Universität für Bodenkultur Wien

Department für Angewandte Genetik und Zellbiologie

SPECIFICITY OF REPORTER PLANTS FOR AND *IN VIVO* METABOLISM OF ZEARALENONE

Diplomarbeit zur Erlangung des Diplomingenieurgrades an der Universität für Bodenkultur Wien

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Abstract

On one hand this thesis is dealing with the specificity of zearalenone (ZON) reporters in the model plant *Arabidopsis thaliana* and on the other hand with the *in vivo* metabolism of zearalenone. The objective was to obtain well responding reporters for future mutagenesis and genetic studies. Furthermore a functional correlation during the metabolisation of ZON of two ZON induced genes was determined. To achieve these aims several methods were chosen including quantitative real time PCRs (qPCR), sterile cultures and microscopic analyses. To improve an existing reporter construct, a new reporter construct was cloned and stably transformed into *Arabidopsis thaliana*. This modified new reporter construct enhanced the expression significantly. The *in vivo* metabolisation experiments with a mutant of an ABC transporter and pleiotropic drug resistant gene (PDR12) and an overexpressor of an UDP-glycosyltransferase (UGT73B4) revealed that both genes have a significant effect.

Kurzfassung

Diese Arbeit beschäftigt sich einerseits mit der Spezifität von transgenen Zearalenon-Reporterlinien der Modellpflanze *Arabidopsis thaliana* und andererseits mit der *in vivo* Metabolisierung des Mykotoxins. Ziel war es spezifisch induzierbare Reporterpflanzen, herzustellen, um diese zu Mutagenisieren und für genetische Analyse zu verwenden. Außerdem wurde versucht, den Mechanismus der Zearalenon (ZON) Metabolisierung weiter aufzuklären. Dafür wurde die Metabolisierung von ZON bei Mutanten bzw. Überexpressionslinien von Genen quantifiziert, die durch ZON induziert waren, , Methodisch wurden quantitative Real time PCR Analysen, verschiedene Mikroskopiertechniken und sterile Zellkulturen verwendet. Zur Verbesserung des interessantesten Reporterkonstrukts wurde ein neuer Vektor mit einem endogenen Terminationssignal kloniert und stabil in *Arabidopsis thaliana* transformiert. Der zeitliche Verlauf der Metabolisierungsversuche zeigte, dass sowohl die ABC-Transporter Mutante (PDR12) als auch die Überexpression der UDP-Glykosyltransferase (UGT73B4) einen signifikanten Effekt auf die Metabolisierung von ZON haben.

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1 Introduction

1.1 A model system for plant pathogen interaction

Arabidopsis thaliana is a small flowering plant which is a member of the *brassicaceae* family. Arabidopsis has no agricultural importance but it is widely used as a model organism. It has numerous advantages like a small genome (125 Mbp) which has been sequenced in the year 2000. Furthermore, Arabidopsis owns a rapid life circle. It takes about 6 weeks from germination to mature seeds. It has a good seed production and uncomplicated cultivation conditions. There is an efficient transformation method using *Agrobacterium tumefaciens*, and a large number of mutant lines is available (TAIR, 2012).

1.2 The mycotoxin Zearalenone

Zearalenone (ZON) is a non-steroidal oestrogenic mycotoxin which is biosynthesized by several Fusarium fungi, including *F. graminearum (Gibberella zeae), F. culmorum, F. cerealis, F. equiseti, F. crookwellense* and *F. semitectum*. These fungi occur usually in the soil. They need temperate or warm conditions and are regular contaminants of cereal crops worldwide (Bennett and Klich, 2003). Cliver (1990) reported that the production of zearalenone on corn and other cereals is favoured by temperatures near freezing for an extended time and by cycles of temperature from low to moderate(Cliver, 1990).

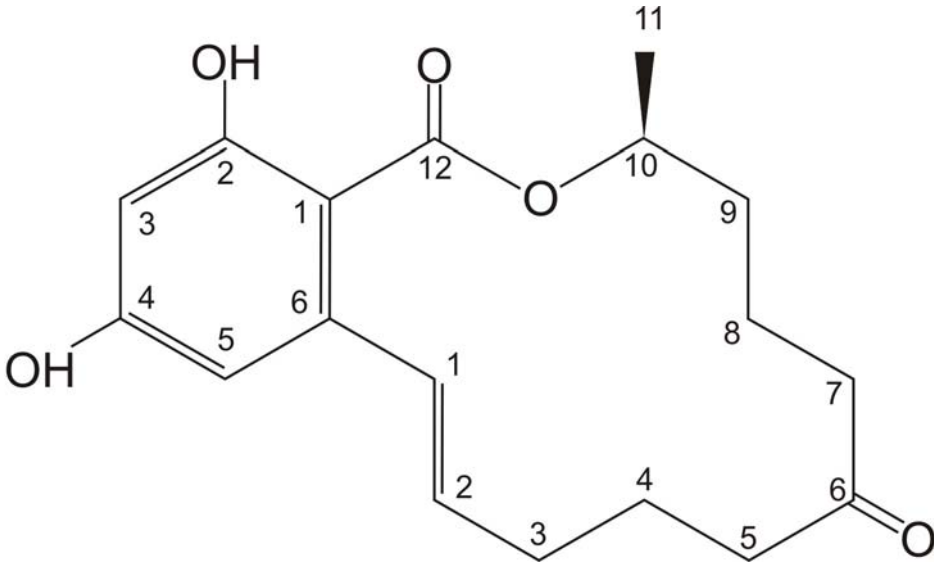

Figure 1: Chemical structure of Zearalenone (6-[10-hydroxy-6-oxo-trans-1-undecenyl]-β-resorcyclic acid lactone). ZON and all its metabolites are all in a trans-configuration at the C1-C2 double bond. ZON is composed of a resorcyclic acid C1-C6 and a lactone C1-C12.

The main toxin production takes place before harvesting, but post harvesting toxin production is also possible due to poor storage conditions (Kuiper-Goodman et al., 1987). If contaminated parts of the plants are taken up by mammals, they are not only exposed to the native form of zearalenone but also to the plant processed metabolites. Chemical zearalenone is described as 6-[10-hydroxy-6-oxo-trans-1-undecenyl]-β-resorcyclic acid lactone. It gains its name as a combination of *G. zeae* (zea), resorcylic acid lactone (ral), -ene (for the presence of the C-1 to C-2 double bond), and -one for the C-6 ketone (Urry and Eyring, 1966). As mentioned before, zearalenone can bind to oestrogen receptors due to its structure. This binding leads to several disorders like problems with conception, ovulation, implantation, fetal development and viability of neonates (Kuiper-Goodman et al., 1987, Kennedy et al., 1998). Furthermore zearalenone is responsible for alterations in the reproductive tract of laboratory animals like mice, rats, guinea pigs and farm animals (Kuiper-Goodman et al., 1987).

1.3 Zearalenone in *Arabidopsis thaliana*

Zearalenone in *Arabidopsis thaliana* leads to a higher than two-fold change in gene expression of 495 genes after 2 hours (Werner, 2005). Genes involved in ZON detoxification like pleiotropic drug resistance gene (*PDR12,* an ABC-transporter) are induced 14 times. Glutathione-S-transferases (GSTs), UDP-glycosyltransferases (UGTs) and several small heat shock protein genes were also strongly induced. A strong down regulation happened to

genes which are involved in cell wall functions like remodeling or reinforcement. Zearalenone furthermore was found to be able to suppress the short root phenotype of a novel allele of the cellulose synthase (CESA3) (Werner, 2005, A., 2009, Acosta, 2009). Berthiller et al (2006) showed that ZON is metabolized into phase I and II metabolites in *Arabidopsis thaliana*.

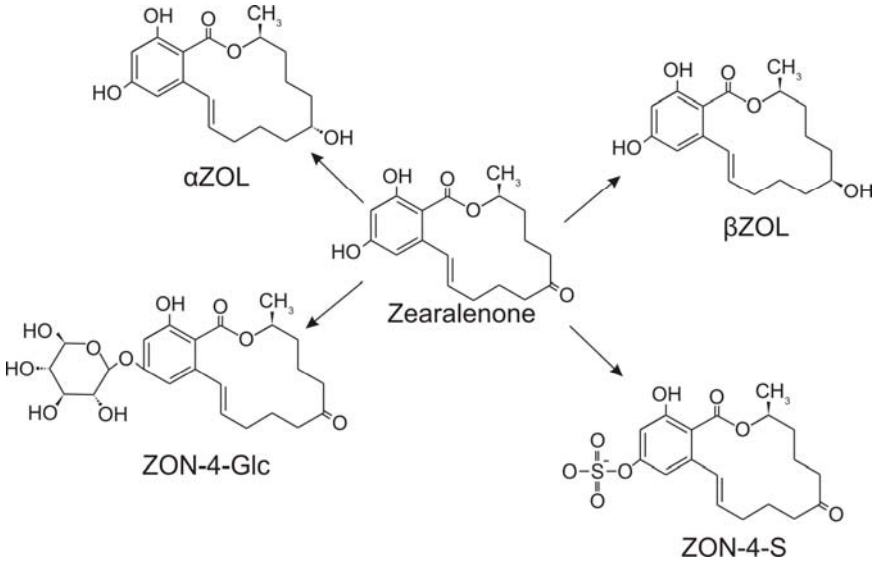

Figure 2: Zearalenone and its main metabolites. ZON is metabolised in its four main metabolites, Zearalenone-4-sulfate, α- and β-Zearalenol and Zearalenone-4-glycoside.

17 different metabolites were found in the plant and the medium including the four most important metabolites, namely zearalenol (αZOL and βZOL), Zearalenone-4-glycoside (ZON-4-Glc) and Zearalenone-4-sulfate (ZON-4-S). (Berthiller et al., 2006). Figure 3 shows the degradation of ZON and the time dependent synthesis of its metabolites in *Arabidopsis thaliana*. Compared to the other components concerning the absolute mass, ZON-4-G is produced most.

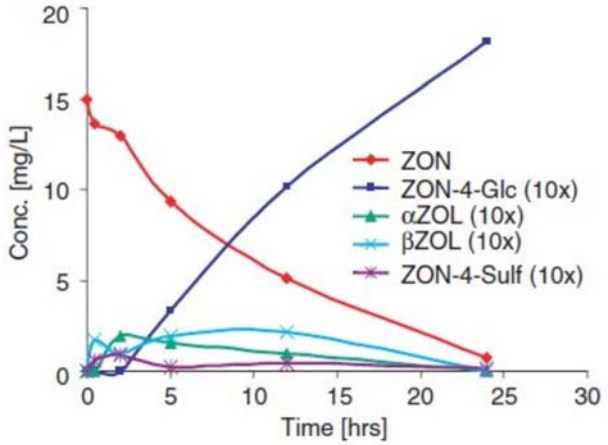

Figure 3: ZON degradation and metabolite forming over a time course in the growth media (Berthiller et. al. 2006).

It has been shown that ZON-4-Glc is completely cleaved to ZON during swine digestion (Gareis et al., 1990). In this way ZON is able to affect the animal's organism. Gareis coined the term "masked mycotoxins". These are substances which are not usually detected in a routine analysis but which count to the total mycotoxin load. Furthermore, experiments with radiolabelled ^{14}C-ZON in maize cell suspension showed that over 50% of the initial radioactivity was incorporated as insoluble bound residue in the plant matrix. But this bound ZON is maybe still bioavailable (Engelhardt, 1999).

1.4 Green fluorescent protein (GFP) as reporter

The protein was first purified and characterized from the jellyfish *Aequorea victoria* in the year 1961. The protein was initially described as the green protein and is now called green fluorescent protein (GFP) (SHIMOMURA et al., 1962, Chalfie, 1995). GFP is a 27 kDa protein consisting of 238 amino acids (SHIMOMURA, 1979). GFP is excited with UV- and blue visible light because of its two (395 nm and 470 nm) excitation peaks (Chalfie, 1995).

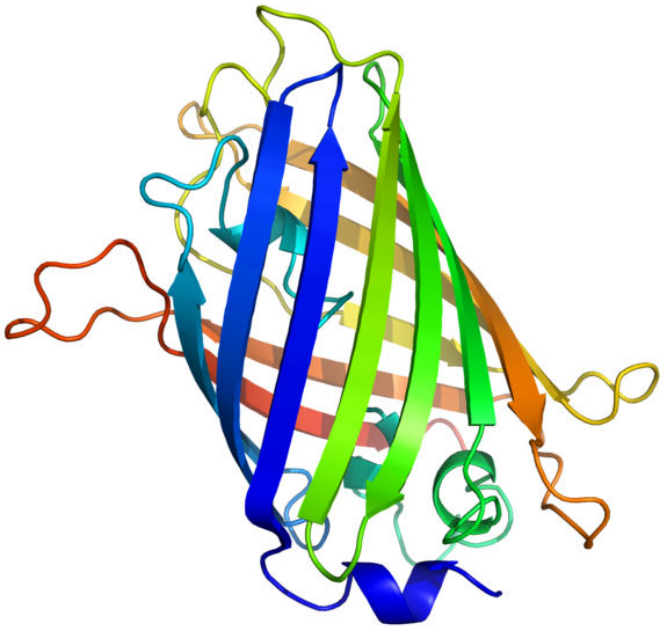

Figure 4: Schematic view of GFP. The protein fold consists of an 11-stranded beta barrel with a coaxial helix, with the chromophore forming from the central helix.

This feature enables a direct visualization of gene expression in vital cells. A cell lysis and subsequent biochemical analysis is not necessary. Furthermore, it is relatively resistant against photobleaching. These points make GFP an attractive fluorescent tag for observing protein interaction, traffic and localization. GFP used with strong promoters and universal transcription and translation elements gives a detectable signal in Arabidopsis (Chalfie, 1995). Nowadays various versions of improved fluorescent proteins exist. They are brighter, cover a broader spectral range, have enhanced photostability, have reduced oligomerization, pH sensitivity and fast maturation rates. The wild type GFP was quickly modified and gave different types like BFP (blue fluorescent protein), CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) (Heim et al., 1994, Ormö et al., 1996, Tsien, 1998). The first red fluorescent protein was detected surprisingly in a non-bioluminescent reef coral (Matz et al., 1999). The most common GFP is an enhanced wild type GFP (EGFP) as well as the enhanced yellow and cyan version (EYFP and ECFP) (Shaner et al., 2007). Recombinant EGFP could be expressed and purified from *E. coli*. The protein is a 32.7 kDa monomer with 293 amino acids tagged with 6 histidine residues (C- and N-terminus). In this way EGFP can for example be detected by a His-tag antibody and a GFP antibody (BioVision, 2012). Continuing efforts in protein engineering of the existing FPs, coupled with advanced new screening technologies, should further expand the available colour palette and ultimately provide rapidly maturing, bright and photostable proteins in every spectral class (Shaner et al., 2007).

1.5 The Zearalenone induced genes

To select *Arabidopsis thaliana* genes upregulated upon ZON exposure, a microarray experiment was done by Ulrike Werner (2005). The best responding genes were chosen.

1.5.1 The ABC transporter and pleiotropic drug resistance gene PDR12

At1g15520/PDR12 This gene encodes for an ATP-binding cassette transporter (ABC-transporter) protein. The protein is also known as ATP-BINDING CASSETTE G40 (ABCG40) *and PDR12* (Pleiotropic drug resistance gene). The transporter is located in the plasma membrane and is responsible for transmembrane movement of abscisic acid (ABA) which is an ubiquitous phytohormone involved in many developmental processes and stress responses of plants (Kang et al., 2010). The gene is located on chromosome 1.

1.5.2 Glutathione-S-transferase

55 glutathione transferases (GSTs) are known in *Arabidopsis thaliana*, which are divided in seven groups. The popular phi and tau classes are often highly stress inducible. GSTs are enzymes that typically add, or substitute, the non-ribosomally synthesised tripeptide glutathione (GSH; γ-Glu–Cys–Gly) to an electrophilic centre contained within a small molecule acceptor. Arabidopsis contains 54 soluble GSTs and one membrane protein associated with this activity (Dixon and Edwards, 2010).

At1g17180/GSTU25 This gene encodes a tau class glutathione transferase which is located in the cytoplasm. Tau class glutathione transferases are plant specific and have also a role in herbicide detoxification (Wagner et al., 2002). The gene is located on chromosome 1.

1.5.3 UDP-glycosyltransferase (UGTs)

Uridin diphosphat glycosyltransferases (UGTs) induce the transfer of glycosyl residues from activated nucleotide sugars to acceptor molecules (Ross et al., 2001). UGTs in higher plants are thought to encode enzymes that glycosylate a broad array of aglycones (non sugar containing compounds) including plant hormones, all major classes of plant secondary metabolites and xenobiotics such as herbicides (Vogt and Jones, 2000). This transfer

influences the properties of the acceptor molecule such as bioactivity, solubility, and transport within the cell and throughout the organism. There are more then 100 genes encoding for a UGT in *Arabidopsis thaliana*. Every UGT contains a 42 amino acid consensus sequence. Until now none of these UGTs has been crystallized and their three-dimensional structures are not known (Ross et al., 2001).

At2g15480/UGT73B5	The gene At2g15480 codes for the UDP-glucosyl transferase 73B5. The protein is transferring glucose to small molecular weight compounds such as secondary metabolites (Lim et al., 2002). The expression of UGT73B5 is induced by O_2^- and during the hypersensitive response of Arabidopsis to *Pseudomonas syringae pv tomato* (Pst) carrying the AvrRpm1 gene (Langlois-Meurinne et al., 2005). The AvrRpm1 gene is required for virulence on Arabidopsis (Ritter and Dangl, 1995) In addition, UGT73B5 can accept kaempferol (natural flavonol) diglycosides as substrates resulting in kaempferol triglycosides (Sirikantaramas et al., 2010). It is expressed in 9 plant organs such as the hypocotyl, root and stem.
At2g15490/UGT73B4	UGT73B4 is involved in the metabolism of the explosive 2,4,6-trinitrotoluene (TNT). This UGT is able to degrade the TNT and form its metabolites (Gandia-Herrero et al., 2008). Compared to At2g15480 it is mainly expressed in roots. The gene is located in tandem with the At2g15480 gene on the second chromosome.

Both genes were up-regulated during menadione (member of vitamine k group)-induced oxidative stress in 10-day-old Arabidopsis seedlings (Sirikantaramas et al., 2010).

1.5.4 Small heat shock proteins (sHSPs)

Small heat shock proteins and the related α-crystallins are stress proteins. The sHSPs belong to the Hsp20 family. The range of size is approximately from 16 - 42 kDa (Scharf et al., 2001). Two families of smHSPs are located in the cytosol (class I and II), and three distinct families in the chloroplast, the endoplasmic reticulum and the mitochondria (Helm et al., 1995). They can act as chaperons and prevent irreversible protein aggregation. sHSPs have a α-crystallin domain in common. This domain consists of approximately 90 amino acids and shows a conserved β-sandwich structure (Van Montfort et al., 2001). For example,

it had already been shown that sHSPs enhance the refolding of chemically denatured citrate synthase and lactate dehydrogenase in an ATP-independent manner (Lee et al., 1995).

At2g29500/HSP17.6B-Cl	HSP17.6B is a small heat shock protein, which is involved in the oxidative stress response. Reactive oxygen species (ROS) cause oxidative stress which is a challenge faced by all aerobic organisms. Environmental stress like high light intensity, drought, extreme temperatures, heavy metals, and UV radiations all enhance photosynthetic ROS generation. ROS are also produced in the chloroplasts through the photoreduction of the herbicide methyl viologen (MV). HSP17.6B is induced by MV in a time-dependant manner (Scarpeci et al., 2008). The expression of At2g29500 is not affected by apoplastic H_2O_2 or H_2O_2 produced by the plasma membrane (Bechtold et al., 2008). This indicates, that chloroplastic ROS contribute to the signaling cascade of this gene (Scarpeci et al., 2008).
At5g12030/ HSP 17.6A	At-HSP17.6A encodes a smHSP belonging to the cytosolic class II family in *Arabidopsis thaliana* The gene is expressed during development and stress The expression of HSP 17.6A is regulated by heat shock and osmotic stress whereas the heat stress induce the expression in a much stronger way then salt stress or polyethylenglycol treatment. Expression of HSP17.6A in vegetative tissues was undetectable in the absence of stress. It is suggested that At-HSP17.6A may has a function, together with other developmentally regulated smHSPs, in protecting cellular components during seed desiccation and subsequent rehydration (Sun et al., 2001).
At5g59720/HSP18.2	The gene HSP18.2 is an important tool in plant genetics. The 5'untranslated region of HSP18.2 mRNA is known to play an important role in determining the rate of translation. An experimental approach done 2003 including the CaMV35s (Cauliflower mosaic virus 35 S RNA) promoter the 5'UTR gained from HSP18.2, and a GUS (β-glucuronidase) reporter

system in a pBI223 vector transfected into *Nicotiana tabacum* (tobacco) BY-2 protoplasts, showed a 10 fold higher protein level than in the unmodified pBI223 vector (containing CaMV35s and GUS reporter) (Dansako et al., 2003). Additional the terminator of the gene shows better results in efficiency of gene expression in transfected Arabidopsis protoplasts then the widely used CaMV35s terminator (Nagaya et al., 2010).

Experimental data had shown that the Hsp20 family exhibited the most stress-general response pattern compared to Hsp70 and Hsp100 families. These results suggest that this protein family is of special importance for stress tolerance in plant species (Swindell et al., 2007).

1.6 Transcriptional regulation of gene expression in eukaryotic cells

The transcriptional gene expression is influenced by several factors, which are important to achieve good results in protein expression in genetically modified organisms (GMOs).

1.6.1 Promoters

The region upstream the starting point of transcription is called promoter and is located upstream the non coding area on the 5' end of the gene. A well known promoter element is the so called TATA-box. It is located 26 to 34 bp upstream the transcription start point and it is a binding site for transcription factor TFIID (Transcription factor for RNA polymerase II). This factor recognizes the TATA box with its TBP (TATA box binding protein) subunit and initiates the binding of other transcription factors and RNA polymerase II. Another occurring promoter element is the initiator element (Inr)-element. This element is located mostly at the starting point of transcription. This starting nucleotide, normally an adenine nucleotide, is located frequently in the initiator element (Knippers, 2006). It has the consensus sequence YYAN(T/A)YY and is functionally similar to TATA in facilitating TFIID (transcription factor II D binding (Xi et al., 2007). Most of the transcriptions start at an adenine nucleotide, which is normally flanked by pyrimidines (cytosine and thymine). The DPE (downstream promoter element) is like the Inr-element. It is located 28 to 32 bp downstream the transcriptions starting point and the motif is A/G G A/T C/T G/A/C. To summarize, it is possible that genes contains one or all of these elements. Many regulating genes contain a TATA-box in contrast to housekeeoing genes. Housekeeping genes are genes which are expressed throughout the

cell cycle. The are important for maintenance the basic functions like metabolism, cytosceleton (Knippers, 2006).

1.6.2 Untranslated region (UTR)

The coding sequence of a gene is sometimes flanked by untranslated regions (UTRs). The 5' UTR is upstream of the translation codon ATG end and the 3' UTR is downstream the stop codon of the transcript. The gene based mRNA (messenger RNA) can be divided into three parts: a 5' untranslated region (5' UTR), the polypeptide coding region, sometimes called open reading frame (ORF), and the 3' untranslated region (3'UTR). Many mechanisms modulating the ability to translate mRNAs, operate through interactions of regulatory molecules with sequences in the 3' ends of transcripts. If certain sequences are present within the 3' UTR of an mRNA, that mRNA will be degraded very rapidly. In other cases, sequences in the 3'UTR do not cause degradation but can lead to lower level of translation. For example a gene (lin-4) coding for a small mRNA in *Caenorhabditis elegans* is known for repression of translation of another gene (lin-14). Lin-4 encodes not for a protein but for a mRNA. This mRNA has a complementary sequence to the 3'UTR in lin-14 mRNA. If the lin-4 mRNA is expressed it forms a double helix with the 3'UTR in lin-14 mRNA. In this way the lin-14 mRNA is not translated (Griffiths et al., 2000). Another example for powerful expression control by 3'UTR is the 3'UTR of the gene cyclooxygenase-2 (COX-2) in renal mesangial cells. Insertion of the entire 3'-UTR of COX-2 into the 3'-UTR of luciferase resulted in a 70% decrease in luciferase enzymatic activity Measurement of steady-state reporter gene mRNA levels suggested that the loss of activity was due to decreased translational efficiency (Cok and Morrison, 2001). Another interesting property is the regulation of expression of mRNA by the length of the 3'UTR. It has been showen that the insertion of a polyA tail in a luciferase (lcu) reporter mRNA in transiently transfected Chinese hamster ovary (CHO) cells caused a 97-fold higher expression when the length of the 3'UTR was 19 bases. Its stimulatory effect was only 2.3 fold when the length of the 3'UTR was increased to 156 bases. The effect of the luc 3'UTR on poly(A) tail function was orientation independent, suggesting that its length and not its primary sequence was the important factor (Tanguay and Gallie, 1996). 3' UTRs are substantially longer than their 5' counterparts, indicating a significant potential for regulation (Mazumder et al., 2003). In most cases, translational control mechanisms result from the interaction of RNA-binding proteins with 5'- or 3'-untranslated regions (UTRs) of mRNA. This protein interaction leads to the formation of an RNA loop in organisms ranging form viruses to humans, which means a termination signal for translation. As a result, such RNA loops are thought to increase the translational efficiency (Mazumder et al., 2003).

1.6.3 Capping and polyadenylation

In an early stage of transcription (25 to 30 nucleotides length) several enzymes process the 5' end of the RNA strand. A RNA-triphosphatase clips the terminal phosphate group. Thereafter, a guanylyltransferase transfers a GMP (guanosine mono phosphate) to the cleaved end. Finally, the ribose is methylated and the mRNA is capped.

The RNA polymerase stops hundreds of base pairs after the last exon. 10 - 30 nucleotides upstream the polyadenylation starting point, a poly-A signal is located. It is a sequence of 6 bases (AAUAAA rarely AUUAAA), which is detected by CPSF (cleavage and polyadenylation specificity factor). CPSF cleaves the mRNA and polyadenylation starts, catalyzed by polyadenylate polymerase. A polyA-tail protects mRNA from enzymatic degradation in the cytoplasm, aids in transcription termination and translation (Knippers, 2006).

1.6.4 Enhancer

Enhancers are classically defined as *cis*-acting DNA regulatory elements that stimulate transcription, independent of their position and orientation (Banerji et al., 1981). In higher eukaryotes transcriptional enhancers play critical roles in the integration of cellular signaling (Arnosti and Kulkarni, 2005). Enhancers are DNA-segments with regulatory features and are located outside the translated region (Knippers, 2006).

1.7 Agrobacterium tumefaciens

Agrobacterium tumefaciens is a gram-negative, aerobic, soil born bacterium which is able to transfer T-DNA into dicot plants. If a dicot plant is wounded it excretes compounds like acetosyringone, which can be detected by the VirA sensor of the bacterium. The activated VirA sensor phosphorylates the VirG regulator which leads to the expression of the *vir* gene located on the Ti-plasmid (> 200 kb). After the expression of the T-DNA it gets covalently bound on its 5' end to VirD2. This relaxase leads the T-DNA-VirD2 complex and VirE2 trough a type-IV-secretion system into the recipient cell. In the cell VirE2 binds to the T-DNA-VirD2 complex. The VirE2 coated T-DNA-VirD2 complex is guided through core pores to the nucleus. There the T-DNA becomes integrated into the chromosomal DNA of the target cell. The plant now expresses phytohormone synthesis (*onc* genes) and produces indol-3-acetic acid and cytokinin which leads to an increased callus growth (tumor growth). Furthermore, the recipient cell produces modified amino acids called opine. These opines are taken up by the *Agrobacterium* and are used as C-, energy- and N-source (Fuchs, 2007).

