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# Elucidating interactors of bZIP19, the secret of the ZDRE

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# Contents

# Summary

Zinc is an essential element for plants and humans, zinc deficiency can pose a serious problem for human health. Regulation of the zinc deficiency response in *Arabidopsis thaliana* is two transcription factors, bZIP19 and bZIP23. These transcription factors bind a zinc deficiency response element and activate amongst others zinc transporter genes. Here, I describe the search for interactors of bZIP19 using electrophoretic mobility shift assays. I find that possibly TCP21 (At5G08330) is an interactor of bZIP19, linking zinc deficiency to the circadian clock. I also report the specific binding of At5g26280, a member of the TRAF-like protein family, and SUVH9 (At4G13460), a suppressor of variegation, to the zinc deficiency response element. This suggests that the zinc deficiency response element is not necessarily zinc responsive, but could also be a more general response element.

# Chapter 1

# Introduction

## 1.1 General Intro

Zinc is an essential element for all plants. It is a component of many proteins and many other proteins require zinc binding for their function. This makes plants sensitive for a deficiency of zinc. However, also an excess amount of zinc is detrimental for the plant (Scaife et al., 1983). Excess amounts of zinc in the soil mostly occur around industrial areas and is often caused by dumping of waste in nature (Nriagu, 1996). Zinc deficient soils can be divided in two categories, soils with a natural low amount of zinc and soils with a low available amount of zinc for the plant. This occurs for example when zinc binds other soil components (Barrow, 1993). Zinc deficiency affects the plant in multiple ways, like chlorosis, necrosis and reduced growth (Marschner and Rimmington, 1988; Hussain et al., 2004) resulting in reduced crop yield.

Zinc deficiency can also affect humans. In humans Zn deficiency can cause symptoms like diarrhoea, a higher risk to inflammation and Zn plays an important role in development (Hambidge, 2000). The uptake of zinc for humans comes partially from plants. Especially in third wold countries where, for example, rice or maize are used as a staple food, zinc uptake comes mainly from their staple diet. Therefore zinc deficiency in plants is also a serious problem for human health. WHO estimates that around one third of the world population is suffering from zinc deficiency (Caulfield and Black, 2004). The areas with widespread zinc deficiency mostly overlap with soils with low (available) zinc content (Alloway, 2009).

Over the last decade lots of research has been done to try and discover the mechanisms of zinc homeostasis in the plant (Sinclair and Krämer, 2012). Most of this research used the plants *Arabidopsis thaliana* or a zinc hyper accumulator like *Arabidopsis halleri* or *Nocceae caerulescens*. These are ideal species for this research (Assunção et al., 2003; Bert et al., 2000), because they are closely related to *A. thaliana* and share some of its characteristics, like their small size. It is known that *ZIP*-genes (*ZRT*, *IRT*-like Proteins) are involved in the uptake and transport of zinc (Guerinot, 2000). 15 *ZIP*-genes have

been identified in *A. thaliana*. ZIP 1 and 3 are necessary for the uptake of zinc and ZIP 4 is important for the transport of zinc through the plant (Grotz et al., 1998). ZIP7, ZIP11 and ZIP12 are also involved in zinc transport and ZIP5, ZIP6, and ZIP9 can transport manganese (Milner et al., 2013). In addition to the *ZIP* genes, other gene families are also important for zinc homeostasis. HMA2 and 4 (Heavy metal ATPase) are involved in transporting zinc into the xylem from neighbouring root cells. YSL (yellow stripe like) family proteins transport metal-nicotianamine (NA) complexes (Bloß et al., 2002). Thus nicotianamine synthase (NAS) also influences YSL2 as YSL2 needs NA to function. *YSL2* is known to be down regulated under zinc deficiency (DiDonato et al., 2004). Also a NAS protein is influenced by zinc deficiency, *NAS2* expression is strongly increased under zinc deficiency. Recently, two transcription factors, bZIP19 and bZIP23 were identified that are crucial for genes involved in the zinc deficiency response (Assunção et al., 2010).

## 1.2 bZIP19 and bZIP23

In this research I will focus on the bZIP transcription factors. bZIP19 and bZIP23 are transcription factors from a large gene family in *Arabidopsis*. The bZIP family consists of 75 genes in *Arabidopsis* so far. The proteins are divided in classes based on sequence similarity and the presence of certain motifs. bZIP19 and bZIP23 are in the F-class together with bZIP24 (Jakoby et al., 2002). The F-class is a fairly uncharacterized group. It was shown that bZIP19 and bZIP23 have a very similar amino acid sequence and they both also share considerable similarity with bZIP24, albeit to a lesser extent. The three members of the F-class are also slightly different in size; bZIP19 is 261aa, bZIP23 is 249aa and bZIP24 is 229aa. Most bZIP transcription factors form dimers. They can form either homodimers or heterodimers with other bZIPs (Hurst, 1993). It has been shown that bZIP19 and bZIP23 form homodimers (Assunção et al., 2010), and although it is difficult to demonstrate it is widely assumed that they also form heterodimers. bZIP19 and bZIP23 induce expression of ZIP-genes, including *ZIP1, ZIP3 ZIP4, ZIP5* and *ZIP9* as well as other genes involved in the zinc homeostasis. Here, bZIP19 and bZIP23 differ from bZIP24 as bZIP24 functions in the soil salinity stress response (Yang et al., 2009) and not in the zinc deficiency response.

bZIP19 and bZIP23 regulate the gene expression by binding to a palindromic sequence 'RTGTCGACAY', called the zinc deficiency response element (ZDRE) (Assunção et al., 2010). This differs from other bZIPs that mostly bind to short palindromic sequences with a core of 'ACGT' (Izawa et al., 1993). Most of the genes that are known to be influenced by bZIP19 and bZIP23 have a ZDRE in their promoter. bZIP19 and bZIP23 are mostly redundant in function. Only a double knockout mutant of bZIP19 and bZIP23 is unable to control the zinc deficiency response. However, it seems that the bZIP19 single mutant is slightly more sensitive to zinc deficiency (Assunção et al., 2010). This demonstrates

there is a difference between bZIP19 and bZIP23, but it is not yet understood what this difference is. It is also still not understood how bZIP19 and bZIP23 sense zinc deficiency and respond to it (Assunção et al., 2013) and whether bZIP19 and bZIP23 interact with other proteins to influence gene expression.

## 1.3 Putative interactors

Previously, an immunoprecipitation experiment coupled to mass spectrometry identified several putative interactors of bZIP19 (appendix A.1). Herein, a bZIP19 and bZIP23 double mutant line was transformed with bZIP19-GFP. bZIP19-GFP was able to complement the zinc deficiency phenotype in the *m19m23* line (unpublished data). Three putative interactors were chosen for validation of interaction with bZIP19. These were chosen based on function, location in the cell and other available information on the TAIR database, like certain domains or zinc binding quality.

### 1.3.1 TCP21

TCP21 (At5G08330) is a member of the TCP gene family. The TB1, CYC, PCFs (TCP) family is named after the first three members of the family (Kosugi and Ohashi, 2002). TCP21 is also known as CCA1 hiking expedition (CHE). TCP21 is present in the nucleus of the cell. It is a transcription factor and negatively regulates *circadian clock associated 1 (CCA1)* (Pruneda-Paz et al., 2009). CCA1 is a transcription factor that can form a dimer with long hypocotyl (LHY). As a heterodimer CCA1 and LHY can bind the promotor of *Timing of Cab expression 1 (TOC1)* and act as repressors of *TOC1* gene expression (Alabadı et al., 2001). CHE/TCP21 interacts with TOC1 and binds the promotor of *CCA1*, acting as a repressor of gene expression (Pruneda-Paz et al., 2009). The four genes involved are in a negative feedback loop with each other.

### 1.3.2 SUVH9

Suppressor of variegation 3-9 homolog 9 (SUVH9, At4G13460) is a protein that functions in RNA directed DNA methylation. SUVH9 has a SET (Su(var)3-9, Enhancer-of-zeste and Trithorax) domain, which is involved in histone binding. SUVH9 also has a pre-SET domain involved in zinc binding. SUVH9 functions especially in Histone 3 lysine 9 dimethylation (H3K9Me2) (Kuhlmann and Mette, 2012). SUVH9 is localized in the nucleus.

### 1.3.3 At5G26280

At5G26280, hereafter to be called TRAF280, is a TRAF-like family protein. TRAF stands for Tumor necrosis factor receptor associated factor. Members of this family are often involved in signal transduction during stress response (Chung et al., 2002). It is however unknown what the function of TRAF280 is. It is speculated that TRAF280 functions in metal transport (Heyndrickx and Vandepoele, 2012). TRAF280 is probably localized on the plasma-membrane, and is not known to be present in the nucleus (Mitra et al., 2007). TRAF280 also seems to be associated with the Arsenate stress response (Abercrombie et al., 2008). TRAF280 is closely related to At5G26290 (TRAF290), another TRAF-like family protein, which is adjacent to TRAF280 in the genome.

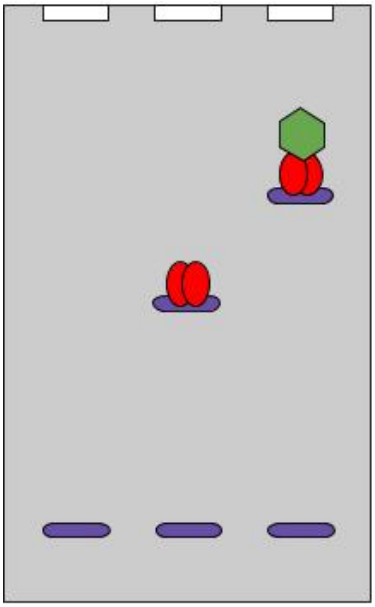

Figure 1.1: EMSA explanation. The blue bars represent the tagged DNA fragments, the red ovals are bZIP19 and the green hexagon is a putative interactor of bZIP19. They are depicted on the gel (grey and white).

