Regulation of class III peroxidases and respiratory burst oxidase homologs by biotic and abiotic stress in maize (*Zea mays* L.)

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1 Abbreviations

Chemicals

2,4 D	*2,4-Dichlorophenoxyacetic acid*
$(NH_4)_2SO_4$	*Ammonium sulphate*
$CaCl_2$ x $2H_2O$	*Calcium chloride dihydrate*
$CoCl_2$ x $6H_2O$	*Cobalt (II) chloride hexahydrate*
Cd	*Cadmium*
$CdCl_2$	*Cadmium chloride*
$Cd(NO_3)_2$	*Cadmium nitrate*
CSPD	*Chemiluminescence substrate for alkaline phosphatase detection Disodium 3-(4-methoxyspiro {l,2-dioxetane-3,2'-(5'-Chloro)Tricyclo[3.3.1.13,7]Decan}-4-yl) Phenylphosphat*
$CuSO_4$ x $5H_2O$	*Copper (II) sulphate pentahydrate*
DIG	*Digoxigenin*
EDTA	*Ethylenediaminetetraacetic acid*
EtBr	*Ethidium bromide*
EtOH	*Ethanol*
H_3BO_3	*Boric acid*
HCl	*Hydrochloric acid*
KH_2PO_4	*Potassium dihydrogen phosphate*
KJ	*Potassium iodide*
KNO_3	*Potassium nitrate*
MeOH	*Methanol*
MES	*2-(N-morpholino)ethanesulfonic acid*
$MgSO_4$ x $7H_2O$	*Magnesium sulphate heptahydrate*
$MnSO_4$ x H_2O	*Manganese (II) sulphate hydrate*
Na_2MoO_4 x $2H_2O$	*Sodium molybdate dihydrate*
NaCl	*Sodium chloride*
NaFe-EDTA	*Ethylenediaminetetraacetic acid iron(III) sodium salt*
NaOH	*Sodium hydroxide*
NH_4NO_3	*Ammonium nitrate*
PMSF	*Phenylmethylsulfonylfluorid*
SDS	*Sodium dodecyl sulphate*
TAE	*Tris-acetatic-EDTA*
TE	*Tris-EDTA*
TRIS	*Tris (hydroxymethyl)-amino methane*
Tween	*Polyethylene glycol sorbitan monolaurate*
$ZnSO_4$ x $7H_2O$	*Zinc sulphate heptahydrate*
H_2O	*Water*

Prefixes and Units

%	*Per cent*
°C	*Degree Celsius*
µ	*Micro*
A	*Ampère*
bp	*Base pair*
cm	*Centimetre*
g	*Gram*
h	*Hour*
kb	*Kilo bases*
L	*Litre*
M	*Molar (mol per L)*
mg	*Milligram*
min	*Minute*
mL	*Millilitre*
mM	*Millimolar*
nm	*Nanometre*
s	*Second*
U	*Unit*
V	*Volt*
x g	*x-acceleration of gravity*
¤	*Self-pollinated*

Abbreviations

35S	*Promoter from cauliflower mosaic virus*
APX	*Ascorbate peroxidase*
Bar 1	*Glufosinat resistance Bar gene exon 1*
Bar 2	*Glufosinat resistance Bar gene exon 2*
BLAST	*Basic local alignment search tool*
cDNA	*complementary DNA*
CoCu	*Co-cultivation media*
Cre1	*Cre (causes recombination) gene artificial exon 1*
Cre2	*Cre (causes recombination) gene artificial exon 2*
DNA	*Deoxyribonucleic acid*
dNTPs	*Desoxynucleotide triphosphate*
et al.	*And others (latin: et alii)*
etc.	*And so on (latin: Etcetera)*
for	*Forward*
gDNA	*Genomic DNA*
HSP	*Heat shock promoter of glycine max*
Inf.-Med	*Infection media*
Lox A	*Recombination side A*
Lox B	*Recombination side B*
mRNA	*Messenger RNA*
MS	*Murashige Skoog*