The Ti-plasmid has a major importance for the green biotechnology. To obtain modified plants, the Ti-plasmid is used to transfer T-DNA of interest into the target plant cell. This system was first described in 1977 (Schell and Van Montagu, 1977). Therefore, the most important feature is that the transfer and integration do not rely on genes encoded by the T-DNA itself. The only necessary sequences on the T-DNA are the border sequences (right and left) that edge it. Between these sequences genes of interest can be cloned (Valentine, 2003). An overview is given in Figure 5.

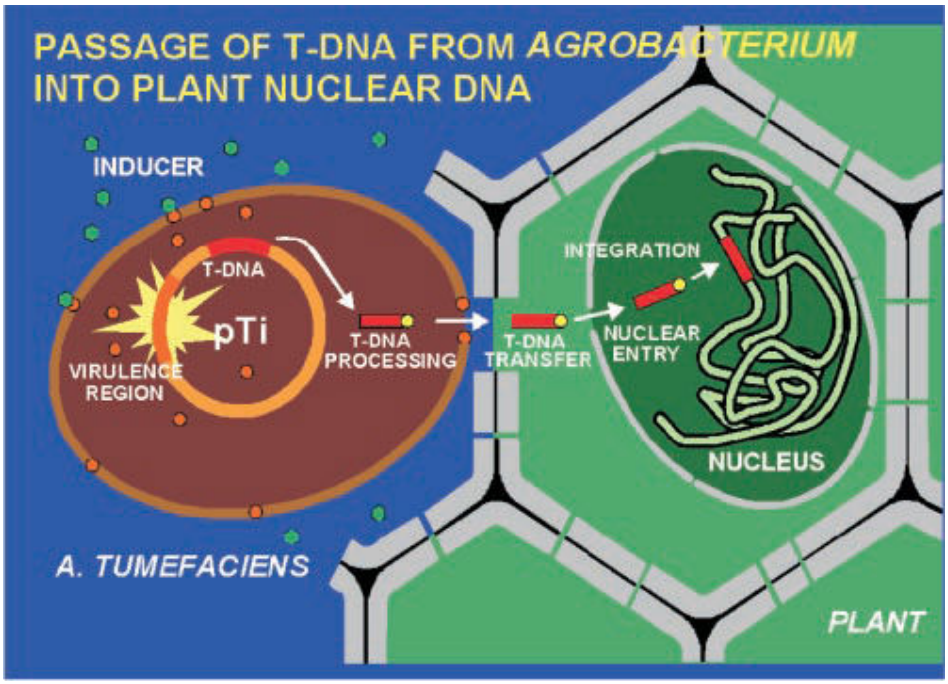

Figure 5: The path of the T-DNA. The T-DNA travels after processing from the Ti-plasmid into the plant cell and nucleus where it is stably integrated into the genome (Rossi L et al., 1998).

2 Objectives and aims

2.1 Bioinformatic data analysis

The objective was to summarize the knowledge of the gene expression of the seven highly ZON inducible genes. Therefore, the data were obtained from the free access version of the Genevestigator V3 (www.genevestigator.com). The main focus was on the organ specificity , their expression pattern upon different conditions and during the developmental stages of the plant.

2.2 Screening a reporter line library of nine transcriptional reporter constructs for their response to zearalenone

Before testing the ZON induction the amount of inserts per T2 line were determined. Therefore the seedlings were plated on a selective medium containing kanamycin and the amount of inserts was determined by segregations analyses. Afterwards, a time course expression analysis was done to confirm the transient inducibility upon ZON exposure. Furthermore, the lines were reevaluated in T3 generation for their homo- or heterozygosity.

2.3 Modification and testing of a promising constructs

After screening of the first reporter line it was decided to modify a construct. The existing constructs were done by Mona Ayad and David Merz. They assembled the promoter region of the target gene with an EGFP reporter. Due to weak results of the reporter line pm2g29500::GFP (sHSP), the idea was to attach the endogenous termination signal. Therefore, the 3' UTR of the gene was added to the existing construct to enhance the expression level (Figure 6). A cloning strategy was developed and the obtained construct was transferred into the plant via an *Agrobacterium* binary vector. The obtained Arabidopsis line was screened for stronger signals.

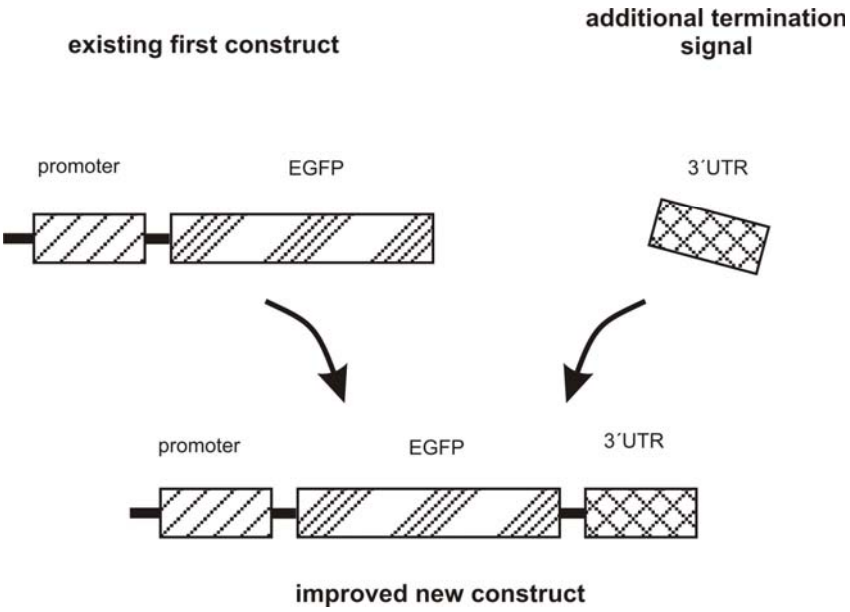

Figure 6: The cloning overview. The first construct contained a promoter and eGFP (enhanced green fluorescent protein). An additional termination signal (3'UTR) was added. The improved new construct consisted of a promoter eGFP and 3'UTR (untranslated region)

2.3.1 Quantitative real-time PCR

The aim was to quantify the levels of gene expression after ZON treatment in a time course. Therefore, *Arabidopsis thaliana* Columbia wild type plants were kept in liquid culture and treated with ZON and/or DMSO. The plants were harvested after certain time points and RNA was isolated. cDNA was synthesized and a quantitative real-time PCR was executed. To obtain quantitative data, standard series with known copy numbers of the genes were generated. To compare ZON induced expression levels the genes of interest were normalized with at least two housekeeping genes.

2.3.2 Metabolisation of ZON

Based on the findings that Arabidopsis seedlings can metabolize ZON *in vivo* through reduction, conjugation of sugars and sulfates, the goal was to analyze the effect of the constitute overexpression of a ZON inducible UDP-glycosyl transferase and a knock-out mutant of the ABC transporter, PDR12. Therefore, plants extracts and liquid growth media were analyzed in a time course after ZON exposure.

3 Material and Methods

3.1 Plant handling

3.1.1 Surface sterilization and plating of Arabidopsis seeds

Approximately 40 seeds were taken out of the seed stock tube of the respective plant line. These seeds were transferred into a new tube using a sheet of paper. After that, the laminar flow working bench was prepared. Therefore, all needed items were disinfected using ethanol (denatured, 70%). Low melting agarose (1 %) was heated for 20 seconds in a microwave oven until it became viscous. 10 ml sterilization solution, containing 5% sodium-hypochlorite and 1 drop Tween 20, was prepared. About 1.2 ml of the sterilization solution was added to the tube containing the seeds and then incubated for 3 minutes inside the working bench. Thereafter, the tube was centrifuged shortly to get the seeds down. The sterilization solution was removed by using a disposable pipette. The seeds were washed twice with sterile deionized water (diH_2O). Therefore, 1.2 ml of water were added, then the tube was centrifuged and the washing water was removed. After that, 6 to 8 drops of low melting agarose were added to the seeds. For spreading of seeds, they were sucked out of the tube by using a disposable pipette. Now the seeds were placed in straight lines on MSK 100 agar plates (20 seeds per row and 6 rows per plate).. The plates were sealed with parafilm and incubated at 4 °C for 2 days. After this imbibition step the plates were transferred into a continous light growth chamber and 22 °C (Rumed).

Table 1 Composition of MSK medium

Murashig and Skoog salt	4.5 g
Plantagar	10 g
Sugar	45 g
diH_2O	replenish to 1000 ml

A pH ranging from 5.6 to 5.8 was necessary. Thereafter, the agar was autoclaved. Kanamycin (50 mg/ml) was added to reach a target concentration of 100 ng/µl

3.1.2 Transfer of *in vitro* grown seedlings to soil

The soil was prepared the day before planting. It contained 50 % potting soil and 50 % perlite. The flowering pots were filled with the mixture and watered plentifully. The next day a

small hole was dug with a pencil in the middle of each well. The small plants in the age of 14 to 21 days were extracted from the germination plates and transferred to the prepared pots. This had to be done carefully because of the fine roots. After planting, the seedlings were covered with clingfilm to obtain a greenhouse climate and avoid dehydration. The pots were transferred into a growth chamber with a 16/8 h light/dark cycle and 20 °C.

3.1.3 Harvesting of seeds

The mature plants were cut close to the soil with scissors. The plastic tube of the Aracons was placed vertical on a sheet of paper and the previously cut plant was pulled out under the tube. In this way, the siliques were split up by the bottom edge of the plastic tube. The whole material on the sheet of paper was sieved and the obtained seeds were transferred into a new tube (1.5 ml). The tubes were stored for 3 days with open caps for seed drying.

3.2 Polymerase Chain Reaction (PCR)

The goal of the PCR is to amplify a desired DNA fragment. Therefore, the DNA has to be denatured at high temperatures. Two primers are needed, which are binding to the denatured DNA strands. One primer binds to the coding strand, the other one to the complementing strand. After the primer annealing, the provided DNA polymerase starts to synthesize the new complementary strands by using provided dNTPs. The cycle of denaturation, primer annealing and elongation was repeated about 30 times. This method gives theoretically 2^{30} copies of the target DNA. The efficiency depends on various factors (e.g. primer quality, temperature profile, polymerase quality, buffer conditions, template composition, etc.).

3.2.1 Home-made Taq DNA polymerase

The following Table shows the standard PCR approach. The different primers which were used are listed in 3.1.1. A homemade Taq DNA polymerase was chosen. The master mix ingredients are listed below.

Table 2 Composition of the 10x PCR-buffers

TrisHCl	100 µl
KCl	500 µl
$MgCl_2$	25 µl
100 % Trition X	15 µl
diH_2O	360 µl

equals 1000 µl 10x PCR-buffer

Table 3: Homemade Taq DNA polymerase approach

	Sample	**negative Sample**
	Volume [µl] for one sample	Volume [µl] for one sample
10 x PCR Buffer	1	1
2 mM dNTPs	1	1
20 µM Primer (Forward)	0.25	0.25
20 µM Primer (Reverse)	0.25	0.25
di H_2O	6.3	7.3
TAQ DNA Polymerase	0.2	0.2
Template	1	-
Final Volume	10	10

Each tube was covered with the drop (20 µl) of mineral oil.

Table 4: Used PCR machine

PCR Program:	SSLP 55
PCR-Machine	Mastercycler Eppendorf
Volume:	30 µl

The chosen PCR program was operating under the following conditions:

Table 5: PCR operating conditions

Cycle steps:	**Temperature [°C]**	**Time [s]**
Initial denaturation	94	180
Annealing	55	15
Elongation	72	30
Denaturation	94	15

35 cycles were usually carried out.

3.2.2 Dream Taq DNA polymerase

There were no big differences to the standard PCR approach normally used. The amount of Dream Taq DNA polymerase was less and the amount of H_2O is higher.

Table 6: Dream Taq DNA polymerase approach

	Sample	**negative Sample**
	Volume [µl] for one sample	Volume [µl] for one sample
10 x PCR Buffer (20mM $MgCl_2$)	1	1
2 mM dNTPs	1	1
20 µM Primer (Forward)	0.25	0.25
20 µM Primer (Reverse)	0.25	0.25
di H_2O	6.3	7.3
DNA Polymerase	0.04	0.04
Template	1	-
Final Volume	10	10

The operating conditions and the used PCR machine were the same as with 3.2.2.1. 10x DreamTaq Green Buffer contains KCl and $(NH_4)_2SO_4$ at a ratio optimized for robust performance in PCR and includes $MgCl_2$ at a concentration of 20mM.

3.3 Real-time PCR

The real-time PCR is based on the principle of normal PCR (3.2.2). The difference of the method using EvaGreen® is based on a fluorescence signal which can be quantified by using standards with known amount of single-stranded DNA amounts. The real-time PCR was done with the EvaGreen® real time PCR Kit (company?). The contents of the master mix for one assay are shown below.

Table 7: Quantitative Real time PCR approach

	Sample	**negative Sample**
	Volume [µl] for one sample	Volume [µl] for one sample
5x EvaGreen® qPCR master mix	2.8	2.8
20 µM Primer (Forward)	0.25	0.25
20 µM Primer (Reverse)	0.25	0.25
di H_2O	9.7	10.7
Template	1	-
Final Volume	14	14

Two real-time PCR machines, Rotor-Gene 2000 and 3000 were in used. To get the standard curves and for sample analyzing, Rotor-Gene machines (former Corbett Research, Australia, and now Qiagen, Germany) were used.

Table 8: Rotor-Gene 2000 used for standard curves

Real-time PCR machine	Rotor-Gene 2000
Volume:	14 µl

Table 9: Rotor Gene GR3000 A used for sample analyzing

Real-time PCR machine	Rotor-Gene GR3000 A
Volume:	14 µl

Each machine was driven under the following conditions:

Table 10: Operating conditions PCR machines

operating conditions:	Temperature [°C]	Time [s]	
Initial denaturation	95	900	
Cycle denaturation	95	5	
Cycle annealing	55	5	
Cycle elongation	72	12	Channel A
	79	10	Channel B
	84	10	Channel C

The signal was recorded at three different temperatures. The melting happened from 65 °C to 99 °C. Every degree was one single step of the procedure and lasted 2 seconds.

3.4 Assembly of real-time PCR standards

The goal was to obtain a standard solution with a known amount of copies of generated cDNA templates. Therefore, each standard was calculated as follows:

$$x = \frac{y\,[g/\mu l]}{z\,[bp] \times 660\,[g/mol]} * 6{,}022 * 10^{23}$$

y = measured amount of DNA

z = number of base pairs

660 [g/mol] = average mass of a base pair

$6.022 * 10^{23}$ = Avogadro constant

x = number of copies/µl

Table 11: Calculation of quantitative real time standards

Standard (AGI code or gene)	Base pairs	Stock concentration [ng/µl]	DNA fragments per µl	Dilution factor to get 10^9
1g15520/PDR12	263	12.53	$4.35 * 10^{10}$	1:42.5
1g17180/GSTU25	312	12.9	$3.77 * 10^{10}$	1:36.7
2g15480/UGT73B5	485	31.23	$5.88 * 10^{10}$	1:57.8
2g15490/UGT73B4	603	21.98	$3.33 * 10^{10}$	1:32.3
5g12030/sHSP	316	8.59	$2.48 * 10^{10}$	1:23.8
5g59720/sHSP	284	25.51	$8.2 * 10^{10}$	1:81
2g29500/sHSP	332	4.48	$1.23 * 10^{10}$	1:11.3
UBQ5	425	26.43	$5.67 * 10^{10}$	1:55.7
PP2A	401	15.45	$3.52 * 10^{10}$	1:3402
GFP	207	15.87	$6.99 * 10^{10}$	1:68.9

After that, the sample had to be diluted to 10^7, 10^6, 10^5, 10^4 and 10^3 copies μl^{-1}. The listed factors were used to get a 10^9 dilution. 5 µl of the 10^9 dilution were mixed properly with 495 µl diH_2O to obtain the 10^7 dilution step. The further dilution was done by taking out 10 µl of the previous dilution step and mixing it with 90 µl diH_2O. In this way all real-time PCR standards were produced.

3.5 Agarose gel electrophoresis

3.5.1 Preparation of gels

To obtain 1.5 % and 0.8 % agarose gels, 1.5 g and 0.8 g low electroendosmosis (LE) agarose were solubilized in 100 ml 1x TAE buffer per gel with a microwave oven. Therefore, the flasks were heated for 30 seconds four times. A Petri dish, used as cap, was applied to prevent evaporation. The flasks were cooled down by cold water from the pipe. At approximately 60 °C, 5 µl ethidium bromide (from a 10 mg/ml Stocksolution) were added. These mixtures were poured, without creating bubbles, into prepared gel tanks, where the desired combs were already put in. After 20 min cooling time, the gels were ready for use.

Table 12 Composition of 50x TAE-buffer

TRIS base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M) pH 8	100 ml

replenished with diH_2O to 1000 ml complete solution.

For use, the 50x TAE-buffer had to be diluted to 1x TAE-buffer. Therefore 20 ml of 50x TAE-buffer were mixed with 980 ml diH_2O.

3.5.2 Execution of electrophoresis

The amount of the sample which was decided to load was mixed with 1:9 loading dye. The gel was loaded with the samples, a size marker and a negative control and was run with 100 Volt for more or less 45 min. Subsequently the gel was photographed by a CCD imaging system under UV excitation. Most of the time the size marker was at position one, followed by the samples and the negative control at the end.

3.6 DNA extraction from agarose gels (QIAEX II-Kit)

For this procedure the known amount of excised gel was important. Therefore, the desired bands were cut out with a scalpel and transferred into already weighed 1.5 ml reaction tubes. The tubes were weighed again and the amount of the gel piece was calculated. After that, three times the volume of Buffer QX 1 (Qiagen) was added. The tube was then vortexed for 30 sec. Afterwards, 30 µl of QIAEX II (Qiagen) were added (10 µl < 2 µg DNA, 30 µl > 2 µg DNA) and the tube was vortexed again just for blending. The mixture was now incubated at 50 °C for 10 minutes, with vortexing every two minutes. Now the tube was centrifuged in an Eppendorf Centrifuge at 13.000 rpm for 30 seconds. The supernatant was discarded by decantation and the pellet was washed with 500 µl of Buffer QX1 (Qiagen). Thereafter, the pellet was dissolved again in Buffer QX1 (Qiagen), vortexed, and centrifuged again under the same conditions as before. The supernatant was discarded again and the received pellet was washed twice with 500 µl Buffer PE (Qiagen) following the same procedure like the Buffer QX1 washing. After the final washing step, the supernatant was removed as much as possible with a pipette. Now the pellet was air-dried until it became almost white (approximately 10-15 min). To resolve the pellet, 20 µl autoclaved and purified water was added. The tube was vortexed and incubated at room temperature for five minutes. The mixture was centrifuged at 13.000 rpm for 30 seconds. The supernatant was transferred into

a new, labeled tube. This last solving step was repeated in the same manner with just 15 µl eluent.

3.7 Plasmid DNA preparation (Miniprep) from *Escherichia coli*.

To prepare plasmids from *E. coli*, 2 ml of an overnight culture were necessary. The 2 ml broth was transferred into 2 ml tubes and centrifuged at 13.000 rpm for 5 minutes. The supernatant was discarded and 2 ml broth was added one more time. The tube was centrifuged again at 13.000 rpm for 5 minutes. The supernatant was discarded once more, and 200 µl of GTE buffer (GET buffer consits of 50 mM glucose (MW 180), 10mM EDTA, 25 mM Tris-HCl pH 8, diH_2O) were added and mixed. Now the tube was incubated on ice for 5 minutes. Meanwhile the solution for alkaline cell lysis was prepared. Therefore, 1 ml 1 M NaOH, 250 µl 20 % SDS and 3.75 ml of deionized water were mixed together. 400 µl of this prepared solution was added to the sample tube. After that, the tube was turned five times for mixing. Afterwards, 300 µl of sodium acetate (3M, pH 4.8) were applied, carefully shaken and incubated on ice for 10 minutes. After the incubation, the tube was centrifuged at 13.000 rpm at 4 °C for 10 minutes. Meanwhile, 600 µl isopropyl alcohol were provided in new 1.5 ml tubes. After centrifugation, 800 µl of the supernatant were added to the prepared 600 µl isopropyl alcohol. This mixture was vortexed and incubated on ice for 10 minutes, followed by centrifugation at 13.000 rpm at 4 °C for 15 minutes. The obtained supernatant was discarded and the pellet was washed with ethanol (70%). Therefore, 500 µl ethanol were added, followed by vortexing, and centrifugation at 13.000 rpm at 4 °C for 15 minutes. The supernatant was discarded through decantation and the tube was left reverse for air drying. In the meantime the DNAse free RNaseA solution (10 mg/ml) was prepared. Therefore, 40 µl purified water and 2 µl of DNAse free RNaseA were mixed together. Once there was no ethanol left in the sample tube, 42 µl of DNAse free RNase solution was added and incubated on a shaker at room temperature for 10 minutes. Afterwards the sample was further incubated at 37 °C for 30 minutes. Thereafter, the sample was ready for further processing.

3.8 Cloning into pDrive

3.8.1 Preparation

The linearized vector (pDrive) carries a single uridine (U) overhang at the 3' end. Therefore, the sample needs an adenosine (A) overhang on the 3' end for hybridization. A master mix was prepared, containing:

Table 13: pDrive cloning approach

	Volume [µl] for one sample
10 x PCR Buffer	1
2 mM dATP	1
Taq DNA polymerase	0.2
diH_2O	3.8
PCR template	4
Final volume	10

The used buffer was Dream Taq buffer (Fermentas). The used polymerase was a Dream Taq DNA polymerase (Fermentas).

3 to 5 times molar excess of the PCR product in relation to the vector was incubated.

The PCR product was provided in a 0.5 ml tube and the master mix was added. Afterwards, the tube was put into a PCR machine (Eppendorf Mastercycler) and was kept at 72 °C for 10 min. After the incubation, the tube was stored on ice.

3.8.2 Ligation

The following approach was used for ligation:

Table 14: Ligation approach

	Volume [µl] for one sample
pDrive cloning vector (50 ng/µl)	0.5
Ligation Master Mix (Qiagen)	2.5
PCR product (3' A - overhang) ng/µl	1.3
diH_2O	0.7
Final volume	5

The plasmid (25 ng) was provided in 0.5 ml tubes. The calculated amount of PCR product (65 ng) and the ligation master mix (Qiagen PCR Cloning System) were added. The tube was incubated at 4 °C for 30 min.

3.8.3 Transformation of competent cells

To reach a transformation into competent *E. coli* DH5α cells, the prepared ligation assay was mixed with 5x KCM buffer and diH_2O.

Table 15: Transformation of competent cells approach

	Volume [µl] for one sample
Ligation assay	10
5x KCM	20
diH_2O	70
Final volume	100

This mixture was now added to 100 µl competent DH5α (homemade) cells. After careful shaking by flicking with a finger, it was incubated on ice for 30 minutes. After this incubation, the transformation was done by heat shock. The sample was heated to 42 °C for 90 seconds. Thereafter, the cells were kept on ice for 5 minutes. 800 µl of YT medium was added and the whole sample was kept at 37 °C and shaken for one hour. After that, the cells were ready for plating on YT medium plates containing the 50 µg/L antibiotic selection marker kanamycin.

Table 16 Composition of YT-medium

Yeast extract	10 g
Casein hydrosylate	10 g
Agar-agar	15 g
NaCl	5 g
diH_2O	replenish to 1000 ml

If the medium was used as liquid culture, the agar agar part had to be excluded.

3.9 Transformation of competent *Agrobacterium tumefaciens* cells

The transformation was done via electroporation. Therefore, the cuvette had to be prepared and stored on ice. Competent *Agrobacterium tumefaciens* (GV3101 pMP90) cells, stored at -80 °C, were used. Before pipetting the cells, a small piece of the pipette tip was cut off to avoid cell injuries. The cells were defrosted on ice. 50 µl of the competent cells were

transferred into a tube. The target plasmid was added (2 µl) and carefully mixed. The cell plasmid mixture was transferred into the ice cold cuvette, using again a cut off tip. The cuvette was dried carefully on the outside and placed in the UV-C cross-linker for sterilization. The cross-linker had been started before and was adjusted to 6.000 kJ. For electroporation, the transformation program Bacteria/AGR in the Biorad electroporator was executed. After the pulse, the cuvette was again placed on ice. 700 µl YT medium was added, carefully mixed and transferred into a fresh tube. For recovery, the tube was incubated at 30 °C for 1 hour on a shaker. The cells were then ready for plating.

3.10 Plasmid DNA preparation from *Agrobacteria tumefaciens* (Miniprep)

The goal was to extract the target plasmid from the *Agrobacterium tumefaciens* cell culture. Therefore, 2 ml of the liquid culture were centrifuged at 5.000 rpm for 5 minutes. The supernatant was discarded and 200 µl of GTE buffer were added. The pellet was resuspended by pulling the tube over the rack. After that, the sample was incubated at room temperature for 5 minutes. Meanwhile, a solution was prepared containing 1 ml NaOH (1 M), 250 µl SDS (20 %) and 3.75 ml diH_2O (sterile). After the incubation step, 400 µl of the freshly prepared NaOH/SDS solution were added. The tubes were carefully flipped several times for blending and were incubated on ice for 15 minutes. Then 300 µl of potassium acetate (3 M pH 4.8) were added. The mixing was done by inverting the tubes by hand. Afterwards, the sample was centrifuged at 13.000 rpm at 4 °C for 15 minutes. The supernatant was saved and transferred into a new tube. The next step was done under an exhaust hood. 500 µl phenol:chloroform:isoamyl alcohol (24:24:1 volume ratio) were added. The tubes were shaken for one minute and then centrifuged at 13.000 rpm at 4 °C for 5 minutes. Meanwhile, 1.2 ml isopropyl alcohol was prepared in a new 2 ml tube. After the centrifugation step, the supernatant was transferred into the prepared 2 ml tube and the sample was mixed by using a vortex. After that, the sample was centrifuged at 13.000 rpm at 4 °C for 30 minutes. The obtained supernatant was discarded, 500 µl 70 % ethanol were added and the sample was vortexed. Thereafter, the sample was centrifuged at 13.000 rpm at 4 °C for 10 minutes. The obtained supernatant was discharged and the remaining liquid was removed using a pipette. The pellet was air dried and solved in 40 µl TE buffer, containing 2 µl DNAse free RNase A (200 µg/ml). To obtain a good solving, the tube was incubated at room temperature for 10 minutes on a shaker, followed by incubation at 37°C for approximately 30 minutes. Now the sample was ready for further procession.

3.11 Sequencing reaction

To sequence a sample, the following approach (BigDye®) was used.