## 1.4 Aim of the research

The aim of this research is to validate the interactors of bZIP19 described before in section 1.2. I will try to confirm these interactors via the electropheritic mobility shift assay (EMSA). EMSA is a technique to study binding of proteins to DNA. When a protein is bound to the DNA element, it will migrate slower through a gel, causing a 'shift'. In the presence of a successful interaction between a putative interactor and bZIP19 the DNA-protein complex should migrate even slower, causing a 'supershift'. A model of EMSA is shown in figure 1.1. I decided to elaborate on previous findings for bZIP19 and bZIP23 where bZIP19 as a transcription factor binds to the ZDRE. I will try this by proving bZIP19 interactions, while bZIP19 is bound to the ZDRE.

# Chapter 2

# Materials and Methods

## 2.1 Cloning

The coding sequence (CDS) of *TCP21, SUVH9* and *TRAF280* was amplified from an *Arabidopsis thaliana* cDNA library of *bzip19bzip23* double mutant plants transformed with bZIP19-GFP grown on low zinc medium. The plants suffered from zinc deficiency and all genes involved in the zinc deficiency response should be expressed. Thus the transcripts of the interactors of bZIP19 should be present when all this is extracted. The CDS was cloned into the vector pSPUTK, insertion sites and a vector map are shown in figure 2.1.

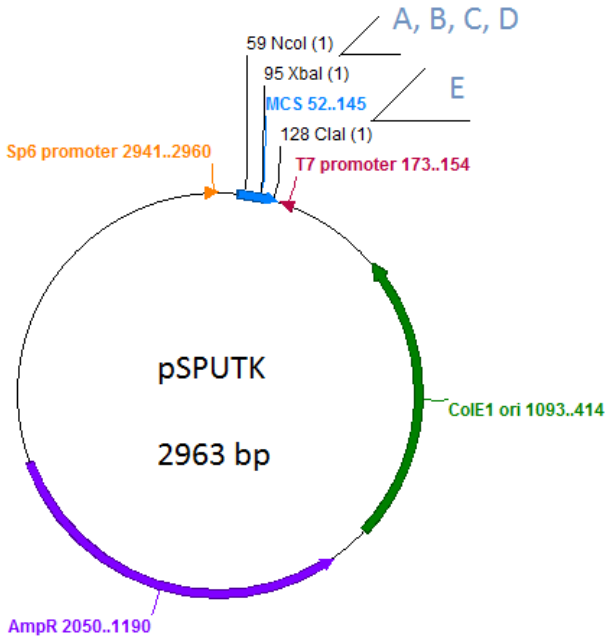


Figure 2.1: Vector map of pSPUTK, with insertion site of the genes between NcoI and XbaI; and XbaI and ClaI. A = bZIP19, B = bZIP23, C = TCP21, D = SUVH9, E = TRAF280. AmpR is the site of ampicillin resistance. ColE1 ori is the origin of replication. MCS is the multiple cloning site. The Sp6 and T7 promoter sites act as promoter for expression of genes cloned into the MCS.

### 2.1.1 Primer Design

Primers were designed to generate the full length transcripts including restriction sites. Where possible we used the same restriction sites as previously described by Assunção et al. (2010) used for cloning bZIP19 and bZIP23, namely NcoI and XbaI. Because the CDS of TRAF280 did not start with ATGG which is required for NcoI digestion the forward primer got extended with an XbaI site and the reverse primer with a ClaI site. The primers are listed in table 2.1.

Table 2.1: Primers. Capital letters represent part of the CDS, the underlined part is the restriction site and small letters are added to accommodate binding of the restriction enzymes. The primers were ordered at Biolegio.

| **Gene** | **Forward primer (5'-3')** |
|---|---|
| TCP21 | gttaccATGGCCGACAACGACGGAG |
| SUVH9 | gttaccATGGGTTCTTCTCACATTC |
| TRAF-like (At5g26280) | tgtatctagaATGAATAATCAAAAATGGAG |

| **Gene** | **Reverse primer (5'-3')** |
|---|---|
| TCP21 | tcgctctagaTCAACGTGGTTCGTG |
| SUVH9 | tcgctctagaTTAATTACAAATGGCAAGCT |
| TRAF-like (At5g26280) | tcgcatcgatTTAAGCGGAGACGAT |

### 2.1.2 PCR

Gradient PCRs were performed to find optimal conditions for cloning of the genes. A mix for PCR was prepared as stated in table 2.2 2 µL template cDNA was added to 18 µL mix. The mixture was subjected to the programme as described in table 2.3 in a Bio-Rad T100 thermal cycler. The PCR was performed at four different temperatures, namely 65, 63, 58.8 and 55.7 °C.

**Gel-electrophoresis**

3 µL of the PCR-product was mixed with 7 µL mQ and 2 µL 6x Loading Dye (Thermo-Scientific). This mix was loaded on a 1 % agarose gel and run at 100 V for 45 min. Also a DNA ladder (Thermo-Scientific) was loaded on gel. The gel was post-stained with gel red for 30 min. After staining a picture was made of the gel.

Colony PCR was performed on the colonies retrieved from transformation (section 2.1.6). To each 25 µL reaction an *E. coli* colony was added with a sterile toothpick/pipette tip. The same primers were used as for the PCRs before. A different mix was used for the colony PCR. the new mix is as stated in table 2.4. The PCR conditions were as described above in table 2.3. The PCR-product was analyzed on a 1 % agarose gel.

Table 2.2: PCR-mix (Thermo-Scientific)

| **Component** | **20 µL reaction** |
|---|---|
| 5x Phusion buffer | 4 µL |
| 10mM dNTPs | 0.8 µL |
| 10 µM Forward Primer | 1 µL |
| 10 µM Reverse Primer | 1 µL |
| cDNA | 2 µL |
| Phusion DNA polymerase | 0.2 µL |
| MilliQ | 11 µL |

Table 2.3: PCR conditions

| **Cycle step** | **Temperature** | **Time** | **Cycles** |
|---|---|---|---|
| Initial Denaturation | 98 °C | 30s | |
| Denaturation | 98 °C | 10s | 34x |
| Annealing | 65-55 °C | 30s | |
| Extension | 72 °C | 1min. | |
| Final Extension | 72 °C | 5min. | |
| Hold | 12 °C | $\infty$ | |

Table 2.4: Colony PCR-mix (Promega)

| **Component** | **25 µL reaction** |
|---|---|
| 5x GoTaq buffer | 5 µL |
| 10 mM dNTPs | 0.5 µL |
| 10 µM Forward Primer | 0.5 µL |
| 10 µM Reverse Primer | 0.5 µL |
| GoTaq DNA polymerase | 0.125 µL |
| MilliQ | 18.375 µL |

### 2.1.3 DNA clean-up and nanodrop

The PCR-product was purified with the nucleospin, gel and PCR cleanup from Macherey-Nagel according to the manufacturer instructions and eluted with 15 µL buffer NE from the kit. 1 µL of sample was pipetted on a nanodrop to measure the DNA concentration.

### 2.1.4 Digestion and ligation

pSPUTK and the PCR-products were digested with the corresponding restriction enzymes (pSPUTK with both XbaI+ClaI and XbaI+NcoI). The digestion was performed in a mix consisting of 1 µL of each restriction enzyme (NEB), 5 µL NEBuffer Cutsmart, 1 µg DNA

and complemented to a total of 50 µL with milliQ. This mix was incubated at 37 °C for 1 hour and the reaction was inactivated at 80 °C for 20 min. The digested DNA was purified and the concentration measured on nanodrop as described before in section 2.1.3.

Ligation was performed with a desired vector:insert ratio of 1:3. The amounts to be used for the ligation were calculated by formula 2.1. The vector and insert were mixed together in a total of 8 µL, if the concentration of the products were so that the desired amounts together were less than 8 µL, the mix was complemented with mQ. To this mixture 1 µL 10x T4 Ligase Buffer from Promega and 1 µL Ligase were added. The mix was incubated o/n at 4 °C.

$$\text{ng Insert} = 100 \text{ ng Vector} * \frac{\text{kb Insert}}{\text{kb Vector}} * \frac{3}{1} \tag{2.1}$$

### 2.1.5 Making competent cells

An *E. coli* TOP10 strain was plated on a LB agar plate without antibiotics and grown o/n at 37 °C. A colony from the plate was inoculated in 5 mL liquid LB medium (10 g/L Bacto-Tryptone (BD), 5 g/L Bacto-Yeast extract (BD), 10 g/L NaCl) and incubated o/n at 37 °C shaking at 250 rpm. The preculture was added to 500 mL LB medium without NaCl to reach an OD600 of 0.1. The *E. coli* culture was grown at 37 °C shaking at 250 rpm until the OD600 was just below 0.6. The culture was transferred to two sterile precooled 250 mL tubes. All the following steps are performed with sterile ice-cold material. The culture was centrifuged for 10 min. at 4000 rpm in a Beckmann centrifuge at 4 °C. After spinning the supernatant was poured off and the bacterial pellet was resuspended by swirling in 2x250 mL mQ. The centrifugation and resuspension step were repeated and the culture was centrifuged again. After pouring off the supernatant the pellet was resuspended in 40 mL 8.7% Glycerol. The mixture was transferred to 50 mL tubes and centrifuged again. The supernatant was poured off again and the pellet was resuspended in 1.5 mL 8.7% Glycerol. The competent cells were divided in 80 µL aliquots in precooled Eppendorf tubes and quickly frozen in liquid nitrogen. The frozen aliquots were stored for use at -80 °C.

### 2.1.6 Transformation

The ligated plasmids were transformed into *E. coli* (TOP10 (Thermo-scientific, NEB5alpha or NEB10beta (NEB)).

**Heatschok**

The competent cells were thawn on ice and ligation mixture was added (1-5 µL, max 100 ng). Cells were incubated on ice for 30 min. A heat shock of 42 °C was applied for 30 s, afterwards the cells were incubated on ice again for 5 min. 950 µL of SOC medium (2% bacto-tryptone (BD), 0.5% yeast-extract (BD), 10 mM NaCl, 2.5 mM KCl, 10 mM

MgCl2, 20 mM glucose) was added and the mix was transferred to a 12 mL tube and incubated at 37°C shaking (250 rpm) for one hour. 100 µL of this mix was plated out on agar plates containing 100 µg/mL Ampicillin (Amp). The rest of the mix (900 µL) was centrifuged for 3min. at 8000 rpm. 800 µL of the supernatant was discarded and the pellet was resuspended in the residual 100 µL. This 100 µL was plated out as stated above. The plates were placed o/n at 37 °C.

