KpnI and EcoRI, thus giving the pCREGsas plasmid. The XbaI/BamHI fragment (~ 500-600 bp) from pCREGsas was then cloned into the

pPT2M plasmid for transformation in *Agrobacterium tumefaciens*. The hairpin construct in its final form is: 35S promoter (from Cauliflower Mosaic Virus – CaMV) – CREG sense fragment – AthXylT intron 2 – CREG antisense fragment – terminator. The 35S promoter mediates constitutive transcription of the construct throughout the whole plant. Upon formation of dsRNA the endogenous CREG-mRNA is degraded because of RNA interference. The transformed *A. tumefaciens* were then used for floral dip of *A. thaliana* Col-0 wildtype plants.

After transformation, *A. thaliana* seedlings that have incorporated the hairpin construct in their genomes were selected and the endogenous CREG level determined by means of Reverse Transcription (RT) PCR, using aCREG-fw (5'-AGTATGGAACTTCAAGTCCTTGTTCTTCG-3') and aCREG-rev2 (5'-AAGGGTTAGTTTAGAGCAAGTAGGATTCA-3') primer. Figure 5 shows the knock-down of endogenous CREG in the aCREG RNAi line in the first generation (T1).

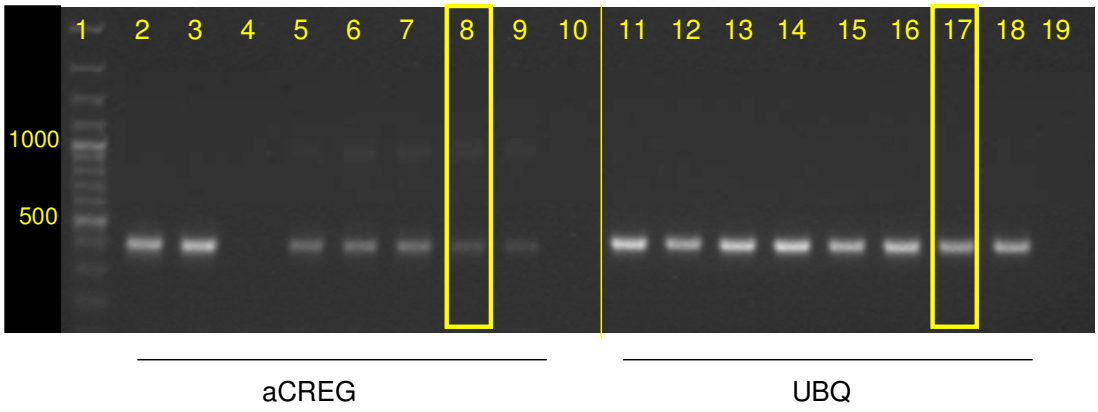

Figure 5: Reverse transcription PCR data from aCREG RNAi lines. Lane 1: marker (100 bp DNA Ladder Plus), lanes 2, 3 and 11, 12: Col-0 wildtype, lanes 4 – 7, 9 and 13 -16, 18: aCREG RNAi knockdown lines that were not continued, lane 8 and 17: aCREG RNAi T1, lane 10 and 19: negative control. aCREG ~ 400 bp, UBQ ~ 400 bp. As expected, Col-0 shows a stronger aCREG signal than aCREG-RNAi T1. RT-PCR data obtained using aCREG-fw (5'-AGTATGGAACTTCAAGTCCTTGTTCTTCG-3') and aCREG-rev2 (5'-AAGGGTTAGTTTAGAGCAAGTAGGATTCA-3') primer for amplification of the aCREG sequence and UBQ-5U (5'-CTCCTTCTTTCTGGTAAACGT-3') forward and UBQ-5D (5'-AACCCTTGAGGTTGAATCATC-3') reverse primer for amplification of the Ubiquitin sequence.

The CREG mRNA levels in several aCREG RNAi lines were found to be reduced. For example in lane 4 (aCREG RNAi #2, not continued) and lane 8 (aCREG RNAi T1) CREG levels are significantly reduced when compared to wildtype expression levels (lane 2 and 3), whereas Ubiquitin (UBQ) levels (lane 13 and 17) used as loading control stay constant. The aCREG

RNAi T1 line was continued and two lines of the 4th generation (aCREG RNAi T4/ 1 & 2) were used for the experiments in this project.

Construction of the p20F aCREG-GFP vector

For subcellular localization studies in *N. benthamiana* and *A. thaliana* using an aCREG-GFP fusion protein the p20F aCREG-GFP vector was constructed (work done by Christiane Veit, Department of Applied Genetics and Cell Biology, BOKU, Vienna). For this, full-length aCREG was amplified by PCR from aCREG cDNA isolated earlier using the aCREGfw5 (XbaI) primer (5'- TATATCTAGAATGGAACTTCAAGTCCTTGTTC-3', restriction sites underlined) and the aCREGrev4 (BamHI) primer (5'-TATAGGATCCTAAAAAGGAAGCGAGTTTGATCG-3'). The PCR product was digested with XbaI and BamHI and cloned into the PCR4-TOPO vector (giving the PCR4-TOPO-aCREG vector) and subsequently cloned into the XbaI/BamHI site of the p20F-GFP vector (Figure 6) thus yielding a C-terminal fusion of GFP to aCREG.

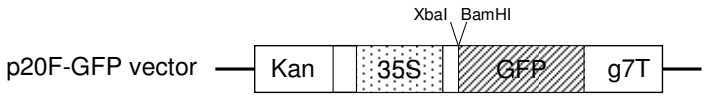

Figure 6: p20-GFP construct for subcellular localization studies. Kan, kanamycin resistance gene, 35S, CaMV 35S promoter, XbaI and BamHI, restriction sites, GFP, green fluorescent protein, g7T, gene 7 terminator.

The p20F aCREG construct was introduced into *A. tumefaciens* and used for floral dip of *A. thaliana*, thus generating stable transformants expressing the aCREG-GFP fusion protein. Two lines of the 4th generation (T4) were used in this project: p20F aCREG T4/ 1 & 2. The *A. tumefaciens* strain harbouring the construct was also used for infiltration studies in *N. benthamiana*.

3.2 Construction of the p31 aCREG-mRFP vector

The construction of the p31 aCREG-mRFP vector was performed in a similar way to the construction of the p20F aCREG-GFP vector. The full-length aCREG sequence was subcloned from the PCR4-TOPO-aCREG vector into the p31-mRFP plasmid (Figure 7) thus yielding the p31 aCREG-mRFP vector.

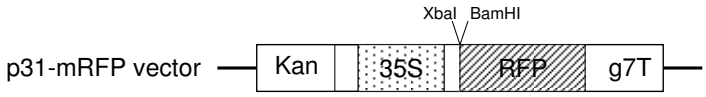

Figure 7: p31-mRFP construct for subcellular localization studies. Kan, kanamycin resistance gene, 35S, CaMV 35S promoter, XbaI and BamHI, restriction sites, RFP, red fluorescent protein, g7T, gene 7 terminator.

For this, the p31-mRFP vector was digested with XbaI and BamHI and the PCR4-TOPO-aCREG vector also was digested with the same restriction enzymes. The digestion products were loaded on an agarose gel (Figure 8) and the required bands were cut out. After purification of the DNA from the gel bands the ~ 650 bp aCREG fragment was ligated into the ~ 12.0 kb p31-mRFP vector.

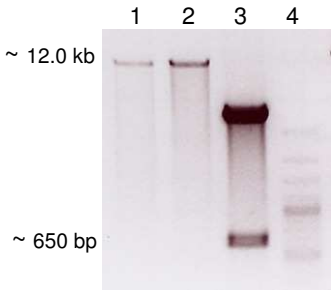

Figure 8: Restriction enzyme digest products. Lane 1: 70 ng p31-mRFP, lane 2: 135 ng p31-mRFP (~ 12.0 kb); digested with XbaI and BamHI. A small ~ 70 bp fragment that is removed by the restriction enzyme digest is not visible. Lane 3: 5 µl pCR4-TOPO-aCREG vector digested with XbaI and BamHI. The aCREG insert is visible at ~ 650 bp. Lane 4: 1 µl marker (100 bp DNA Ladder Plus)

The vector was introduced into *Escherichia Coli* and the resulting colonies were screened by PCR. Figure 9 shows that out of 28 screened colonies, 16 were positive transformants. Six of the clones (#1, #2, #4, #19, #23 and #24) were continued.

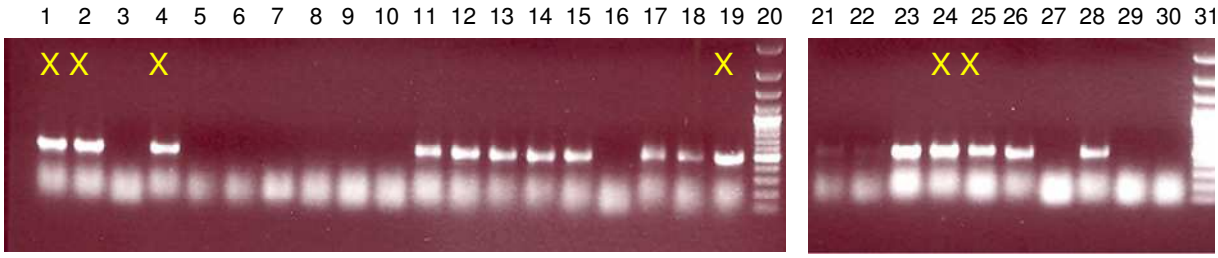

Figure 9: Agarose gel electrophoresis of PCR samples (colony screening). Lanes 1, 2, 4, 11-15, 17-19, 23-26 and 28 have an insert of the expected length (~ 500 bp). Clones #1, #2, #4, #19, #23 and #24, marked with an "X" were continued. Lane 30: negative control of PCR. Lanes 20 and 31: 10 µl marker (100 bp DNA Ladder Plus).

From the six selected clones, the plasmid DNA was isolated and digested with XbaI and BamHI to prove that the plasmid carried the right insert of ~ 650 bp (Figure 10).

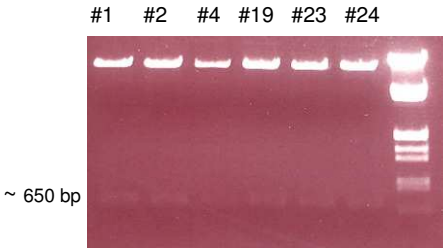

Figure 10: Test digest of the six selected *E. coli* clones. Restriction enzyme digest was performed with XbaI and BamHI. Lane 7: 10 µl marker (λ DNA/EcoRI and HindIII DNA Ladder). All appear to have the right insert (~ 650 bp) incorporated. Clones #1 and #24 were selected to be continued.

The clones #1 and #24 were selected and sequenced. After proving that the sequence contained no mutations, both constructs were used for *A. tumefaciens* transformation. The *A. tumefaciens* strain harbouring the p31 aCREG-mRFP #1 construct was used for subsequent *A. thaliana* floral dip and *N. benthamiana* infiltration studies.