Table 17: Sequencing approach

	amount in µl
Terminator Ready Reaction Mix	4
Primer (4µM)	0.8
Sample (~ 200 ng)	1
diH_2O	14.2
Final volume	20

This mixture was prepared in 0.5 reaction tubes. Thereafter, the tubes were transferred into a Eppendorf Mastercycler PCR machine. The following program was chosen:

Table 18: Eppendorf Master Cycler sequencing operation conditions

Cycle steps:	Temperature [°C]	Time [s]
Denaturation	96	30
Annealing	50	15
Elongation	60	240

25 cycles were completed

For further sample processing, 50 µl ethanol (95 %) and 2 µl sodium acetate (3 M, pH4.8) was provided in a 1.5 ml tube. The 20 µl sequencing reaction was added to the provided ethanol/sodium acetate mixture and incubated on ice for 15 minutes. After that, the sample was centrifuged at 4 °C for 25 minutes at 13.000 rpm. The supernatant was discharged and the pellet was washed with 500 µl ethanol (70 %). The sample/ethanol mixture was centrifuged at 4 °C for 10 minutes at 13.000 rpm. The supernatant was again discharged and the pellet was dried for 4 min in a vacuum centrifuge concentrator. After this processing, the sample was ready for the sequencing. This was done by Susanne Neubert.

3.12 Agrobacterium-mediated plant transformation

3.12.1 Preparation of the plant material

The plants (colwt) were cultivated and transferred to soil as described in 4.1.1 and 4.1.2. The plants were ready for transformation at the time when they had plenty of unopened flowers. If the plant was too old, the shoot axis was cut to remove mature siliques and new shoots were formed by the plants (approximately after two weeks the plant was ready again for transformation). The plants were not watered the day before the transformation.

3.12.2 Transformation - Dipping

Colonies of *Agrobacterium tumefaciens*, which contained the recombinant binary plasmid, were transferred to 5 ml YT liquid medium (spectinomycin 100 mg/l, gentamycin 50 mg/l, rifampicin 100 mg/l). The tubes were incubated at 160 rpm at 30 °C for 48 hours. After this incubation, 1 ml of the culture was transferred to 4 ml YT liquid culture. This new culture again was incubated at 160 rpm at 30 °C for 48 h. 300 ml YT liquid medium were inoculated with 2 ml of the o/n culture and were incubated in a 1 liter flask at 160 rpm at 30 °C for 72 hours. Thereafter, the culture was centrifuged at 22 °C at 6.000 rpm for 20 minutes. The supernatant was discharged and the pellet was solved in 100 ml sucrose solution (5 %). The solution was transferred into a 1 liter beaker and was diluted with sucrose solution (5 %) to get a OD_{600} of 0.8. To increase the transformation efficiency, Silwet L-77 was added (300 µl per 1000 ml sucrose solution). Now the plants were dipped into the suspension for two minutes. The dipped plants were provided with a plastic coverage and were kept in the dark for 24 hours at room temperature. Thereafter, the plants were transferred to the growth chamber (16 hrs light/8 hrs dark, 20 °C).

3.13 Zearalenone-induced reporter expression (microscopy)

To check for ZON induction, the plants were treated with a ZON solution and a DMSO mock control. Therefore, 300 µl ZON (50 µM) and 300 µl DMSO (1:200 of 0.05 %) solution per well was prepared in a 24 well plate. Three plants (8 dag) were transferred into each well and incubated at 22 °C for 2 hours in a light chamber (Rumed). After the incubation step, the plants were examined by using an UV fluorescence microscope. To excite GFP fluorescence, a mercury metal halide bulb was used and emission was made visible with a

GFP filter set. The plate was put back into the light chamber and was again examined after 4 and 6 hours, respectively. Photos were taken of the plants sample.

3.14 Heat shock experiment

To check for heat induction, plants (11 dag) where transferred to a MS 4.5 agar plate. The plate was incubated at 38 °C for 3 hours. After that, the plate was incubated at 22-25 °C for 24 hours in a light chamber to let the plants regenerate. Subsequently, the plants were tested for GFP expression using the same UV microscope as for the ZON experiment.

3.15 RNA isolation

To extract RNA from plants, the plants were harvested and transferred into reaction tubes. The tubes were frozen in liquid nitrogen. Therefore, the tubes simply were thrown into a box containing liquid nitrogen and taken out again after approximately one minute. After, this freezing procedure, the samples were homogenized by the use of a sterile pestle which fits to the 1.5 ml reaction tubes. Afterwards the samples were again put to the liquid nitrogen. Now the samples were ready for RNA extraction. During the whole RNA extraction procedure, the samples were kept on ice.

To extract the RNA, 1 ml TRI reagent (company?) was added to the sample. After that, the samples were incubated for 5 min at room temperature. After the incubation step, 0.2 ml of chloroform was added and immediately vortexed for 15 seconds. Afterwards, the sample was incubated on ice for 8 minutes. Then the sample was centrifuged for 15 minutes at 4 °C at 12.000 rpm. The clear supernatant was transferred into new tubes and mixed with 0.5 ml isopropyl alcohol. This mixture was again vortexed properly. After that, the sample was incubated on ice for 7.5 minutes. Immediately afterwards, the sample was centrifuged for 8 minutes at 4 °C at 12.000 rpm. The obtained supernatant was discharged and 1 ml ethanol (75 %) was added to the remaining pellet and vortexed properly. Afterwards, the sample was centrifuged for 5 min at 4 °C at 12.000 rpm. The supernatant was discharged and the remaining pellet was air-dried until it became almost white (approximately 10 -15 min). Up to that point, all steps were done under an exhaust hood. For solving the pellet, 50 µl autoclaved RNAse free ddH_2O was added. The sample was shortly vortexed and stored at -80 °C.

3.16 RNA quantification

The RNA quantification was done by a Kit (Invitrogen Quant-it RNA Assay Kit). The isolated RNA was diluted 1:10. 2 µl of the sample were mixed with 18 µl of RNAse free ddH_2O. Then

a working solution was prepared. Therefore, the quant-it RNA reagent was diluted 1:200 with the provided buffer. 198 µl of this working solution were transferred into a fresh tube. 2 µl of sample was added and mixed properly. The measurements were started with a zero control and a sample of known RNA concentration provided by the kit. The fluorescence was measured at excitation/emission maxima of 644/673 nm. To calculate the correct amount of RNA, the dilution steps had to be considered.

3.17 DNase digestion and cDNA synthesis

To obtain cDNA, RNA samples were needed. In our case, 2.5 µg of RNA were used for synthesis. The RNA sample was diluted with ddH_2O to get a sample volume of 6.65 µl containing 2.5 µg RNA. Then 0.3 µl RNAse free DNase and 0.3 µl $MgCl_2$ (1 M) was added. After that, the sample was incubated for 10 minutes at 75 °C in a Thermocycler. After the incubation the assay was chilled on ice for several minutes. Meanwhile, a master mix was created. The master mix consisted of:

Table 19: cDNA synthesis

	Sample
	Volume [µl] for one sample
5 x RT Buffer	3
10 mM dNTPs	1
0.1 M DTT	1.5
25 mM Oligo dT_{18}	1.25
200 u/µl Superscript III	1
Final volume	7.75

The master mix was added to the sample and carefully mixed. The end volume of this approach was 15 µl. The sample was incubated for 70 minutes at 37 °C. After this incubation period, the enzymes were inactivated for 5 minutes at 94 °C. Immediately after that, 135 µl ddH_2O were added (giving a 1:10 dilution). These 150 µl were split into three new tubes and stored at -20 °C.

3.18 Zearalenone metabolisation experiment

3.18.1 Preparation of the experiment

Arabidopsis seedlings (13 dag) were transferred from agar plates into 10 ml MS 2.5 liquid culture. The liquid cultures were incubated for 3 days with continous light at 22 °C and on a rotary shaker with 50 rpm.

Table 20 Composition of MS 2.5 liquide culture

Murashig and Skoog salt	4,33 g
Sugar	25 g
diH_2O	replenish to 1000 ml

pH 5,7

3.18.2 Execution of the experiment

The prepared liquid cultures were treated with 50 µM ZON and further incubated at 22 °C at 50 rpm. After 0.5 h, 2 h, 5 h, 12 h, 24 h and 36 h the plants were obtained from the liquid culture. The plants were rinsed with 10 ml sterile H_2O using a 5 ml pipette. After that, the harvested plants were weighed and transferred into a fresh 50 ml tube containing 4 ml of 75 % acetonitrile. The plant material was homogenized for 1 minute using a Turrax T25. Afterwards, the homogenized plants were centrifuged at 5.000 rpm for 10 minutes. The obtained plant extract was filtered with a round filter (Macherey-Nagel). The remaining washing water was mixed with the remaining liquid culture and was also filtered. The volume of this filtered mixture was measured and the same volume of methanol was added. The samples were stored at 4 °C following a HPLC-MS which was carried out by Franz Berthiller (Department of Agrobiotechnology, IFA-Tulln).

Figure 7: Experimental setup of the metabolism experiment. Abbreviations: MS 2.5 liquid media (Murashig and Skoog medium), ZON (zearalenone)

3.19 Cryostocks

To keep a bacterial (*E.coli* or *Agrobacterium*) culture for a longer period of time, it is necessary to store it as cryostock. 800 µl of an over night culture with appropriate antibiotics were transferred into a fresh tube. 400 µl of sterile glycerine (86 %) were added to the sample and mixed by flipping the tube. This mixture was put into liquid nitrogen for shock frosting. After that, the sample was transferred into a -80 °C deep freezer.

4 Results

4.1 Bioinformatic data analysis

The analysis was done with the freely available tools at the Genevestigator webpage (ETH, 2008) and is summarized here for better reflection. The original data can be found in the appendix.

4.1.1 Gene expression during the developmental stages of the plant

The following Figures show the expression levels of the genes during the different developmental stages of the plant.

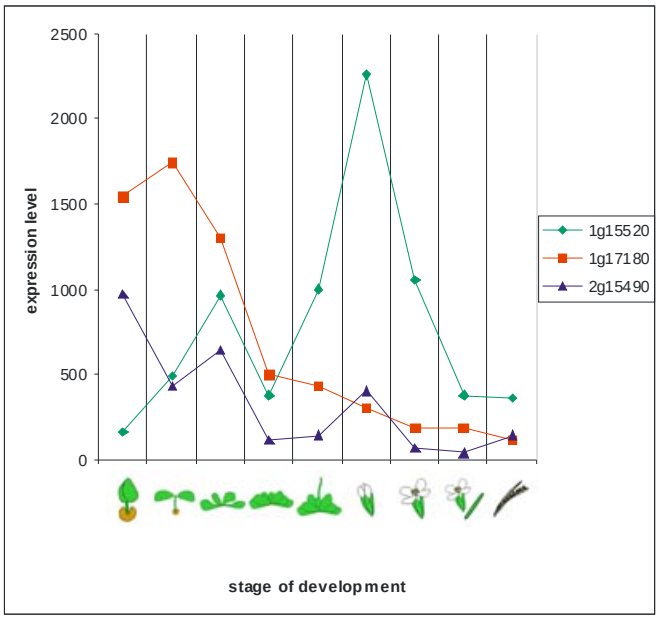

Figure 8: Summary of the micro array expression analyses at the Geninvestigator webpage of genes At1g15520/PDR12, At1g17180/UGT73B5 and At2g15490/UGT73B4. The genes expression level concerning the developmental stage of *Arabidopsis thaliana* is displayed.

Ad Figure 8: At1g15520/PDR12 has its expression maximum when the flowering starts. On the opposite, At1g17180/UGT73B5 shows the highest expression level in the two leaf stadium while At2g15490/UGT73B4 is expressed mostly in the seedling stadium.

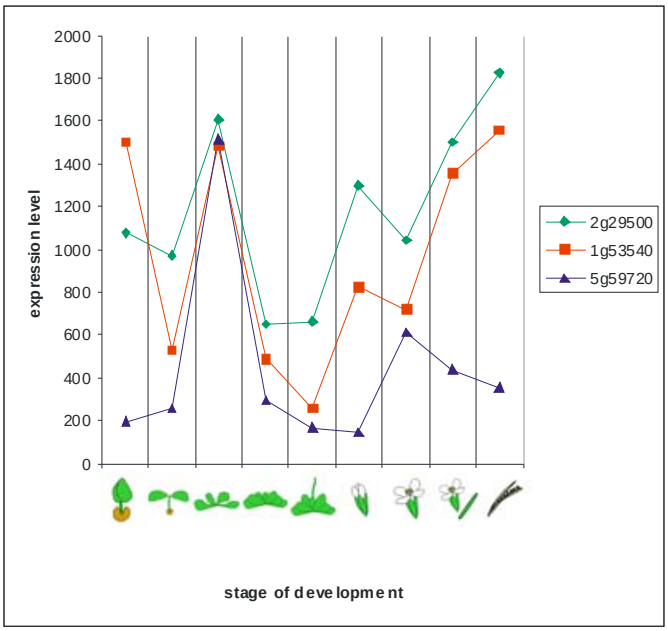

Figure 9: Summary of the micro array expression analyses at the Geninvestigator webpage of genes At2g29500/HSP17.6B-CI, At1g53540/HSP17.6C-CI and At5g59720/HSP 18.1-CI. The genes expression level concerning the developmental stage of *Arabidopsis thaliana* is displayed.

Ad Figure 9: At2g29500/*HSP17.6B-CI* and At1g53540/HSP17.6C-CI show two nearly equally behaving expression graphs. They are expressed mostly in the four leaf stadium and in the mature silique/seeds. The gene At5g59720/HSP18.1-CI is expressed mostly in the four leaf stadium. It has no further maxima.

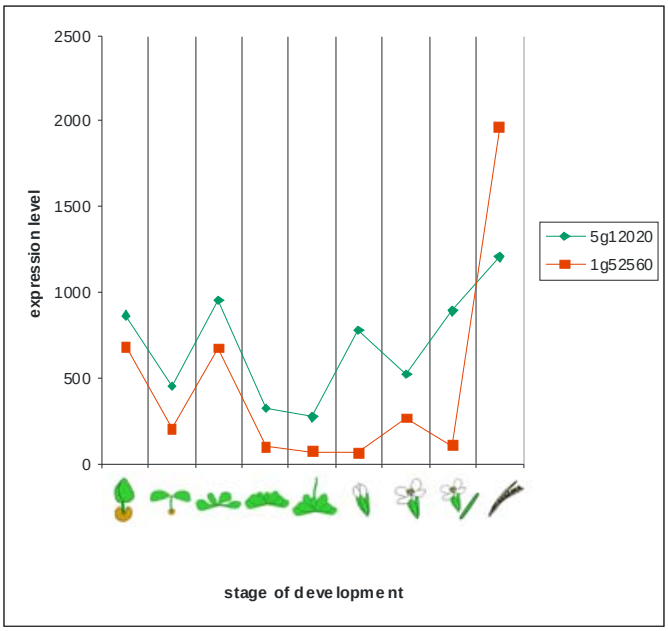

Figure 10: Summary of the micro array expression analyses at the Geninvestigator webpage of genes At5g12020/HSP17.6-CII and At1g52560/HSP 18.1-CI. The genes expression level concerning the developmental stage of *Arabidopsis thaliana* is displayed.

Ad Figure 3: At5g12020/HSP17.6-CII and At1g52560/HSP26.5-P(r) exhibit nearly the same expression graphs. Both have their maximum expression level in the mature silique/seeds.

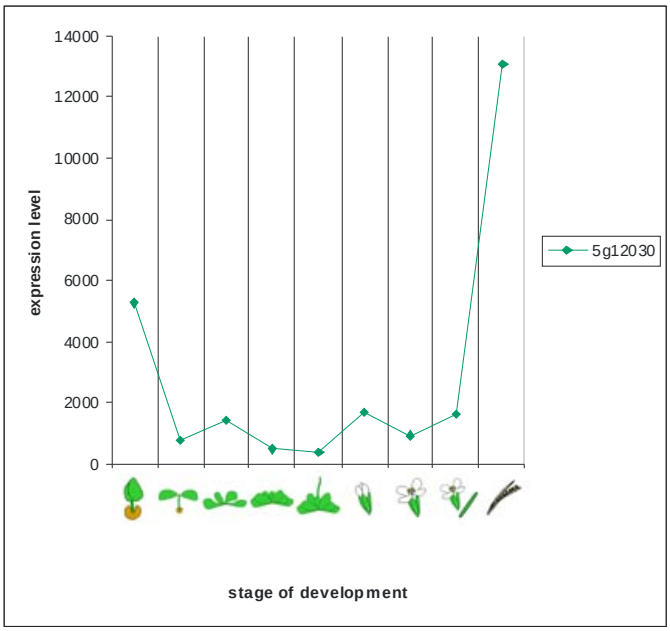

Figure 11: Summary of the micro array expression analyses at the Geninvestigator webpage of gene At5g12030/ HSP 17.6A The gene expression level concerning the developmental stage of *Arabidopsis thaliana* is displayed

Ad Figure 11: At5g12030/HSP17.6 A is depicted alone because of its crucial higher expression level compared to all other genes. It has its maximum expression in the mature silique/seeds stadium.

4.1.2 Localization of gene expression

To obtain a good overview, a heat map was rendered. The original data can be found in the appendix.

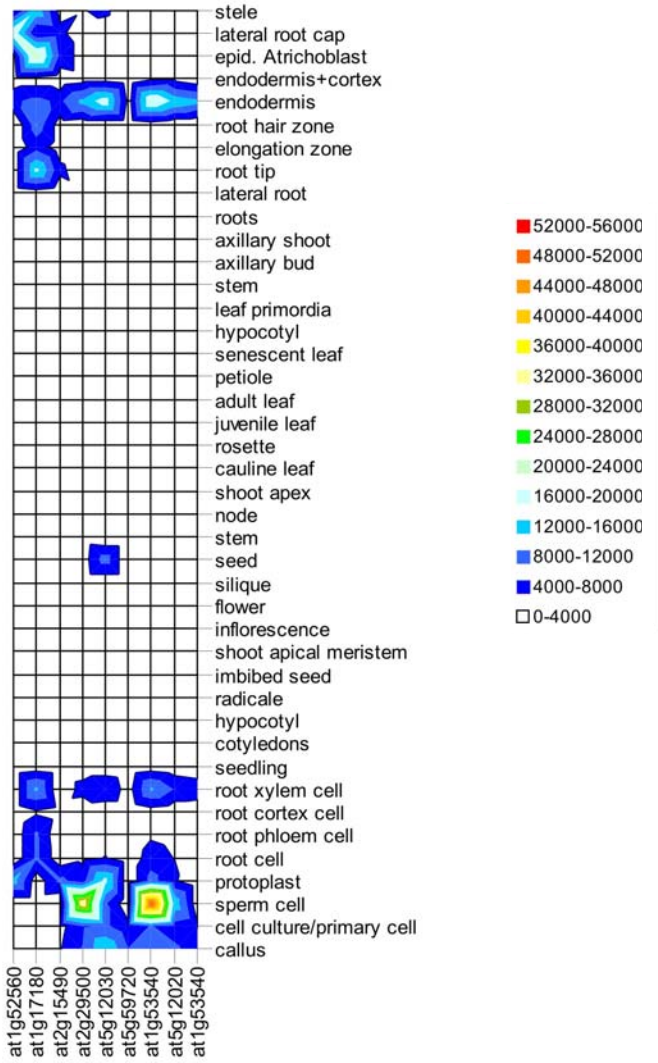

Figure 12: Localization of gene expression of At1g15520/PDR12, 1g17180/UGT73B5, 2g15490/UGT73B4, 2g29500/HSP17.6B-CI, 1g53540/HSP17.6C-CI, 5g59720/HSP18.1-CI, 5g12020/HSP17.6-CI, 1g52560/HSP18.1-CI and 5g12030/HSP17.6A in *Arabidopsis thaliana* compartments. The intensity of expression is displayed by colors.

The heat map (Figure 12) shows the location of the gene expression. Furthermore, the intensity of expression is displayed with different colours. For example, At5g53540/ is mostly expressed in sperm cells (pollen). Most of the genes show a high gene expression in the sperm cell. Nearly all genes are expressed in the endodermis.

4.2 Assembling of the promoter constructs with the 3' UTR

4.2.1 Preparation of 3' UTR of At2g29500/ *HSP17.6B-CI* and cloning into pDrive

To obtain the 3' UTR of the gene At2g29500, it was necessary to design primers. It was done by David Merz using the program DNAstar. The chosen area of the gene included a part of the 3' end of the gene and the following untranslated region.

5'
agagagagataga**gagttgttgaagcgagtgtg**ttttgttcgccgagtctttcgtcgaagttgtttttttttttcccaaaaaaaaaaaacata
tctttttgtggccgacggatttagtaaaaaaaacagtataatttttataaagatgggcccaatagtagaatcgtacgctcaagattccgtaatg
aacgcgggaagttacgtcattgtgaccctacttacttagtggagctcaatgtccaatatacaccgttggatttgtttaatcctcctttagatgg
gcccagtttttagtaatattctattcaattcagcacgttatacaattgcttttaaagcccatggcccataatactcagacagccttaaatgctt
cagattaacaaagaaaatgttttagattttatggatagtgtgattagagtccaattattttggaatttaattcggttaaccagtcgaaccggat
aatctgtagaaatccagtttttttttttggaagactacatatttgtctaaagcccatgaaggcccattaaacccaacgaacacaaacaatcttca
aaatgctcctagaattttctggattcacctgatttttctcatagacgtcctctagatgtttctgtattccccataacaactcgagaagctcgag
aaatttcggttagtacctttaaatagcttcaaaaatccttcgtgtgttct**atcagaaatcgaaaaatcaaagttctcaagaagatatcaacaaa**
aaaaaaagtaaattctttaaaATGTCGATGATTCCAACTTTCTTCAACAACAACAGACGAAGCAACATCTTTGATCCATTCTCTCTTGACGTAT
GGGATCCATTCAAGGAACTAACATCATCATCACTTTCTCGTGAGAACTCAGCGATCGTGAACGCACGTGTGGACTGGAGAGAGACGCCTGAGGC
CCACGTGTTTAAAGCTGACTTGCCTGGATTGAAGAAGGAGGAAGTTAAAGTTGAGATTGAGGAGGATAGTGTTTTGAAGATCAGTGGAGAGAGA
CACGTGGAGAAAGAAGATAAGAATGACACGTGGCACCGTGTGGAGAGATCGAGTGGACAGTTTACGAGGAGGTTTAGGTTGCCGGAGAATGTGA
AGATGGATCAGGTTAAGGCTGCGATGGAGAATGGTGTGTTGACTGTTACGGTGCCTAAGGCTGAGACTAAGAAGGCTGATGTTA**AGTCTATTCA**
GATCTCTGGTTGAgtaatgggttcgagttttatcatcggagttgcttgtgtttttgtcatggttatggttcatgttttacttgagtgtgtgagt
actctatctaaattataataatctccgattgagctatgaattatgatgtatcggatacatttgatcctaatgaagtatggaataaataataatt
attaacctttgtctaaattcaaaagaagtttaaccgacgagatgatttcgcttagagttcgatttcggtttaactaaaccaaatcaaccgatac
cgtgaatcaagacggaatcaaacccaaaaactgaaaaggttcacattatataaatgttatattttgatttagattattacatcatttctttcaa
aatgattaacattttgtttgataactgaatatttcttctgttttacaatgtcattcatttcaaatgtttggcagacaatgaattggccaaaaaa
tagactcttcaaaacaaaaaa
3'

Figure 13: The genomic region of the gene At2g29500 with up- and downstream sequences. Red and orange dyed bases belongs to the gene. Yellow and turquoise dyed bases displays the forward and reverse primer of the promoter and 5' UTR of the gene. Brown and red dyed bases display the forward and the reverse primers of the 3'UTR region. Orange letters stands for the coding region of the gene. Red dyed bases display the overlapping regions of the gene and the chosen segments of promoter and 3'UTR.

The depicted code (Figure 13) represents the gene At2g29500 and the up- and downstream intergenic regions. The red and orange dyed nucleotides belong to the gene, whereas red nucleotides are the 5’ and 3’ UTRs and included into the promoter or the 3' UTR construct. The black nucleotides belong to the intergenic regions. The brown labeled nucleotides display the forward primer and the red dyed the reverse primer of the 3' UTR construct. The yellow and turquoise dyed nucleotides illustrate the forward and reverse primers of the promoter region including the 5' UTR of the gene.

The 3' UTR was amplified by PCR from genomic *Arabidopsis thaliana* DNA and ligated into pDrive. The pDrive cloning system provides high cloning efficiency of PCR products based on a U/A hybridization. The vector is distributed with an uracil overhang at each 3' end which hybridizes with high specificity to the A overhang of PCR products (QUIAGEN, 2013). The construct was sequenced to be sure that the correct DNA of interest was ligated (sequence analysis can be found in the appendix).

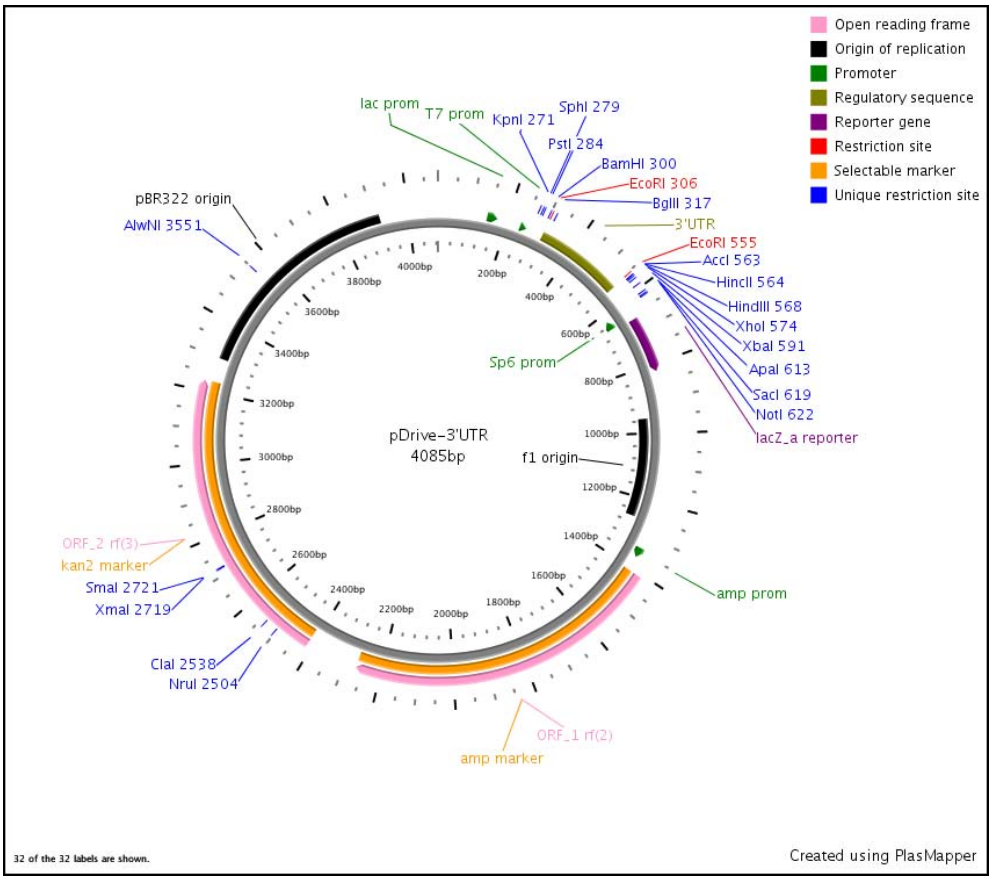

Figure 14: The pDrive cloning vector containing 3'UTR. The fragment of interest (3'UTR) is inserted between two EcoRI restriction sites (red displayed). The pDrive cloning vector owns an ampicillin (AmpR - orange) and a kanamycin (KanR) gene for selection. A blue/white screening is possible because of the lac_Z reporter.