**Electroshock**

The home made competent cells were thawn on ice and divided into 40 µL aliquots. A plasmid (1-10 µL, max 100 ng) was added to the cells. The cells were transferred to an electroporation cuvette of 0.1 mm (BioRad). It was made sure that no air was underneath the sample in the cuvette. The cells were given a pulse of 2500 V by a bio-rad micropulser and immediately after 950 µL SOC medium was added. The cells were transferred to a 12 mL tube and incubated at 37 °C shaking at 250 rpm for an hour. Plating out was done in the same way as described above.

The recipe of the agar plates per 20 plates of 25 mL was: 0.5 L H2O, 5 g Bacto-Tryptone (BD), 2.5 g Bacto-Yeast extract (BD), 5 g NaCl, 4 g Agar (Duchefa). The pH of this mix was adjusted to 7 with either HCl or NaOH and the mixture was autoclaved. After cooling the mixture to 60 °C 500 µL 100 mg/mL Ampicillin was added. The media was poured in 9 cm petri dishes in a fumehood and allowed to solidify.

### 2.1.7 Miniprep

Colonies with a confirmed plasmid insert were selected from the plate and incubated in 5 mL liquid LB (10 g/L Bacto-Tryptone (BD), 5 g/L Bacto-Yeast extract (BD), 10 g/L NaCl) with Ampicillin (100 µg/mL) in a 12 mL tube o/n at 37 °C shaking (250 rpm). A miniprep was performed to retrieve the plasmid from the o/n culture. Using the Miniprep kit from Qiagen, the miniprep was performed to manufacturers instruction, only pelleting by centrifugation (the first step) was done at 4000 rpm for 6 min instead of 8000 rpm for 3 min. The concentration of the plasmids were measured by nanodrop.

### 2.1.8 Sequencing

The plasmids were send for sequencing at GATC biotech. The sample was prepared according to requirements by GATC biotech. Sequencing was executed with a Sp6 (5'-ATTTAGGTGACACTATAGAA-3') and T7 (5'-TAATACGACTCACTATAGGG-3') primer.

## 2.2 Protein work

### 2.2.1 Protein synthesis

The constructed plasmids were used to produce protein. This was done with the TnT SP6 High-Yield Wheat Germ Protein Expression System by Promega. The protocol described by Promega was downscaled five times to a 10 µL reaction, containing 4 µL plasmid and 6 µL Sp6 mix. The protein concentration was between 250 ng/µL and 500 ng/µL, the highest available concentration was used. The proteins used for the western blot had a slightly different protocol, in this reaction 0.25 µL transcend RNA (Promega) was added to the mix to be able to visualize the proteins (see section 2.2.3). The complete mix was incubated at 25 °C for two hours. For the EMSA the synthesis was altered. Since the goal was to make protein complexes, two plasmids (bZIP19 and an interactor) were mixed together in equimolar concentrations to a total of 4 µL.

### 2.2.2 SDS-PAGE

Two gels were prepared for SDS-PAGE. The SDS-PAGE gels contained the following ingredients as listed in table 2.5 These were mixed together, except for TEMED (Bio-rad) and Acrylamide/Bis (Bio-rad). These should be added to the mixture in a fumehood. After addition of Acrylamide/Bis the resolving gel was poured between two glass plates with a spacer of 1 mm and left to polymerize for at least 30 min. For a uniform surface 150 µL isopropanol was added on top of the gel. After polymerization the isopropanol was removed and the stacking gel was added on top and polymerized for 30 min. Pouring and polymerizing was also done in a fumehood.

Table 2.5: SDS-PAGE gel ingredients

| **Ingredients** | **12% resolving gel** | **4% Stacking gel** |
|---|---|---|
| H2O | 4,95 mL | 4,475 mL |
| 30% 29:1 Acrylamide/Bis | 6 mL | 1 mL |
| Tris-HCl | 3,75 mL | 1,875 mL |
| SDS | 150 µL | 75 µL |
| TEMED | 15 µL | 7,5 µL |
| APS | 150 µL | 75 µL |

Running buffer for SDS-PAGE contained 100 mL 10x Electrophoresis buffer (142.6 g/L glycine, 30.3 g/L Tris), 10 mL 10% SDS and 890 mL demiwater. The proteins (with tRNA) were prepared for SDS-PAGE by adding 10 µL of the protein to 60 µL SDS sample buffer (50 mM Tris-HCl (pH 6.8), 50 mM DTT, 1% SDS, 1 mM EDTA (pH 8.0),

0.005% bromophenol blue, 10% glycerol). This mixture was incubated for 10 min. at 95 °C. 14 µL of the sample mix was loaded on the gel. All elements were assembled in a miniprotean tetra sytem (Bio-rad) set-up. The gels were installed, running buffer was poured in between the gels and in the large compartment. The samples and a protein marker (pageruler, Thermo-Scientific were loaded on the gel). The gel was run at 20mA constant for 1 hr. Timing was determined by tracking of the protein marker.

### 2.2.3 Western blot

The SDS-PAGE gel was removed from the glass plates and placed upon three whatmann papers. On top of the gel a PVDF membrane was placed and on top again three whatmann papers. All the elements were wetted in blotting buffer (20% ethanol, 142.6 g/L glycine, 30.3 g/L Tris), before being stacked. The stack was assembled in a mini trans-blot (bio-rad) set-up. Blotting was done in a 4 °C room overnight running at 30 V.

**TnT blot**

The blot was removed from the set-up and incubated in TBS-T (0.02M Tris-HCl pH 7.5, 8.766 g/L NaCl, 0.05% Tween-20) shaking for one hour. The blocking solution was poured off and replaced with 15 mL TBS-T with 6 µL Streptavidin Alkalin Phosphatase and incubated for one hour shaking. The solution was discarded and the blot was washed two times with TBS-T and two times with H2O. 3 mL Western blue stabilized Substrate for Alkaline phosphatase was added on top of the blot and incubated for 15 min. in the dark. The blot was washed with H2O and a picture was made.

**IP sample**

The blot was removed from the set-up and incubated in 20 mL blocking solution (3% non-fat dry milk, TBS-T) for four hours at room temperature. The blocking solution was poured off and the blot was cut in lanes. The different lanes were incubated in 20 mL blocking solution with different 1:2.000 rabbit antibodies (CHE R1255 Rabbit Polyclonal Antibody, abiocode) overnight at 4°C. The blocking solution with primary antibodies was discarded and the blot was washed three times with TBS-T. Blocking solution with 1:15.000 secondary antibodies (goat anti-rabbit, company) was added to the blot and this was incubated for two hours on a roller. The blocking solution with secondary antibodies was discarded and the blot was washed three times with TBS-T. On top of the blot 1 mL 1:1 Stable Peroxide solution / Enhancer solution was added. Pictures were taken of the blot using a camera.

### 2.2.4 Binding of bZIP19 to ZDRE

Single-stranded oligo's with a DY-682 label were ordered from Biolegio to construct a labelled 3x ZDRE and a labelled 3x mutated ZDRE, in which the core 'TCGA' sequence was changed to 'TAGA'. The sequence of the non-mutated 3Z oligo's was: Forward

5'-DY682-TAATTCATGTCGACATATGTCGACATATGTCGACACGAGCT-3' and reverse 5'-DY682-TAGCTCGTGTCGACATATGTCGACATATGTCGACATGAATT-3'. The sequence of the mutated 3mZ oligo's was: Forward 5-DY682-AATTCATGTAGACATATG TAGACATATGTAGACACGAGCT-3 and reverse 5-DY682-AGCTCGTGTCTACATAT GTCTACATATGTCTACATGAATT-3 The two single-stranded oligo's were annealed by mixing 10 µL 50 µM forward and reverse oligo together with 5 µL anneal mix (50 mM Tris-HCl pH 7.5, 5 mM EDTA pH 8.0, 250 mM NaCl). This mix was incubated at 95 °C for 10 min. The annealed probe was slowly cooled down with 1 °C/min until 12 °C. The resulting probe is double labelled and present in a concentration of 20 mM. For the EMSA the probe was diluted to 4 pmol/µL (4mM).

Binding of the protein complexes to the probe was done in a binding mix consisting of 20 mM Tris-HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, 0.25 µg/µl BSA, 1 mM DTT and 0.25 µg/µl salmon sperm DNA. First a 2x buffer containing double the concentration of the binding mix was prepared. The final binding mix is made with 6 µL 2x buffer, 1.2 µL 80% glycerol, 2 µL protein (complex), 1 µL probe and 1.8 µL mQ. This mix is incubated for 30 min at RT.

### 2.2.5 EMSA set-up

Two gels were prepared for EMSA. The ingredients of the gel are as listed in table 2.6. The first ingredients are mixed and in a fumehood TEMED and Acrylamide/BIS was added. The gel was then poured between two glass plates with a spacer of 1.5 mm and allowed to polymerize for at least two hours.

Table 2.6: EMSA gel ingredients

| **Ingredients** | **5% gel** |
|---|---|
| H2O | 14.67 mL |
| 30% 37.5:1 Acrylamide/Bis | 3.33 mL |
| 10x TBE | 2 mL |
| TEMED | 16 µL |
| 10% APS | 200 µL |

The gel was assembled in the miniprotean set-up and the compartments were filled with TBE buffer (108 g/L Tris base, 55 g/L Boric acid, 7.4 g/L EDTA).

## 2.3 T-DNA analysis

To investigate the effect of the candidate genes on zinc deficiency, knock-out mutants of these genes in a Col-0 background were made by T-DNA insertion by 'The European Arabidopsis Stock Centre' (http://arabidopsis.info/). The specific T-DNA inserts per gene were found in the TAIR database. For TCP21 SALK_143403 was ordered, for TRAF280 SALK_042138 was ordered, for SUVH9 SALK_048093 and SALK_048033 was ordered. Also knockouts of TRAF290 were ordered, because of the possible redundancy in function between TRAF290 and TRAF280. The T-DNA lines that were ordered for TRAF-290 were SALK_146328 and SALK_059768.