3.3 Subcellular localization of CREG

3.3.1 Expression of CREG-GFP and CREG-mRFP in *N. benthamiana*

Transient expression of CREG-GFP and CREG-mRFP was achieved by infiltrating *N. benthamiana* leaves with *A. tumefaciens* carrying the respective constructs at an OD ranging from 0.03 to 0.3. The CREG fusion constructs were expected to be localized in the vacuoles

of the leaf epidermal cells. It has been reported that vacuolar GFP in higher plants is being degraded in the light due to a conformational change of the protein (Tamura *et al.*, 2003). The conformational change induced by light leads to exposure of protease recognition sites in the otherwise compact structure of the GFP protein and thus makes it susceptible for degradation. Therefore plants expressing CREG-GFP were cultivated in light and dark conditions after infiltration.

Results

When kept in the dark, CREG-GFP fluorescence could be observed in the vacuoles of the leaf epidermal cells (Figure 11C). In some cases the fluorescence was not restricted to the vacuoles but also visible in the apoplast surrounding the cells (Figure 13C). On the other hand, when plants were cultivated in the light, the GFP fluorescence was generally diminished and no vacuolar staining could be observed (Figure 11A). Instead, the fluorescence was reminiscent of cytoplasmic or even apoplastic staining and was probably due to small amounts of GFP that was not targeted to the vacuole, and therefore not degraded in the light.

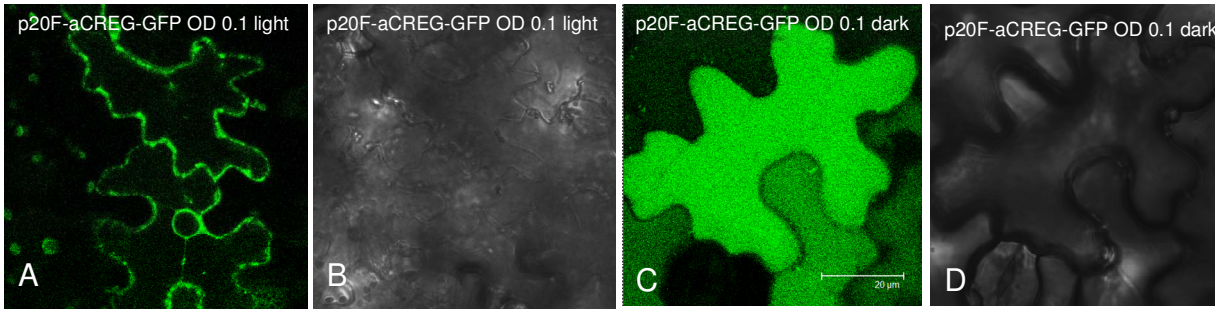

Figure 11: aCREG-GFP expression in *N. benthamiana*, 48 h after infiltration. **A**: GFP-fluorescence in leaves cultivated in the light. Fluorescence is observed mainly in the cytoplasm. **B**: Transmission light image corresponding to A. **C**: GFP-fluorescence in leaves cultivated in the dark. Fluorescence is observed in the vacuole. **D**: Transmission light image corresponding to C.

Transient expression of CREG-RFP was additionally used to validate the vacuolar localization of CREG, but the subcellular localization of CREG-RFP in *N. benthamiana* was never observed in vacuoles. In fact, only apoplastic staining (the apoplast is the free diffusional space outside the plasma membrane, characterised by bulges in the continuous surrounding line) in leaf

cells could be detected, regardless of whether the plants were kept in the dark after infiltration or not (Figure 12).

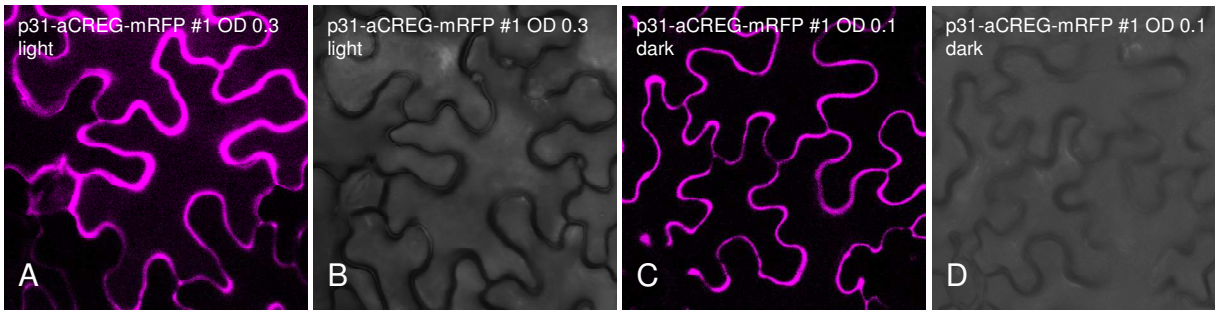

Figure 12: aCREG-mRFP expression in *N. benthamiana*, 48 h after infiltration. **A**: RFP-fluorescence in leaves cultivated in the light. Fluorescence is observed in the apoplast. **B**: Transmission light image corresponding to A. **C**: RFP-fluorescence in leaves cultivated in the dark. Fluorescence is again observed only in the apoplast. **D**: Transmission light image corresponding to C. Higher fluorescence intensities in A are due to higher OD (0.3) of *A. tumefaciens* cultures used for infiltration (OD = 0.1 in C).

3.3.1.1 *Perturbation of intracellular protein transport using Brefeldin-A*

To examine why CREG-mRFP was found in the apoplast whereas CREG-GFP localized in the vacuole, we treated the cells with *brefeldin-A* (BFA), a lactone antibiotic that interferes with intracellular transport from the Endoplasmic Reticulum (ER) to the Golgi apparatus. In *N. benthamiana* BFA leads to the fusion of ER membranes with Golgi membranes and proteins targeted to the secretory pathway accumulate in the ER (Nebenfuhr *et al.*, 2002). For BFA treatment, pieces of *N. benthamiana* leaves were incubated for approximately 1 h in 100 µg/ml BFA solution before being observed under the microscope.

Results

After incubation in the presence of BFA the fluorescence of the GFP and mRFP fusion constructs became visible in the ER network. Cells expressing CREG-GFP that were exposed to light showed the typical faint fluorescence in the absence of BFA (Figure 13A). After BFA treatment fluorescence was also detectable in the ER (Figure 13B), probably due to newly synthesized fusion protein accumulating in this compartment. Expression under dark

conditions without BFA was mainly observed in the vacuole (Figure 13C), after BFA treatment also in the ER (Figure 13D).

Before incubation with BFA, CREG-mRFP was found mainly in the apoplast (Figure 13E), whereas thereafter, it was also found in the ER (Figure 13F). Therefore it can be said that both proteins take the same transport route in the beginning, namely the secretory pathway via the ER, but that CREG-mRFP then mus take another route (possibly the secretory pathway instead of the vacuolar targeting pathway), ending up in the apoplast.

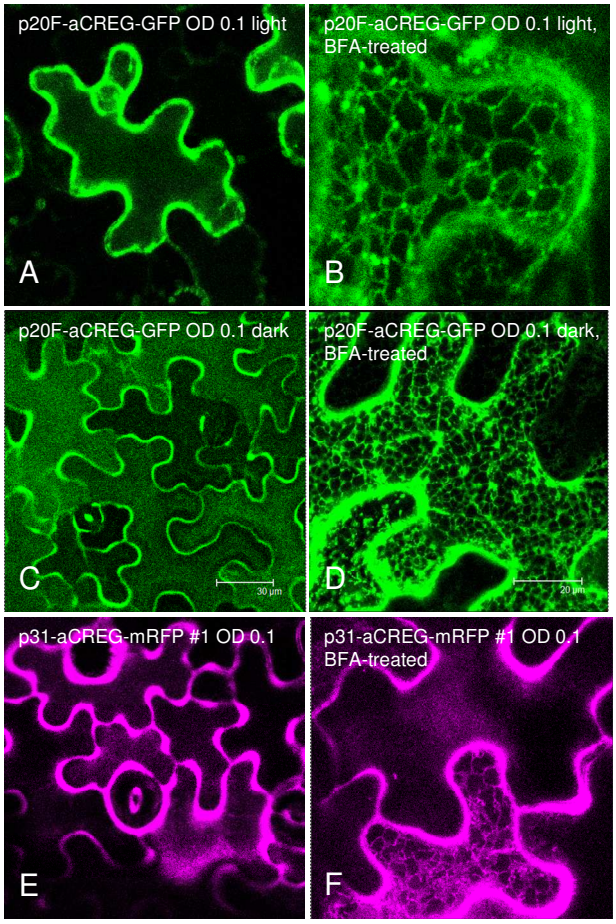

Figure 13: BFA treatment of *N. benthamiana* leaf epidermal cells expressing fusion proteins. **A, C** and **E**: Fluorescence in epidermal leaf cells before BFA treatment. GFP fluorescence in leaves cultivated in the light (A) is observed faintly in the cytoplasm and apoplast. When cultivated in the dark (C) it is visible in vacuoles and the apoplast. RFP fluorescence is observed mainly in the apoplast (E). **B, D** and **F:** corresponding images obtained after 1 h incubation in BFA. In all cases fluorescence is observed in the ER. BFA treatment was conducted 48 h post infiltration.

3.3.1.2 *Protease inhibitor treatment*

Analysis of the fusion proteins by immunoblotting revealed that the fusion proteins were largely degraded into a degradation product of ~27 kDa recognized by α-GFP and a degradation product of ~27 kDa recognized by α-mRFP (Figure 15, first lanes). Therefore the

attempt to stabilize the fusion proteins in planta was undertaken using E64d ((2S,3S)-*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester). E64d is a membrane-permeable cysteine protease inhibitor (PI) that has been shown to stabilize vacuolar GFP in the light (Tamura *et al.*, 2003).

Results

Figure 14B shows that vacuolar GFP fluorescence appears in plants cultivated in the light, thus proving the stabilizing effect of co-infiltrated E64d in this case. Infiltration of 1% DMSO (Figure 14C and F) was performed as negative control and shows no stabilizing effect on GFP or RFP expression. CREG-mRFP fluorescence remains unchanged by PI treatment and is still visible only in the apoplast (Figure 14E).