NADPH / $NADP^+$	*Nicotinamide adenine dinucleotide phosphate (reduced / oxidised)*
NCBI	*National Center for Biotechnology Information*
PAGE	*Polyacrylamide gel electrophoresis*
PAR	*Photosynthetically Active Radiation*
PCR	*Polymerase chain reaction*
PRX	*Peroxidase*
qPCR	*Quantitative polymerase chain reaction*
RBOH	*Respiratory burst oxidase homolog*
RDRP	*RNA dependent RNA polymerase*
REME	*Resting media*
rev	*Reverse*
RISC	*RNAi induced silencing complex*
RNA	*Ribonucleic acid*
RNAi	*Ribonucleic acid Interference*
RNase	*Ribonuclease*
ROS	*Reactive oxygen species*
rRNA	*Ribosomal ribonucleic acid*
RT	*Room temperature*
RT-PCR	*Reverse transcriptase-polymerase chain reaction*
SEME I	*Selection media I*
Seme II	*Selection media II*
SNP	*Single nucleotide polymorphism*
SSC	*Standard saline citrate*
T	*Temperature*
T35S	*Terminator from cauliflower mosaic virus*
Taq-Polymerase	*Polymerase of Thermus aquaticus*
Tocs	*Terminator octopin synthase*
Ubi Int	*Ubiquitin promoter from solanum tuberosum*
UV	*Ultraviolet*
YEB	*Yeast extract beef*

2 Abstract

The plasma membrane-bound peroxidases (PRX) *zmprx01*, *zmprx66* and *zmprx70* and the respiratory burst oxidase homologs (RBOH) *rbohA*, *rbohB*, *rbohC* and *rbohD* were analysed in this study. The distribution of the genes inside the roots was investigated by real-time-qPCR. Therefor four different segments (root tip, elongation zone, differentiation zone and lateral roots) were in focus of the analyses. It could be observed that the genes are differently distributed in the root. The peroxidases were predominantly expressed in the elongation zone and almost not in the root tip. The *rboh* genes were more inhomogeneous distributed. For each RBOH a specific expression pattern could be detected. *rbohA* was mostly expressed in the differentiation zone. *rbohB* was more even expressed in the root. *rbohC* was even distributed as well but predominantly in the elongation zone. *rbohD* was mostly expressed in the differentiation zone. For a further investigation of the peroxidases plants were exposed to cadmium (short-term and long-term trial). The plants grew in cadmium contaminated hydroponics. *zmprx66* and *zmprx70* were upregulated after 15 minutes (quick response). Subsequently the expression went back to normal. Through the long-term trial a decrease of each peroxidase was detected after three days of exposure. RNAi mutants were produced to analyse the lack of each peroxidase. RNAi was mediated by a heat shock inducible RNAi construct with double opposing promoters. This experiment was not finished, yet. By now it could be concluded that the down-regulation of *zmprx66* decelerated the development of the whole plant. Further investigations are necessary. To find out more about the triggers for each gene and correlations between protein and mRNA abundance a stress profiling experiment was performed in accordance to the proteomic approach of Mika *et al.,* 2010. The plants grew in hydroponics while the stress factors (chitosan, H_2O_2, NaCl, salicylic acid) were applied into the nutrition media. Additionally, mechanical wounding was performed. By the stress profiling it could be concluded that every gene has different triggers. The expression of the peroxidases did decrease by almost every treatment except *zmprx70,* which was positively affected by salicylic acid and wounding. Results suggest no correlation between protein abundance and mRNA, after 1 h or more. *rbohC* and *rbohD* were upregulated by H_2O_2, NaCl affected *rbohB*, *rbohC* and *rbohD* positively. Salicylic acid almost did not affect any RBOH except *rbohC,* which was slightly

upregulated. *rbohD* was significantly upregulated through wounding. In association with Dr Meisrimler the genes were analysed under the impact of waterlogging. Waterlogging was performed with 28 days old potted maize plants. Mature and immature leaves were analysed separately. At that developmental stage it could be detected, that *zmprx66* and *zmprx70* were not expressed in leaves (control individual were analysed, preliminarily) but the protein (ZmPrx66) was found in that tissue. In this case no qPCR studies for *zmprx66* and *zmprx70* could be performed. It was observed that *zmprx01* was predominantly expressed in immature leaves. The waterlogging had an impact on mature leaves. The expression was increased. For *rbohB* the same observation was made. The remaining RBOH seemed not to be affected by waterlogging, significantly.

Because of this study many new information could be gained for *zmprx01*, *zmprx66* and *zmprx70* and *rbohA*, *rbohB*, *rbohC* and *rbohD*. Their triggers, co-regulations and involvements in different processes could be identified or more clarified.