4.2.2 Subcloning into pPZP211MCSpUC-GFP

The pPZP is a binary vector which is relatively small, stable and fully sequenced. The plasmid owns pTiT37 T-DNA regions and also the pBR322 ori site. These features enable mobilization from *E. coli* to *Agrobacterium tumefaciens*. Furthermore, the plasmid contains

the ColE1 and pVS1 origins of replication, which allow a replication in *E. coli*. Spectinomycin is used as a drug resistance marker (Hajdukiewicz et al., 1994).

The 3' UTR was cloned into the pre-existing pPZPpm2g29500-GFP vector for transformation into *Agrobacterium tumefaciens*. Therefore, the 3' UTR was inserted at a PstI restriction site. The plasmid was transferred into *E. coli* DH5α and verified by a control digestion.

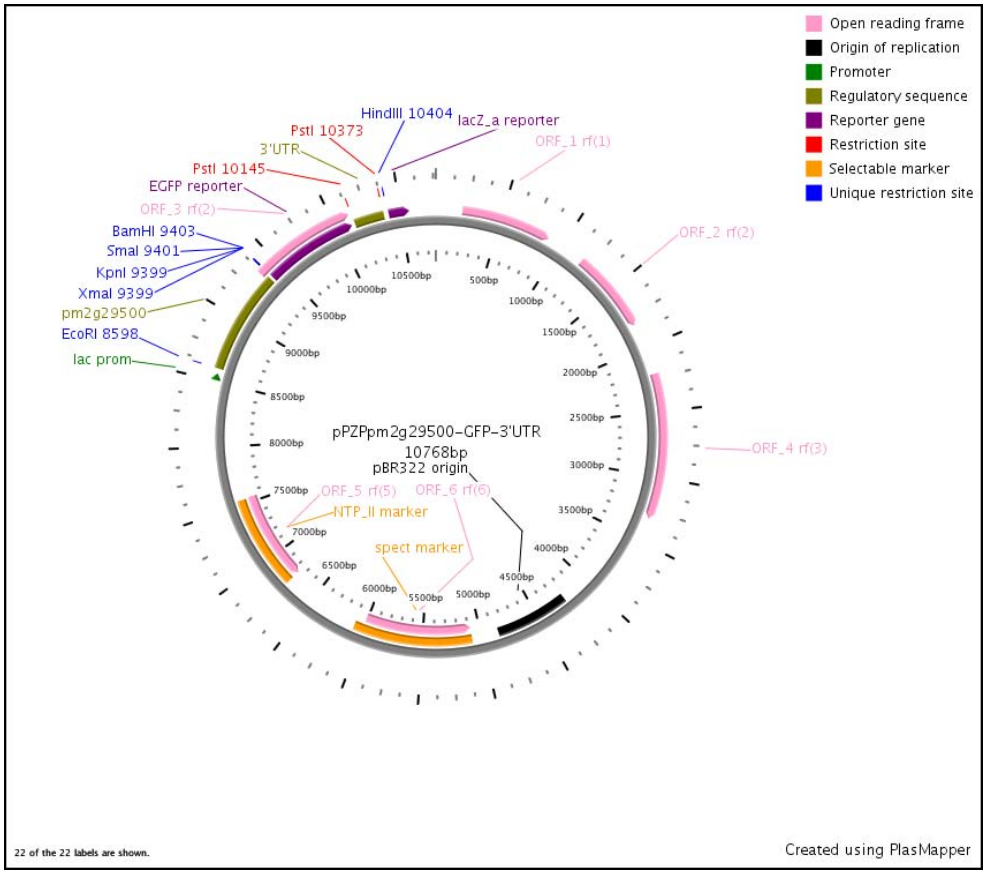

Figure 15: Final construct pPZPpm2g29500-GFP-3' UTR. The inserted promoter sequence pm2g29500 is displayed (green) after its EcoRI (blue) restriction site. The 3'UTR (green) was inserted at a PstI restriction site (red). The reporter (eGFP-purple dyed) is located between the promoter and the 3'UTR.

In Figure 15 the plasmid pPZP containing the preexisting construct and the 3' UTR are shown. The 3' UTR is flanked by two PstI restriction sites (red).

The target plasmid was transferred into *Agrobacterium tumefaciens* and via this bacterium into *Arabidopsis thaliana.*

4.3 Agrotransformation

The goal was to determine the amount of inserts and subsequently the determination of the ratio of homozygous and heterozygous plants.

Table 21: Overview transgenic lines concerning amount of inserts

		number of lines containing			
construct	number of tested lines	1 insert	2 inserts	1 or 2 inserts	more or less then 1 or 2 inserts
pPZPpm2g29500-GFP	**22**	12	7	3	0
pPZPpm2g29500-GFP-3'UTR	**22**	13	7	0	2
pPZPpm1g15520-GFP	**15**	9	3	1	2
pPZPpm1g53540-GFP	**13**	9	3	1	0
pPZPpm1g17180-GFP	**28**	18	6	2	2
pPZPpm5g59720-GFP	**20**	16	3	1	0
pPZPpm5g12030-GFP	**20**	15	1	2	2
pPZPpm2g15490-GFP	**18**	14	4	0	0
pPZPpm5g12020-GFP	**19**	9	10	0	0
pPZPpm1g52560-GFP	**12**	9	2	1	0
total number of lines containing inserts		124	46	11	8

Table 21 gives an overview on the tested lines concerning the amount of inserts. Overall nearly 10500 plants were tested in the T2 generation. The major part, 65 %, of the lines contained one insert and nearly 25 % contained two inserts. The amount of inserts was determined by Mendel's law with a segregation analyses of the dominant selection and kanamycin resistance marker. 25 % of sensitive plants stand for one insert. On the opposite 1/16 of sensitive plants mean two inserts. Sometimes the estimated amount of sensitive plants did not match the expected value. In this case no exact statement could be done referring to the amount of inserts. To sum up, 90 % of the examined plants had one or two inserts.

4.3.1 Determination of hetero- and homozygocity of transgenic T3 lines

To find homozygous Arabidosis lines with the inserted construct, the T3 generation was examined. On, average five offsprings of an insert containing T2 plant were put to soil. In

Table 22 a detailed list of line 4 of pPZPpm2g29500-GFP is displayed. If there are only resistant and no sensitive plants, the line is homozygous. In this example line 4-1 and 4-2 are homozygous.

Table 22: T3 Segregation analysis of the progeny of the T2 line 4 with the pPZPpm2g29500-GFP construct

line	resistant	sensitive	not germinated	total	homozygous line
4-1	20	0	0	20	yes
4-2	20	0	0	20	yes
4-3	16	2	0	18	no
4-4	14	4	0	18	no
4-5	18	2	0	20	no

Table 23 gives an overview of the whole pPZPpm2g29500 analysis. 89 lines, on average 20 plants per line, were examined. Nearly 1800 plants were observed to determine the homozygocity of pPZPpm2g29500-GFP T3 lines.

Table 23: Overview of the segregation analysis of pPZPpm2g29500-GFP construct in the T3 generation

tested T2 line	number of tested offspring (T3) lines per T2 line	number of homozygous lines (T3)
1	5	0
2	4	2
3	5	2
4	5	2
5	4	0
6	5	2
7	5	1
8	5	3
9	5	3
10	4	1
11	4	2
12	4	1
13	5	1
14	5	2
15	4	1
16	4	0
17	3	0
18	5	3
19	5	0
20	1	0
22	2	1
	total number of homozygous lines	27

27 lines approximately 30 % of the tested lines of pPZPpm2g29500 are homozygous originating from 16 independent transformants.

Table 24: Overview of complete T3 analysis

construct	total number of tested lines (T3)	total number of homozygous lines (T3)
pPZPpm2g29500-GFP	89	27
pPZPpm2g29500-GFP-3'UTR	69	18
pPZPpm1g15520-GFP	38	14
pPZPpm1g53540-GFP	15	3
pPZPpm1g17180-GFP	80	46
pPZPpm5g59720-GFP	71	28
pPZPpm5g12030-GFP	39	13
pPZPpm2g15490-GFP	15	6
pPZPpm5g12020-GFP	6	3
pPZPpm1g52560-GFP	14	4
	total number of homozygous lines	162

In Table 19 an overview of the complete T3 analysis is given. Finally 436 lines were evaluated and 162 homozygote lines were observed. About 9000 plants were necessary to obtain this data.

4.4 Quantitative expression analysis with Real time-PCR

To obtain quantitative expression data, standard series with known amount of gene copies, were done. Columbia wild-type plants (colwt) were used to monitor the ZON induced gene expression in time-course experiments. Therefore, steril seedlings were treated with 50 µM ZON and DMSO as solvent control. After certain time points, the seedlings were harvested and the RNA was isolated. cDNA was synthesized and quantitative real-time PCRs were done. The sample measurement was done in triplicate.

4.4.1 Standards

In Table 26 the ct-values of the standards are displayed. The ct-value (cycle threshold) marks the number of cycles whereby the level of fluorescent signal is raising compared to the background signal. The DNA increase in this point is exponential. In theory, it is possible to calculate back for the starting amount of DNA if the efficiency of the PCR is known. In practice, the amplification is influenced by several factors which make this complicated. As a consequence, it is easier to carry along a standard series with known amount of DNA fragments and to compare these known ct-values to the obtained sample ct-values (Mülhardt, 2009). In this case, dilution steps from 10^2 to 10^7 copies were done. With these obtained ct-values it was possible to draw a standard curve which was necessary to calculate the amount of sample fragments. The difference between the ct-values for each step should be around 3.5. The ct-value depends on the quality of the cDNA, the primers and the operation conditions. From the ct-values the efficiency of the PCR reaction can be determined.

Table 25: ct-values of quantitative real-time PCR standards

ct-value	1g15520	1g17180	2g15480	2g15490	5g12030	5g59720	UBQ5	PP2A	GFP	TUB9
10^7 copies	11,22	9,06	9,71	10,36	9,96	13,74	9,03	9,03	9,91	11,6
10^6 copies	14,82	12,46	13,33	-	13,57	17,67	12,88	12,87	13,7	15,33
10^5 copies	18,44	16,04	16,83	17,66	17,4	21,32	16,49	16,43	17,09	18,5
10^4 copies	22,12	19,56	20,29	21,26	20,76	24,98	20,23	19,67	20,64	22,64
10^3 copies	25,65	23,1	24,02	24,64	24,04	28,39	24,02	23,27	23,99	25,83
efficiency	88,9 %	92,3 %	92,1%	90,4 %	94,1 %	87,9 %	85,2 %	91,1 %	92,6 %	91,9 %

All detailed standards can be found in the appendix

4.4.2 Housekeeping and reference genes

Transcripts of stably expressed genes are crucial internal references for normalization of gene expression data. This is especially the case for quantitative reverse transcription PCR studies (Czechowski et al., 2005). For normalization, often so called housekeeping genes (HKGs) are used. These include genes such as TUBULIN and UBIQUITIN that play essential cellular roles and that are therefore thought to be stably expressed (Huggett et al., 2005). Ideally, the conditions of the experiment should not influence the expression of these internal control/reference genes (Schmittgen and Zakrajsek, 2000). For the model plant *Arabidopsis thaliana* it has been shown that such genes are not necessarily stably expressed (Czechowski et al., 2005). The use of unvalidated or unstable reference genes can have significant impact on the results obtained and could lead to erroneous conclusions (Huggett et al., 2005, Gutierrez et al., 2008). One resource that has been exploited is data from gene expression studies using micro arrays in wide range of developmental stages and environmental conditions. Micro array data sets can be mined for genes that are stably expressed over a diverse set of conditions and have been used in the medical field (e.g. (Kidd et al., 2007, Monaco et al., 2010)). This strategy has also been successfully employed in several plant species such as Arabidopsis (Czechowski et al., 2005). In this case, three housekeeping genes were used:

At4g20890/TUBULIN β9	*TUBULIN* β *9* is one of the subunits of microtubules. The basic structural unit of microtubules is a heterodimer containing one α-tubulin and one β-tubulin molecule, both with a molecular weight of approximately 50 kDa (Knippers, 2006). There are nine β-*tubulin* genes in Arabidopsis (Snustad et al., 1992), two to three more than in vertebrates (Wang et al., 1986, Monteiro and Cleveland, 1988). Microtubules have different functions like transport of vesicles while exo- or endocytosis, maintaining complex compounds like central bodies or spindel apparatus, and facilitating elongated cells like fibroblasts (Plattner and Hentschel, 1997). Genomic DNA 470 bp, cDNA360 bp

At3g62250/UBIQUITIN 5 Ubiquitin is a is a highly conserved, 76 amino acid protein that appears to be present in all eukaryotes. Ubiquitin bet characterized duty is a covalently bound recognition signal for proteolysis (Callis et al., 1990). This process contains of three, enzyme involved, steps. This enzyme cascade results in a transfer of ubiquitin to the target protein via a E3-ubiquitin-ligase and the degradation of the target protein (Knippers, 2006). In higher plants and animals, ubiquitin genes can be divided into three types of genes: the polyubiquitin genes, the ubiquitin-like genes and the ubiquitin-extension protein genes (Callis et al., 1989). The first two types encode ubiquitin polyproteins, with the latter encoding at least one repeat with amino acid replacements. The ubiquitin-extension protein genes contain one ubiquitin coding region followed by one of two different ribosomal proteins and have been described previously (Callis et al., 1990). The Arabidopsis ubiquitin gene family of 14 members consists of a total of five polyubiquitin genes, five ubiquitin-like genes and four ubiquitin-extension genes.

At1g69960/PP2A At1g69960 encodes for a subunit of the so called serine/threonine protein phosphatase 2a (*PP2A*). Reversible protein phosphorylation is an essential regulatory mechanism in many cellular processes which can be done by PP2A. The core enzyme ($PP2A_D$) is a dimer consisting of a 36 kDa catalytic subunit ($PP2A_C$) and a regulatory subunit of molecular mass, 65 kDa, termed PR65. A third regulatory B subunit can be associated with this core structure. Four subunits are already known (B', B'', B''' and B'''')(Janssens and Goris, 2001). Due to its properties PP2A is a well known reference gene in Arabidopsis (Czechowski et al., 2005).

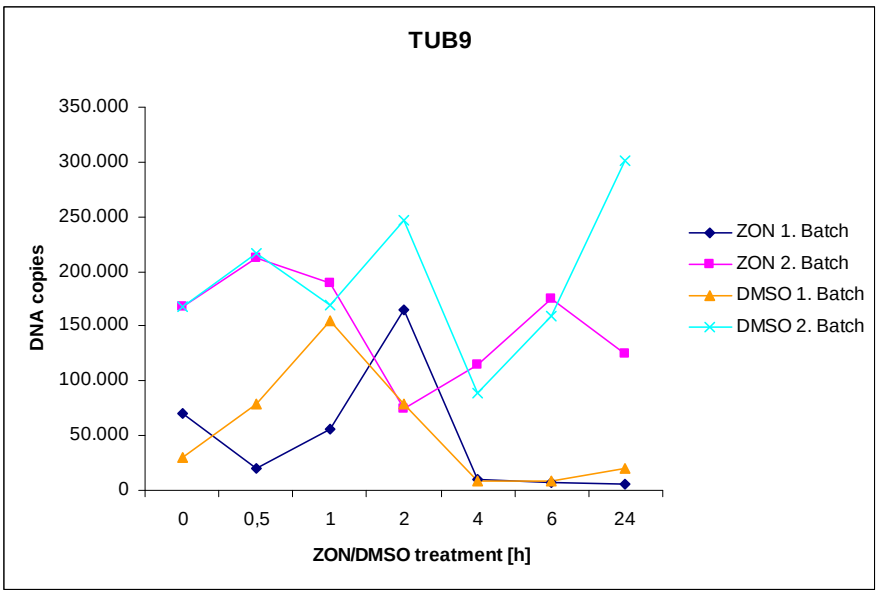

Figure 16: Time course experiment of the house keeping gene At4g20890/TUB9 upon ZON and DMSO treatment.

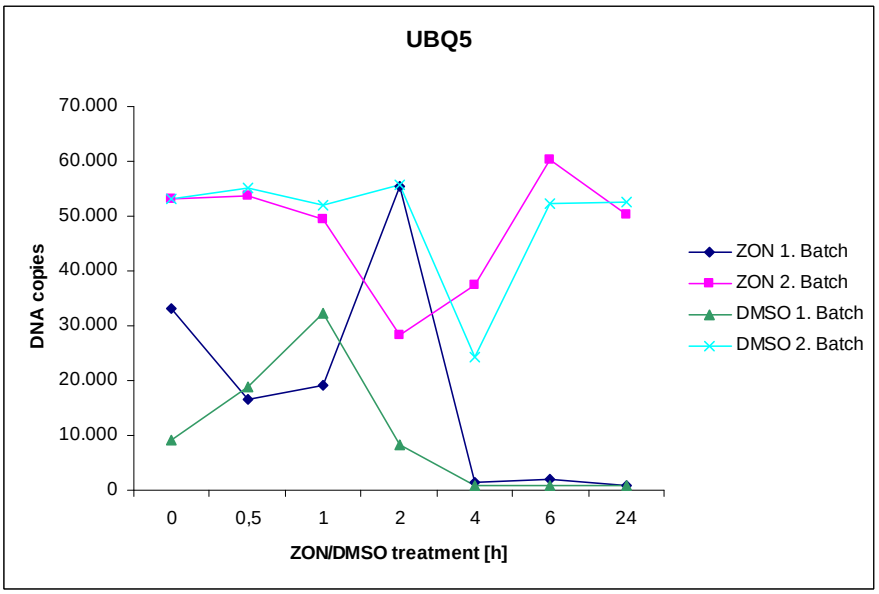

Figure 17: Time course experiment of the house keeping gene At3g62250/UBQ5 upon ZON and DMSO treatment.

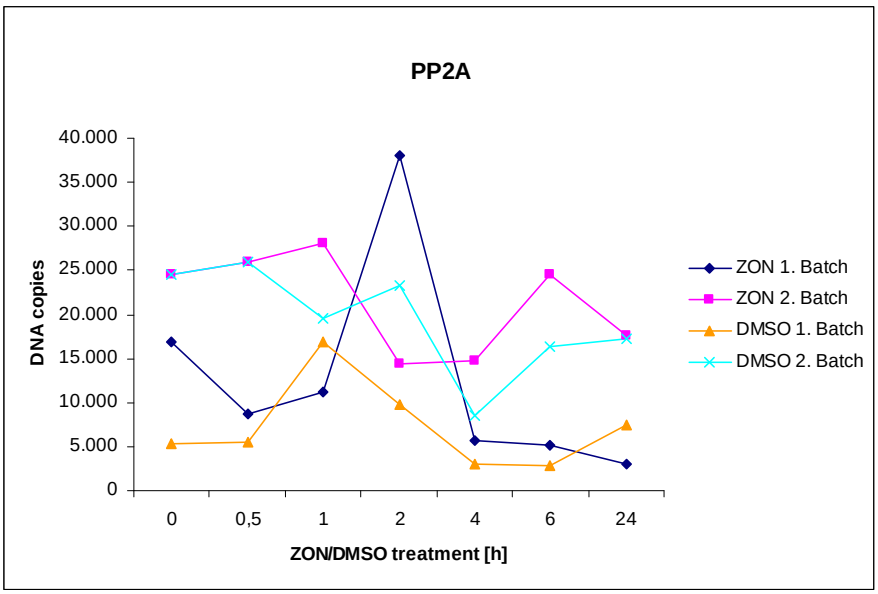

Figure 18: Time course experiment of the house keeping gene At1g69960/PP2A upon ZON and DMSO treatment.

In Figure 16, 17 and 18 the results for the reference genes are displayed. In general the second experiment revealed higher amounts of cDNA copies than the first experiment. *UBQ5* (Figure 17) and *PP2A* (Figure 18) showed an outlier at two hours ZON treatment in the first experiment. These outliers may have distorted the evaluation of the data but the effect is reduced because of two independent experiments carried out and three housekeeping genes were used for normalization. In contrast, the DMSO control showed a divergent value but not as strong as the ZON value. Apart from that, the second experiment revealed that ZON and DMSO are closer together. No clear ZON or DMSO maximum could be observed. *TUB9* (Figure 18) showed a DMSO maximum after one hour and a ZON maximum after two hours. These two maxima ranged nearly at the same level. In general the second experiment also showed a higher amount of copies. Two maxima of DMSO occurred simultaneously with two minima of ZON.

To check for outliers in Figure 16, 17 and 18 the housekeeping genes were normalized (Figure 19, 20 and 21) with each other. If the ratio of the compared values stay nearly on the same level it would be an evidence for varying amounts cDNA in the sample, what could explain outliers in Figure 16, 17 and 18. A varying level of the ratio values indicates an influence of the ZON/DMSO stress on the expression level of the housekeeping genes.

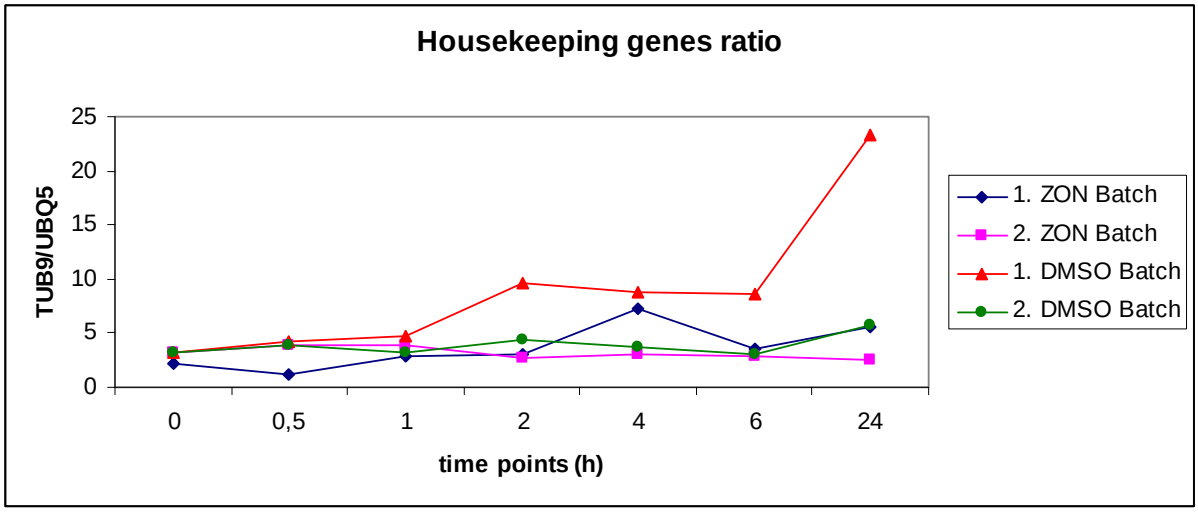

Figure 19: Comparison of the two housekeeping genes At4g20890/TUBULIN β9 and At3g62250/UBIQUITIN 5. The ratio of the first ZON batch is displayed blue, the ratio of second ZON batch is displayed pink, the ratio of first DMSO batch is displayed red and the ratio of the second DMSO batch is displayed green. The values range approximately on the same level except the 24 hour first DMSO batch.

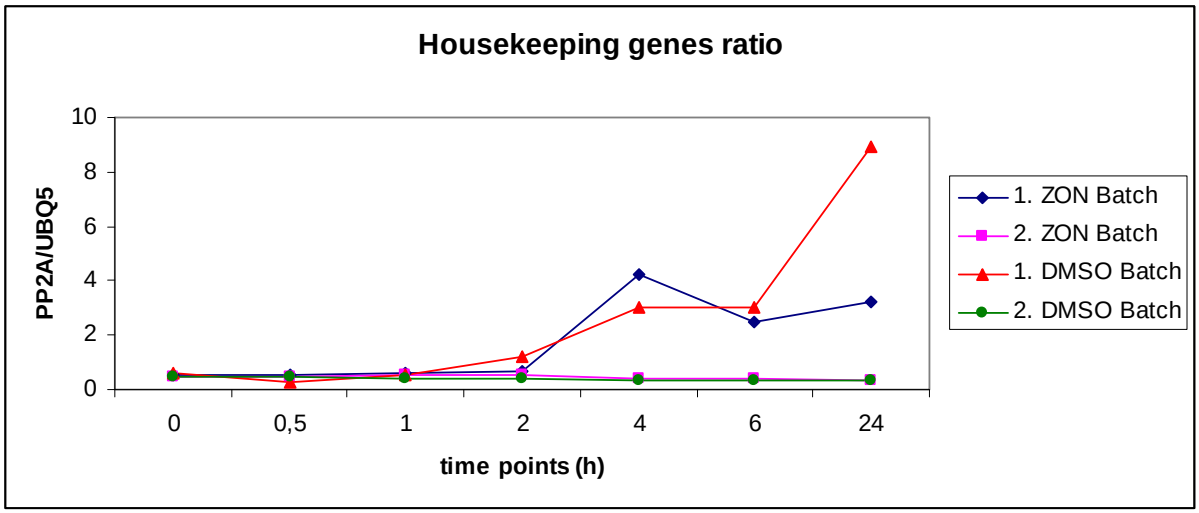

Figure 20: Comparison of the two housekeeping genes At1g69960/PP2A and At3g62250/UBIQUITIN 5. The ratio of the first ZON batch is displayed blue, the ratio of second ZON batch is displayed pink, the ratio of first DMSO batch is displayed red and the ratio of the second DMSO batch is displayed green. The values range approximately on the same level except the 24 hour first DMSO batch.

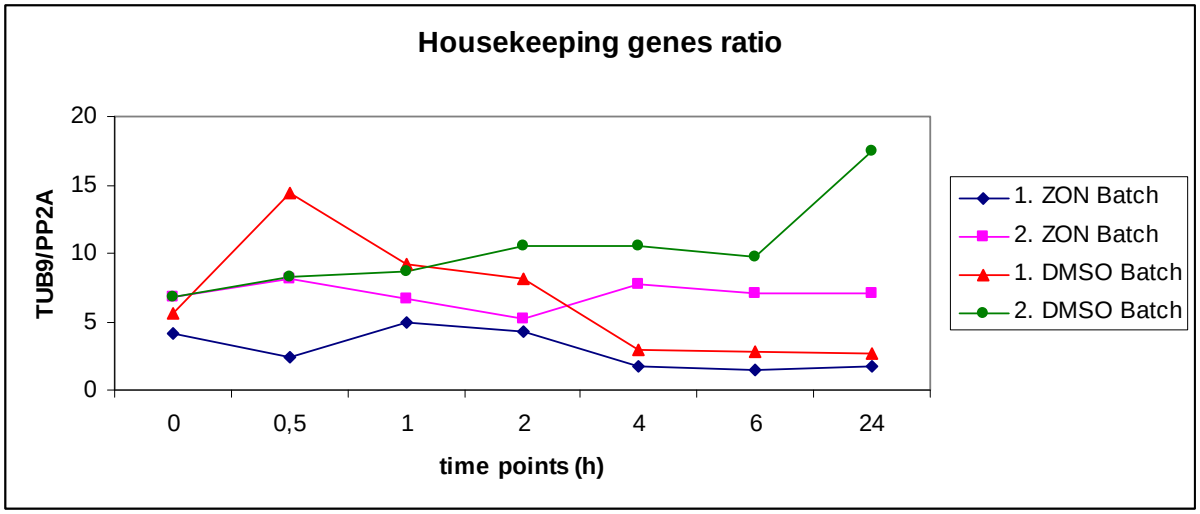

Figure 21: Comparison of the two housekeeping genes At4g20890/TUBULIN β9 andAt1g69960/PP2A. The ratio of the first ZON batch is displayed blue, the ratio of second ZON batch is displayed pink, the ratio of first DMSO batch is displayed red and the ratio of the second DMSO batch is displayed green. The values range approximately on the same level except the 24 hour second DMSO batch and half a hour first DMSO batch.