### 2.3.1 Growing plants

Seeds were germinated in small petridishes with a wet filter paper inside. The petridishes were placed in a cold (4 °C) dark room for three days. After three days the petridishes with seeds were moved to an incubator at 24 °C and 16 hours light. After two days twelve seedlings per line were transferred to rockwool blocks and placed in the greenhouse and grown on Hyponex and watered regularly. After four weeks leaf material was harvested from the plants for DNA isolation.

### 2.3.2 DNA isolation

Plant material was harvested by cutting a leaf from the base. The leaf was put in a tube with 2 3 mm-glass beads and frozen in liquid nitrogen. After freezing the leaf tissue was disrupted by shaking for 2 min. at 30/s. 300 µL CTAB (20 g/L CTAB, 81.82 g/L NaCl, 0.1 M Tris, 20 mM EDTA) was added to the leaf tissue and incubated at 65 °C for 30min. The mixture was cooled to room temperature. It was centrifuged briefly and 300 µL chloroform was added in a fumehood and mixed. This mixture was centrifuged for 15 min. at 3250 rpm and the upper layer was transferred to new eppendorf tubes with 200 µL 2-propanol. The new mixture was centrifuged again for 15 min. at 3250 rpm. The supernatant was removed and the pellet was washed twice with 200 µL 70% ethanol and centrifuged for 10 min. at 3250 rpm. After the second wash the pellet was allowed to dry and when the pellet was completely dry, it was resuspended in 100 µL mQ and stored in a -20 °C freezer. Concentration of the isolated DNA was measured with a nanodrop.

### 2.3.3 Determining genotype

The genotype of the plants was determined by PCR. Per line one PCR was performed with the LP and RP primer and one with the BP and RP primer. PCR was performed as described in table 2.3 and 2.4 Primers were: BP SALK 5'-ATTTTGCCGATTTCGGAAC-

3' BP SAIL 5'-TGTATTGGTAATTTGGGAAGATATAATAGGAAGC-3', the individual LP and RP primers are listed in table 2.7.

Table 2.7: t-DNA primers

| **Gene** | **T-DNA line** | **LP (5'-3')** |
|---|---|---|
| TCP21 | SALK_143403 | AGACTTGATTGAATGGCAATGATCAC |
| SUVH9.A | SALK_048093 | GTCCTTCAATCCTCTCCAACC |
| SUVH9.B | SALK_048033 | GTCCTTCAATCCTCTCCAACC |
| TRAF280 | SALK_042138 | GGTCTGTTTTGGCTTCTGTTG |
| TRAF290.A | SALK_146328 | TCACAGTTCACGAATGGTACG |
| TRAF290.B | SALK_059768 | ATCTTTTCCACCGTCCTTTTC |

| **Gene** | **T-DNA line** | **RP (5'-3')** |
|---|---|---|
| TCP21 | SALK_143403 | TAGTCAACACCGGAGTCAAGC |
| SUVH9.A | SALK_048093 | CAAACAAAACCCATTTCTTCG |
| SUVH9.B | SALK_048033 | CAAACAAAACCCATTTCTTCG |
| TRAF280 | SALK_042138 | TGAAAGTGGATTAGTCGTCGG |
| TRAF290.A | SALK_146328 | CCAGTATCGGTCTCCAACAAG |
| TRAF290.B | SALK_059768 | CCAAACTTCGAATACAAAAATCTG |

### 2.3.4 Acquiring seeds

The plants were dried for two weeks. The dry plants were crushed between paper. The seeds were filtered out of the crushed material by a seed collector. The seeds were collected in small plastic bags and kept for storage at RT.

# Chapter 3

# Results

## 3.1 Cloning

The putative interactors and bZIP19 and bZIP23 were cloned into pSPUTK using the method as described by Assunção et al. (2010). A vector map showing the insertion sites of the genes is shown in figure 2.1.

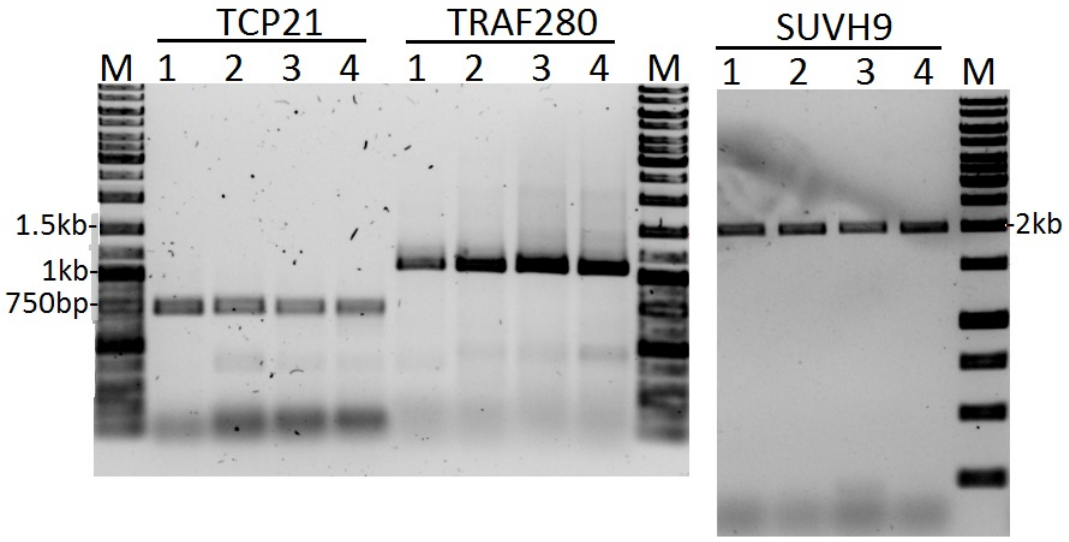


Figure 3.1: Gradient PCR gel, the numbers at the top stand for the different annealing temperatures, where 1 is 65 °C, 2 is 63 °C, 3 is 58.8 °C, 4 is 55.7 °C. The PCR products were analyzed on a 1% gel.

### 3.1.1 PCR

To amplify the CDS of the genes, cDNA from *A. thaliana* was used in PCR. The PCR product from the gradient PCR was analyzed on gel and this is shown in figure 3.1. TCP21 is visible on gel at a height of 750 bp, SUVH9 is visible around 1100 bp and TRAF280 around 2000 bp. This corresponds to the expected size of the products; 736 bp for TCP21, 1073 bp for TRAF280 and 1969 bp for SUVH9. It can be seen that the

amplified product is more intense at some temperatures. For further amplification of the genes the following annealing temperatures were chosen per gene; for TCP21 65°C, TRAF280 63°C and SUVH9 65°C.

### 3.1.2 Restriction and ligation

To check whether the restriction enzymes used for cloning are functional and whether the pSPUTK vector was alright, the pSPUTK vector was digested and analyzed on gel. The digestion of pSPUTK was successful as can be seen in figure 3.2. The expected size of the pSPUTK vector is 2963 bp. The digested vector shows a band at the correct size of about 3 kb. The undigested product runs in three different conformations on the gel. No other bands seem to be present with the digested vector, showing that there was no partial digestion. Only with the single ClaI digestion a vague band is present at 2.7 kb.

The PCR-products were also digested for cloning. The restriction sites used for this digestion are located at the very end of the PCR-product as they are not part of the CDS of the gene, but added by the primer. After digestion only about 8 bp were cut off at each side of the PCR-product. Therefore this was not put on gel, since the difference would be too small to see on gel.

**Ligation**

To adhere the digested vector and PCR-product, they were ligated. No confirmation of this was performed, but linear DNA cannot be transformed into *E. coli*. If colonies are present after transformation this is also a partial confirmation that ligation was successful. It is possible that the vector re-annealed. The chance of this is small, as the sequence overhangs created by digestion do not match. Transformation was performed successfully. After overnight incubation at 37 °C, colonies were growing on the plates with antibiotics.

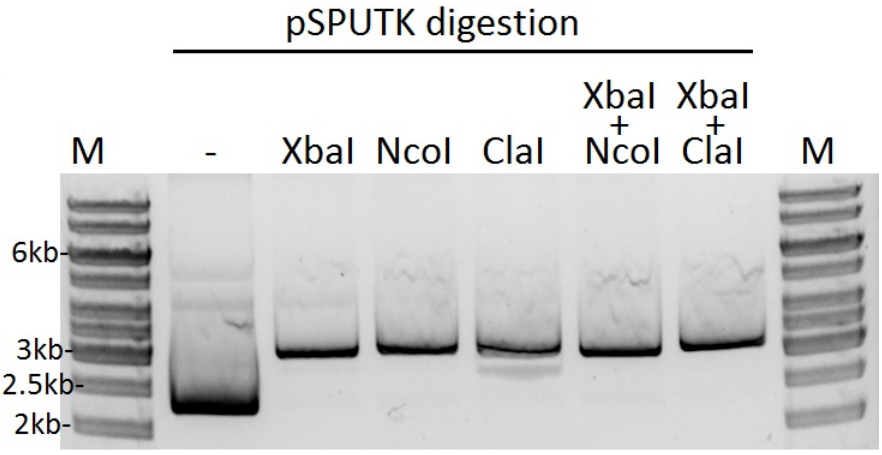


Figure 3.2: Digestion of pSPUTK. Single and double digestion with the required restriction enzymes for cloning.

### 3.1.3 Confirmation

After miniprep the plasmids were subjected to digestion. The restriction enzymes used for digestion were the same as the ones used XbaI + NcoI and XbaI + ClaI . With these restriction enzymes a clear seperation between backbone and insert will be made, resulting on gel in a band of the size of the backbone and one for the insert. This is easy to confirm on gel. The digested plasmids were analyzed on gel as can be seen in figure 3.3. For the TCP21.1 sample only single digestion took place. The other restriction enzyme probably did not work. However it can be seen that the plasmid is at a height of around 4 kb, which is the expected size for a linearized TCP21-pSPUTK plasmid (2969bp + 736 bp). In TCP21.2 both restriction enzymes worked. The upper band corresponds to pSPUTK and the lower band to TCP21. SUVH9.1,3 and 4 all show the expected bands. SUVH9.2 contains a partial digestion where one of the two restriction enzymes did not work optimal. So the top most band is a single digestion of SUVH9-pSPUTK and the bands below represent the double digestion. The digestion of TRAF280 looks as expected with the upper band representing pSPUTK and the lower band TRAF280.