3 Introduction

3.1 Peroxidases

Peroxidases are enzymes, which catalyse the reduction of peroxides and belong to a large multigene family. Peroxidases (PRX) are involved in both, production and detoxification of reactive oxygen species (ROS). They can control the level of ROS together with other antioxidant systems, sensitively (Passardi *et al*., 2005; Lüthje *et al*., 2011). Apart from ROS homeostasis PRX are also involved with many other fields such as cell growth, hormone signalling, programmed cell death, stomata opening and regulation of gene expression (Passardi *et al*., 2005).

For class III peroxidases approximately 142 peroxidases have been found in maize (*Zea mays* L.), 73 in *Arabidopsis thaliana* (L. [Heynh.]), 138 in *Oryza sativa* (L.), 86 in *Solanum tuberosum* (L.), 138 in *Triticum aestivum* (L.) (Peroxibase, 2015); they can furthermore be divided into soluble and membrane-bound isoforms (Passardi *et al*., 2005; Lüthje *et al*., 2011). By transcriptional and posttranslational modification, several other iso-enzymes are generated (Tognolli *et al*., 2002; Welinder *et al*., 2002). Four plasma membrane-bound peroxidases have been identified to be differentially regulated by oxidative stress in maize (Mika *et al*., 2008; Mika *et al*., 2010).

According to Welinder (1992), plant peroxidases are divided into three classes (class I, class II and class III). Class I peroxidases are suggested to be the evolutionary origin of the other classes (Passardi *et al*., 2007). They do not belong to the secretory pathway. The detoxification of H_2O_2 is their major function. The class I peroxidases can again be separated into three different groups. First, ascorbate peroxidases (EC1.11.1.11) have a high affinity to ascorbate and are found in photosynthetic organisms. Second, cytochrome *c* peroxidases (EC1.11.1.5) are found in the intermembrane space of mitochondria and use cytochrome *c* as an electron donator. Finally, catalase-peroxidases (EC1.11.1.6) are able to oxidise H_2O_2 just like peroxidases, but can also use other molecules as a substrate. They do not have disulphide bonds, no glycosylation or no signal peptide, which leads to an affinity to the endoplasmic reticulum. Class II peroxidases are only known in fungi, were they are mainly involved in the degradation of soil debris

(Piontek *et al*., 2001; Martinez *et al*., 2005). They can be divided into manganese peroxidases (EC 1.11.1.13), lignin peroxidases (EC 1.11.1.14) and versatile peroxidases (EC1.11.1.16) (Ruiz-Duenas *et al*., 2001).

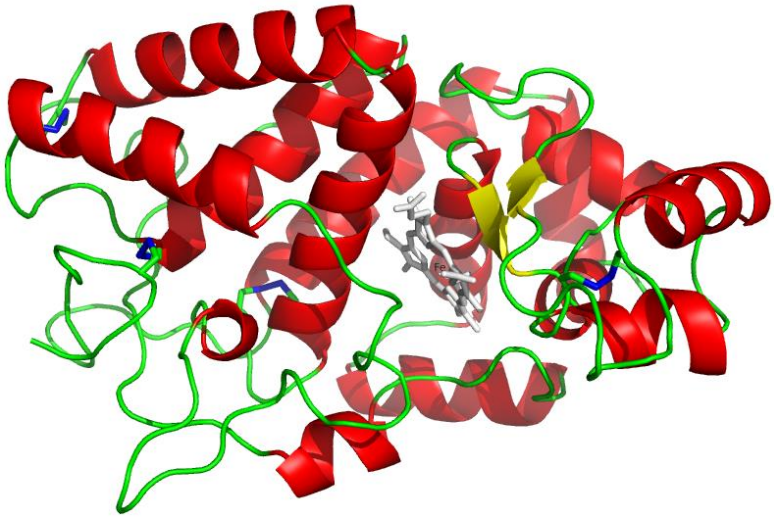


Figure 1: Predicted 3D model of the PRX ZmPrx70. Helices are displayed in red and beta-sheets are displayed in yellow. The backbone is displayed in green. The haem (grey) is located in the centre. In class III peroxidases the haem is not covalently bound.

This study deals with the class III plant peroxidases (EC 1.11.1.7). Most of them are induced by stress (Passardi *et al*., 2004). Class III peroxidases were firstly described in 1855. They are involved in plenty of processes in plants triggered by stress and during the development (Hiraga *et al*., 2001; Passardi *et al*., 2005; Cosio & Dunand, 2009). In addition to the known fact that class III peroxidases are soluble apoplastic and cell wall bound enzymes, four plasma membrane-bound peroxidases could be detected (Mika & Lüthje, 2003; Mika *et al*., 2008). Analysis of the sequence of the "new found" peroxidases (ZmPrx01; ZmPrx66; ZmPrx70) revealed a function in oxidative stress on the apoplastic side of the plasma membrane (Mika *et al*., 2008).