In Figures 19, 20 and 21 the ratio of the housekeeping is displayed. Concerning the Tubulin9/Ubiquitin5 ratio (Figure 19) reveals quite similar ratio values except the first DMSO batch value after 24 hours. This outlier also occurs in Figure 20. This means a low value of UBQ5 in the first DMSO batch after 24 hours. In Figure 21 (TUB9/PP2A) a high value after 24 hours second DMSO batch is noticeable. The striking ZON values of the first batch after two hours (Figure 16, 17 and 18) is attributable to a higher amount of cDNA in the samples.

4.4.3 Quantitative expression analyses of the genes used for constructing the reporter lines.

To obtain valid data, two independent experiments were carried out. The sample measurement was done in triplicate. A mean value was calculated and became normalized with each of the three reference genes (TUB9, UBQ5, PP2A).

$$\frac{value\ sample}{value\ reference} \times 100$$

After this calculation the ZON and DMSO values were displayed as a multiple of the reference genes in percent. This was done twice because of two independent experiments. To bring this to experiments together, The data of the ZON experiment were displayed as a fold of DMSO.

$$\frac{normalized\ ZON\ value}{normalized\ DMSO\ value}$$

This happened for each of the three reference genes and for both experiments. Now a mean value was calculated to bring the two experiments together. Finally average values of all reference genes and standard deviation were calculated. The result is displayed in Figure 22 and 23. In Figure 22 the lower expressing genes are depict and in Figure 23 the higher expressing genes are shown.

Concerning the lower expressing genes (Figure 22) At2g15480/UGT37B5 showed a clear maximum after two hours of ZON treatment. At2g29500/HSP17.6 B-CI also revealed a maximum after two hours and a still high value after four hours whereas At2g29500/HSP17.6 B-CI was induced twice as much as At2g15480/UGT37B5. At2g17180/GSTU25 had its maximum after four hours of ZON treatment. The only gene without a clear maximum was At5g59720/HSP18.2. Compared to the other genes it showed a slow in- and decrease of the expression level. The other genes, except At5g59720/HSP18.2, showed a rapid increase and decrease of expression. After 24 hours of ZON treatment, the expression reached the starting level.

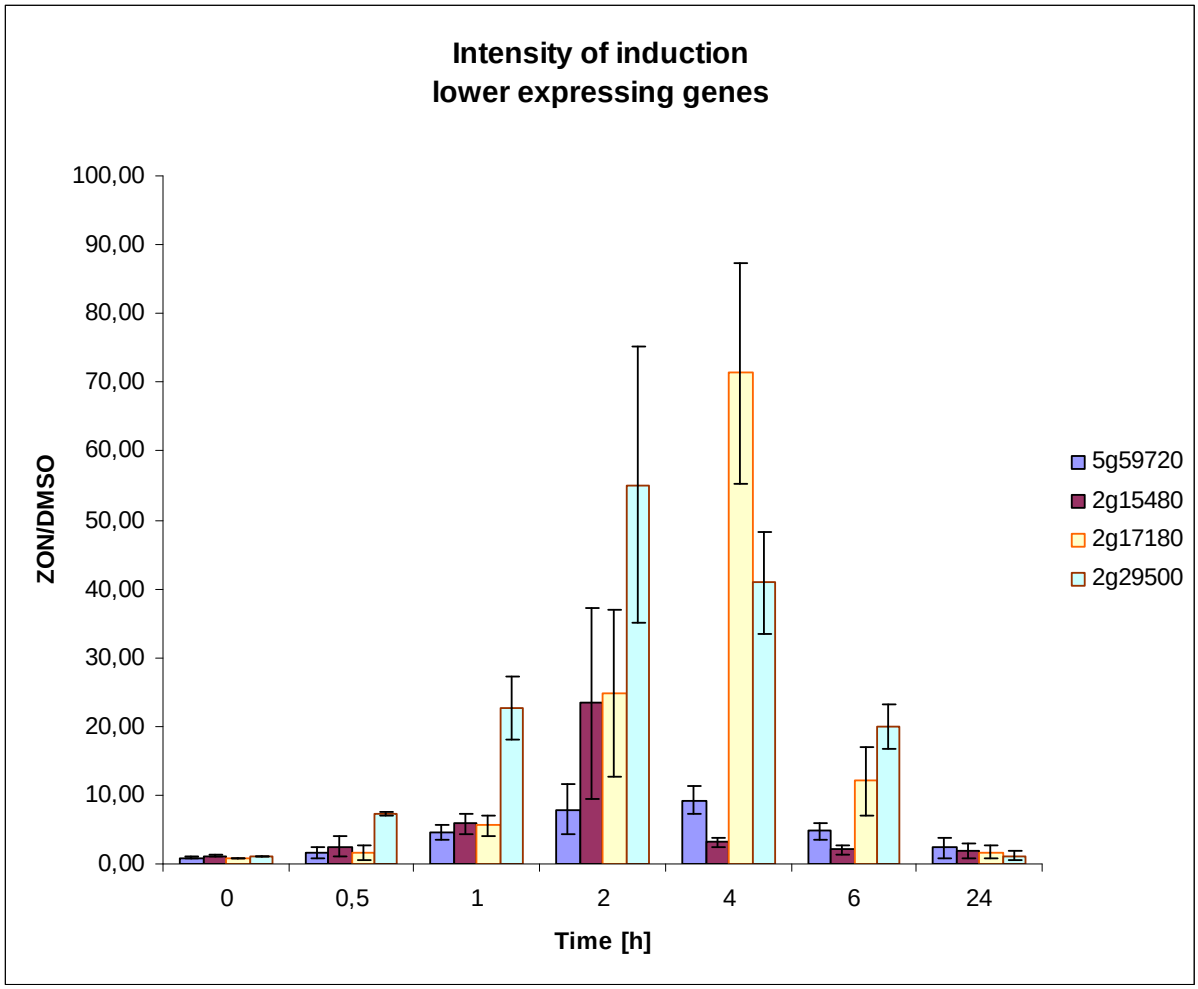

Figure 22: Intensity of induction of lower expressing genes. The ZON induced gene expression is displayed as a fold of DMSO over a time course for gene At5g59720/HSP18.2, gene At2g15480/UGT37B5, gene At2g17180/GSTU25 and gene At2g29500/HSP17.6-CI.

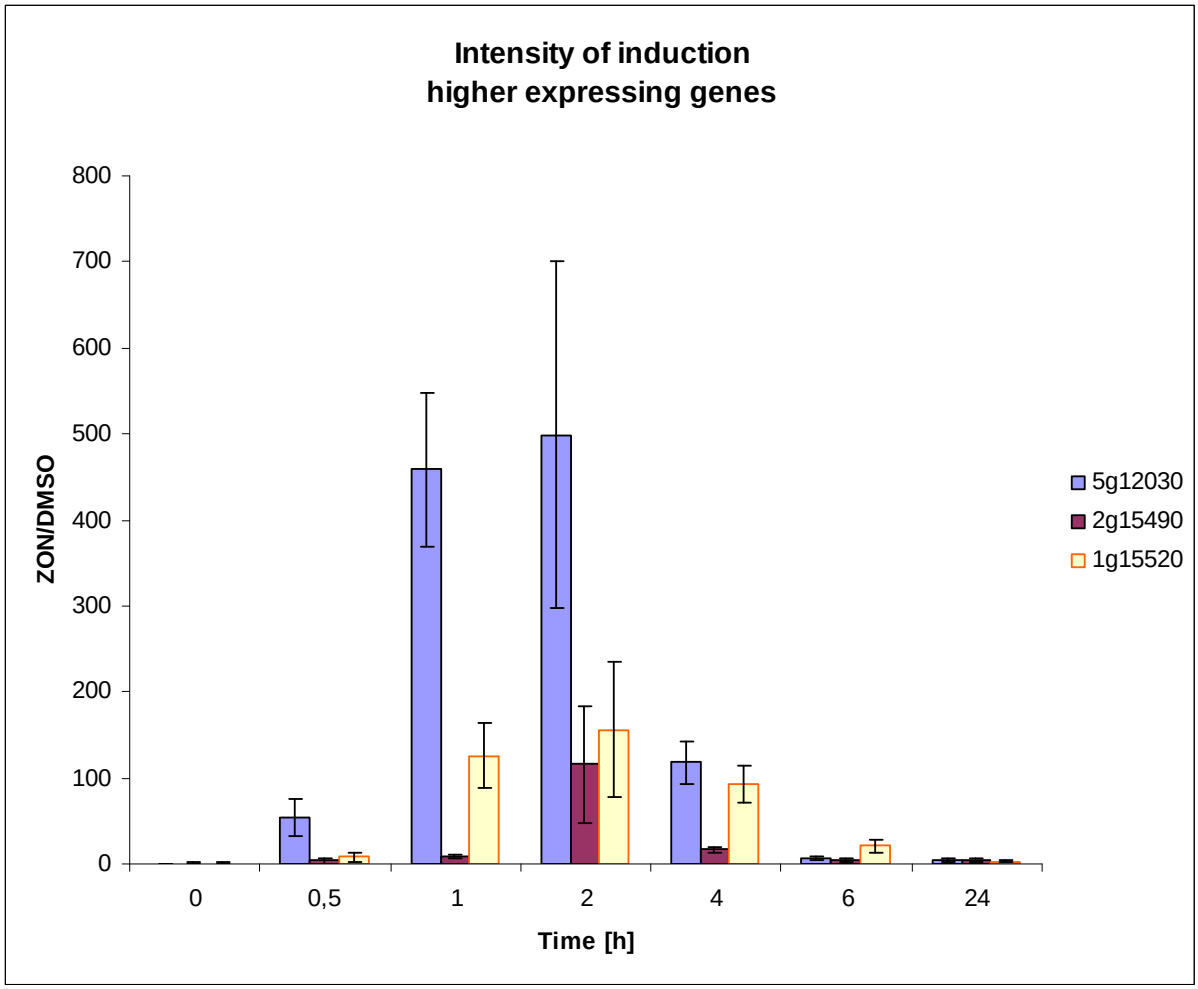

Figure 23: Intensity of induction of higher expressing genes. The ZON induced gene expression is displayed as a fold of DMSO over a time course for gene At5g12030/HSP17.6A, gene At2g15490/UGT37B4 and gene At1g15520/PDR12.

In Figure 23 the higher induced genes are displayed. The genes At2g15490/UGT37B4 and At1g15520/PDR12 showed a quite similar ZON maximum after two hours. They are nearly twice as much induced compared to the less induced genes of Figure 22. At2g15490/UGT37B4 revealed the clearest ZON induction after two hours and was induced about 100 fold. Before and after the maximum expression, the intensity of induction is almost at the starting level. At1g15520/PDR12 featured a maximum after two hours, an already high value after one hour and a still high value after 4 hours. The increase and decrease of the expression level was not as fast as At2g15490/UGT37B4 had shown. These data suggests a "longer answer" (3 hours of gene expression) of the plant to the ZON stress compared to the short answer the plant gave with the gene At2g15490/UGT37B4. At5g12030/HSP17.6A revealed a nearly five time higher induction then the other genes. The increase happened not as immediately as with At2g15490 but the decrease also occurred rapidly. All genes reached their starting level after six hours.

To sum up, the induction maximum occurred after two hours, except for At2g17180/GSTU25 which had its maximum after four hours and At5g59720/HSP18.2 which had no clear maximum. Furthermore, the genes showed a fast increase and decrease of the expression level. The expression nearly reached starting level after six hours of ZON treatment. The obtained data predict the best ZON induced GFP signal after three to five hours. The intensity of the excitation (e.g. At5g12030/HSP17.6A) of the genes indicates strong ZON induced GFP signals. This experiment confirmed the micro array data from Ulrike Werner.

4.5 ZON- and heat shock experiment

In Table 20 an overview is given about all the performed ZON- and heat exposure results. The results were not as good as expected after the real-time experiment. The obtained signals were weak to moderate. For example, the constructs pPZPpm5g59720, pm5g12030 and pm2g15490 exhibited the best signals. The improved construct pPZPpm2g29500-GFP-3' UTR showed a moderate better signal than its counterpart pPZPpm2g29500-GFP. A signal intensity improvement of the construct with the fused 3' UTR termination signal could be observed. In contrast to ZON results, the heat induction concerning the sHSP promoters revealed strong signals.

Table 26: Overview of ZON- and heat-excitability. Following color code was used; white - no excitability, light green/ yellow - weak excitability, dark green/orange - strong excitability, red - best excitability

construct	ZON inducible	Heat inducible
pPZPpm2g29500-GFP	x	x
pPZPpm2g29500-GFP-3'UTR	x	x
pPZPpm1g15520-GFP	x	
pPZPpm1g53540-GFP	x	x
pPZPpm1g17180-GFP	x	
pPZPpm5g59720-GFP	x	x
pPZPpm5g12030-GFP	x	x
pPZPpm2g15490-GFP	x	
pPZPpm5g12020-GFP	x	x
pPZPpm1g52560-GFP	x	x

4.5.1 pPZPpm2g29500-GFP

The unmodified pPZPpm2g29500-GFP construct showed no induction after ZON exposure. After two hours, no difference between ZON and DMSO induction could be observed (B and C). Even after four hours no ZON induction occurred (E and F). In contrast, the heat induction gave a good signal. GFP was produced in several plant organs like roots (D) and shoot axis (A). The fluorescent GFP is clearly identifiable compared to the ZON and DMSO results.

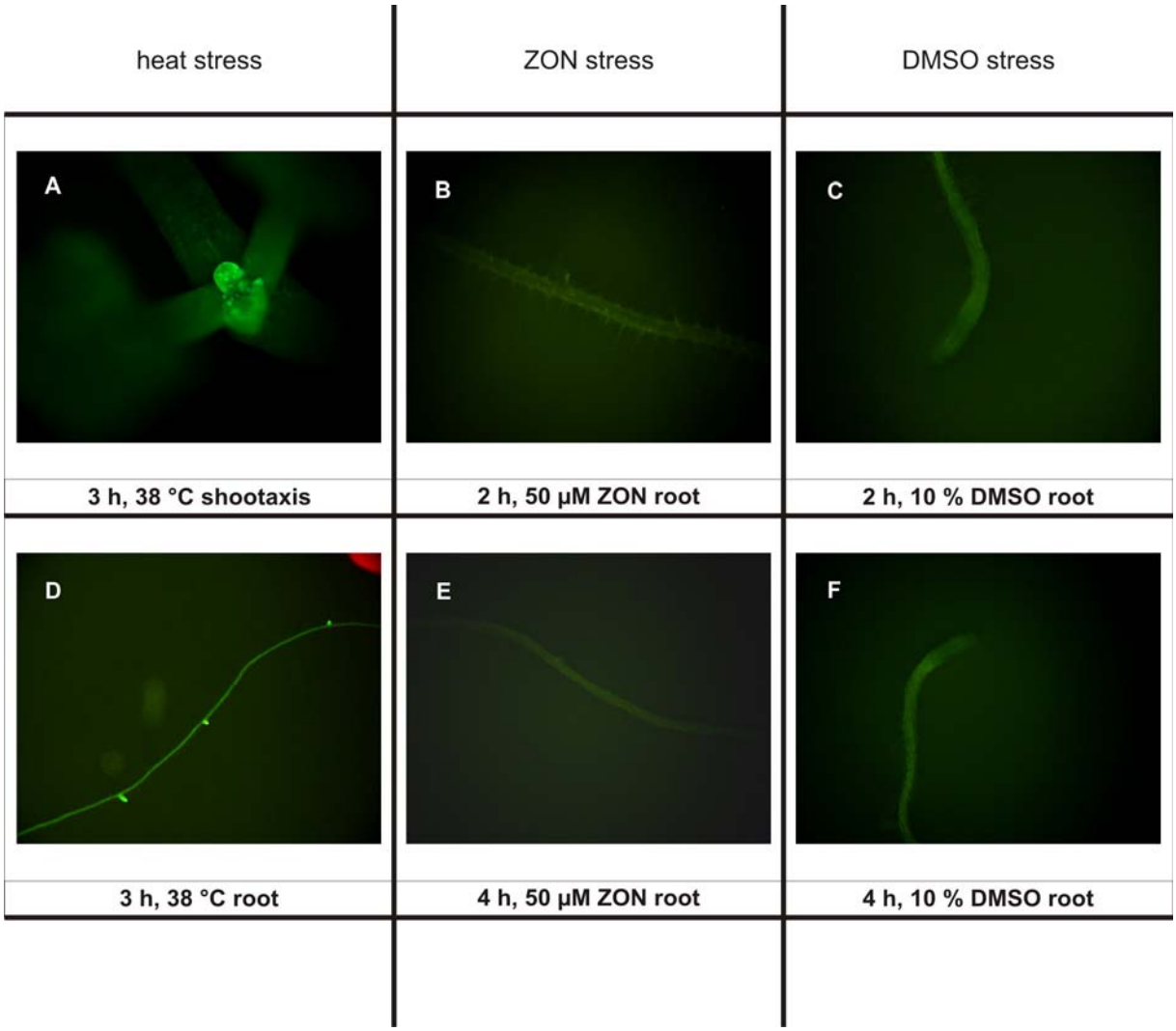

Figure 24: ZON/Heat induction of a homozygous pPZPpm2g29500-GFP T3 plant. In picture A and D the heat induction (38°C, 3h) is displayed. In picture B and E the ZON stress (50 µM, 4h) is depicted. The DMSO control (10 %) plants are shown in picture C and F.

4.5.2 pPZPpm2g29500-GFP-3'UTR

Due to the poor ZON induction results of the pPZPpm2g29500-GFP, the 3' UTR with the terminator was fused to improve the GFP production. Figure 17 shows the results for the different stress conditions. At2g29500 codes for the sHSP protein *HSP17.6B-CI* which exhibit a good heat stress response. There was a strong GFP expression in the plant, the leaves (Figure 17A), and all compartments of the root (Figure 17D). The heat induced signal obviously improved compared to pPZPpm2g29500-GFP. Furthermore, the ZON induction was also improved. A clear GFP expression is visible in the roots and the root tip (Figure 17E). The DMSO control (Figure 17C and F) and the wild type (Figure 17G and H) exhibit no

GFP expression. Compared to pPZPpm2g29500-GFP, a signal enhancement could be reached and the result was repeatable. Nearly no basal expression could be observed.

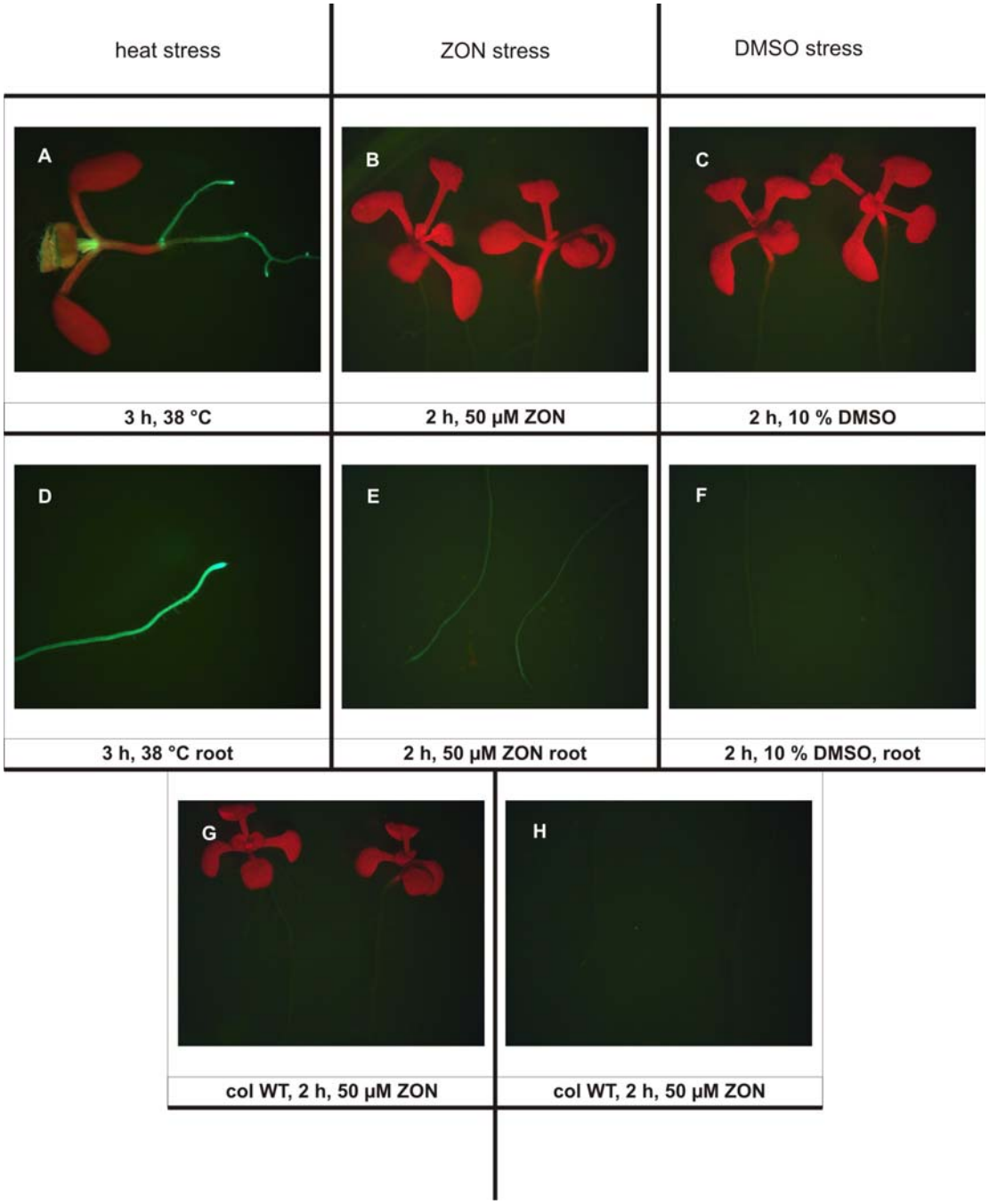

Figure 25: ZON/Heat induction of a homozygous pPZPpm2g29500-GFP-3'UTR T3 plant. In picture A and D the heat induction (38°C, 3h) is displayed. In picture B and E the ZON stress (50 µM, 4h) is depicted. The DMSO control (10 %) plants are shown in picture C and F. A columbia wild type plant exposed to ZON (50µM, 2 h) stress is shown in picture G and H.

4.5.3 pPZPpm1g15520-GFP

In Figure 18 the results of the pPZPpm1g15520-GFP construct are displayed. No specific signal could be observed, instead a high level of autofluorescence. The ZON treatment (Figure 18A and C) revealed no visible GFP production in the plant. The level of fluorescence is the same as in the DMSO control plant.

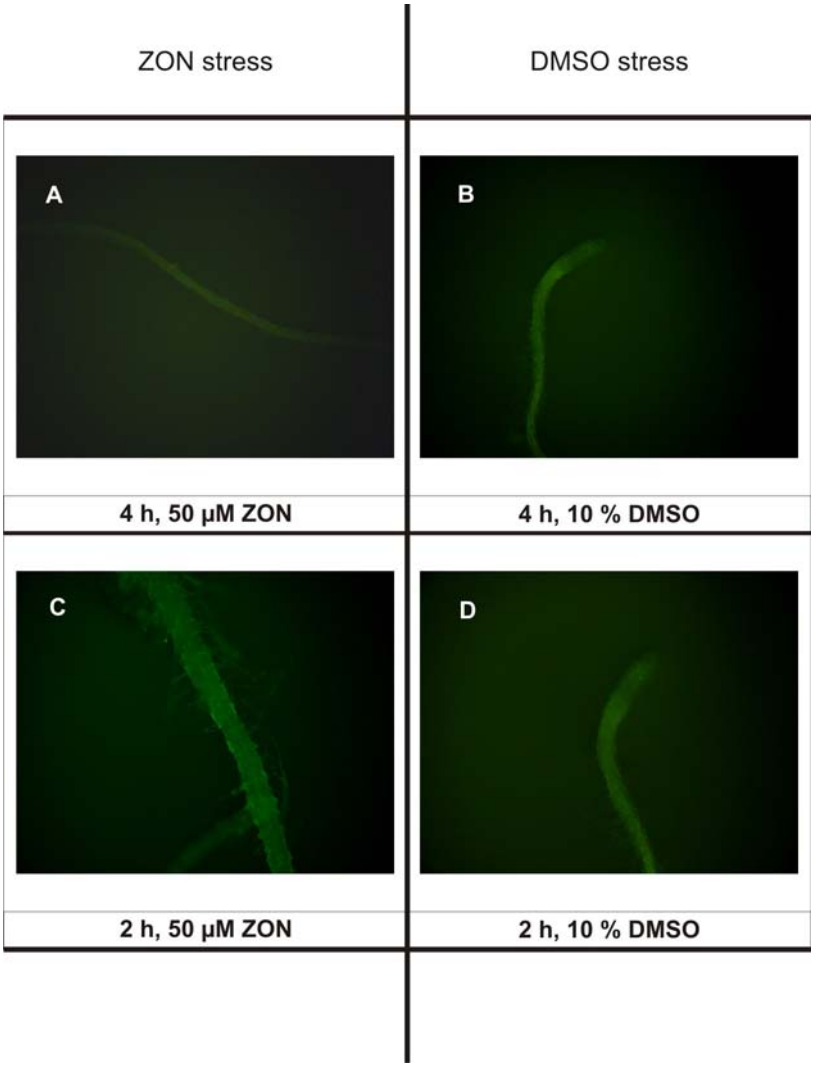

Figure 26: ZON induction of a homozygous pPZPpm1g15520-GFP T3 plant (roots). In picture A and C the ZON stress (50 µM, 4h) is depicted. The DMSO control (10 %) plants are shown in picture B and C

4.5.4 pPZPpm1g53540-GFP

Transgenes expressing pPZPpm1g53540-GFP showed no specific ZON induced GFP production. Figure 19 displays the obtained data. No GFP expression after ZON treatment (Figure 19A and C) could be observed. A slight basal GFP expression occurred in the DMSO control (Figure 19D).