As a second confirmation all the plasmids were also send for sequencing. This was also to see whether no mutations were introduced during PCR. No mutations were detected for the putative interactors.

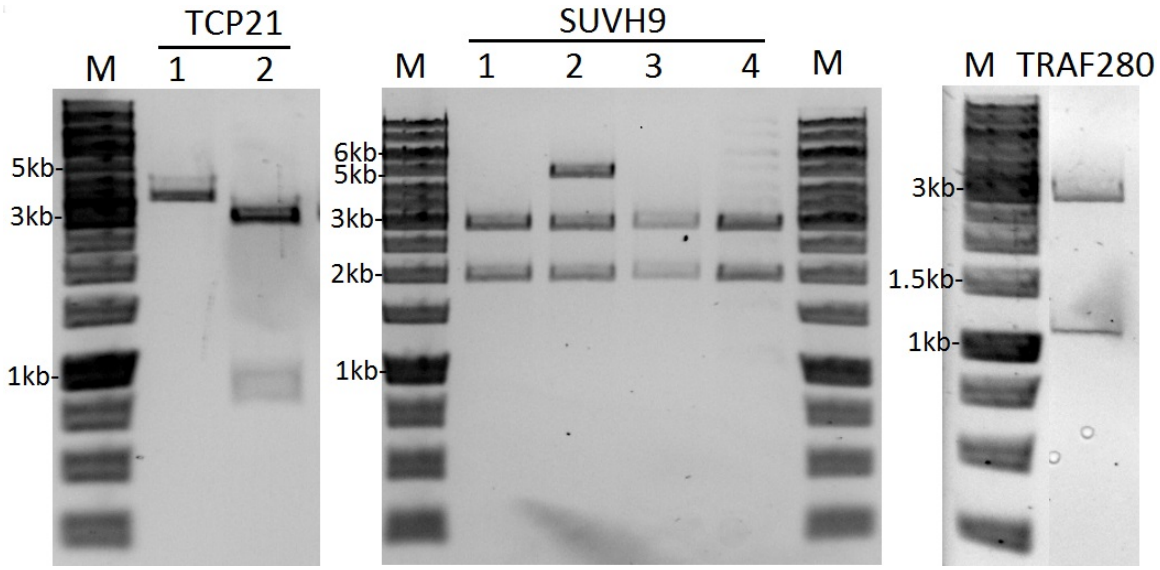


Figure 3.3: Digestion of the plasmids with the same restriction enzymes as used for cloning. With two plasmids for TCP21, four for SUVH9 and one for TRAF280. The upper band corresponds to the pSPUTK backbone and the lower band corresponds to the gene insertion. Only TCP21.1 shows just one band.

## 3.2 Western Blot

To verify the success of the *in vitro* synthesis of proteins, the presence of the proteins was verified with SDS-PAGE followed by western blotting. The proteins were visualized by their biotin label. The picture of this blot is shown in figure 3.4 . All proteins were present on the blot. However, the size of the proteins on the blot does not correspond to the expected size. bZIP19 is annotated to have a size of 28.6 kDa, but is on the blot at about 38 kDa. Also bZIP23 is present on the blot 10 kDa larger than its expected size; 36 kDa versus 27.3 kDa. The difference for TCP21 is a bit smaller, it is present on the blot at a height of 28 kDa, while its expected size is 24.7 kDa. SUVH9 has the biggest difference in size. It is expected to be 72 kDa, but on the blot it is present at 37 kDa. However it may also be present at 70 kDa, but at 70 kDa also a background band is present which might mask the presence of SUVH9. The predicted size of TRAF280 is 39.4 kDa and on blot it is around 36 kDa.

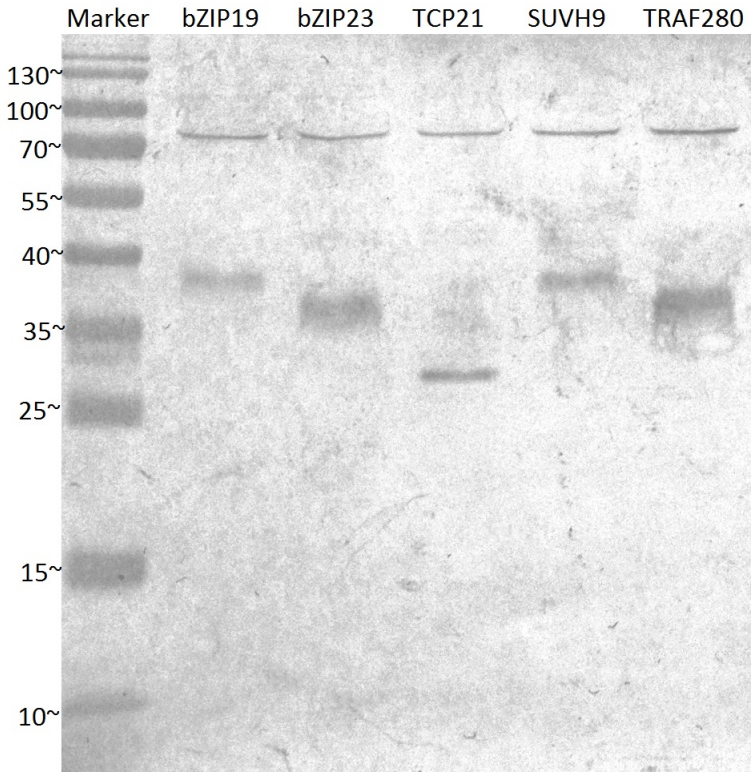


Figure 3.4: Western blot. Western blot of in vitro translated proteins. The lower band in each lane is suspected to be the protein. The upper band present in each lane is background coming from the synthesis of the proteins.

Figure 3.4 shows a newly cloned bZIP19. When the experiment started another pSPUTK-bZIP19 plasmid was used, that was already present in the lab. The old bZIP19 did not show such a bright band on western blot (figure 3.5C). Thus the bZIP19 plasmid was send for sequencing. The sequencing information revealed that the plasmid contained a strange insert at the start and end of the CDS of bZIP19 (figure 3.5A). There were no

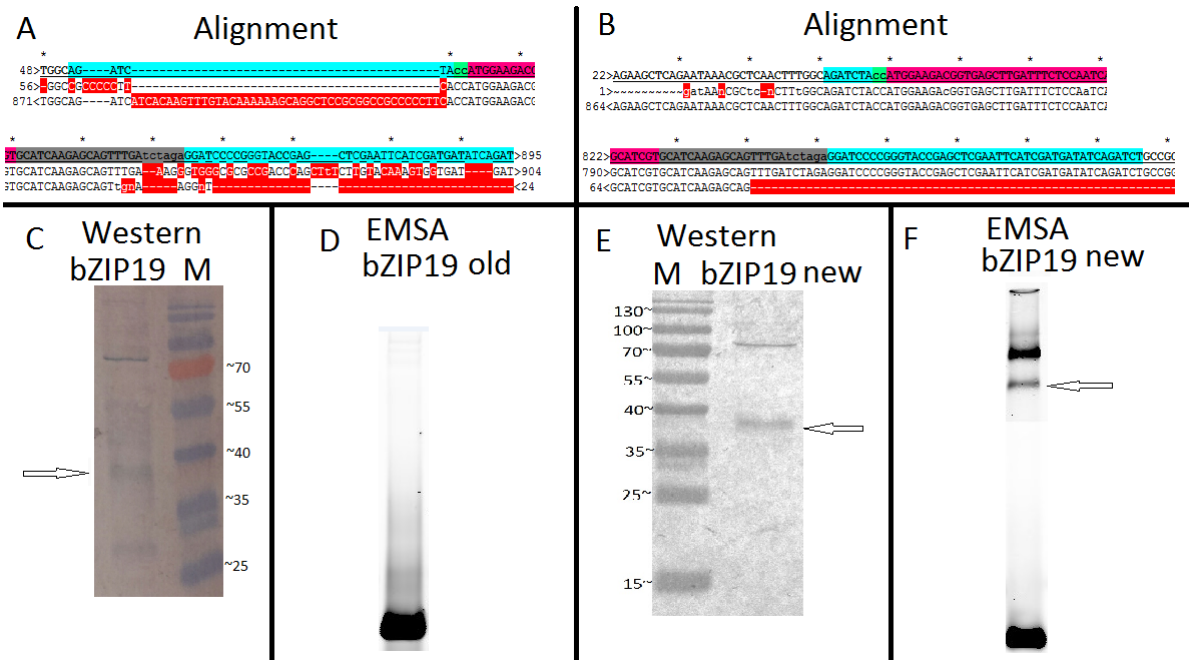


Figure 3.5: bZIP19-pSPUTK. A, B: alignment of pSPUTK-bZIP19 expected sequence (top line) to the sequenced plasmids (second line: Sp6 sequencing primer; third line: T7 sequencing primer). C, E: western blot of bZIP19 old and new. The expected band of bZIP19 protein is marked with an arrow. D, F: EMSA with the 3Z element and bZIP19. Suspected binding of bZIP19 to the ZDRE is marked with an arrow.

changes inside the coding sequence of the genes. However the insertion before the start coding might have caused the gene to be less or not responsive to the Sp6 promoter. This old plasmid was also used for EMSA (figure 3.5D) and no binding to the ZDRE can be observed then. The plasmid was recloned using the method described by Assunção et al. (2010) and send for sequencing again. The new plasmid corresponds to the expected plasmid (figure 3.5B). The new plasmid is more prominently visible on western blot and also shows binding to the ZDRE on EMSA (figure 3.5E, F)

## 3.3 Electrophoretic Mobility Shift Assay

### 3.3.1 EMSA

To validate interactions between bZIP19 and our genes and to reproduce results presented by Assunção et al. (2010) an electrophoretic mobility shift assay was performed. All in vitro produced proteins were used for EMSA. The results can be found in figure 3.6. The figure shows that bZIP19 and bZIP23 can bind to the ZDRE as expected. The binding band disappears when the proteins are mixed with the mutated element, showing that the binding is specific for the sequence of the ZDRE. Strikingly, also TRAF280 and SUVH9 show specific binding to the ZDRE and not to the mutated element. TCP21 does not show binding. In the lanes of bZIP19, bZIP23, SUVH9 and TRAF280 above the band that