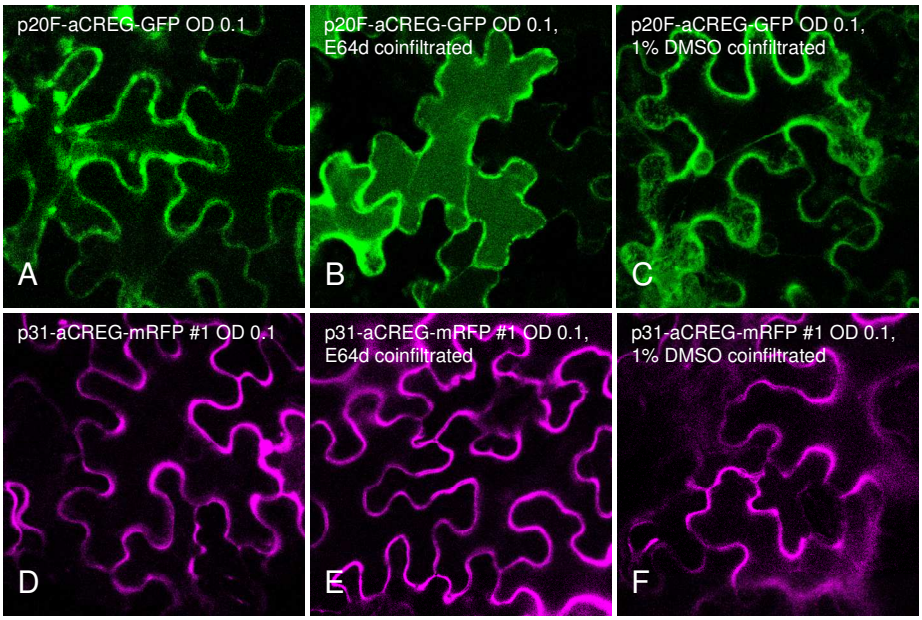

Figure 14: E64d protease inhibitor treatment of *N. benthamiana* leaf epidermal cells expressing aCREG-GFP and aCREG-mRFP fusion constructs. A: aCREG-GFP expression in the light. **B**: aCREG-GFP expression when the fusion construct was co-infiltrated with E64d. Vacuolar fluorescence can be seen. **C**: Expression of aCREG-GFP in epidermal cells co-infiltrated with 1% DMSO (used as solvent for E64d). DMSO alone seems to have no effect on aCREG-GFP localization. **D,E** and **F**: corresponding images obtained for aCREG-mRFP expression. Treatment with E64d seems to have no effect on mRFP fluorescence.

Analysis by immunoblotting of the CREG-mRFP expression levels upon E64-d treatment reveals that the ratio of cleaved mRFP (27.5 kDa) to CREG-mRFP (~50 kDa) remains unchanged. An additional band at ~20 kDa indicates that also an mRFP degradation product seems to become stabilized by PI treatment (Figure 15, lane on the mRFP blot).

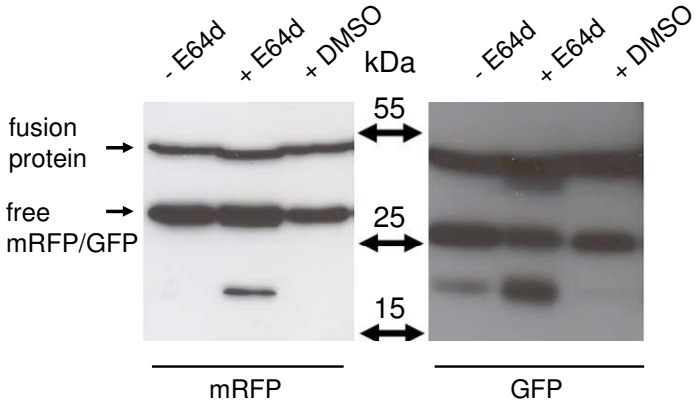

Figure 15: Western blot analysis of aCREG-GFP and aCREG-mRFP expression in *N. benthamiana* before and after protease inhibitor treatment. 20 µl of a protein extract prepared with 1.25 µl extraction buffer per 1 mg plant material were loaded in each lane.

3.3.1.3 *CREG-mRFP is secreted into the interstitial fluid whereas CREG-GFP is retained in the vacuole*

To further analyse why CREG-mRFP is found in the apoplast whereas CREG-GFP is found in the vacuole, *N. benthamiana* leaves were subjected to vacuum infiltration and subsequent analysis of the obtained interstitial fluid (IF). Additionally, total protein extracts were prepared from the remaining leaf tissue for comparison of secreted versus intracellular proteins. Total protein and IF fractions were analysed side to side on Western blots, where only 1% of the total protein extracts (wet weight) were compared to 10% of the IF fraction (in µl). This measure was taken to avoid protein overload in the total protein fraction and should be taken into account when comparing protein expression levels in Figure 16.

Results

Analysis of the secreted protein fraction of leaves expressing the CREG-GFP construct shows that CREG-GFP (~ 50 kDa) is found only in the total protein extracts (faintly on the anti-CREG blot and clearly on the anti-GFP blot) as well as CREG (~ 18 kDa) alone (Figure 16B). Free GFP (~ 25 kDa) is observed in very small amounts in total protein extracts, probably due to degradation of vacuolar GFP in the light. The very small amounts of free CREG found in the IF

are probably artefacts arising from the IF preparation, where the rupture of cells can cause a mingling of intracellular proteins with the IF. To analyze to what extend such mingling takes place, leaves were also infiltrated with cytoplasmic GFP. This was performed using the p20-GFP vector, where GFP has no further targeting sequences attached to it and therefore is translated in the cytoplasm. Analysis of total protein extracts and IF from such leaves shows that only small amounts of cytoplasmic GFP are found in the IF as shown in Figure 16C on the right, taking into account that only 1% total protein fraction is compared to 10% IF. These results show that intact CREG-GFP is found in the cells, most likely in the vacuoles and no secretion takes place.

Analysis of the IF from CREG-mRFP expressing *N. benthamiana* leaves by immunoblotting revealed that all of the CREG-mRFP (~ 50 kDa) fusion protein is secreted to the apoplast, as well as free CREG (~ 18 kDa) and mRFP (~ 27 kDa), as shown in Figure 16A. Free CREG and mRFP are also found in total protein extracts, but no fusion protein is detectable there. It might very well be possible, that small amounts of fusion protein are also present in the cells, but that the amounts are below the detection limit. The vast majority of the construct that is found inside the cells is in its degraded form (free CREG and mRFP), and probably represents CREG-mRFP that has already been degraded before reaching its final destination in the IF. Where the degradation process of CREG-mRFP takes place and what mechanisms are involved could not be identified during this work. It also remains unclear why the mRFP tagged fusion protein is targeted for secretion whereas the GFP construct is transported to the vacuole when transiently expressed in *N. benthamiana* leaf epidermal cells.

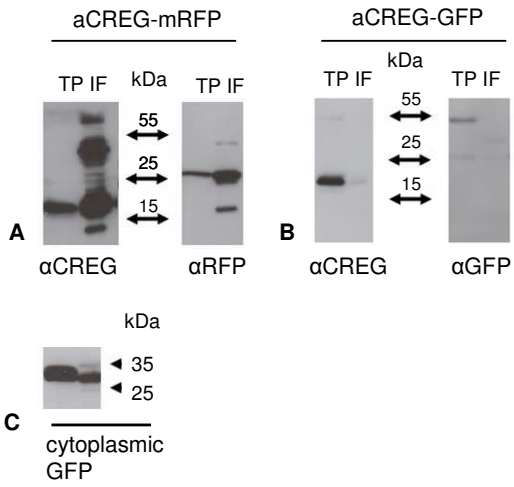

Figure 16: Western blot analysis of aCREG-mRFP and aCREG-GFP expression in *N. benthamiana* total protein extracts and interstitial fluid. 1% of the total protein extracts (wet weight) were compared to 10% of the IF fraction (in µl).

It is possible that different intrinsic sequence motifs in the mRFP and GFP sequences are responsible for the different targeting of the fusion proteins. Also the possibility that the targeting sequence of endogenous CREG is located on its C-terminus exists. In this case, a C-terminal tag would interfere with proper targeting. It has been observed, that a GFP tag on proteins can influence their localization, as GFP tends to accumulate inside the cell, mainly in the ER. mRFP on the other hand has been shown to be secreted when it is expressed with no further targeting sequences. It is therefore possible that the fluorescence tags can influence the subcellular localization of the fusion proteins.

3.3.2 Expression of CREG-GFP and CREG-mRFP in *A. thaliana*

3.3.2.1 *Screening and identification of stable CREG-mRFP transformants*

Two *A. thaliana* lines expressing the CREG-GFP construct had already been obtained at our institute before this work had started. For stable transformation of *A. thaliana* with CREG-mRFP young *A. thaliana* plants were subjected to floral dipping and the produced seeds were grown on agar plates containing kanamycin for selection of transformed seedlings. Seedlings resistant to kanamycin, were further propagated and total protein extracts were obtained from their leaves. These seedlings were supposed to be hemizygous for the CREG-mRFP construct and immunoblot analysis was performed with anti-mRFP antibody to visualize the mRFP expression level. The seedlings were also screened by confocal microscopy and the seedlings with the highest fluorescence levels were selected (data not

shown). In the second generation homozygote lines (25% of the progeny) arise as a consequence of chromosome segregation, as well as 25% that lack the transgene. The remaining 50% of the progeny are again hemizygous and carry only one allele of the transgene.

Results

Most of the 28 homozygous seedlings analysedshowed high expression of free mRFP and also a band at ~20 kDa, also observed earlier in Western blots, that probably represents an mRFP degradation product (Figure 17). Bands of higher molecular weight were also present in most seedlings, but the molecular weight varied and it was not clear which of the bands represents the fusion construct, if any one did at all. In lines #5 and #6 the expression level of free mRFP was lower compared to the other seedlings and an additional band of ~70 kDa was detected. The higher molecular weight band at ~ 70 kDa could represent the CREG-mRFP fusion protein, although its expected molecular weight is ~50 kDa (~20 kDa CREG and 27.5 kDa mRFP). Western blotting with anti-CREG antibody showed that CREG produced in *A. thaliana* has a lower molecular weight (~18 kDa) than predicted (data not shown). This is probably due to additional processing of CREG in *A. thaliana*, but it is unclear whether this processing occurs *N*- or *C*- terminally and where it takes place. C-terminal processing of the fluorescent aCREG fusion proteins could also explain the high amounts of free mRFP and GFP.

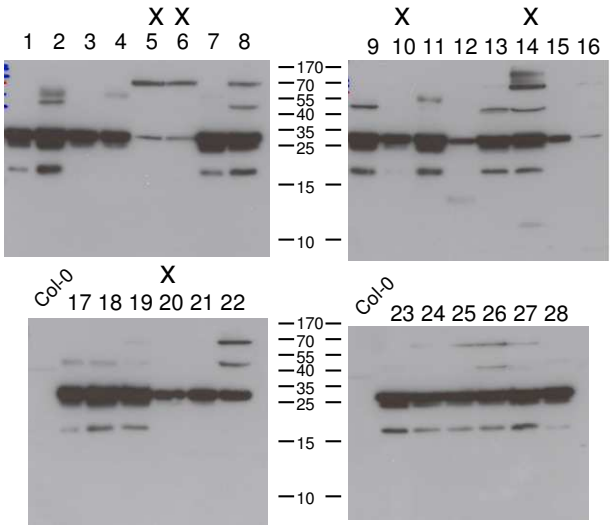

Figure 17: Screening for positive *A. thaliana* transformants by immunoblotting against mRFP. *A. thaliana* lines #5, #6, #10, #14 and #20, marked with an "X" were continued. 20 µl of a protein extract prepared with 1.25 µl extraction buffer per 1 mg plant material were loaded in each lane. Col-0 wt extracts were loaded as negative control.