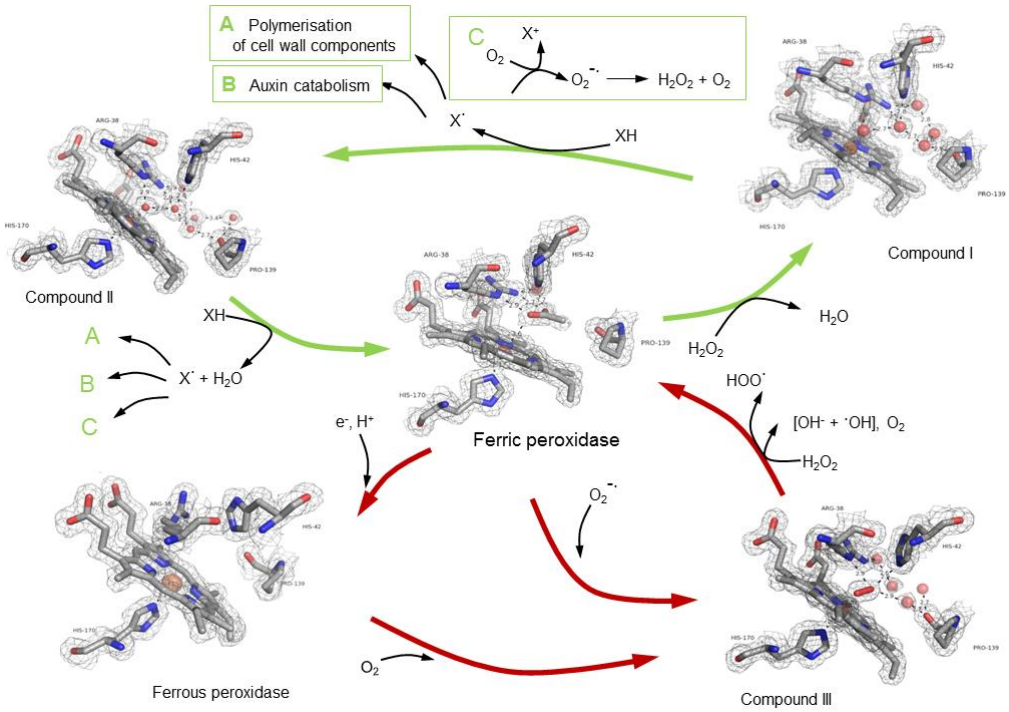


Figure 2: Possible cycles of class III peroxidases in plants. Peroxidases are able to generate or detoxify ROS through the two possible cycles; peroxidative cycle (green arrows) and hydroxylic cycle (red arrows) (Lüthje *et al*., 2013).

Class III peroxidases can underlay two different cycles (figure 2); the peroxidative cycle (green arrows) and the hydroxylic cycle (red arrows). Several substrates can be oxidised by the peroxidative cycle (reduced condition XH), the oxidised condition is marked as X·. Because of a non-catalytic reaction this substrate oxidation plays an important role in the auxin metabolism; polymerisation of cell wall components and NAD(P)H oxidation. By this reaction the superoxide is transformed to H_2O_2 and O_2 by superoxide dismutase or even spontaneously. ROS can be produced by the hydroxylic cycle. Both cycles have the ability to control the level of H_2O_2 (Passardi *et al*., 2005; Lüthje *et al*., 2013). Production of high amounts of H_2O_2 is known for plant-pathogen interaction (Schraudner *et al*., 1996; Minibayeva *et al*., 2009; Minibayeva *et al*., 2015). Cell elongation and expansion processes are under control of ascorbate. This is due to the inhibition of enzymes, which are involved in cell wall stiffening. It was investigated that ascorbate inhibits the activity of peroxidases, which are involved in root elongation. That was proved by the inhibition of root elongation controlling peroxidases by ascorbate (Cordoba-Pedregosa *et al*., 1996).