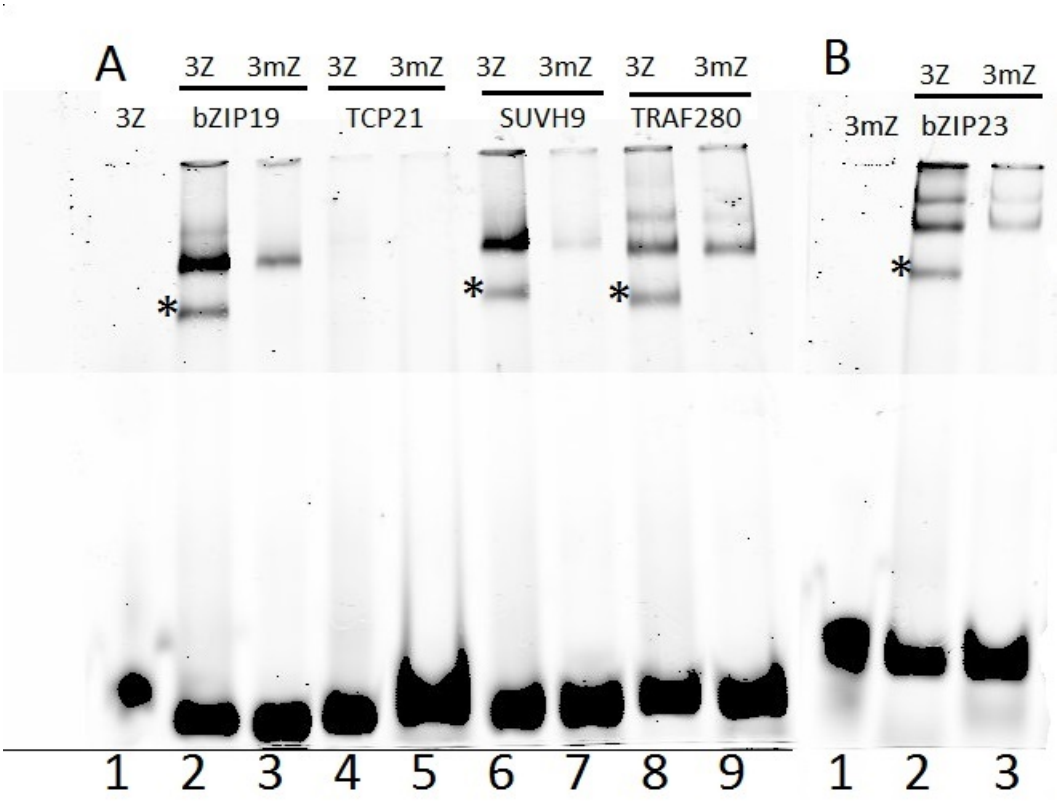


Figure 3.6: EMSA. Two gels are shown (A and B). Binding of a protein to the ZDRE is marked with an asterisk. The band at the bottom of the gel represents the dsDNA of the ZDRE or mutated ZDRE (all lanes). The control lane (lane 1) contains only the ZDRE. The line in the middle of the figure is caused by overlapping two pictures, to show the bands in better intensity.

represents binding another band is present. This band is present with both the normal and mutated element. So it is expected that this band is just background. However since it is not present in the control lane nor in the lanes of TCP21, it is not so easy negligible.

In figure 3.7 the results of the search for protein complexes is shown. The binding band of the protein interactions should be higher than the binding band of bZIP19, corresponding to a heavier molecular weight and thus a slower migration through the gel. However all bands appear at the same size. So in this EMSA no protein interactions are confirmed. The binding band of bZIP19+SUVH9 and bZIP19+TRAF280 is much thicker than the binding band of bZIP19+tcp21, probably due to the binding of both bZIP19 and TRAF280 or SUVH9 to the ZDRE versus the binding of only bZIP19 in the lane of bZIP19+TCP21.

The control lane in figure 3.6 and 3.7 is not a straight band because no glycerol is added to this lane. Glycerol is essential for weighing down the sample causing it to run smoothly and straight.

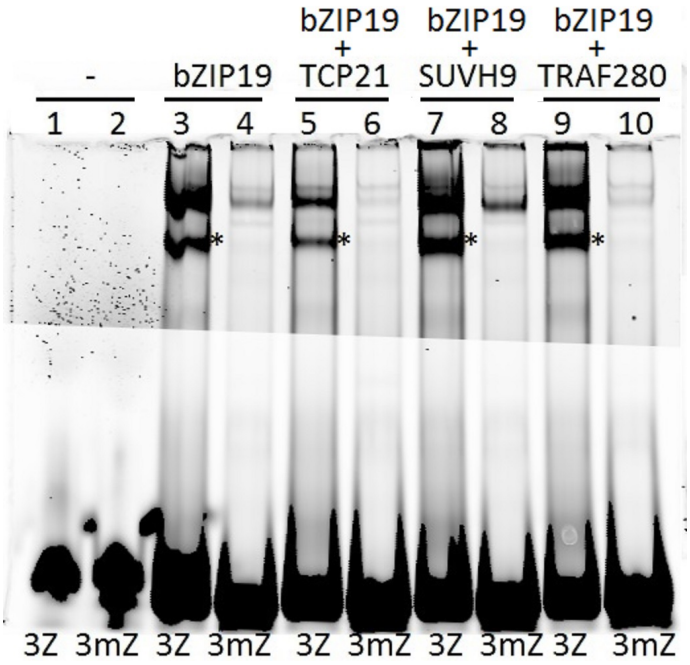


Figure 3.7: EMSA. Lane 1 and 2 contain only DNA. The asterisks represent specific binding to the ZDRE. The bands in lane 3 have a dimple because an air bubble was present in the slot of the gel. The slanting line in the middle of the figure is caused by overlapping two pictures.

### 3.3.2 TCP21

To further test whether TCP21 has an interaction with bZIP19 another experiment was performed. dr R. Akkers performed a Co-IP experiment with bZIP19-GFP following the protocol by Smaczniak et al. (2012), this resulted in a sample that should contain interactors of bZIP19. This sample we subjected to SDS-PAGE and western blot with specific TCP21 antibodies. The results can be seen in figure 3.8. A band at the predicted size of TCP21 is present on the blot.This could mean that contrasting to the results of the EMSA, TCP21 does interact with bZIP19.

## 3.4 T-DNA analysis

In total four T-DNA lines were grown and tested for their genotype. All of these were segregating lines, so these plants should have a segregation ratio of 1:2:1 wildtype (WT):heterozygous (HZ): homozygous (HM) for the t-DNA insert (SUVH9A, SUVH9B, TCP21 and TRAF280). Twelve plants per line were grown and using PCR with primers flanking the t-DNA insert it was tested what their genotype was.

Three of these lines seem to be WT (TRAF280, SUVH9A, SUVH9B). The TCP21 line looks to be all heterozygous. However the results of the other lines are also not so clear as only the TCP21 has confirmed inserts (Appendix B). It might be the case that the primer combination for the PCR of the t-DNA insert failed.

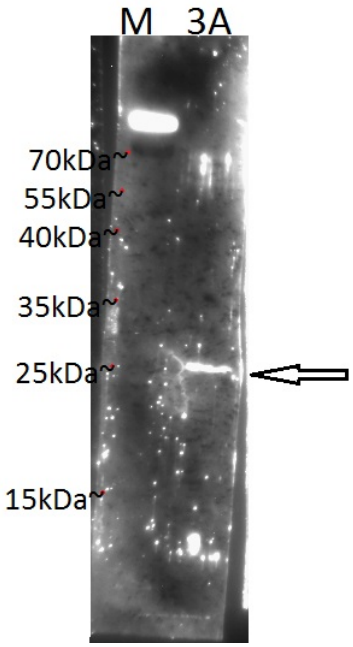


Figure 3.8: Western blot TCP21. The western blot shows a possible band for TCP21 antibody (3A) at a size of 25 kDa.

# Chapter 4

# Discussion

## 4.1 Selection of genes

The data described in this thesis are the first results on the validation of the interacting partners of bZIP19 to uncover part of the mechanisms on how bZIP19 is functioning. Previously an IP-MS experiment resulted in a list of sixty putative bZIP19 interactors (unpublished data, appendix figure A.1). Three proteins were selected for subsequent validation based on their characteristics like location and function. However different or more criteria could have been better in the selection of our genes, like the dependence on zinc or the ability to bind zinc. For example TRAF280 protein is not localized in the nucleus (Mitra et al., 2007), but it is expected that bZIP19 functions only in the nucleus. bZIP19 could however translocated to another part of the cell under normal conditions.

## 4.2 Western blot

To confirm the binding of our putative interactors with bZIP19 I first synthesized the interacting proteins for subsequent use in electrophoretic mobility shift assay (EMSA). My results show that the in vitro translated proteins have a different size on western blot than expected. This might be explained by the incorporation of biotinylated lysine t-RNAs that have an effect on the protein size. This t-RNA labels all lysines in the protein with biotin. Considering a single biotin label has a weight of 244.31 g/mol, this label could have a significant effect on the weight of a protein. For example, bZIP19 contains 15 lysine residues, so when all these are labelled with biotin, this adds a weight of almost 4 kDA to the molecular weight of bZIP19 (28.6 kDa) resulting in a shift to 33 kDa. However, this does not explain the difference for SUVH9. I observe a SUVH9 protein band at approximately 35 kDA, though the expected size is 70 kDa. Moreover, I identify in each protein synthesis experiment a band of 70 kDA. The additional band was also observed before, and is therefore considered background (Hook and Schagat, 2011). It is possible

that SUVH9 is fully present, though not distinguishable due to the background band. Repetitions of this experiment show the same result. Sanger sequencing has confirmed the full length SUVH9 is present in the in vitro translation vector, so a truncated protein is not expected.

## 4.3 Electrophoretic mobility shift assay

EMSA is a powerful tool to study the interaction of a DNA binding protein with its cognate DNA sequence. bZIP19 and bZIP23 bind to a specific region named the zinc deficiency response element (Assunção et al., 2010). However, reproducing these results was challenging because of the following: (i) glycerol, an essential component for EMSA was missing in the material and methods of the Assuncao paper and (ii) available bZIP19 and bZIP23 in pSPUTK had additional parts up and downstream of the coding sequence of the genes. These additional parts caused bZIP19 to be not properly synthesized (figure 3.5).