The p31 aCREG-mRFP lines #10, #14 and #20 (T1 generation) were also selected for further analysis, because those seedlings showed highest fluorescence levels under the microscope. T2 seedlings from the lines p31 aCREG-mRFP #5 and #20 showing no growth impairment in the presence of kanamycin were then transferred to soil for further propagation. These seedlings were also analysed by confocal microscopy to visualize aCREG-mRFP subcellular localization.

3.3.2.2 *Expression of p31 aCREG-mRFP in A. thaliana*

Analysis of p31 aCREG-mRFP #5 and #20 expression in *A. thaliana* was performed with whole seedlings that were mounted on a glass slide and observed under the confocal microscope.

Results

In these *A. thaliana* seedlings fluorescence is observed only in cell vacuoles, indicating that the fusion protein is targeted to the vacuoles (Figure 18).

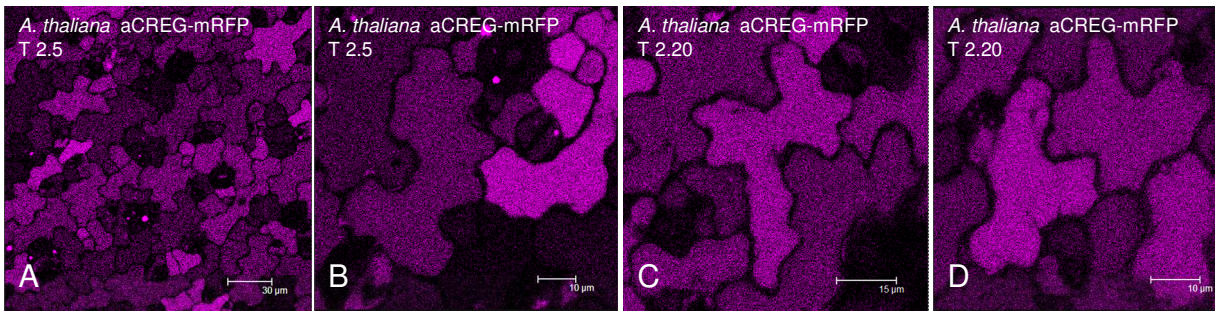

Figure 18: aCREG-mRFP expression in *A. thaliana*, as seen under the confocal microscope. **A** and **B**: *A. thaliana* aCREG-mRFP #5, where B shows a higher magnification. **C** and **D**: *A. thaliana* aCREG-mRFP #20, where D again shows a higher magnification image.

Expression of p31 aCREG-mRFP #5 (Figure 18A and B) and #20 (Figure 18C and D) is observed in the whole cell, typical for proteins in the central vacuole, which occupies most of the cell interior. Expression levels vary between different cells, reminiscent of aCREG-GFP expression in *N. benthamiana* (cultivated in the dark).

To exclude the possibility that vacuolar targeting of the fusion protein is due to the mRFP tag, a ST-mRFP (Sialyl Transferase-mRFP) marker construct was also analysed by CLSM. ST-mRFP should locate in the Golgi vesicles of the cells. Figure 19 shows that the marker construct indeed locates to small punctuate structures reminiscent of Golgi vesicles. Therefore it can be excluded that the mRFP tag is responsible for the vacuolar localization of the aCREG-mRFP fusion constructs.

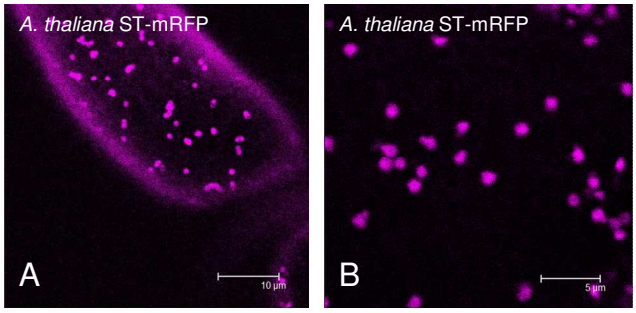

Figure 19: ST-mRFP expression in *A. thaliana*. Fluorescence in punctuate structures shows localization in the Golgi apparatus.

To obtain further evidence that the observed fluorescence of the p31 aCREG-mRFP #5 and #20 constructs was indeed confined to the vacuole, plasmolysis experiments were performed with the aCREG-mRFP expressing seedlings. Plasmolysis was induced by incubation of the seedlings in 0.5 M mannitol for approximately 10 min. The seedlings were then analysed under the confocal microscope. Figure 20A-D shows that the fluorescence is maintained in the shrinking protoplasts, confirming that aCREG-mRFP expression is restricted to the cell interior, most likely to the vacuole. Figure 20E-H shows the expression of PIP 2A-mRFP (plasma membrane intrinsic protein 2A), a plasma membrane marker. Before plasmolysis the expression of PIP 2A-mRFP is seen in the plasma membrane surrounding the cells (Figure 20E) and after plasmolysis the fluorescence is again seen in the membrane surrounding the shrinking protoplasts (Figure 20G and H). This serves as a confirmation, that plasmolysis indeed worked in the expected way.

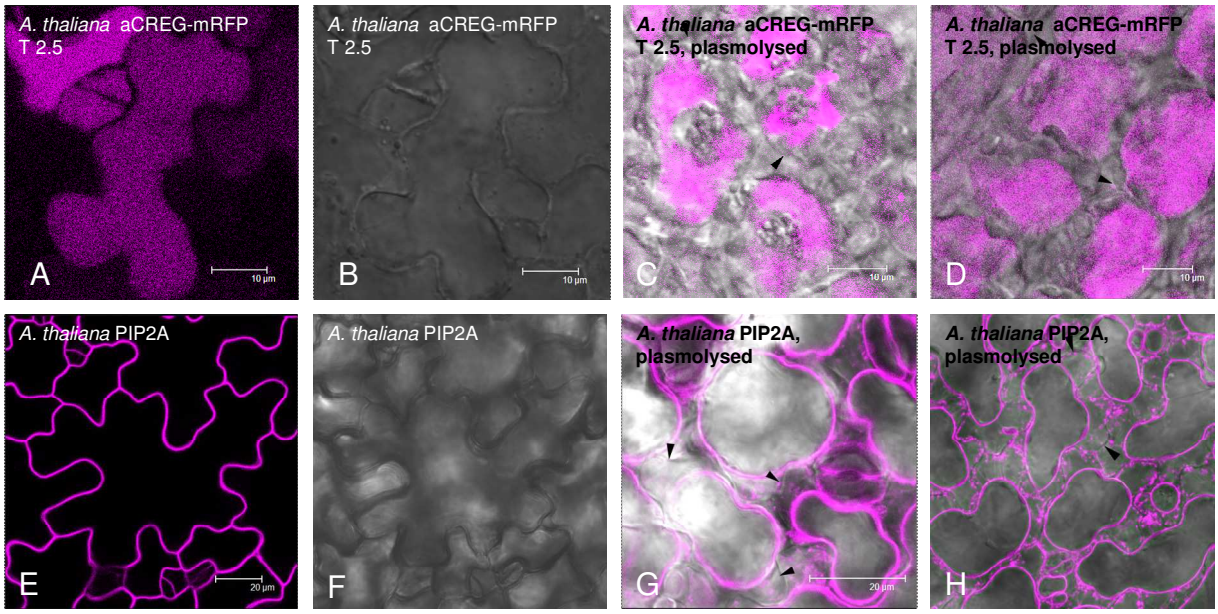

Figure 20: Analysis of subcellular localization in *A. thaliana* by plasmolysis. A-D: *A. thaliana* aCREG-mRFP #5. **E-H:** *A. thaliana* PIP2A. **A** and **E**: before treatment. **B** and **F**: images of untreated cells obtained by transmission light microscopy. **C, D, G** and **H**: cells after plasmolysis (10 min incubation in 0.5 M mannitol), overlay of RFP fluorescence and transmission light images. Arrows shows cell wall boundaries. Fluorescence of aCREG-mRFP is observed only in vacuoles, whereas PIP2A is found in the plasma membrane.

3.3.2.3 *Expression of p20F aCREG-GFP in A. thaliana*

Two p20F aCREG-GFP expressing lines were obtained earlier at our institute. Analysis of the p20 aCREG-GFP lines in the 3rd generation by confocal microscopy showed GFP expression in the cell vacuoles (work done by Eva Liebminger, Department of Applied Genetics and Cell Biology, BOKU, Vienna; data not shown). The lines were also analysed by immunoblotting, and showed high expression levels of ectopic CREG and GFP due to quantitative processing of the fusion protein in planta. Figure 21 (left side) shows aCREG levels in roots for both lines in the 3rd generation. At this stage, the p20F aCREG-GFP lines were considered as aCREG overexpressing lines, due to the strongly increased aCREG levels compared to wildtype.

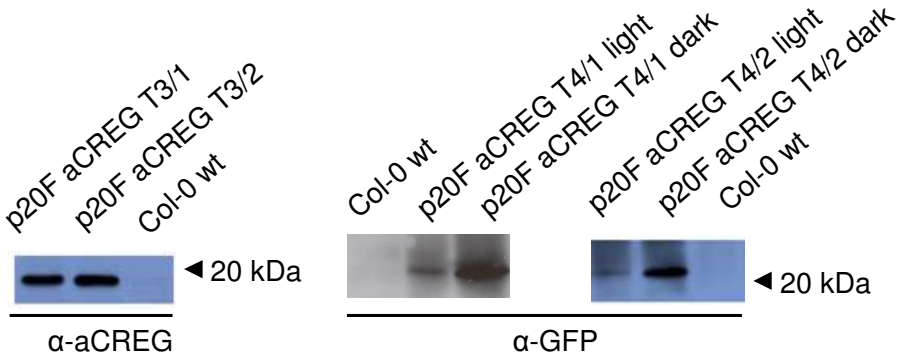

Figure 21: Western blot analysis of aCREG and GFP expression in *A. thaliana* p20F aCREG-GFP lines. Protein extracts were prepared with 1.5 µl extraction buffer per 1 mg root material. On the anti aCREG blot on the left, 20 µl protein extract were loaded in each lane. On the p20F aCREG T4/1 anti GFP blot 10 µl protein extract were loaded in each lane. On the p20F aCREG T4/2 anti GFP blot 11.5 µl for p20F aCREG T4/2 light, 17 µl for p20F aCREG T4/2 dark and 12.1 µl for Col-0 protein extract in each case corresponding to 80 µg of total protein were loaded.

Results

In the 4th generation, immunoblotting with an anti-GFP antibody still shows high GFP-levels in the p20F aCREG T4/1 & 2 lines (Figure 21, right side). The seedlings were cultivated in the light and in the dark to test for light-induced degradation of vacuolar GFP. As can be seen in Figure 21, right side, GFP levels are reduced when seedlings are cultivated under light.