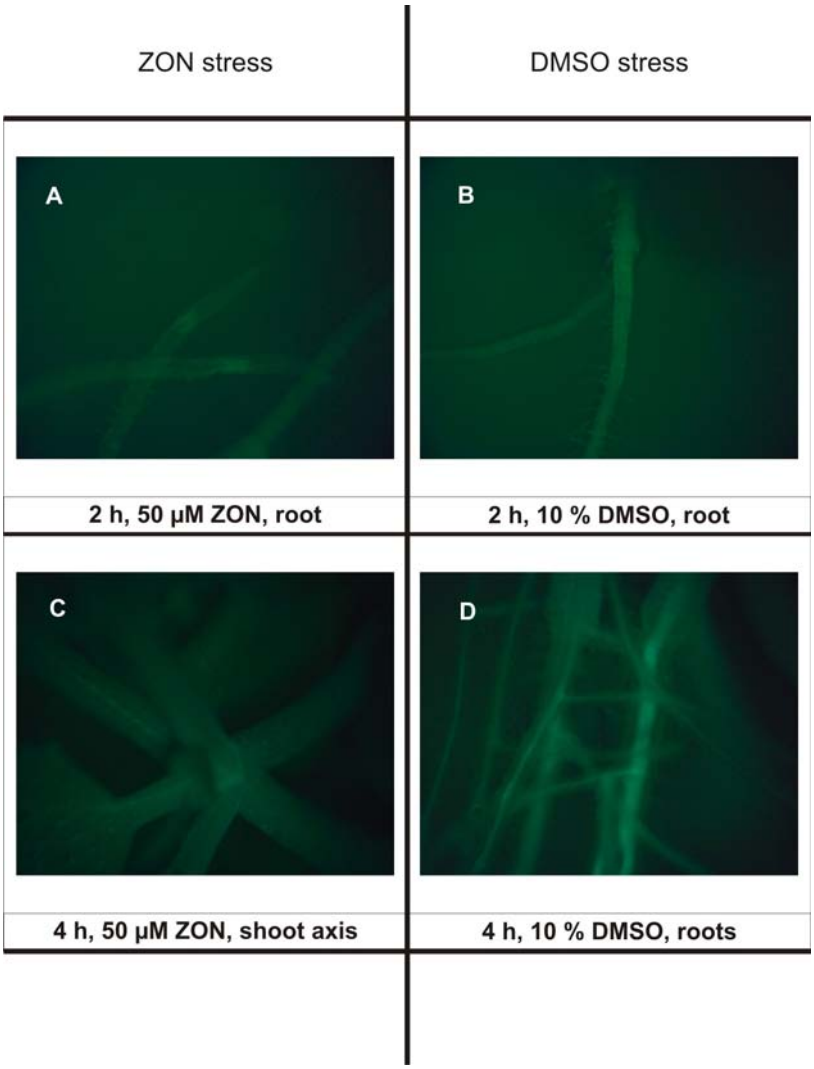

Figure 27: ZON induction of a homozygous pPZPpm1g53540-GFP T3 plant. In picture A and C the ZON stress (50 µM, 2 and 4h) is depicted. The DMSO control (10 % 2 and 4 h) plants are shown in picture B and D.

4.5.5 pPZPpm1g17180-GFP

Seedlings with the construct pPZPpm1g17180 showed a faint signal after ZON induction. As shown in Figure 20, a weak GFP production is noticeable (Figure 20A) compared to the DMSO control (Figure 20B). The GFP is expressed mainly in the root tips (Figure 20C). A basal GFP based fluorescence could be seen in the DMSO controls to a minor extent. Still, the signals were below the expected range.

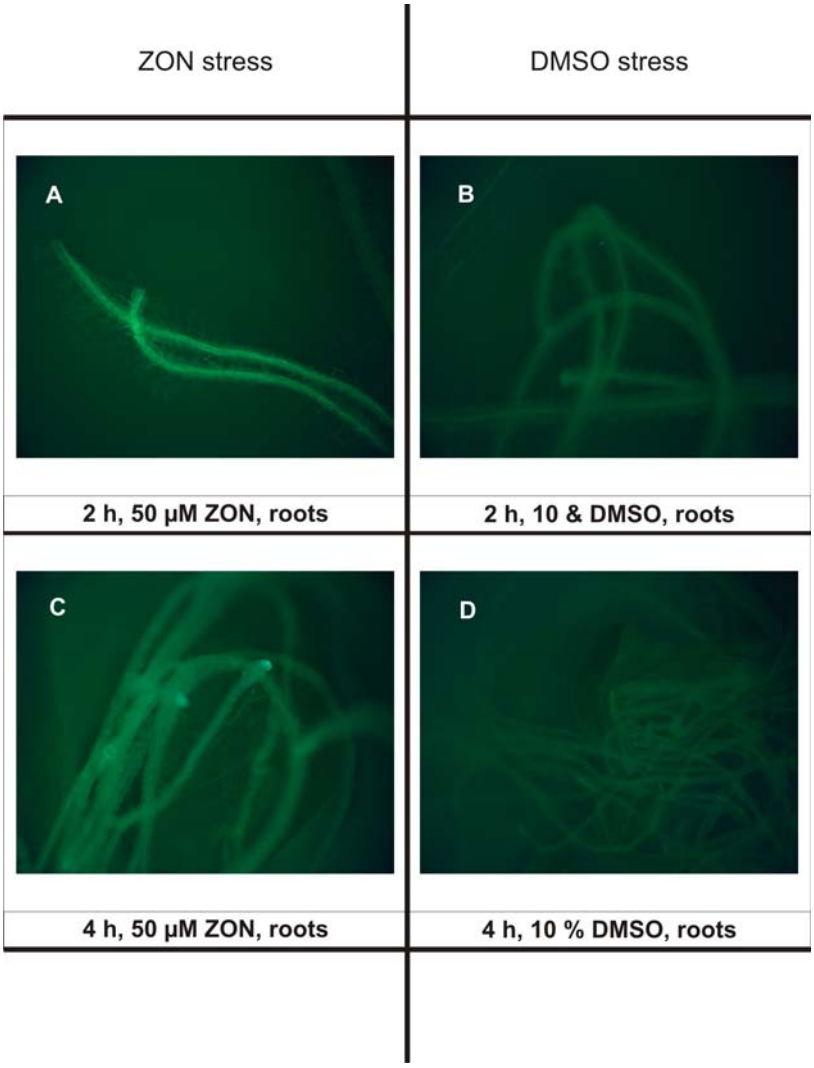

Figure 28: ZON induction of a homozygous pPZPpm1g17180-GFP T3 plant. In picture A and C the ZON stress (50 µM, 2 and 4h) is depicted. The DMSO control (10 % 2 and 4 h) plants are shown in picture B and D.

4.5.6 pPZPpm5g59720-GFP

The gene At5g59720 codes for the small heat shock protein (name) with a good heat inducibility. In Figure 21A and D the response to heat stress is displayed. The obtained results were not as good as obtained from other sHSPs like At2g29500 but still well detectable. The ZON experiment revealed a GFP expression which took place in roots and root tips (Figure 21B and E). In contrast to the quantitative real time results there was an inexplicably strong induction after 24 hours. It was not possible to reproduce this result. The DMSO control (Figure 21F) showed a weak basal GFP expression but it was not as strong as the ZON induced signal.

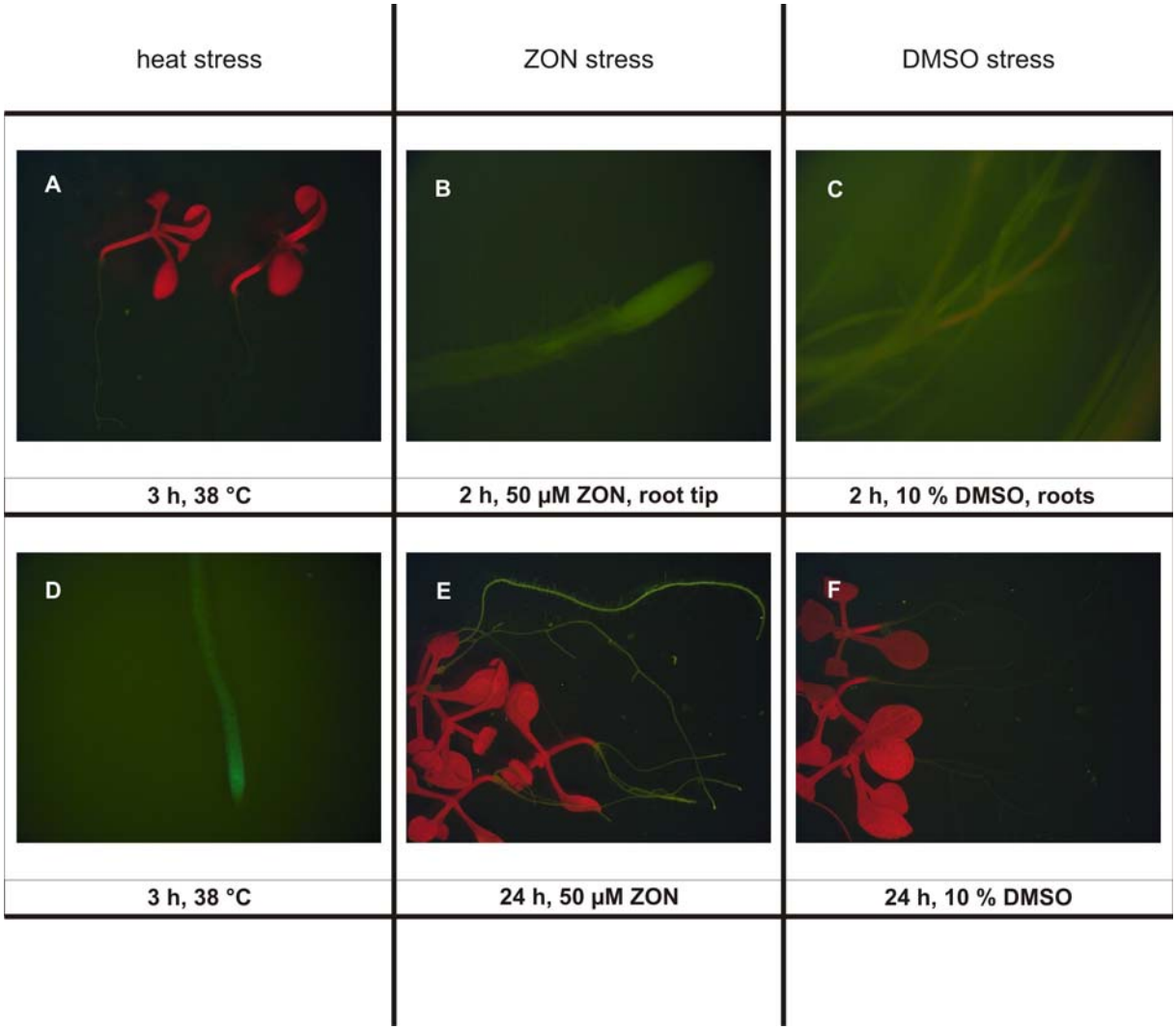

Figure 29: ZON/Heat induction of a homozygous pPZPpm5g59720-GFP T3 plant. In picture A and D the heat induction (38°C, 3 h) is displayed. In picture B and E the ZON stress (50 µM, 2 h and 24 h) is depicted. The DMSO control (10 %, 2 h and 24 h) plants are shown in picture C and F.

4.5.7 pPZPpm5g12030-GFP

The gene At5g12030 belongs to the sHSP family. As a consequence, the construct was induced strongly by heat stress. In Figure 22 (A and D) the heat stress experiment is shown. A well detectable signal in the roots and root tips was observed. Even the ZON based GFP expression showed a strong signal (Figure 22B and E) in the roots and root tips. The DMSO control plants also showed a fluorescent signal in the roots and root tips. The signal (perhaps autofluorescence) was considerably weaker than the ZON based one but still well detectable.

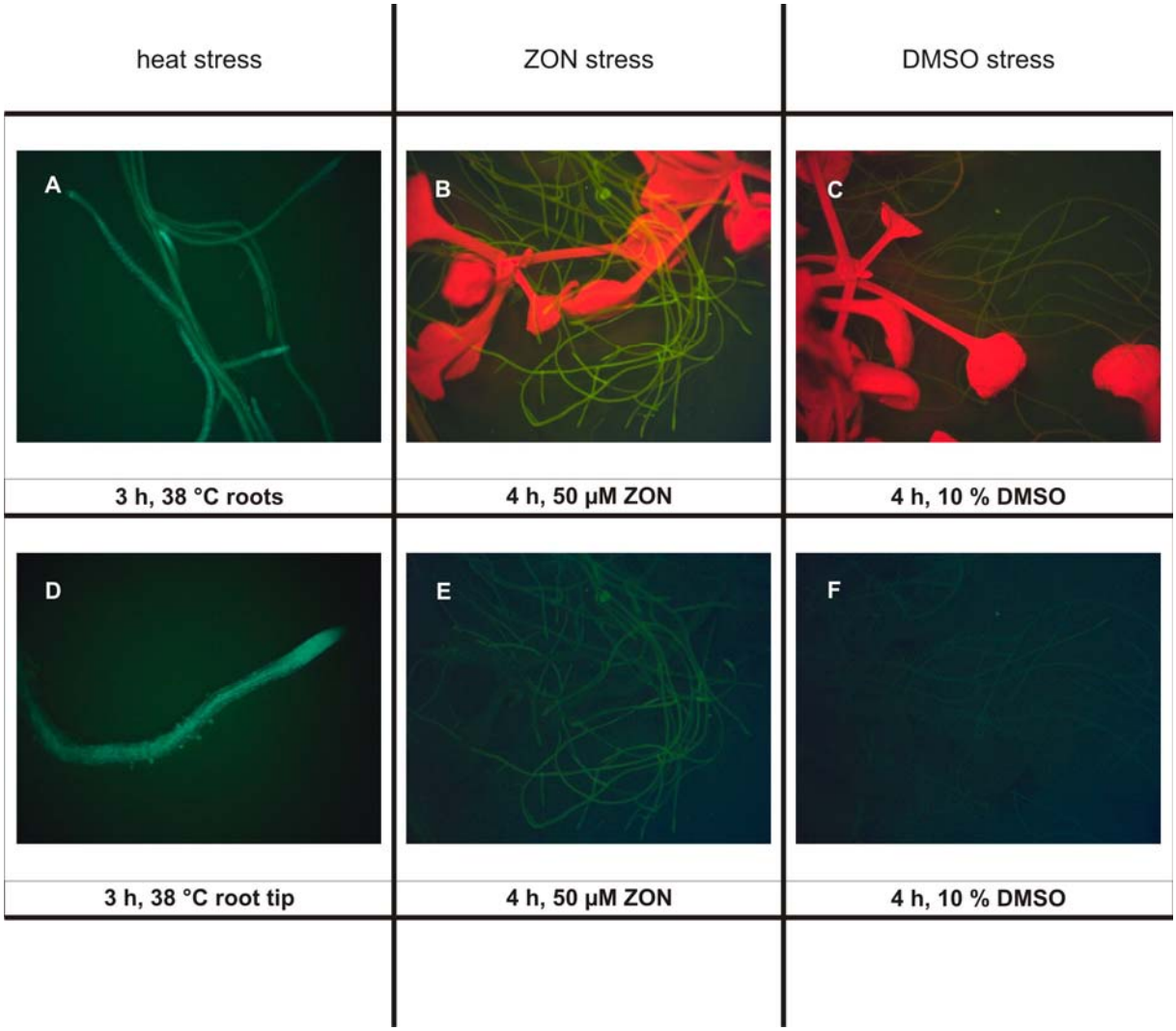

Figure 30: ZON/Heat induction of a homozygous pPZPpm5g12030-GFP T3 plant. In picture A and D the heat induction (38°C, 3 h) is displayed. In picture B and E the ZON stress (50 µM, 4 h) is depicted. The DMSO control (10 %, 4 h) plants are shown in picture C and F.

4.5.8 pPZPpm2g15490-GFP

Seedlings with the construct pPZPpm2g15490-GFP revealed a good ZON induced GFP expression. In Figure 23 (A and C) a moderate signal occurred in the whole root. The DMSO control plants also showed a autofluorescence signal in the root. The detected signal was not as strong as the ZON induced GFP signal but still well detectable.

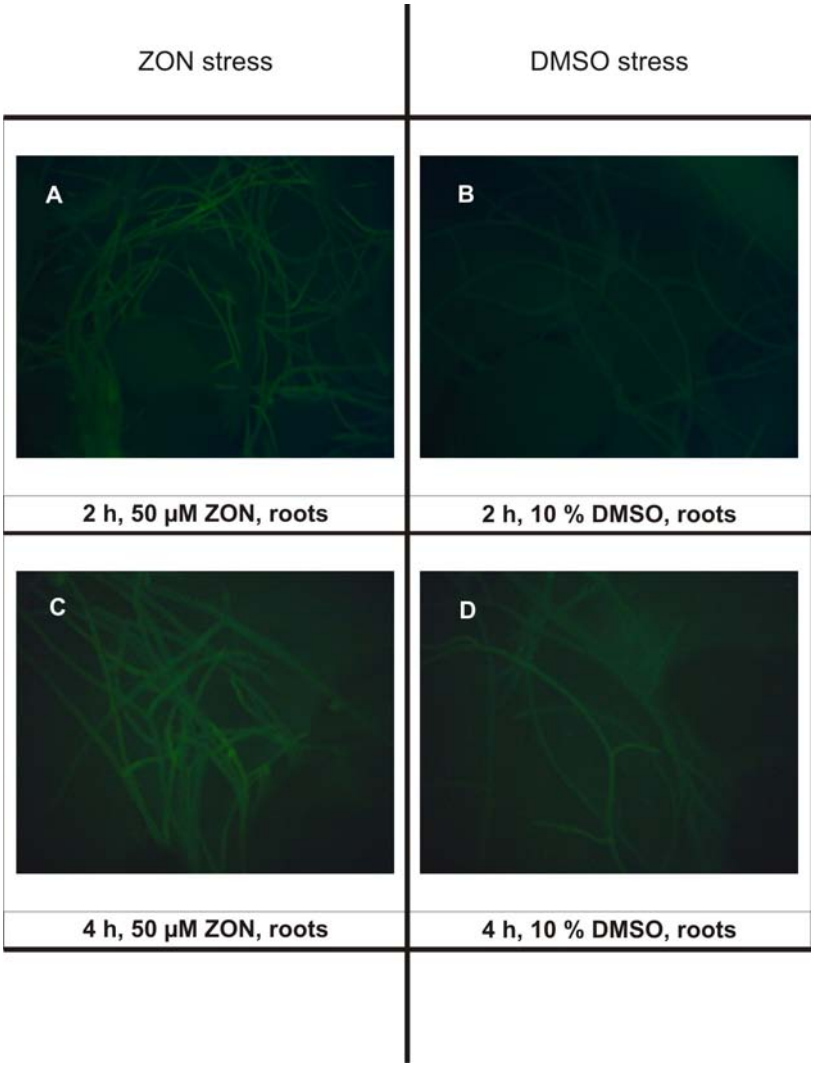

Figure 31: ZON induction of a homozygous pPZPpm2g15490-GFP T3 plant. In picture A and C the ZON stress (50 µM, 2 and 4h) is depicted. The DMSO control (10 % 2 and 4 h) plants are shown in picture B and D.

4.5.9 pPZPpm5g12020-GFP

Seedlings with the construct 5g12020-GFP showed no GFP expression upon ZON stress. Also no signal was detectable in the DMSO control (Figure 24, A-D). This indicates that the reporter construct itself or the transcriptional activation are not functional or too weakly expressed in the tested lines.

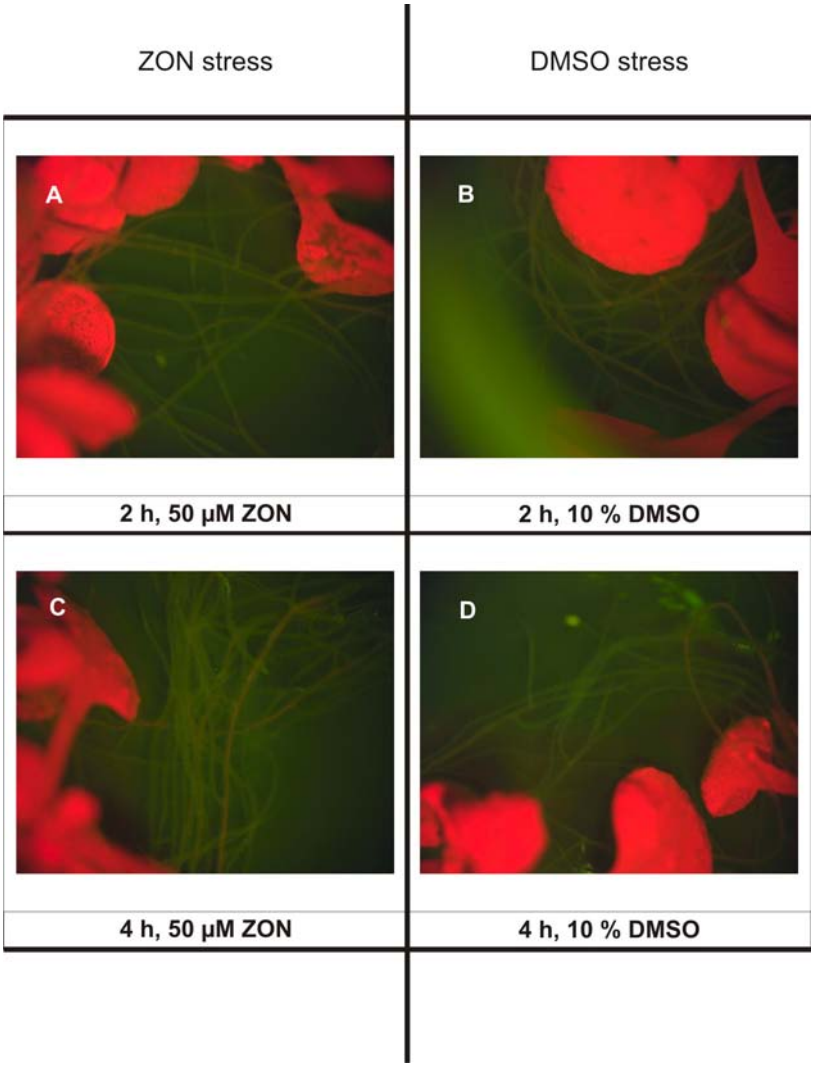

Figure 32: ZON induction of a homozygous pPZPpm5g12020-GFP T3 plant. In picture A and C the ZON stress (50 µM, 2 and 4h) is depicted. The DMSO control (10 % 2 and 4 h) plants are shown in picture B and D.

4.5.10 pPZPpm1g52560-GFP

Similar to pPZPpm5g12020-GFP, seedlings with the pPZPpm1g52560-GFP construct showed no specific ZON induced GFP expression. The ZON and the DMSO experiment showed the same level of, most likely, autofluorescence as displayed in Figure 25 (A-D).

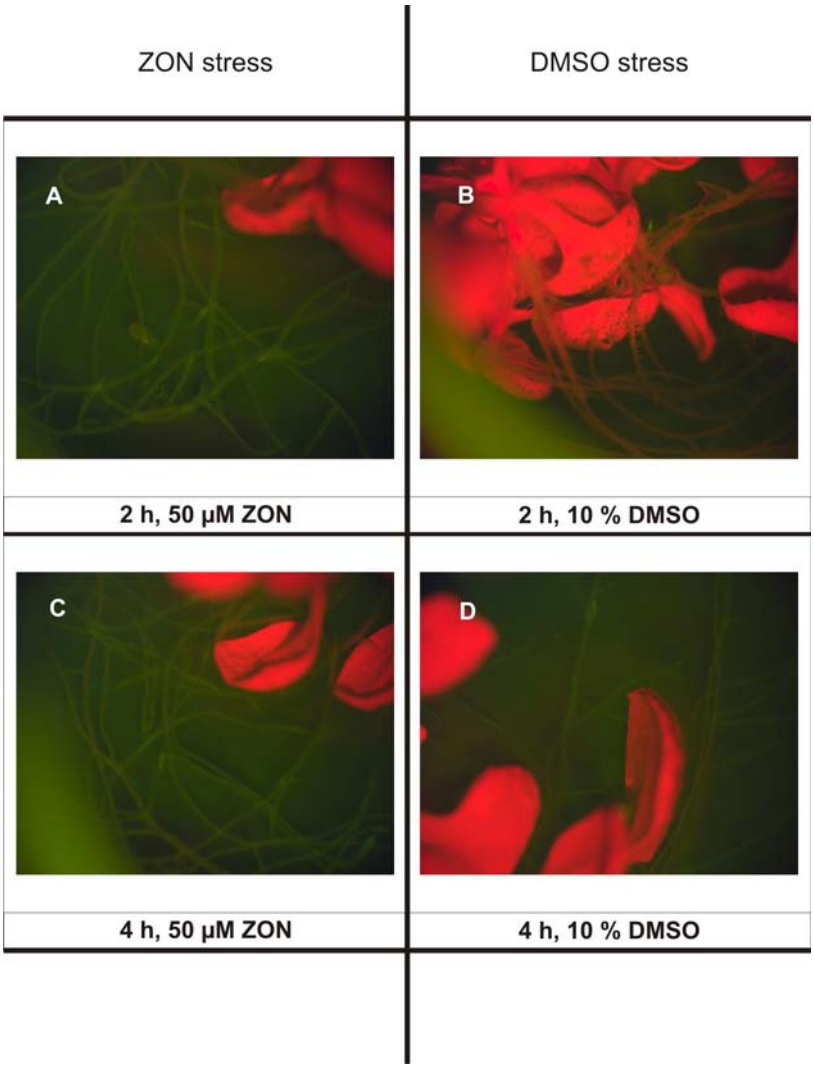

Figure 33: ZON induction of a homozygous pPZPpm1g52560-GFP T3 plant. In picture A and C the ZON stress (50 µM, 2 and 4h) is depicted. The DMSO control (10 % 2 and 4 h) plants are shown in picture B and D.

4.6 ZON metabolisation in *pdr12* mutants and UGT73B4 overexpressors of *Arabidopsis thaliana*

The goal was to track ZON (zearalenone) and its metabolites zearalenol (βZOL), ZON-4-Glc (zearalenone-4-glycoside, ZON4G) and ZON-4-Sulfate (zearalenone-4-sulfate, ZON4S) in the plant and medium over time. To reveal if PDR12 is involved in the ZON transport, *pdr12* mutants were used. This ABC transporter is already known for its detoxification abilities, like the transport of lead out of the cell (Lee et al., 2005). Furthermore it had been shown that At1g15520/PDR12 is involved in the transport of ABA (Abscisic acid), which is an ubiquitous phytohormone involved in many developmental and stress responses of plants (Kang et al., 2010). If *pdr12* is involved in ZON transport, the mutant should not be able to move the ZON as fast out of the cell as the wild type. Furthermore, *UGT73B4* overexpression mutants were included in the experiment. The UGT73B4 protein is a UDP-glycosyltransferase which is able to transfer glycosyl groups. If UGT73B4 glycosylates ZON faster compared to the wild-type, the absolute amount of ZON4G should be higher. To verify these assumptions the living plants were treated with ZON and the plant extract and the remaining plant media were checked via mass spectrometry for their components. A students t-test was performed to check for significant differences.