As a proof of principle I was able to detect bZIP19 and bZIP23 binding to the ZDRE, whereas this binding is absent in the presence of a mutated ZDRE. Surprisingly, SUVH9 and TRAF280 also bind to the ZDRE as shown in figure 3.6. Because SUVH9 and TRAF280 do not bind to the mutated ZDRE, I concluded that SUVH9 and TRAF280 specifically bind to the ZDRE sequence and not just random DNA elements. It is known that SUVH9 can bind DNA (Barrell et al., 2009), however it is not yet known to which specific DNA elements. What is even more suprising is that SUVH9 might not be fully present, as seen on the western blot. However, in previous research it has been shown that truncated versions of proteins can still bind DNA in an EMSA (Riechmann et al., 1996), so this might also be the case for SUVH9. There is still also the possibility that a complete SUVH9 protein is present on the EMSA, this was not tested. TRAF280 is not known to bind DNA, since under normal conditions it is not present in the nucleus, therefore it is unlikely to have a DNA regulatory function.

These findings suggest that the current ZDRE sequence is not only a zinc deficiency response element but perhaps it plays a more general role in gene regulation in *Arabidopsis thaliana*. In addition, 17.6% of all current annotated 27.029 *Arabidopsis thaliana* genes (Swarbreck et al., 2008) contain a ZDRE in their promoter (personal communication). This supports my suggestion that the ZDRE is a universal DNA element. However, the number of ZDREs and their position in the promoter might also be of influence for the functioning of bZIP19. It has not yet been measured how many of the 4768 ZDREs are present 'close' to the start of a gene. Neither has it been quantified how many of the genes that have more than one ZDRE in their promoter are zinc responsive. Also a yeast one-hybrid study with the ZDRE as bait shows only a very small list of binding genes (Assunção et al., 2010). SUVH9 and TRAF280 are not on this list. SUVH9 and TRAF280 might only bind to the 3Z element in EMSA. This experiment is inconclusive about what

happens under physiological conditions. Genes that have a ZDRE in their promoter are enriched for genes involved in the zinc deficiency response in roots (unpublished data). However, many genes involved in the zinc deficiency response do not have a ZDRE in their promoter (Assunção et al., 2010). This implies that it is possible that bZIP19 can bind other elements in the promoter region as well.

Remarkably, all bands of the EMSA appear on the same height (figure 3.6). This is not what I expected, because the proteins subjected to EMSA are not of the same size. I expect that bZIP19 and bZIP23 function as dimers and that TRAF280 and SUVH9 function as monomers. The size difference between a complete SUVH9 and TRAF280 is approximately 30 kDa. This suggests that differences of 30 kDa are not visible in the EMSA experiment. This implies that a supershift of a TCP21 bZIP19 interaction is not discernible from the normal bZIP19 shift. Following this in figure 3.7 no interactions can be determined. However, this does not exclude the possibility that interactions between bZIP19 and one or more putative interactors is still possible. Although I do expect that others have run into the same obstacle as well, I am unaware of of any literature that reports about this, probably due to the irrelevance of such results.

## 4.4 TCP21 western blot

In contrast to the EMSA, indeed a protein complex immunoprecipitation using GFP followed by western blotting with specific antibodies against TCP21 showed that TCP21 was present on the western blot at the expected molecular weight (figure 3.8). Unfortunately the blot shows much background therefore to truly confirm the interaction between TCP21 and bZIP19 this experiment needs to be repeated.

The experiment does suggest that TCP21 interacts with bZIP19. This would confirm that there is a direct link between zinc deficiency and the circadian clock. It is known for iron and manganesium deficiency to affect the circadian clock. Both deficiencies influence CCA1 resulting in a lengthened circadian period (Hong et al., 2013; Hermans et al., 2010; Chen et al., 2013). Our results suggest that zinc deficiency can affect the circadian clock in the same way; lengthening the circadian period by suppressing CCA1. CCA1 contains a ZDRE in its promoter less than 1 kb upstream of the transcription start site. This ZDRE is in close proximity of a TCP21 binding site. Thus I suspect that if TCP21 and bZIP19 interact, they can bind the CCA1 promoter together.

## 4.5 T-DNA lines

Unfortunately, no homozygous plants were found during the genotype analysis of the T-DNA lines. It is expected that approximately one out of every four plants is homozygous within segregating lines. We do not observe this segregation in any of our lines. However,

our results are inconclusive, since the T-DNA LB primer only produces results for the TCP21 line. It is possible that T-DNA inserts are present in some plants, but these could not be confirmed using the current PCR components and protocol.

## 4.6 Future recommendations

I would recommend the following experiments for fur further exploration. First of all it is important to validate the interaction between TCP21 and bZIP19. This could be achieved by repeating the experiment as we performed. Different experiments then EMSA would probably be better for validating the interactors. A follow-up experiment could be a yeast-two-hybrid screening.

The list with putative interactors was not optimal. The IP experiment was performed with a GFP-bZIP19 fusion protein, since no antibodies against bZIP19 are available. The functionality of the protein was confirmed by its ability to complement a *m19m23* mutant line (unpublished data). It is debatable whether bZIP19 could still have its usual interactors, since GFP is equal in size to bZIP19 (26.9 kDa versus 28.6 kDa). GFP could be blocking certain binding domains on bZIP19 in the fusion protein, resulting in an incomplete or erroneous outcome of the IP experiment (Berggård et al., 2007; Dunham et al., 2012). It is important to repeat the IP-MS with optimized settings to enrich for interactors and have no or little aspecific binding. Nowadays it is possible to generate antibodies or produce proteins similar to antibodies, like affimers in a much more cost effective manner. Hopefully they will compile a more plausible list of putative bZIP19 interactors. When interactions with bZIP19 are confirmed, it is also interesting to check whether these interactors also interact with bZIP23 or with a possible heterodimer of bZIP19 and bZIP23.

In this thesis it is questioned whether bZIP19 can only bind the ZDRE or whether it could also bind other DNA elements. A follow-up experiment could be to try to bind bZIP19 to other DNA elements. This could be tested with EMSA. Other than bZIP19 the specificity of DNA binding of TRAF280 and SUVH9 should also be tested. It is interesting to see how many and what DNA elements they could bind. Other than the specificity of the proteins the specificity of the ZDRE should also be tested. It is interesting to see what other proteins have the potential to bind the ZDRE specifically.

Other than this it is intriguing whether bZIP19 bZIP23 heterodimers exists, because of the difference between *bZIP19* and *bZIP23* single knockout plants. Their existence is much speculated by Assunção et al. (2010), but never proven. It should be possible to show these heterodimers with EMSA, considering there is a small size difference between bZIP19 and bZIP23. However as the results above show, the probability of seeing such a small difference is also very small. Other methods to show such an interaction could be Frster resonance energy transfer (FRET) and Bimolecular fluorescence complementation (BiFC).

With FRET bZIP19 and bZIP23 would be tagged with two complementary fluorescence proteins. One fluorescence protein would be excited and if bZIP19 and bZIP23 interact the emission of the excited fluorescence protein would excite the other fluorescence protein resulting in specific emission. With BiFC bZIP19 and bZIP23 would each be tagged with half a fluorescence protein. If the two interact than the fluorescence protein is complete and will emit light. These two methods do not depend on size differences and will probably work better for these two proteins of almost the same size.

# Acknowledgements

I would like to thank Robert Akkers for his daily supervision and suggestions, Cezary Smazcniak for all his help with the EMSA and optimizing the protocols, Mark Aarts for his feedback and support in writing this thesis and the students of genetics for their frienship and 'gezelligheid'.

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# Appendix A

# MS list

| Gene names | present in table Ceza | At number | Fasta headers | Peptides | Peptides |
|---|---|---|---|---|---|
| bZIP19 | N | At4g35040 | >tr\|Q8VY76\|Q8VY76 | 1 | 0 |
| T02O04.4 | N | At3g16460 | >tr\|O04310\|O04310 | 5 | 0 |
| FIB2;FIB1 | Y | At4g25630 | >sp\|Q94AH9\|MD36 | 4 | 0 |
| NRPA2 | N | At1g29940 | >tr\|F4I366\|F4I366_ | 3 | 0 |
| | N | At5g26280 | >tr\|Q93Z83\|Q93Z83 | 2 | 0 |
| F15B8.150 | Y | At3g57660 | >tr\|Q9SVY0\|Q9SVY0 | 2 | 0 |
| BZIP27 | N | At2g17770 | >tr\|F4IPG9\|F4IPG9_ | 1 | 0 |
| | N | At1g16705 | >tr\|F4I4I6\|F4I4I6_A | 1 | 0 |
| | N | At5g26290 | >tr\|F4JZS4\|F4JZS4_ | 1 | 0 |
| | N | At1g30473 | >tr\|Q1G3T0\|Q1G3T0 | 1 | 0 |
| | N | At4g08540 | >tr\|Q8H1E1\|Q8H1E1 | 1 | 0 |
| F23C21.3 | N | At1g60850 | >tr\|Q9C6C2\|Q9C6C2 | 1 | 0 |
| SUVH9 | N | At4g13460 | >sp\|Q9T0G7\|SUVH9 | 1 | 1 |
| HEI10 | N | At1g53490 | >tr\|F4HRI2\|F4HRI2_ | 1 | 0 |
| | N | At1g13635 | >tr\|F4HSJ2\|F4HSJ2_ | 1 | 0 |
| | N | At3g24880 | >tr\|F4J7T3\|F4J7T3_ | 1 | 0 |
| RH15;RH56 | N | At5g11170 | >sp\|Q56XG6\|RH15_ | 1 | 0 |
| T02O04.8 | N | At3g16420 | >sp\|O04314\|PYBP1_ | 1 | 0 |
| ERF008 | N | At2g23340 | >sp\|O22174\|ERF08_ | 1 | 0 |
| SCL34 | N | At2g29065 | >sp\|P0C884\|SCL34_ | 1 | 0 |
| BIO2 | N | At2g43360 | >sp\|P54967\|BIOB_A | 1 | 0 |
| RF298 | N | At4g03000 | >sp\|Q0WPJ7\|RF298 | 1 | 0 |
| | N | At5g65740 | >tr\|Q1ECQ9\|Q1ECQ9 | 1 | 0 |
| SPL16 | N | At1g76580 | >sp\|Q700C2\|SPL16_ | 1 | 0 |
| AGL90 | N | At5g27960 | >sp\|Q7XJK5\|AGL90_ | 1 | 0 |
| At1g58220/F16M22_4 | N | At1g58220 | >tr\|Q8GZ96\|Q8GZ96 | 1 | 0 |
| TCP21 | Y 2X CCA1 PATHWAY | At5g08330 | >sp\|Q9FTA2\|TCP21_ | 1 | 0 |
| TPS15 | N | At3g29190 | >sp\|Q9LS76\|TPS15_ | 1 | 0 |

Figure A.1: output MS list with gene information by dr R. Akkers

# Appendix B

# Genotyping T-DNA lines

Results of the genotyping PCR of the T-DNA lines. 12 plants per line were tested. L+R are the genomic primers and T+R are the primers that should prove the T-DNA insert. the numbers above the gel represent the plant that was tested. Plant 1 in L+R is the same plant as plant 1 in T+R. For SUVH9 only 11 plants were tested.