After the seedlings were continued into the 5th generation, GFP expression was diminished and so were aCREG levels, as shown for p20F aCREG-GFP T5/1 in Figure 22.

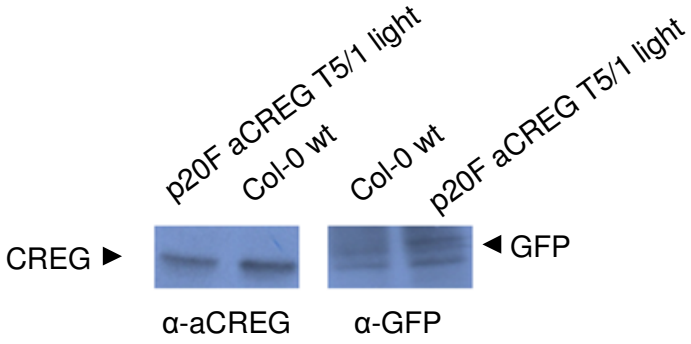

Figure 22: Western blot analysis of p20F aCREG-GFP line 1 in the 5th generation (T5). Anti CREG blot on the left: 10 µl p20F aCREG T5/1 (~67 µg protein) and 9.4 µl Col-0 (~62 µg protein) were loaded. Anti GFP blot on the right: 12 µl Col-0 (~80 µg) and 11.8 µl p20F aCREG T5/1 (~80 µg) protein extracts, as prepared before, were loaded.

GFP was expressed at such low levels, that it appears only as a faint band in the Western blot. Also aCREG levels were now similar to wildtype expression. The GFP fluorescence was diminished to such a high degree, that it could not be detected under the confocal

microscope anymore. The reduced expression of the transgene could be due to gene silencing effects. Hence, in the 5th generation the p20F aCREG-GFP lines can no longer be considered aCREG-overexpressing lines.

3.3.3 aCREG localization analysis by subcellular fractionation

Subcellular fractionation was performed by discontinuous sucrose density gradient centrifugation (SDG), thus separating subcellular compartments according to their density. The method was applied as an independent approach to confirm the vacuolar localization of endogenous aCREG. The lines that were used for SDG were Columbia wildtype (Col-0), the aCREG-RNAi T5/1 line and the p20F aCREG-GFP T5/1 line. The aCREG-RNAi T5/1 line is one of the RNAi-knockdown lines obtained earlier at our institute as described in section 3.1. In the 5th generation, aCREG expression in the knock-down mutant was so low that analysis by immunoblotting gave no signal (expression below detection limit). Figure 23 shows a comparison of aCREG levels between the lines used for SDG.

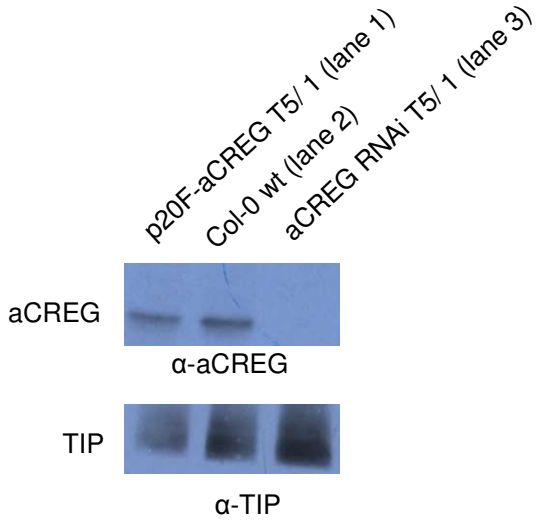

Figure 23: Comparison of aCREG expression in *A. thaliana* aCREG-GFP T5/1, Col-0 wt and aCREG RNAi roots. Lane 1: 10 µl root extract (~67 µg protein), lane 2: 9 µl (~63 µg), lane 3: 12 µl (~48 µg). Root extracts were prepared as described before. The corresponding anti TIP blot is shown below as loading control.

The p20F aCREG-GFP line was originally used in this experiment because it was by then still assumed to be an aCREG-overexpressing line. Unfortunately, in the 5th generation it turned out to have aCREG expression levels similar to wildtype plants, suggesting essentially complete loss of transgene expression. Root extracts from all three lines were subjected to several rounds of centrifugation before being loaded onto the gradient (see section 2.4.7). After SDG centrifugation the gradient was separated into 22 to 23 fractions, with sucrose

concentrations ranging from 20% - 52%. Marker molecules for different organelles show a distinct distribution pattern in the gradient fractions.

First, all fractions were analysed by measuring their *β-N*-acetylhexosaminidase activity (Figure 24). *β-N*-acetylhexosaminidase 1 (Hexo 1) is a soluble vacuolar enzyme, whereas Hexo 3 is resident in the plasma membrane (Strasser *et al.*, 2007). Therefore Hexo 1 is expected to co-localize with aCREG in the gradient.

Results

As expected, Hexo 1 activity was highest in the low-density fractions. Hexo 1 activity peaked in fraction 3 (Col-0 and aCREG RNAi T5/1) or in fraction 4 (p20F aCREG-GFP T5/1). The weak enzyme activity peak appearing in fractions 19 to 21 is due to the activity of Hexo 3, a *β-N*-acetylhexosaminidase residing in the plasma membrane. These results show that subcellular fractionation by SDG was successful and that the soluble vacuolar proteins are confined to the low density fractions 1 to 5.

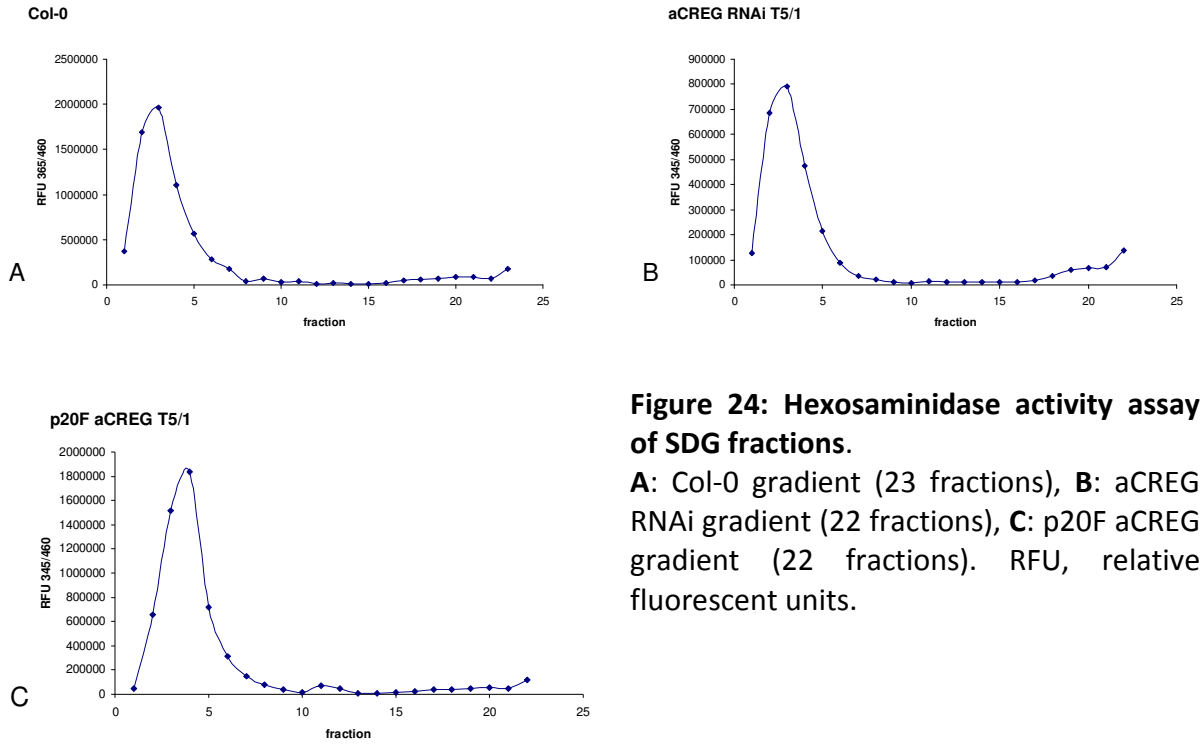

Figure 24: Hexosaminidase activity assay of SDG fractions.
A: Col-0 gradient (23 fractions), **B**: aCREG RNAi gradient (22 fractions), **C**: p20F aCREG gradient (22 fractions). RFU, relative fluorescent units.

After the different fractions were analysed by hexosaminidase activity assays they were subjected to protein precipitation, which was required as a purification step due to the high

sucrose levels in the samples. After the samples were purified, they were analysed by silver staining to show the protein content in the samples. Protein levels are highest in the soluble protein fractions with low density and in fractions 16 to 20, corresponding to sucrose densities of 40% to 47%, probably representing plasma membrane proteins and mitochondria.

The SDG fractions were also analysed by immunoblotting. Western blot analysis with anti-CREG antibody shows, that endogenous CREG in Col-0 wt is restricted to the soluble protein fractions. Same results were obtained for the transgenic aCREG in p20F aCREG-GFP. In the aCREG RNAi knock-down line aCREG was not detectable. To allow quantitative comparison of the individual blots, 30 ng recombinant aCREG (21.4 kDa) produced in *Escherichia coli* were used as a positive control in each blot. The fact that endogenous aCREG from *A. thaliana* migrates at a molecular weight of about 18 kDa (theoretical molecular weight of aCREG would be ~20 kDa) compared to recombinant aCREG shows that the protein is being processed at some point. Figure 25 shows Western blot analysis of SDG fractions. Silver stained protein gels are shown for comparison.