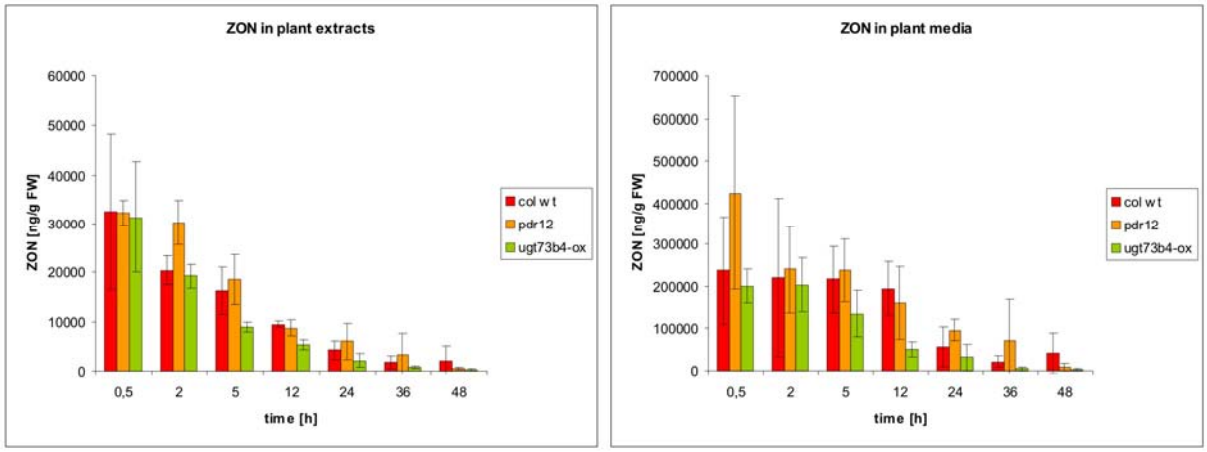

Figure 34: ZON metabolism in *Arabidopsis thaliana* plant extracts (left) and media (right). Arabidopsis seedlings (13 dag) were treated with 50 µM ZON in liquid MS 2.5 culture over a time course. The red column stands for columbia wild type plants (reference), the orange column stands for a PDR12 mutant plant and the green column stands for a UGT73B4 over expresser plant. The displayed variance is the calculated standard deviation.

In Figure 34 the ZON concentration in the plant and in the media is displayed. There is a significant difference after two hours of ZON treatment in the extracts. *pdr12* mutant plants have a higher concentration of ZON in the plant compared to the wild-type and the

UGT73B4-oxerexpressor. During the following measurements no significant difference could be observed but a slightly higher amount of ZON in the extract is recognizable. The significant difference after two hours might confirm the assumption that *pdr12* is involved in the ZON transport. In contrast, the amount of ZON in media of the *pdr12* plants was expected to be lower after two hours because of its incompetence of ZON transport out of cell. This lack of visible difference is explainable due to much higher concentrations of ZON in the media. The small difference, which originated in the plant extract after two hours, is not recognizable because of a tenfold difference concerning the ZON concentration between extract and the media. The results concerning the ZON level in the media revealed one significant difference after 12 hours. The *UGT73B4* overexpressor showed a lower level of ZON which might indicate a faster metabolism of ZON to ZON4G. Overall, the ZON concentration in the plant was decreasing nearly consistently. After 24 hours the greatest fraction of ZON was metabolized. The ZON level in the media was also decreasing nearly consistently because of uptake and metabolism by the plant.

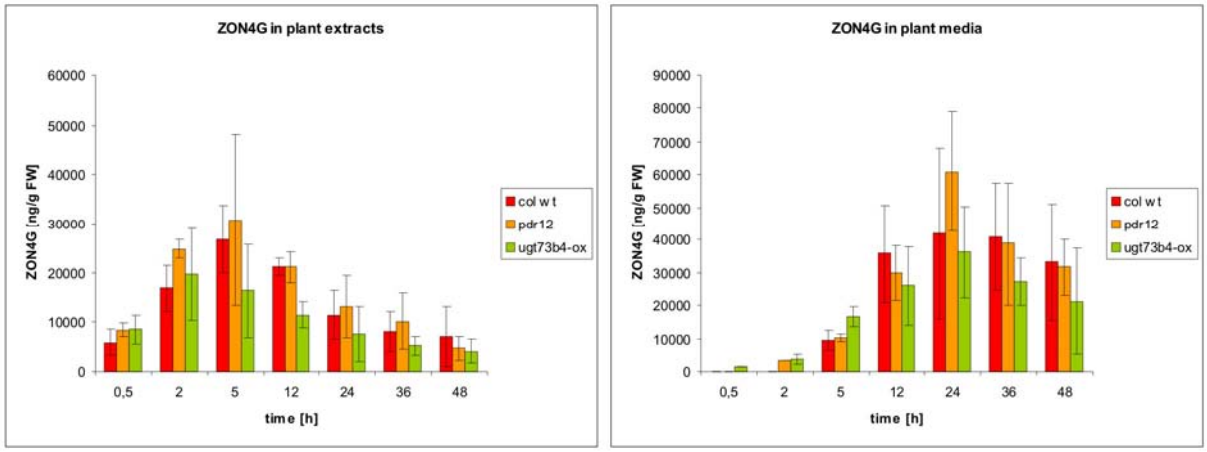

Figure 35: ZON4G metabolism in *Arabidopsis thaliana* plant extracts (left) and media (right). Arabidopsis seedlings (13 dag) were treated with 50 µM ZON in liquid MS 2.5 culture over a time course. The red column stands for columbia wild type plants (reference), the orange column stands for a PDR12 mutant plant and the green column stands for a UGT73B4 over expresser plant. The displayed variance is the calculated standard deviation.

In Figure 35 the metabolite ZON4G is displayed. A significantly higher level of ZON4G concerning the *pdr12* mutant in the extract could be observed after two hours. *pdr12* plants are again delayed in the transport out of the cell similar as with ZON (Figure 34). Furthermore, the *UGT73B4* overexpressor showed generally lower ZON4G concentrations in the extracts. This is an unexpected result because higher ZON4G concentrations in the extract were anticipated due to its glycosyl transferase activity. However, there is a significantly higher ZON4G level of the *UGT73B4* overexpressor in the medium after five hours. After half an hour and two hours, a not significant but slightly higher level of ZON4G

was observed. This could be an evidence for faster glycosylation and transport out of the cell in the *UGT73B4* overexpressor up to five hours.

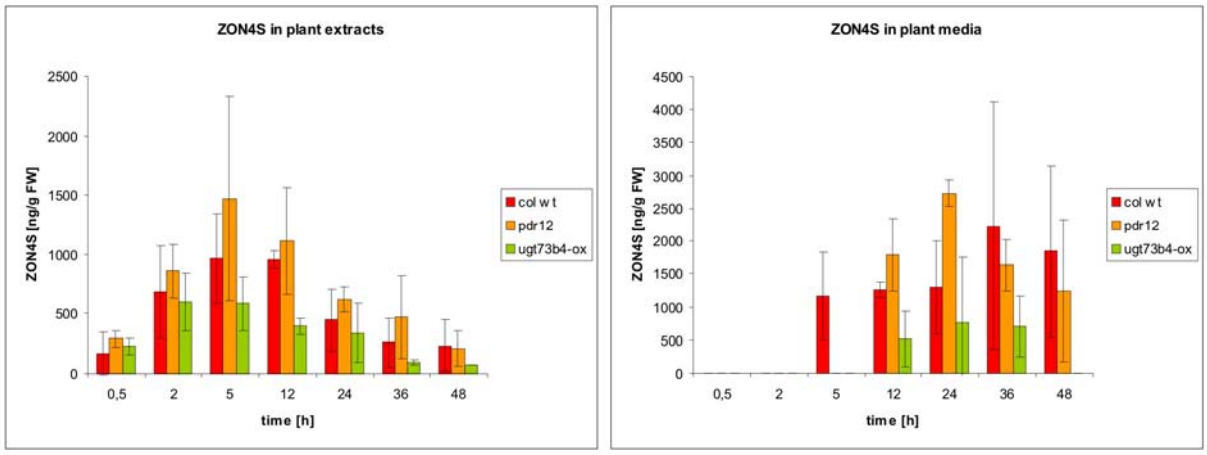

Figure 36: ZON4S metabolism in *Arabidopsis thaliana* plant extracts (left) and media (right). Arabidopsis seedlings (13 dag) were treated with 50 µM ZON in liquid MS 2.5 culture over a time course. The red column stands for columbia wild type plants (reference), the orange column stands for a PDR12 mutant plant and the green column stands for a UGT73B4 over expresser plant. The displayed variance is the calculated standard deviation.

In Figure 36 the ZON4S metabolite is displayed. There was a distinct difference after 12 hours in the extract. The *UGT73B4* overexpressor showed a significantly lower ZON4S value. Overall, it showed lower values of ZON4S in the extracts. This might be caused by a higher glycosylation rate of ZON to ZON4G instead of forming ZON4S. Concerning the *pdr12* mutant plants, a further delay in transport out of the cell, like it occurred in the ZON and ZON4G transport, could be observed. No ZON4S could be detected in the media for the first two hours. After five hours the wild-type plants showed a certain level of ZON4S which was nearly the same level as it occurred in the plant extracts. This indicates a global delay of ZON4S transport out of the cell. The *pdr12* showed again slightly higher but not significant ZON4S values in the extracts, which again indicates a ZON4S retaining effect. The ZON4S level in the media of the *UGT73B4* over expresser was significantly lower after 12 hours and slightly lower after 24, 36 and 48 hours. These results support the assumption of a higher glycosylation rate of ZON to ZON4G. The *pdr12* showed a significantly higher value of ZON4S after 24 hours in the medium. Over all it is more ZON4S in and outside the cell compared to the wild type. Maybe more ZON4S is produced because of the longer residence of ZON inside the cell cause by a delayed ZON transport out of the cell.

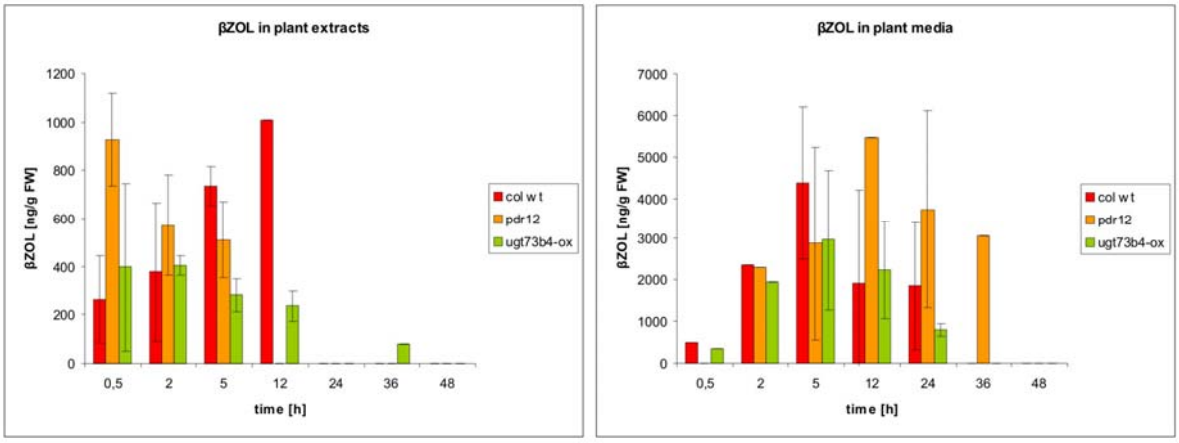

Figure 37: βZOL metabolism in *Arabidopsis thaliana* plant extracts (left) and media (right). Arabidopsis seedlings (13 dag) were treated with 50 µM ZON in liquid MS 2.5 culture over a time course. The red column stands for columbia wild type plants (reference), the orange column stands for a PDR12 mutant plant and the green column stands for a UGT73B4 over expresser plant. The displayed variance is the calculated standard deviation.

In Figure 37 the βZOL results are presented. Again, a strong retention of βZOL in the extract after half an hour is visible in the *pdr12* mutants. Overall, βZOL is formed in all plants until the 12 hours time point. Afterwards nearly no βZOL is detectable in plant extracts. However in the medium βZOL is detectable until 36 hours and with a higher concentration in the *pdr12* mutant.

To sum up, *pdr12* retains ZON and all of its metabolites longer in the cells. The transport of the toxin through the cell wall was delayed for every metabolite. This may indicate a role of PDR12 in ZON metabolite transport and thus detoxification. The UGT73B4-overexpressor had reduce ZON4S and ßZOL concentrations in the plant extracts and secreted also less ZON4S into the medium.

5 Discussion

5.1 Determination of the expression pattern of ZON induced genes during development

The data analysis was done with the free access version of the Genevestigator. Genevestigator is an advanced web-based system, that was designed to perform molecular expression meta-analysis (multiple micro array experiments) using novel concepts of data mining and innovation algorithms (Hruz et al., 2008). Meta-analysis in Genevestigator is based on the large-scale and systematic combination of normalized and quality-controlled expression data with experimental context variables using ontology (e.g. anatomy, genetic background, development) (Zimmermann et al., 2008). It becomes apparent, that all tested genes showed a increase of expression in the cotyledon stage. Furthermore, the Genevestigator analysis concerning the localization of gene expression, showed higher expression in the root (root xylem, root cap) This obtained data was confirmed by the experiments carried out in this thesis. The best ZON/heat-shock results concerning the induce ability of GFP-promoter constructs, were reached in roots and young plants (8 dag). This analysis gave a good overview of gene expression (localization, level of expression and developmental stage of the plant), and allows a rough scheduling of experiments.

5.2 Evaluation of the ZON responsiveness by quantitative Real-time PCR

To validate the results of the micro array experiment quantitative real-time PCR were established and performed. As indicated in the “golden rules of real-time PCR” several reference genes are necessary for a correct determination of the level of gene expression (Udvardi et al., 2008). Therefore three reference genes were evaluated if they are responsive to ZON and the solvent control DMSO. The chosen genes At4g20890/TUBULINβ9 and At3g62250/UBIQUITIN5 are well known and are considered to be relatively stable. They are well established as reference genes (Gutierrez et al., 2008). The gene At1g69960/PP2A was chosen because of its description as stable reference gene (Czechowski et al., 2005). Reference genes were used to avoid bias. These genes should not fluctuate during treatments (Nicot et al., 2005). Concerning the house keeping genes, differences occurred when comparing the first and the second batch. However, these gaps had little influence on the final outcome because of the use of three different housekeeping genes and two independent repetitions. Furthermore these gaps are highly likely caused by varying amounts of cDNA which will not disturb the calculation. The standards (appendix) were made once

and were used for both experiments. The expression analysis of the genes of interest gave a good overview on the expectable ZON treatment induced GFP signals. These results confirmed the ZON and heat shock experiment data. The induction maxima were discovered to occur after two to four hours. At this time points the reporter plants gave the best signals. Summarizing the intended results could be achieved by confirming the micro array data obtained by Ulrike Werner.

5.3 Responsiveness of the different reporter lines upon ZON-treatment and heat shock

The ZON experiment gave moderate results. The first tested construct pPZPpm2g29500-GFP showed no ZON induced GFP excitation. Consequently, it was decided to improve the construct. Therefore, the downstream 3' UTR was attached. This manipulation actually gave better results. But the most important point was the repeatability of the ZON induced GFP excitation. Some constructs, for example the displayed pPZPpm2g15490-GFP (Figure 31), showed a quite good outcome but it was not reproducible. In contrast, the improved construct, pPZPpm2g29500-GFP-3'UTR showed strong and repeatable signals. Therefore, it was chosen for further testing. The experiment was difficult to carry out because of the non-quantitative results. The plants were treated with ZON and DMSO and were observed, pictures were taken and were rated visually and subjectively. That made it hard to say if there was an excitement or just basal expression or autofluorescence. Nonetheless the obtained data coincides with the data received from the quantitative real time PCR. The observed maximum was in both cases after two to four hours. Both experiments confirmed the theory of ZON induction of chosen Arabidopsis genes.

5.4 The impact of the 3' UTR on the expression of the most promising reporter construct

It already has been shown that 3'UTR as an terminator is an effective way to increase the expression efficiency. Most eukaryotic mRNA is cleaved post-transcriptionally at a specific site 10 and 30 nucleotides downstream of a polyadenylation signal (a consensus AAUAAA sequence) in the 3' untranslated region (3'UTR) (Proudfoot and Brownlee, 1976).Subsequent to cleavage a poly(A) tail (e.g. 200 - 300 nt) is added to the RNA at the cleavage site (Brawerman, 1981). This modification has been shown to affect its stability, capacity to be translated and nuclear to cytoplasm export (Zhao et al., 1999). The effect of 3'UTR has already been shown in Arabidopsis. The terminator of the small heat shock protein 5g59720/HSP18.2 which is located within the 3'UTR showed better results in expression than the well known *noapaline synthase* (NOS) terminator which is widely used in plant

expression vector. Furthermore this sHSP terminator was tested with different reporter systems and promoters to except the possibility of coherences of the terminator, reporter and promoter. The increase of expression was caused by an increased mRNA accumulation (Nagaya et al., 2010). In this thesis the goal was to improve the old and not satisfying construct pPZP2g29500-GFP to obtain more expressed protein (GFP) resulting in a better detectable signal. Therefore, the 3' UTR of the small heat shock gene At2g29500/HSP17.6-CI was attached. The results obtained form the new construct showed a better performance (compare Figure 24 and 25) then the first constructs. These results go with the results already published (e.g. Nagaya et al. 2010). It is suggested to improve all of the tested constructs by adding the 3' UTR or other approved terminator sequences to increase the total amount of expressed reporter protein.

5.5 The involvement of PDR12 and UGT73B4 in the metabolism of ZON

The results obtained from the metabolism experiments showed a possible role for PDR12 in the transport of ZON and a slight effect of UGT73B4 concerning the glycosylation of ZON. The gene 2g15490/UGT73B4 is as mentioned above (1.5.3) a glykosyl transferase. For example it is known for its role in the metabolism of 2,4,6-trinitrotoluene (TNT) (Gandia-Herrero et al., 2008). PDR12 is already known for its contribution to toxin resistance against lead in *Arabidopsis thaliana (Lee et al., 2005)*. As mentioned above (1.5.1) PDR12 is additional responsible for trans membrane movement of abscisic acid (ABA) (Kang et al., 2010). As mentioned before the *pdr12* mutant restrained the toxin and its metabolites (ZON4S, ZON4G and βZOL) in the cell compared to the wild-type. The experiment was carried out with four independent repetitions which substantiate the data and the assumption that PDR12 is involved in ZON transport. Overall, the ZON metabolism and assembling of its metabolites followed the data obtained from Berthiller et al (2006, Figure 3). To sum up, the data obtained from the metabolism experiment suggests that PDR12 is involved in the ZON (and its metabolites) transport out of the cell. Clear and significant differences could be observed. A suggestion for further metabolism experiments would be additional time points. They will give a clearer picture of the metabolism in the plant. A disadvantage of additional time points would be the extensive time effort.

6 List of Tables

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8 References

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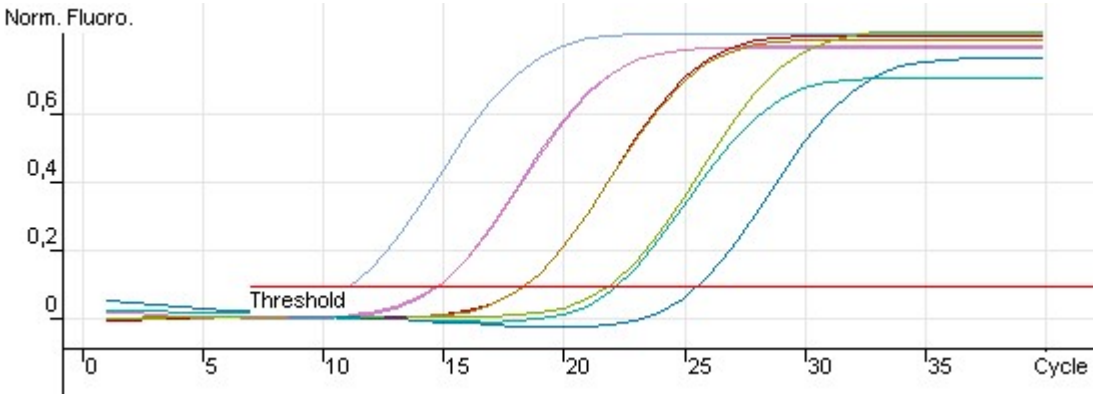
9 Appendix

9.1 Quantitative real-time PCR standards

At1g15520/PDR12:

Quantitation:



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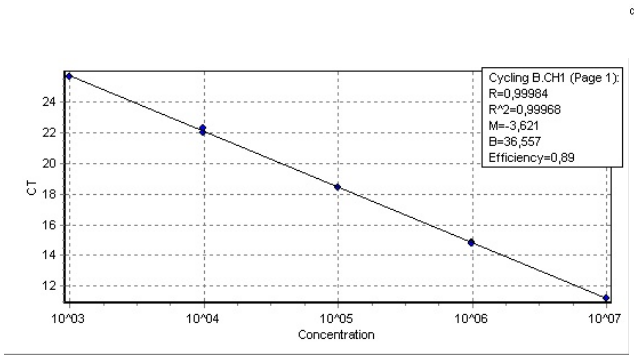

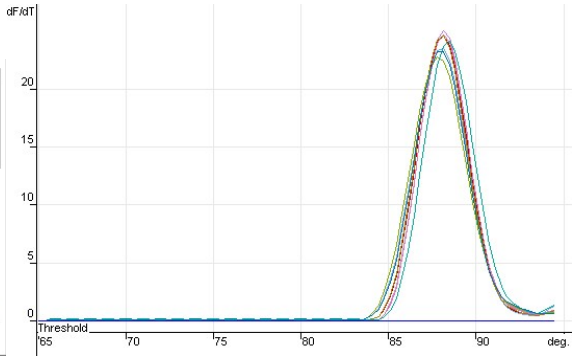

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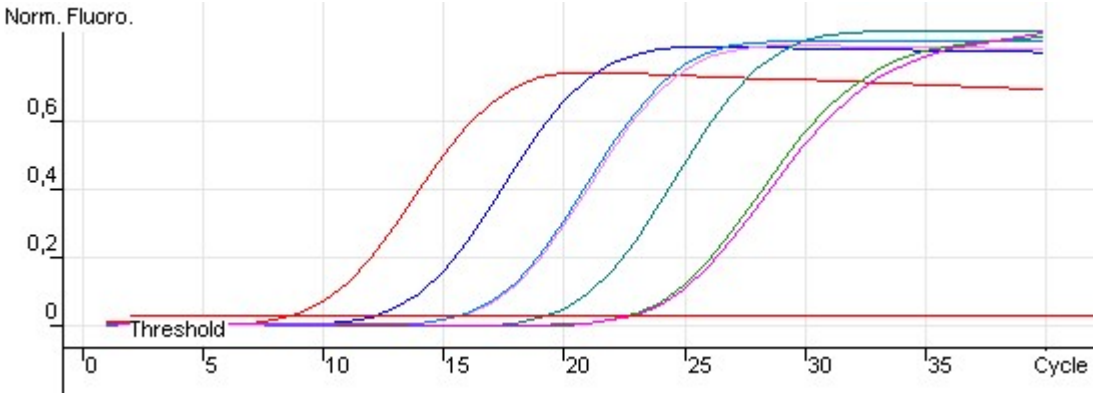
18	10^6	14,84	1000000	996713	0,30%	14,82	1007302
19	10^6	14,81	1000000	1018003	1,80%		
20	10^5	18,44	100000	100902	0,90%	18,44	100922
21	10^5	18,44	100000	100942	0,90%		
22	10^4	21,97	10000	10681	6,80%	22,12	9727
23	10^4	22,27	10000	8859	11,40%		
24	10^3	25,65	1000	1029	2,90%	25,65	1029

At1g17180/GSTU25:

Quantitation:



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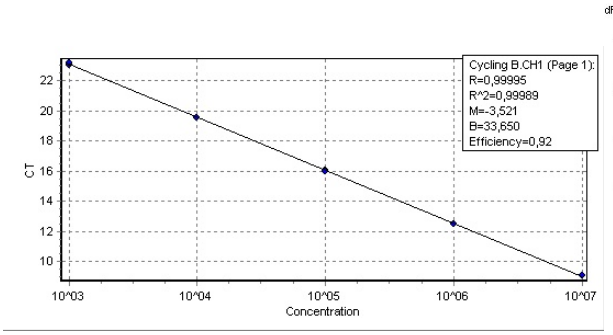

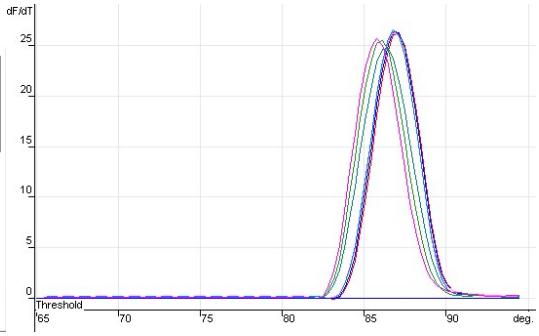

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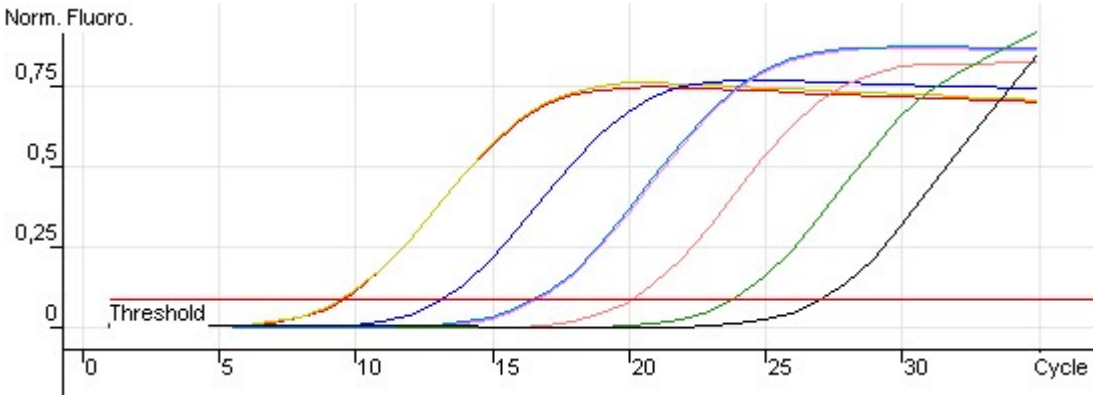
3	10^6	12,46	1000000	1040890	4,10%	12,46	1040890
5	10^5	16,08	100000	97921	2,10%	16,04	100517
6	10^5	16	100000	103182	3,20%		
7	10^4	19,56	10000	10013	0,10%	19,56	10013
9	10^3	23,04	1000	1034	3,40%	23,1	992
10	10^3	23,16	1000	952	4,80%		

At2g15480/UGT37B5

Quantitation:



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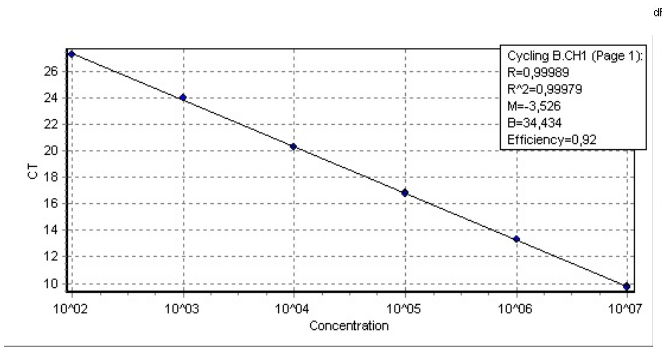