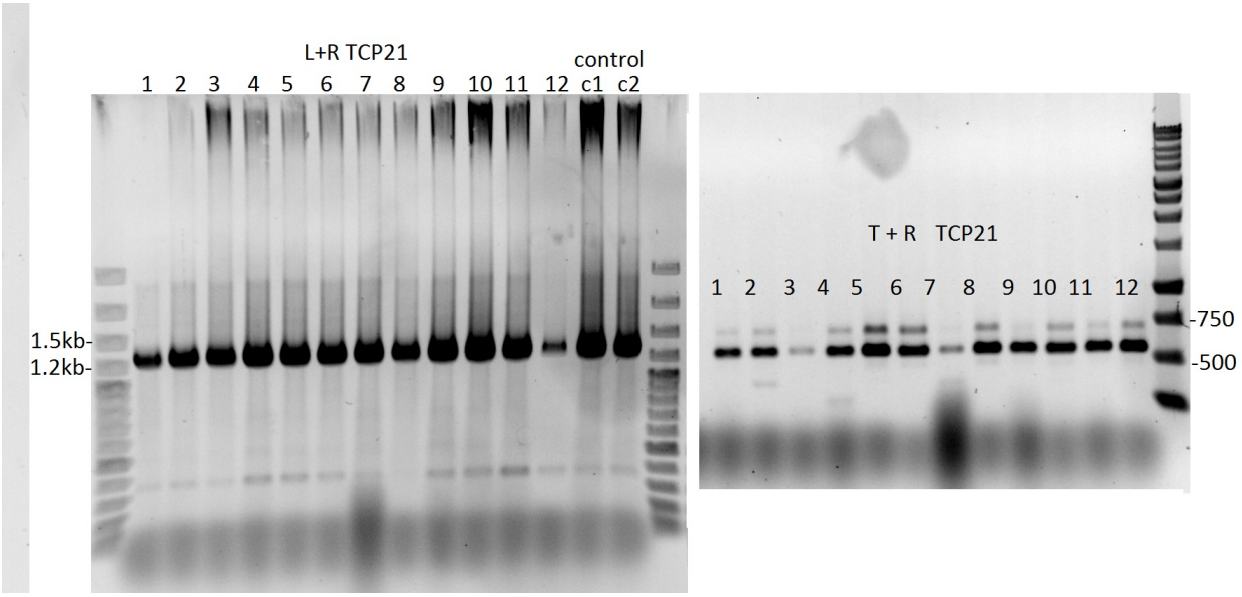


Figure B.1: TCP21

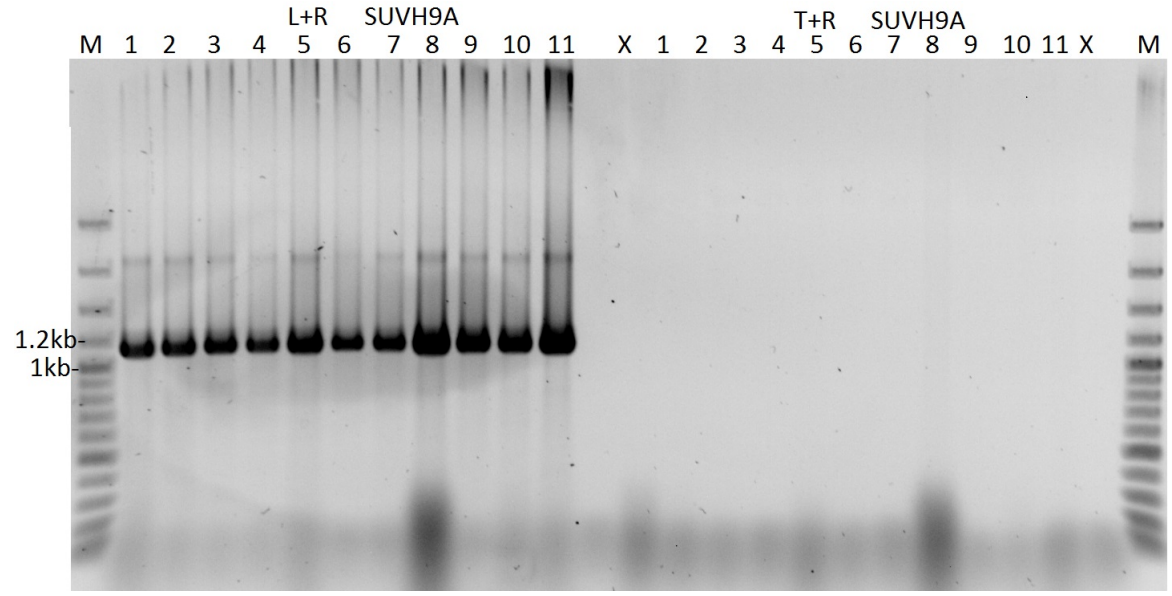


Figure B.2: SUVH9A

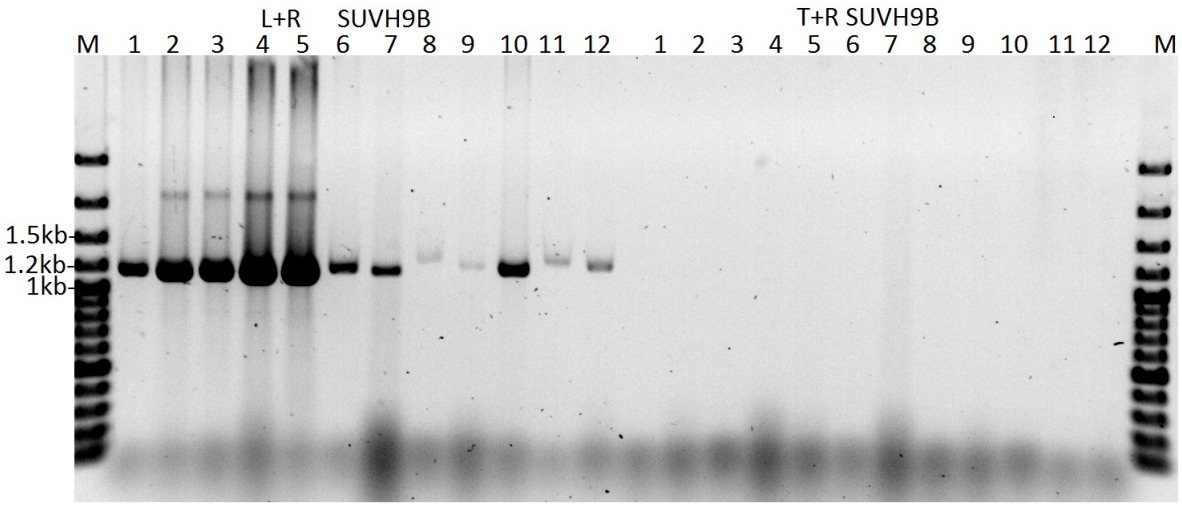


Figure B.3: SUVH9B

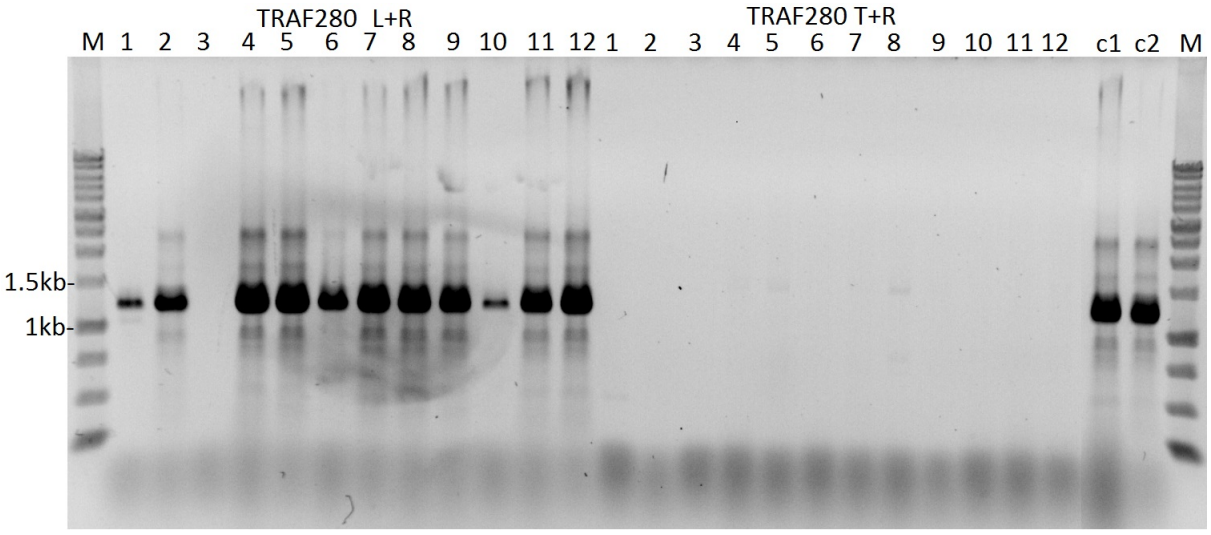


Figure B.4: TRAF280