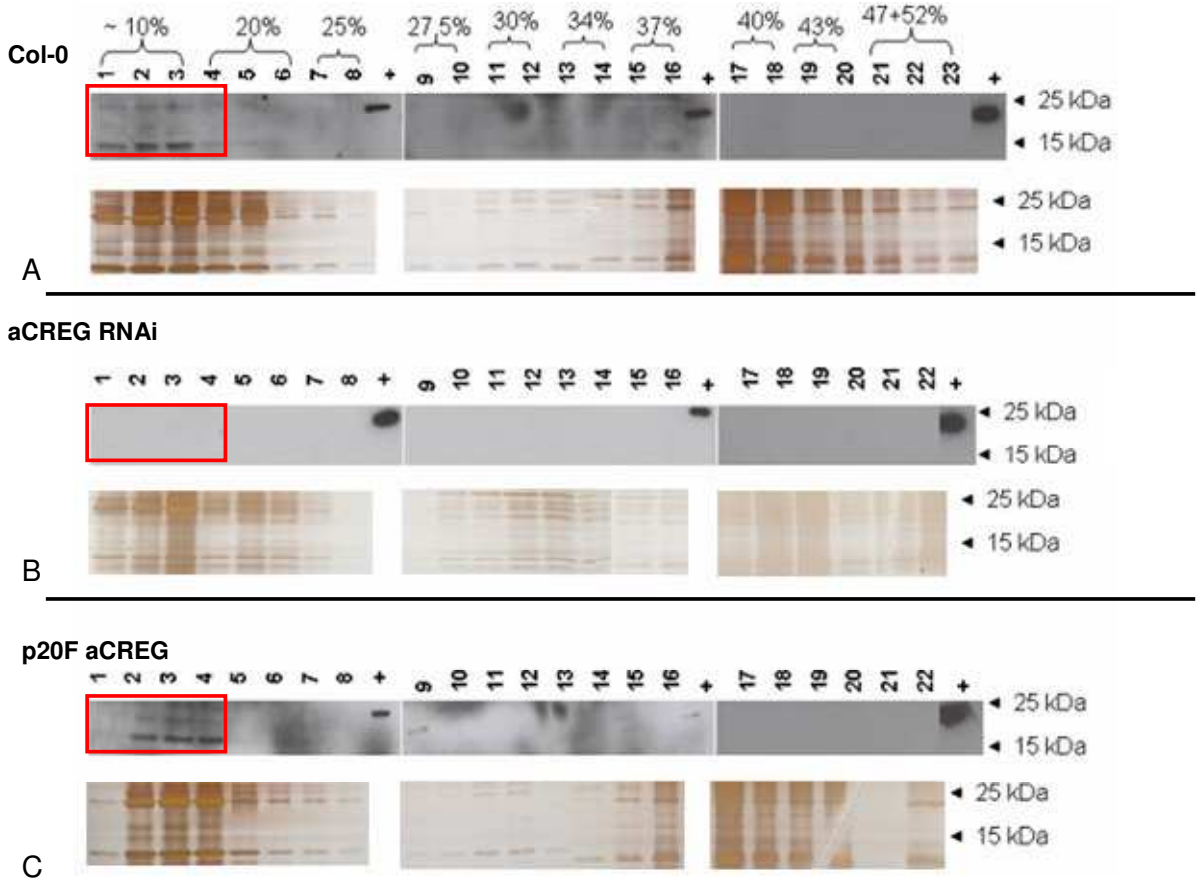

Figure 25: Sucrose density gradient fractionation of *A. thaliana* Col-0 wt, aCREG-RNAi and p20F aCREG-GFP root extracts. Anti-aCREG western blot analysis was performed with 20 µl of protein extracts (preparation described in section 2.4.9). 30 ng recombinant aCREG were used as positive control. Silver stained gels of the corresponding SDG fractions are shown beneath the Western blots. Molecular weights are indicated on the right.

In conclusion it can be said that endogenous aCREG maps to the soluble protein fractions with low density typical for vacuolar proteins, therefore giving another evidence for the vacuolar localization of this protein.

3.4 Functional analysis of aCREG in *A. thaliana*

Functional characterization of aCREG was carried out under abiotic stress conditions. Phenotypic analysis was carried out on the level of total root length. Seedlings were cultivated under stress and under standard growth conditions for comparison. The abiotic stress was elicited by:

- Salt stress: The growth medium contained 140 mM NaCl (control: no NaCl addition)
- Sucrose stress: Seedlings were grown on 0%, 1% (control) and 4.5% sucrose
- Heat stress: A temperature shift from 22°C to 30°C was used to induce heat stress

In these experiments we used two aCREG-knockdown lines (aCREG RNAi T5/1 &2) and two p20F aCREG-GFP lines (p20F aCREG-GFP T5/1 &2). Columbia-0 wildtype seedlings (Col-0) were grown as a control. The two knock-down lines showed diminished aCREG expression (aCREG RNAi T3/1: 25% compared to Col-0; aCREG RNAi T3/2: 20% compared to Col-0) in the 3^{rd} generation. In these experiments we used the 4^{th} and 5^{th} generation. These generations show aCREG levels below the detection limit of Western blot analysis (shown in Figure 23 for aCREG RNAi T5/1, similar results were obtained for the other lines, data not shown). Although the aCREG levels were diminished to such high degrees, the *A. thaliana* knock-down lines showed no visible phenotype when grown under standard conditions. Therefore the stress conditions were thought to be able to induce a phenotype that is not visible under normal growth conditions. The two p20F aCREG-GFP lines were originally thought to represent aCREG overexpressing lines and were therefore also investigated under abiotic stress. As it turned out, aCREG levels were similar to wildtype plants in the 5^{th} generation. The p20F aCREG-GFP lines can therefore only be considered as a further control.

The seedlings in these experiments were first grown on standard MS medium and after 7 days transferred onto the medium for abiotic stress. After 7 more days the total root length (primary root) was determined.

Results

Total root lengths of seedlings grown under standard conditions and such grown on medium supplemented with 140 mM NaCl were compared. Seedlings were grown in the light and in the dark, as lack of light and therefore inhibited photosynthesis was thought to be able to enhance any salt stress phenotype. Figure 26A-C shows three independent repetitions of the experiment; Figure 26D shows mean values of the three experiments. As can be seen, there is no obvious phenotype in the aCREG RNAi knockdown line compared to the wildtype under stress conditions. No phenotype can be observed even when the seedlings are grown in the dark. This shows that aCREG knockdown is not able to provoke a salt stress phenotype.

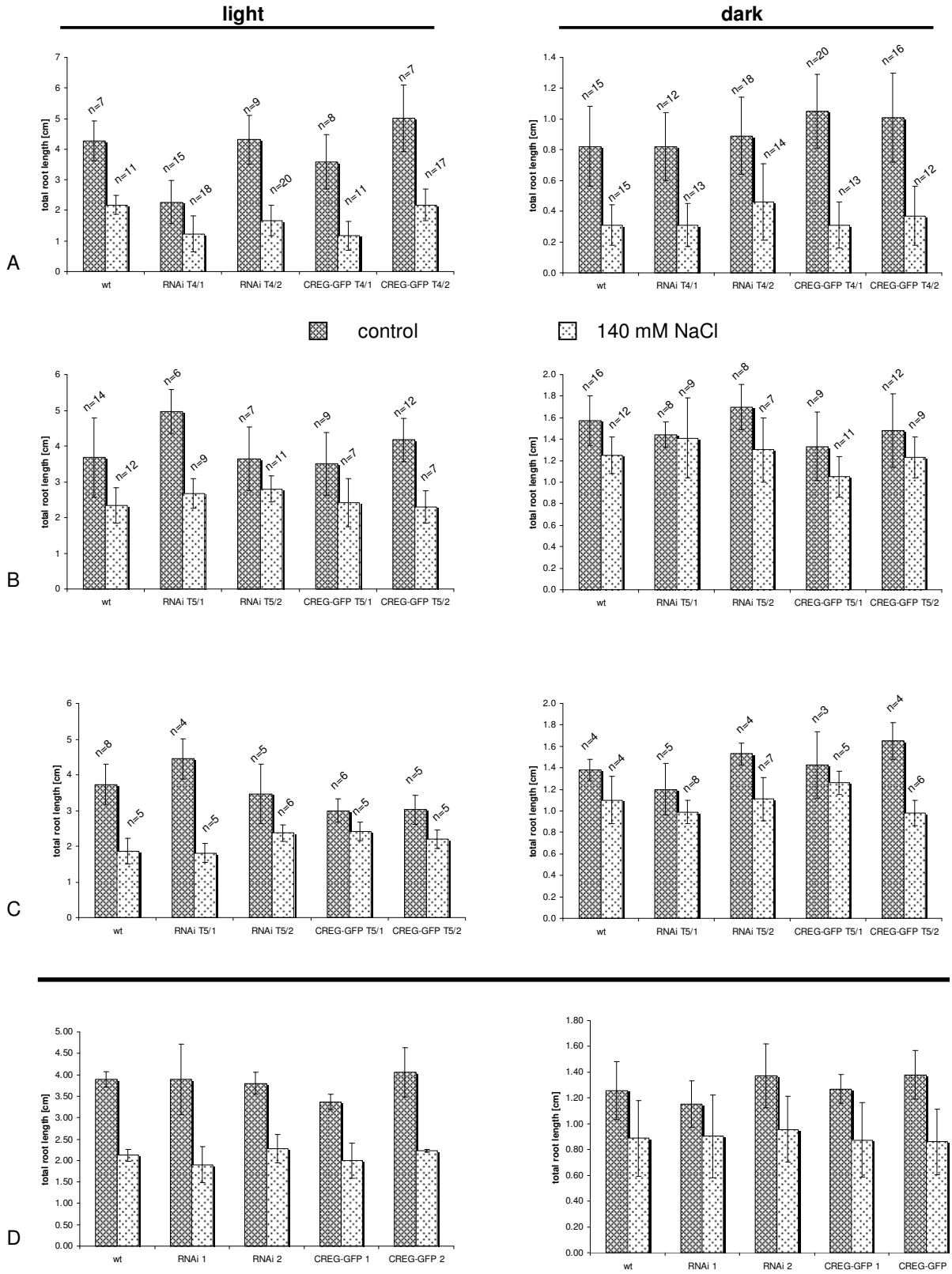

Figure 26: Salt stress experiment in *A. thaliana*. A, B and C: Three independent repetitions of the experiment. Bars show total root lengths. Error bars indicate standard deviation. n, number of seedlings. D: mean of the three experiments A-C. Error bars indicate standard error of the mean. Root lengths were measured on day 7 after induction of abiotic stress.

Figure 27 shows seedlings at the time point when the root length was determined. It also shows that there is no obvious variation in root length between aCREG-knockdown seedlings and wildtype. Also, p20F aCREG-GFP seedlings are shown to behave as wildtype. Although phenotypic analysis was carried out by measurement of the total root length only, other phenotypes were not observed either. All seedlings cultivated on NaCl containing plates generally showed slowed growth but otherwise no differences compared to seedlings grown under standard growth conditions.

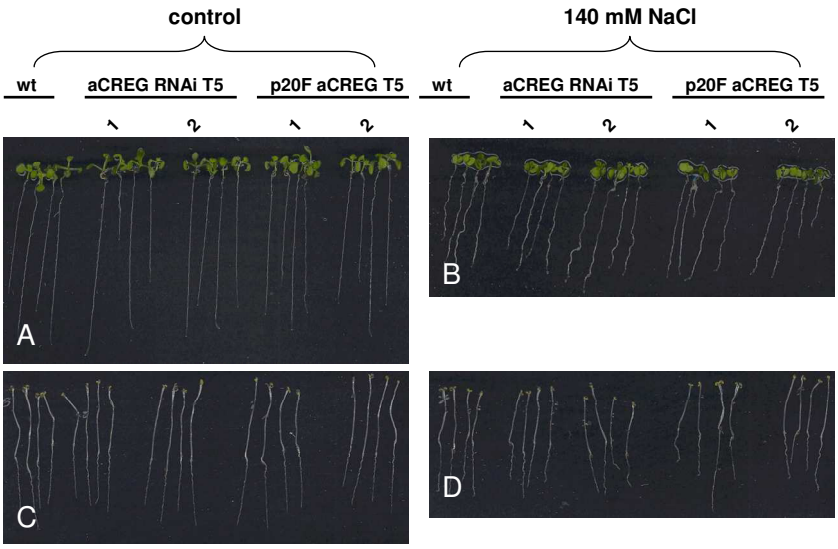

Figure 27: *A. thaliana* seedlings of salt stress experiment. Seedlings were transferred on agar plates and imaged after root length was measured. **A** and **B**: seedlings cultivated in the light. **C** and **D**: seedlings cultivated in the dark.