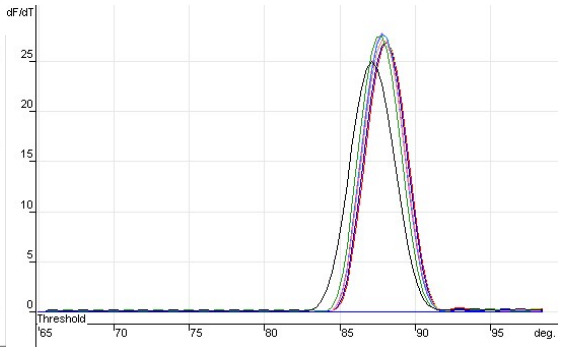

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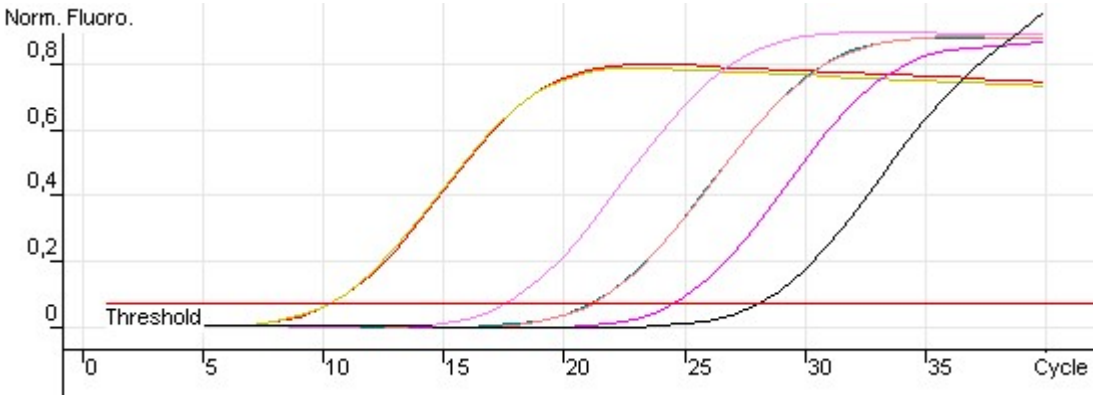
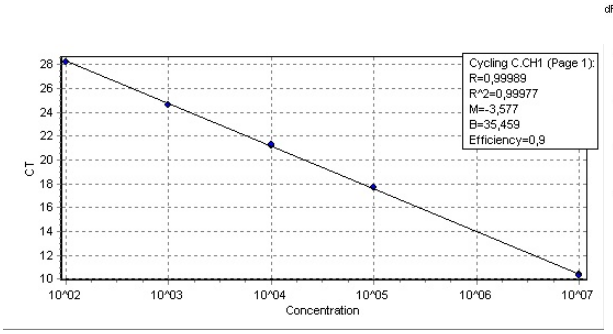
2	10^7	9,68	10.000.000,00	10.464.777,00	4,60%		
3	10^6	13,33	1.000.000,00	966.640,00	3,30%	13,33	966.640,00
5	10^5	16,89	100.000,00	94.631,00	5,40%	16,83	98.441,00
6	10^5	16,77	100.000,00	102.404,00	2,40%		
8	10^4	20,29	10.000,00	10.290,00	2,90%	20,29	10.290,00
9	10^3	24,02	1.000,00	901	9,90%	24,02	901
11	10^2	27,25	100	109	9,00%	27,25	109

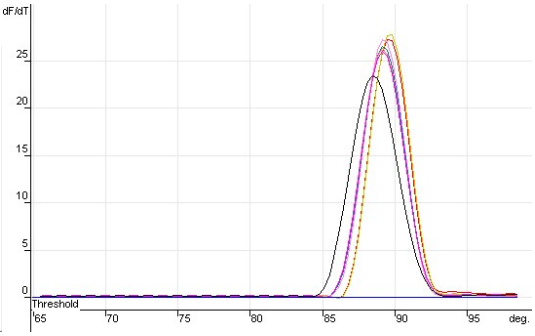
At2g15490/UGT37B4

Quantitation:



Threshold: 0,07

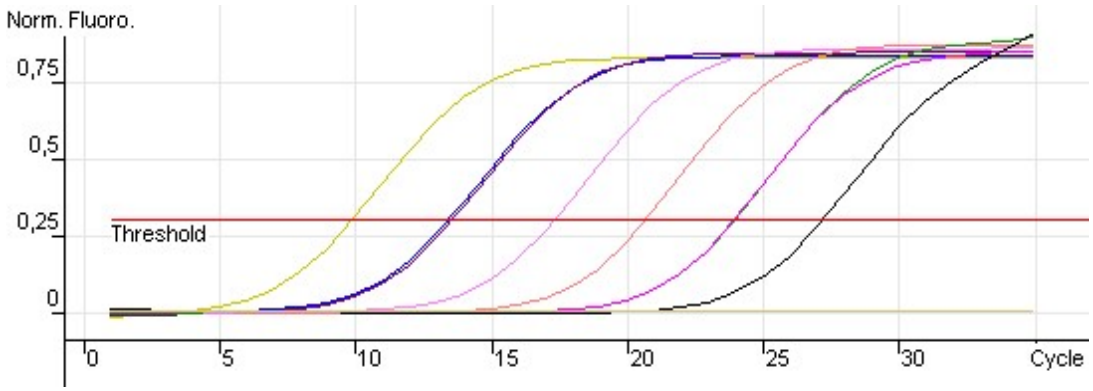


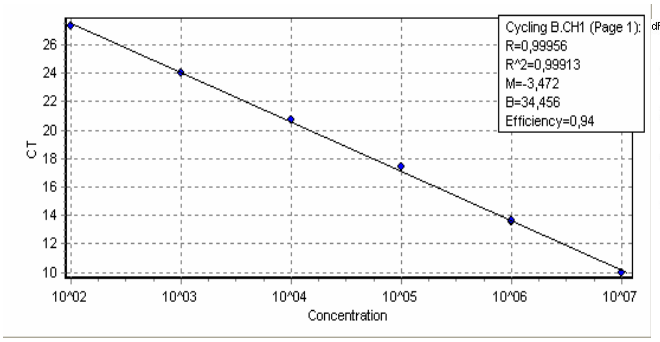


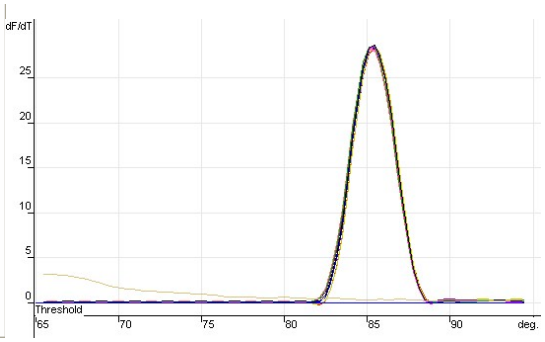
No.	Name	Ct	Given Conc	Calc Conc	% Var	Rep. Ct	Rep. Calc. Conc.
1	10^7	10,42	10.000.000,00	10.023.913,00	0,20%	10,36	10.397.407,00
2	10^7	10,3	10.000.000,00	10.784.816,00	7,80%		
5	10^5	17,66	100.000,00	94.664,00	5,30%	17,66	94.664,00
7	10^4	21,22	10.000,00	9.550,00	4,50%	21,26	9.326,00
8	10^4	21,3	10.000,00	9.108,00	8,90%		
10	10^3	24,64	1.000,00	1.055,00	5,50%	24,64	1.055,00
11	10^2	28,21	100	106	6,50%	28,21	106

At5g12030/HSP17.6A

Quantitation:





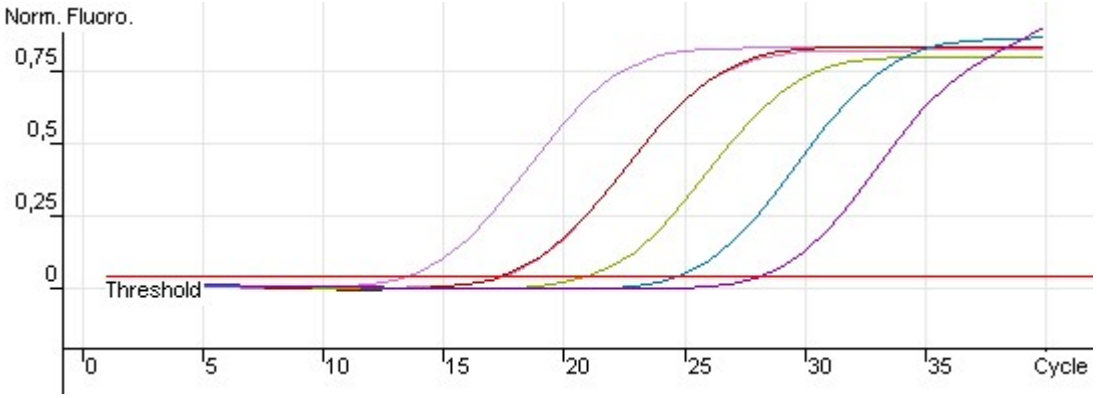


Threshold: 0,0526

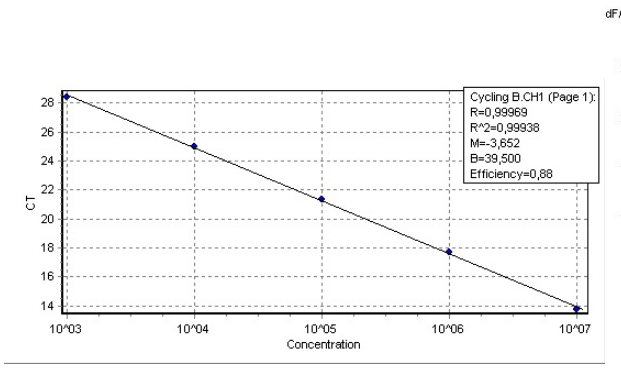
No.	Name	Ct	Given Conc	Calc Conc	% Var	Rep. Ct
2	10^7	9,96	10.000.000,00	11.321.779,00	13,20%	9,96
3	10^6	13,51	1.000.000,00	1.079.837,00	8,00%	13,57
4	10^6	13,64	1.000.000,00	986.635,00	1,30%	
5	10^5	17,4	100.000,00	81.403,00	18,60%	17,4
8	10^4	20,76	10.000,00	8.794,00	12,10%	20,76
9	10^3	24,05	1.000,00	992	0,80%	24,04
10	10^3	24,03	1.000,00	1.009,00	0,90%	
11	10^2	27,29	100	116	15,80%	27,29
13	NTC					

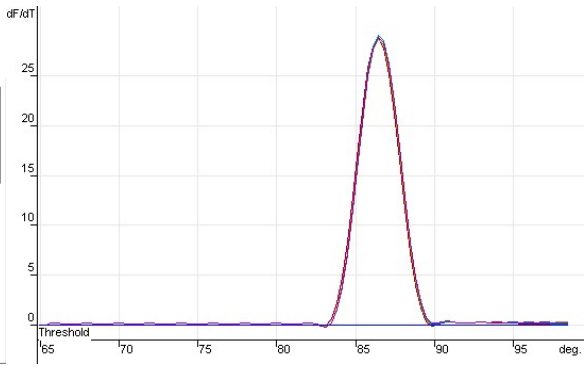
At5g59720/HSP18.2

Quantitation:



Threshold: 0,0436

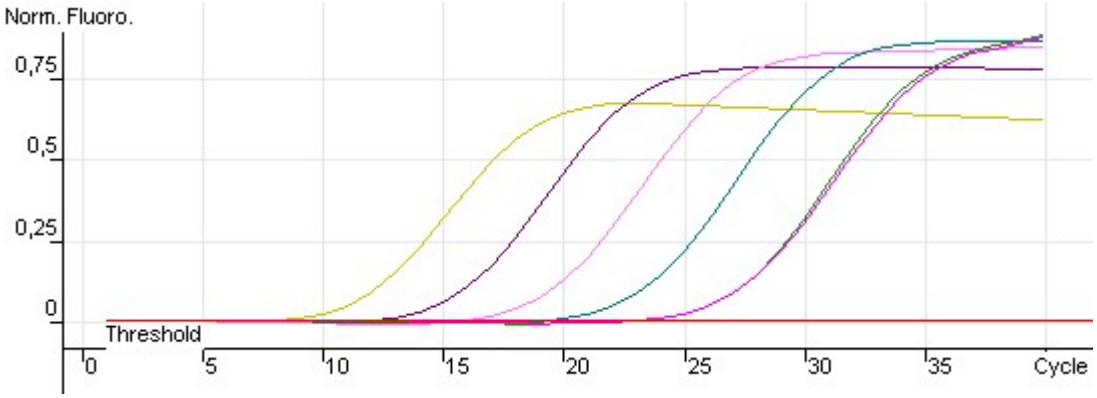




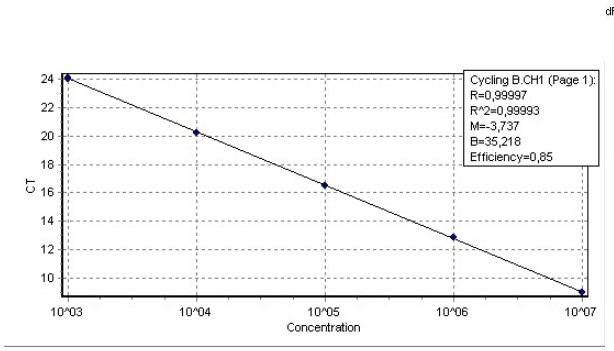
No.	Name	Ct	Given Conc	Calc Conc	% Var	Rep. Ct	Rep. Calc. Conc.
18	10^7	13,74	10.000.000,00	11.300.027,00	13,00%	13,74	11.300.027,00
19	10^6	17,69	1.000.000,00	935.685,00	6,40%	17,67	946.542,00
20	10^6	17,65	1.000.000,00	957.525,00	4,20%		
22	10^5	21,32	100.000,00	94.877,00	5,10%	21,32	94.877,00
24	10^4	24,98	10.000,00	9.474,00	5,30%	24,98	9.474,00
26	10^3	28,39	1.000,00	1.099,00	9,90%	28,39	1.099,00

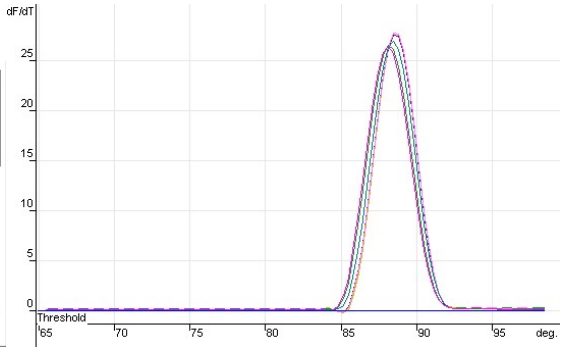
At3g62250/Ubiquitin5

Quantitation:



Threshold: 0,0084

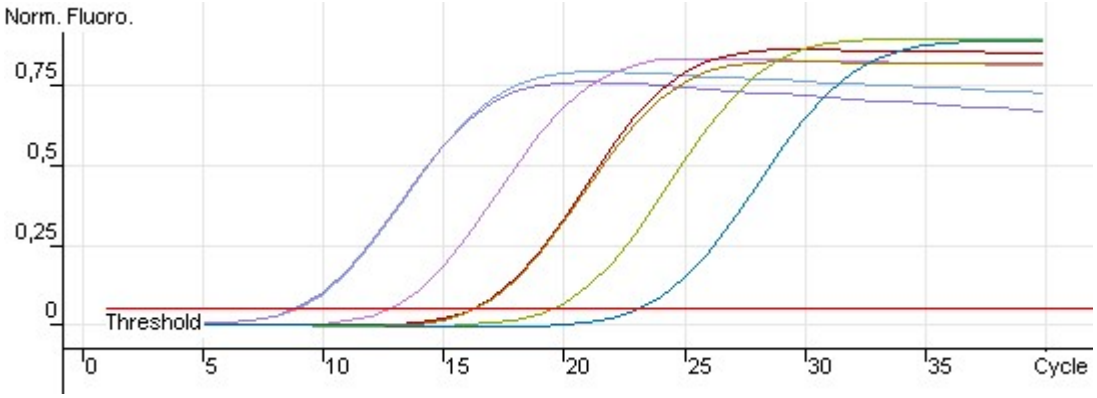




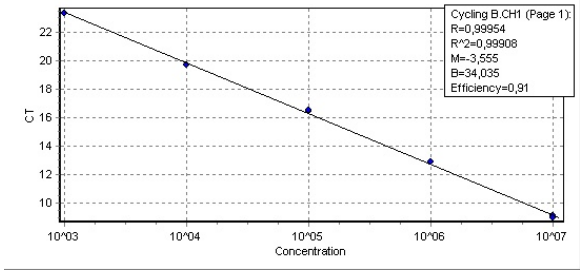
No.	Name	Ct	Given Conc	Calc Conc	% Var	Rep. Ct	Rep. Calc. Conc.
2	10^7	9,03	10.000.000,00	10.181.854,00	1,80%	9,03	10.181.854,00
4	10^6	12,88	1.000.000,00	951.634,00	4,80%	12,88	951.634,00
5	10^5	16,49	100.000,00	102.542,00	2,50%	16,49	102.542,00
7	10^4	20,23	10.000,00	10.268,00	2,70%	20,23	10.268,00
9	10^3	24,04	1.000,00	977	2,30%	24,02	990
10	10^3	24	1.000,00	1.003,00	0,30%		

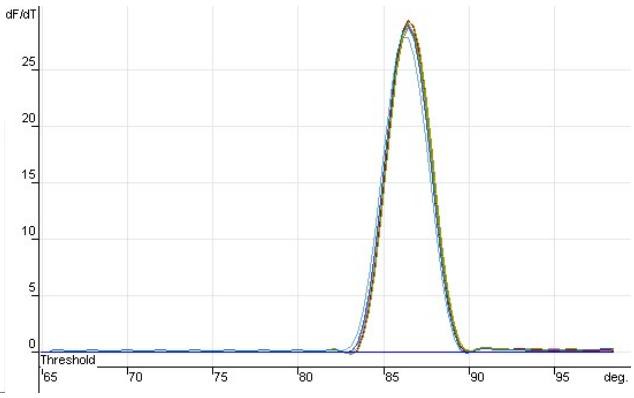
At1g69960/*PP2A*

Quantitation:



Threshold: 0,05334

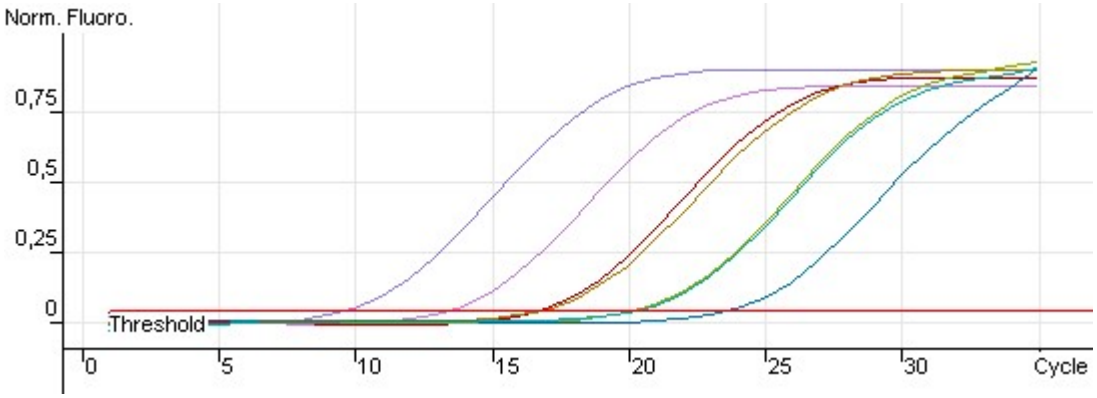




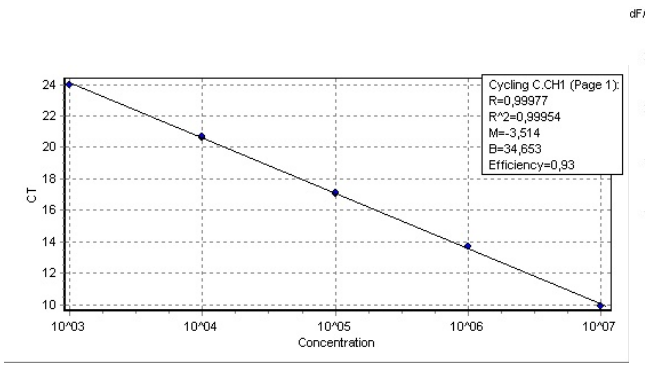
No.	Name	Ct	Given Conc	Calc Conc	% Var	Rep. Ct	Rep. Calc. Conc.
16	10^7	9,1	10.000.000,00	10.352.539,00	3,50%	9,03	10.844.742,00
17	10^7	8,95	10.000.000,00	11.360.346,00	13,60%		
18	10^6	12,87	1.000.000,00	901.473,00	9,90%	12,87	901.473,00
20	10^5	16,39	100.000,00	91.885,00	8,10%	16,43	89.767,00
21	10^5	16,46	100.000,00	87.698,00	12,30%		
22	10^4	19,67	10.000,00	10.988,00	9,90%	19,67	10.988,00
24	10^3	23,27	1.000,00	1.065,00	6,50%	23,27	1.065,00

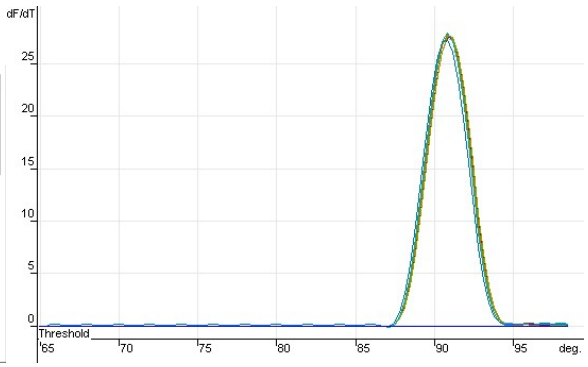
GFP

Quantitation:



Threshold: 0,0452

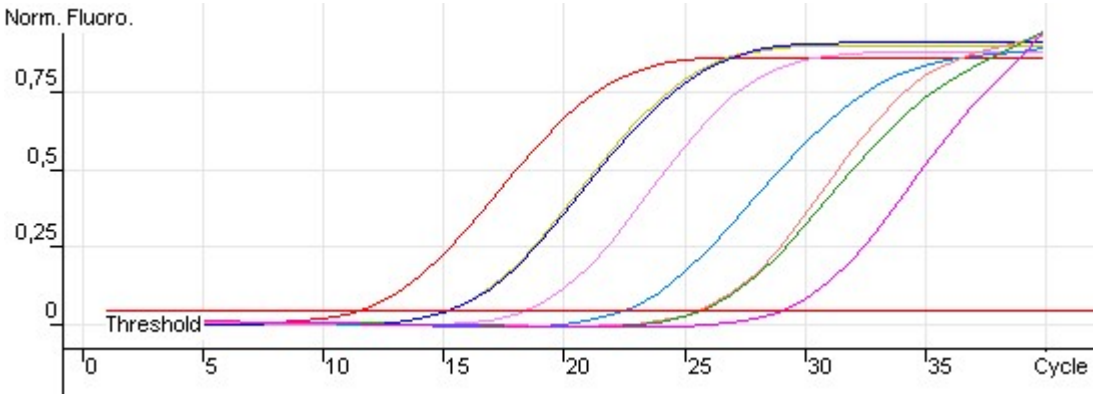




No.	Name	Ct	Given Conc	Calc Conc	% Var	Rep. Ct	Rep. Calc. Conc.
17	10^7	9,91	10.000.000,00	10.965.351,00	9,70%	9,91	10.965.351,00
18	10^6	13,7	1.000.000,00	913.168,00	8,70%	13,7	913.168,00
20	10^5	17,08	100.000,00	100.337,00	0,30%	17,09	99.184,00
21	10^5	17,11	100.000,00	98.045,00	2,00%		
22	10^4	20,6	10.000,00	9.951,00	0,50%	20,64	9.688,00
23	10^4	20,68	10.000,00	9.432,00	5,70%		
24	10^3	23,99	1.000,00	1.082,00	8,20%	23,99	1.082,00

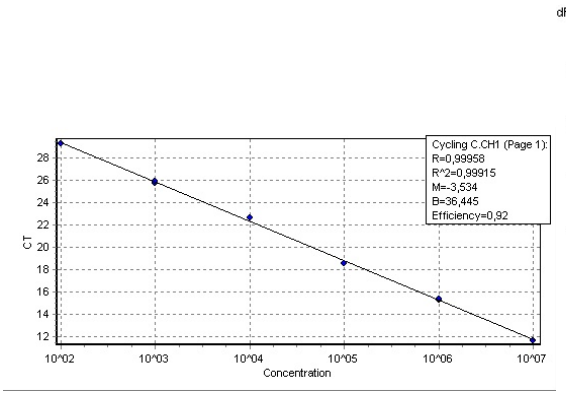
At4g20890/*Tubulin9*

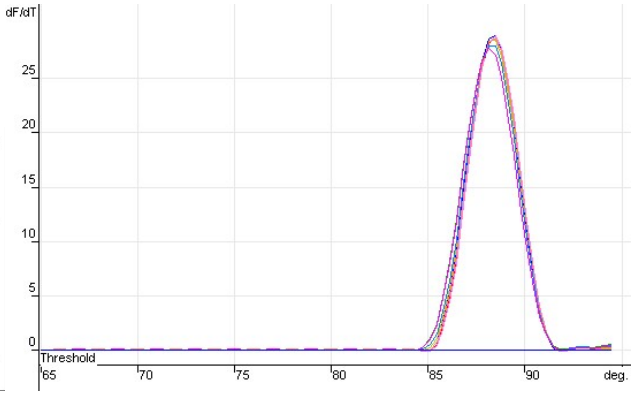
Quantitation:



Threshold: 0,0454

Standardkurve & Schmelzkurve:





Absolute Werte:

No.	Name	Ct	Given Conc	Calc Conc	% Var	Rep. Ct	Rep. Calc. Conc.
1	10^7	11,6	10.000.000,00	10.689.833,00	6,90%	11,6	10.689.833,00
2	10^6	15,28	1.000.000,00	973.262,00	2,70%	15,33	945.367,00
3	10^6	15,37	1.000.000,00	918.272,00	8,20%		
5	10^5	18,5	100.000,00	119.556,00	19,60%	18,5	119.556,00
6	10^4	22,64	10.000,00	8.057,00	19,40%	22,64	8.057,00
8	10^3	25,78	1.000,00	1.044,00	4,40%	25,83	1.006,00
9	10^3	25,89	1.000,00	969	3,10%		
10	10^2	29,27	100	107	7,30%	29,27	107

Summary and comparison of ct-values

	1g15520	1g17180	2g15480	2g15490	5g12030	5g59720	UBQ5	PP2A	GFP	TUB9
ct-Wert 10^7	11,22	9,06	9,71	10,36	9,96	13,74	9,03	9,03	9,91	11,6
ct-Wert 10^6	14,82	12,46	13,33	-	13,57	17,67	12,88	12,87	13,7	15,33
ct-Wert 10^5	18,44	16,04	16,83	17,66	17,4	21,32	16,49	16,43	17,09	18,5
ct-Wert 10^4	22,12	19,56	20,29	21,26	20,76	24,98	20,23	19,67	20,64	22,64
ct-Wert 10^3	25,65	23,1	24,02	24,64	24,04	28,39	24,02	23,27	23,99	25,83
ct-Wert 10^2	-	-	27,25	28,21	27,29	-	-	-	-	29,27