Seedlings subjected to sucrose stress were first cultivated on agar plates supplemented with 1% sucrose. On day 7 they were transferred to plates supplemented with 0%, 1% (control condition) and 4.5% sucrose. Seedlings grown on plates containing no sucrose generally showed growth retardation. Seedlings cultivated on 1% sucrose showed normal growth, whereas seedlings kept on plates containing 4.5% sucrose showed generally shorter primary roots and increased lateral root growth.

Figure 28 shows the total root lengths in the sucrose stress experiment. The experiment was repeated twice (Figure 28A and B) and Figure 28C shows the mean of both experiments. As in the salt stress experiment there is no correlation between aCREG expression levels and

root length under stress conditions. The aCREG knock-down line shows no phenotype under sucrose depletion or sucrose excess (osmotic stress). Figure 29 shows seedlings subjected to sucrose stress on the day when root length was determined. There are no obvious differences between the three lines, confirming the notion obtained by measurement of the root lengths.

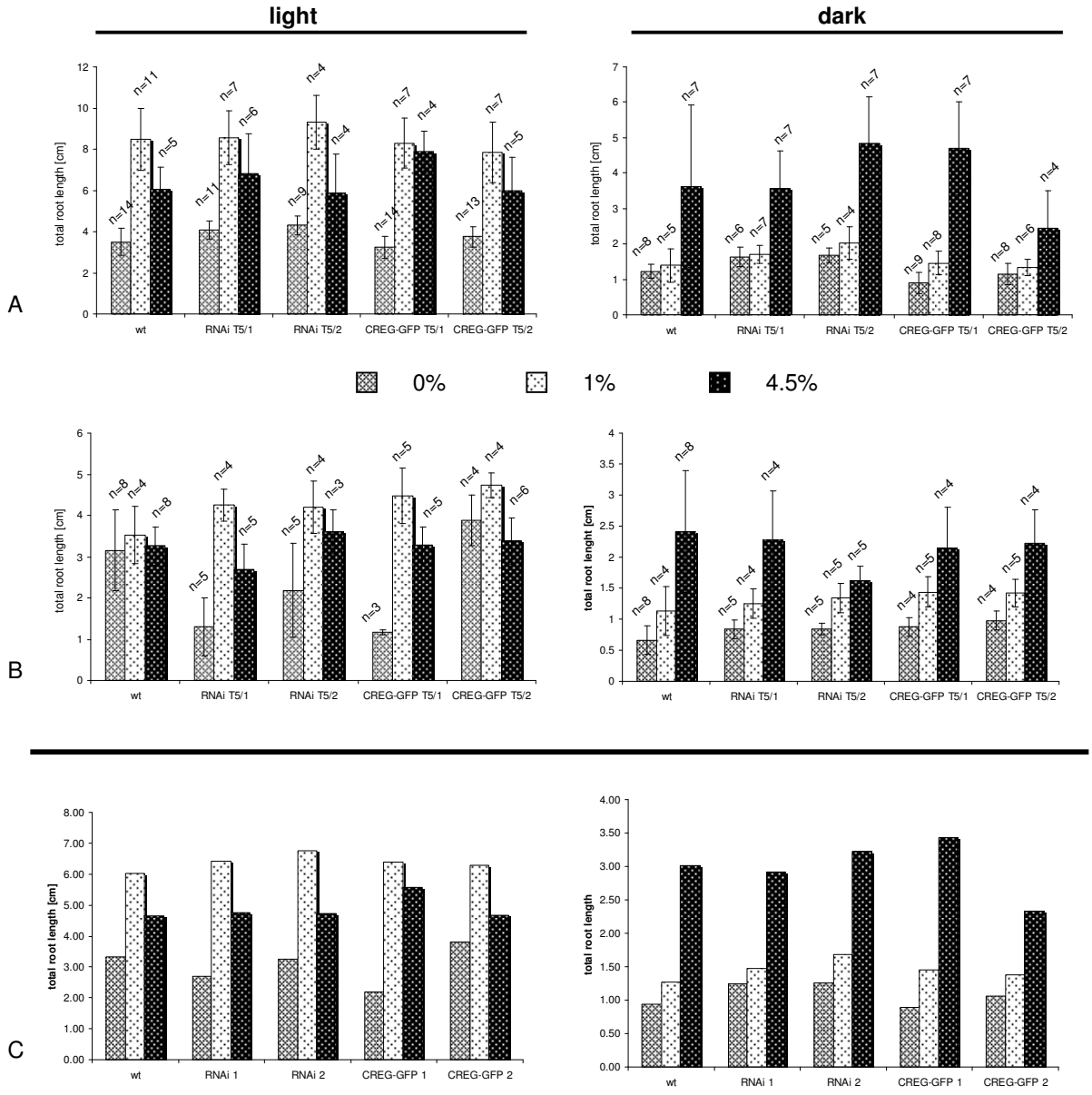

Figure 28: Sucrose stress experiment in *A. thaliana*. A and B: two independent repetitions of the experiment. Bars show total root length. Error bars indicate standard deviations. n, number of analysed seedlings. C: Mean of experiments A and B.

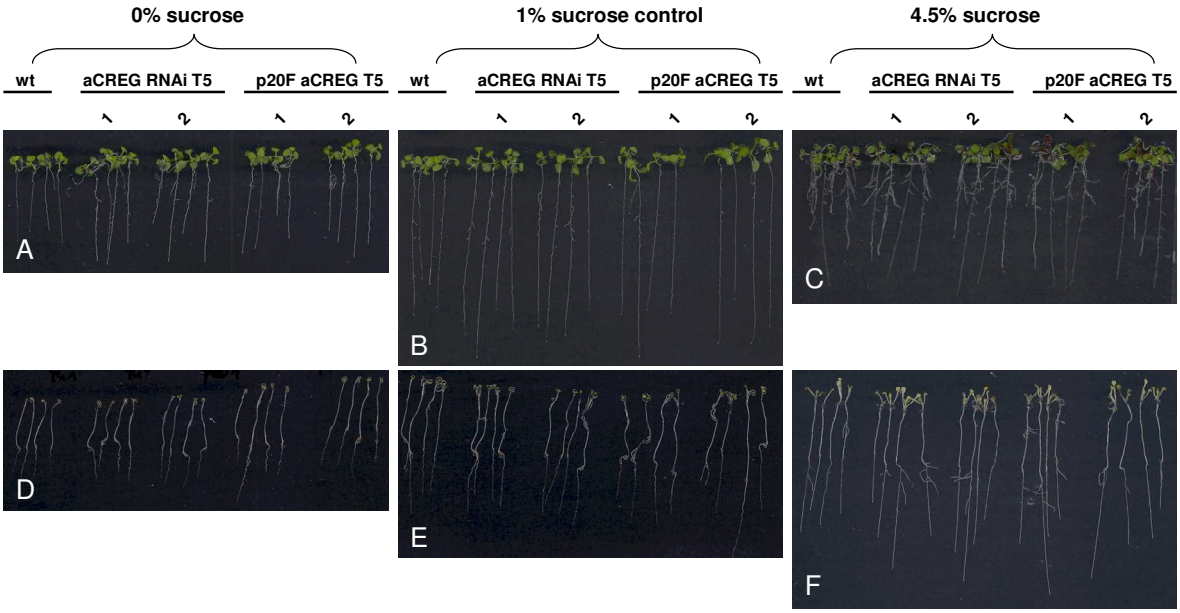

Figure 29: *A. thaliana* seedlings of sucrose stress experiment. A, B and C: seedlings cultivated in the light, D, E and F: seedlings cultivated in the dark. Seedlings were transferred on agar plates and imaged on day 7 after induction of abiotic stress.

Heat stress experiments were carried out in a similar way, cultivating seedlings at 22°C (control conditions) for the first 7 days and then changing their growth conditions. The seedlings that were to be subjected to heat stress were then transferred to H+ plates containing a medium supplementation more suitable for higher temperatures, and grown on 30°C for another 7 days. Again, seedlings were grown in light and dark conditions to see whether light depletion can further reinforce a potential phenotype. Figure 30A and B show total root lengths of the analysed seedlings. Figure 30C shows mean values of the two independent repetitions. As in the stress experiments discussed above, there is also no aCREG-dependent phenotype. Seedlings cultivated at 30°C generally showed an increased number of lateral roots but there was no difference between aCREG-RNAi lines and wildtype or p20F aCREG-GFP seedlings.

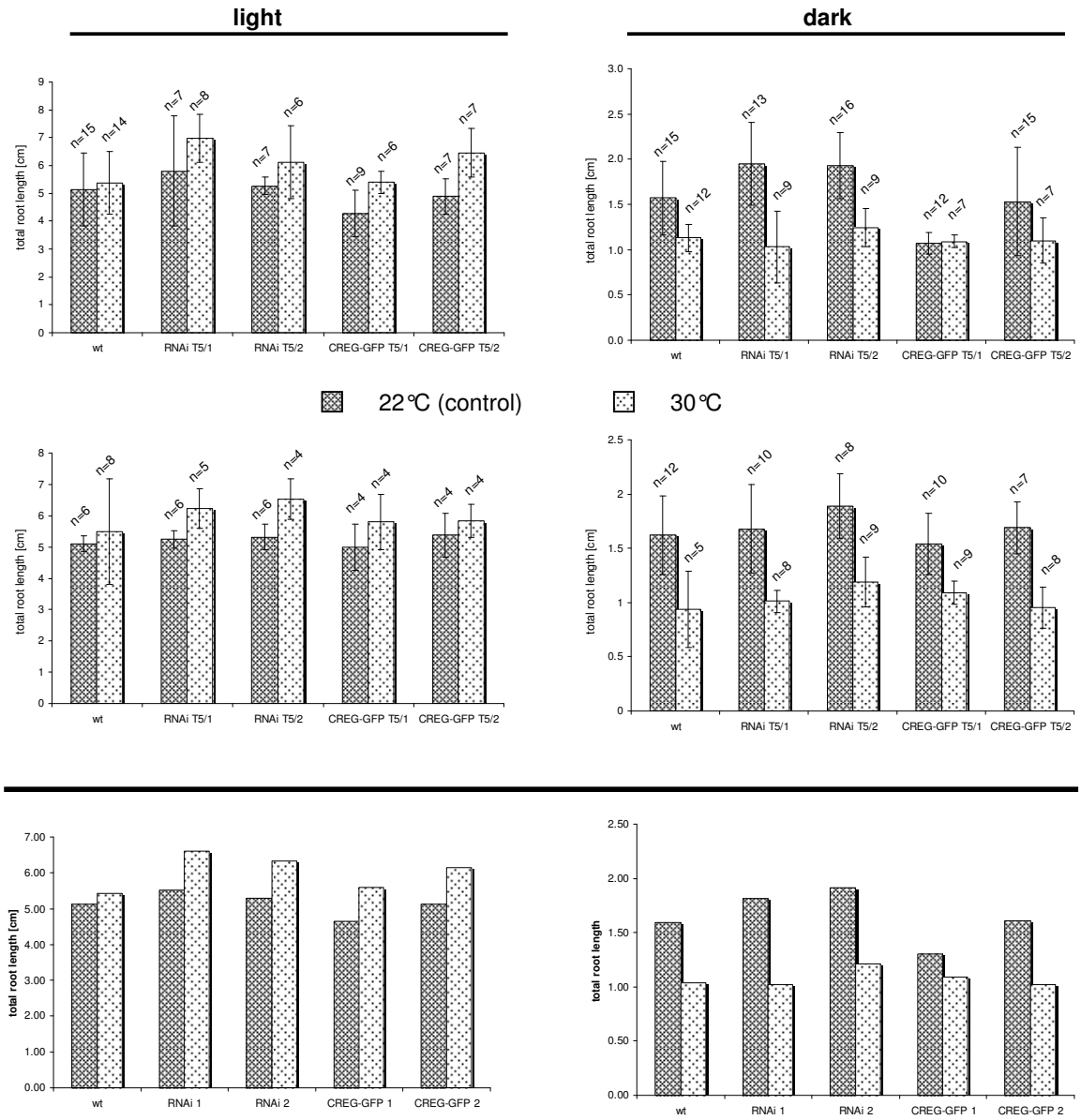

Figure 30: Heat stress experiment in *A. thaliana*. A and B: two independent repetitions of the experiment. Bars show total root lengths. Error bars indicate standard deviations. n, number of analysed seedlings. C: mean of experiments A and B.

Figure 31 shows *A. thaliana* seedlings of the heat stress experiment. No obvious phenotype can be seen in the aCREG knock-down seedlings. As there is also no variation in the root lengths of the different lines, there is probably no involvement of aCREG in the heat response in *A. thaliana*.

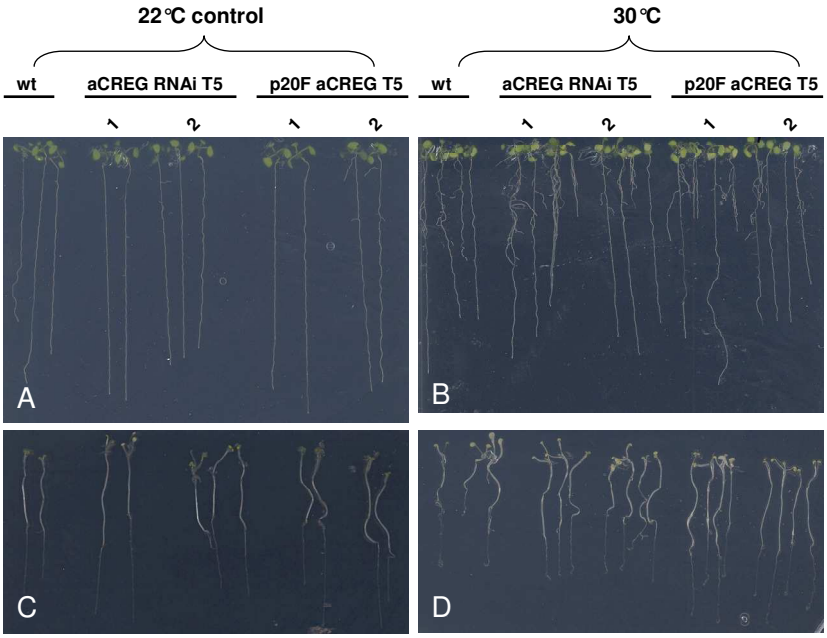

Figure 31: A. thaliana seedlings of heat stress experiment. Seedlings were transferred on agar plates and imaged after root length was measured. **A** and **B**: seedlings cultivated in the light. **C** and **D**: seedlings cultivated in the dark.

Discussion

4.1 Subcellular localization of aCREG

In mammalian cells, CREG has been shown to reside in the lysosome, but the way it influences cell growth and differentiation is still unclear. In plant cells two proteomic studies have led to the identification of CREG in different subcellular fractions: Once it was found in the vacuole and once in the cell wall (Carter *et al.*, 2004, Irshad *et al.*, 2008). Microarray analysis has demonstrated that CREG is expressed in most *A. thaliana* tissues with upregulation in seeds (www.arabidopsis.org). It is supposed that plant CREG resides in vacuoles, as these compartments are the plant homologues of mammalian lysosomes, although the sequence of the aCREG gene contains no known vacuolar targeting signals (Marty, 1999). To prove that aCREG is localized in the vacuole, two fusion constructs (aCREG-GFP and aCREG-mRFP) were constructed and expressed in *N. benthamiana* and *A. thaliana*.

Transient expression in *N. benthamiana* has the advantages of high expression levels and the possibility to generate high resolution images due to the large size of leaf epidermal cells. A possible disadvantage is that intracellular protein trafficking can be negatively affected by the achieved high expression levels. Stable expression in *A. thaliana* has the advantage of the transgene to be transmitted stably to the progeny, as it is integrated into the plant genome, but less efficient transformation rates, compared to transient expression in *N. benthamiana*, make the method more labour intensive and time consuming. In this project, both approaches were used to analyse the subcellular localization of CREG in plants.

Localization studies of aCREG-GFP in *N. benthamiana* show vacuolar localization of aCREG. When plants were incubated in the light, GFP-degradation rendered localization analysis under the confocal microscope impossible. In this case, addition of E64d, a cysteine protease inhibitor, led to slight inhibition of light-induced degradation and thus stabilized the fusion construct. After addition of E64d, aCREG-GFP was detected in the vacuoles even when the plants were cultivated in the light. E64d has been shown earlier to be able to stabilize vacuolar GFP (Tamura *et al.*, 2003).

Expression of aCREG-mRFP in *N. benthamiana* gave a different outcome: aCREG-mRFP is located in the apoplast. Analysis of the interstitial fluid, where secreted proteins can be found, proved aCREG-mRFP to be actively secreted. The question, why aCREG-mRFP is being secreted whereas aCREG-GFP is transported to vacuoles, could not be answered during this work. It is possible that most of the detected fluorescence is caused by free / cleaved mRFP or GFP. Treatment of the cells with *Brefeldin-A* (BFA) proved both fusion constructs to be targeted to the secretory pathway. BFA treatment of *N. benthamiana* cells leads to fusion of the ER membranes with the Golgi network. Interference with the formation of COPI vesicles leads also to an inhibited anterograde protein transport mediated by COPII, and secretory proteins thus become trapped in the ER. As both aCREG-GFP and aCREG-mRFP became trapped in the ER, they are both targeted to the secretory pathway, but for some reason only aCREG-GFP is then targeted to the vacuole.

Western blot analysis of the expressed fusion proteins in *N. benthamiana* revealed that a vast amount of them is being degraded into free aCREG and GFP/mRFP. Treatment of the leaves with E64d could not stabilize the aCREG-mRFP fusion protein, but stabilized aCREG-GFP. Therefore it can be said, that light-induced degradation of aCREG-GFP is probably due to the action of cysteine proteases, whereas aCREG-mRFP degradation is carried out by other proteases.

Subcellular localization of aCREG-mRFP in *A. thaliana* again showed aCREG to reside in the vacuole. Further evidence for the vacuolar localization was obtained by plasmolysis of the cells as aCREG-mRFP fluorescence was observed only in the shrinking protoplasts.

Localization of the aCREG-GFP construct was unfortunately impossible, as the expression of the transgene was too low to be detected by CLSM analysis of stably transformed *A. thaliana* plants. This is probably due to gene silencing effects. The transcription of the transgene is under the control of the 35S promoter from cauliflower mosaic virus. This ensures a high-level constitutive transcription of the transgene but can also lead to transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) in plants (Waterhouse and Helliwell, 2003). TGS is associated with methylation of the promoter region and involves inhibition of transcription, whereas PTGS is associated with methylation of the coding region

of the gene and the genes are transcribed but their mRNA is degraded. Both effects have been associated with high-copy-number transgenes and could present mechanisms of plants to safeguard themselves from viruses, as infected plants are known to accumulate viral transcripts at very high level.

The vacuolar localization of aCREG was further confirmed by subcellular fractionation of *A. thaliana* root extracts from Columbia-0 wildtype and an aCREG-RNAi line (negative control). Subcellular fractionation showed endogenous aCREG to be localized in the fractions with low density as typical for soluble vacuolar proteins. This approach also revealed endogenous aCREG to be processed in the cells as the aCREG form detected was of a lower molecular weight that expected.

In conclusion it can be said that in three out of four cases studied, aCREG has been found to be located in the vacuole, which provides strong evidence for its localization in this compartment.

4.2 Functional characterization of aCREG

As CREG has been implicated to participate in signalling pathways in mammalian cells, its influence on the metabolic response to abiotic stress in plants was examined. For this, *A. thaliana* seedlings were kept under salt, sucrose and heat stress conditions. Plant mutants in several signalling pathways have been shown to be defective in salt and heat tolerance (Larkindale *et al.*, 2005, Quesada *et al.*, 2000). It has been also demonstrated that plants change their transcriptional program in response to sucrose (Koch, 1996).

The results obtained during this work do not prove any participation of aCREG in metabolic responses to salt, sucrose or heat stress. The phenotypic analysis of wildtype and knock-down seedlings shows no aCREG-dependant effects. Either aCREG is not involved in these processes, or the residual aCREG levels are sufficient to maintain its function. Also, the p20F aCREG-GFP line, which was believed to be an aCREG overexpresser, turned out to have aCREG expression levels comparable to wildtype and could therefore not be used in the intended way. Therefore it is impossible to make a statement on the physiological function

of aCREG yet, and further investigations into the biological processes in which it participates have to be undertaken.

4.3 Future Prospects

It would be of great interest to finally define the physiological function of CREG in plants. Phenotypic analysis would be facilitated if knock-out *A. thaliana* lines were available for analysis. The knock-down plants used in this work show aCREG expression below detection limit, but expression may still vary between different generations, plants of the same generation or even cells of the same plant, and although low, could be enough to fulfil its function in the cell. A knock-out mutant line, created by T-DNA insertion mutagenesis or point-mutations (e.g. by TILLING) could provide more insight into the biological function of CREG. For the same reason a true overexpressing line would be useful.

Another approach to investigate the signalling pathways in which aCREG participates would be to find possible interaction partners. This could be achieved by yeast-two hybrid screens or co-immunoprecipitation. It can be envisaged that the identification of interaction partners would lead to new insights into the functions of aCREG.

Substantial advance has been made in the research into the functions of CREG in mammalian systems and the results obtained there may also help to understand the role of CREG in plants.

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