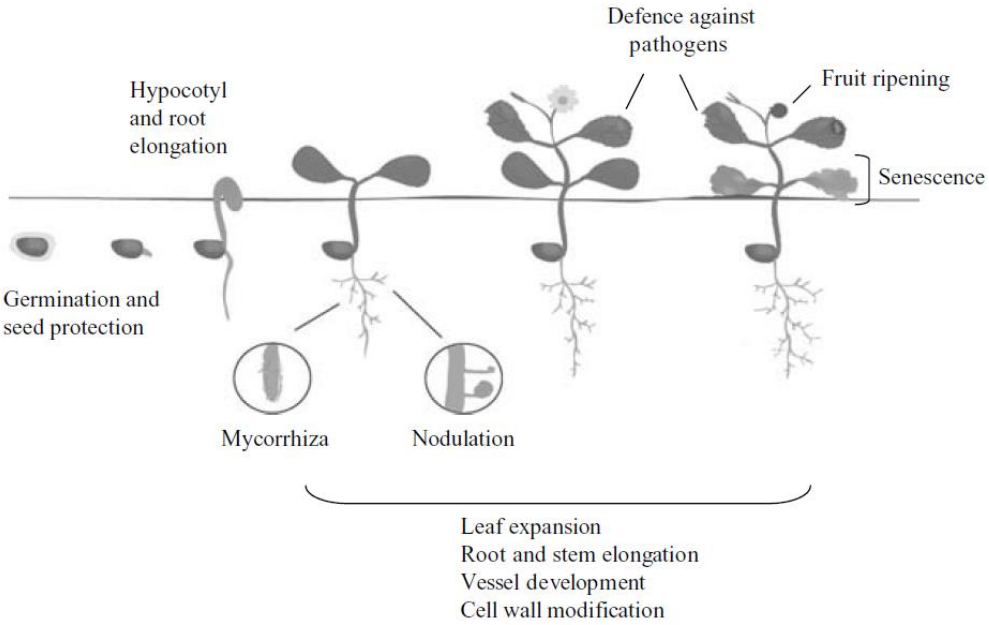


Figure 3: Schematic overview of the whole lifespan of a plant. Class III peroxidases are involved in the whole lifetime of a plant (Passardi *et al*., 2005).

Class III peroxidases were detected in every developmental stage of a plant during the lifespan in every tissue (figure 3) (Passardi *et al*., 2005). Plant peroxidases are involved in many processes. In tomato seeds a first activity of peroxidases was detected very early (24 h after imbibition) (Morohashi, 2002). The elongation of cells is very important during this stage. Lots of investigations were made to determine the involvement of peroxidases in stress response, in the past (Passardi *et al*., 2004; Mika *et al*., 2010; Wang *et al*., 2015; Zámocký *et al*., 2015).

In 2015 another categorisation of peroxidases was published where peroxidases are separated by their reconstructed phylogeny, their sequence signature and essential amino acids in the haem cavity and not by occurrence or species. Four groups (superfamilies) are mentioned i) peroxidase-catalase superfamily, ii) peroxidase-cyclooxygenase superfamily, iii) peroxidase-chlorite dismutase superfamily and the iv) peroxidase-peroxygenase superfamily (Zámocký *et al*., 2015). The previous classification of Welinder (1992) remains unaffected.

It is evident that peroxidases are differentially expressed by biotic and abiotic stress. The latter is caused by industrial or agronomic pollution of the environment, flooding,

dehydration and high light intensities. Biotic factors are pathogenic infections like fungi, bacteria and viruses or herbivorous insects (Foyer *et al*., 1997; Cuypers *et al*., 2010; Cosio & Dunand, 2009; Mika *et al*., 2010; Lüthje *et al*., 2013; Meisrimler *et al*., 2014; Minibayeva *et al*., 2015; Wang *et al*., 2015). One function of class III peroxidases is lignification during normal growth and during stress (El Mansouri *et al*., 1999; Lux *et al*., 2010). During stress the lignification is made to reinforce cell walls. In maize roots it was observed that the lignification was triggered by cadmium. However, this was detected only in tissues directly exposed to cadmium (Lux *et al*., 2010). Observations were made in rice leaves that by the infection of rice blight (*Xanthomonas oryzae* pv*. oryzae*) one peroxidase (PO-C1) was upregulated to the xylem parenchyma and secreted to the xylem vessels. That caused a secondary wall thickening where the pathogen was entering the organism (Hilaire *et al*., 2001).

The expression of peroxidases is highly upregulated in the beginning of stress events followed by a slow decrease. It was suggested that under normal conditions they are constantly expressed to perform "housekeeping" functions like cell elongation and lignification (Liszkay *et al*., 2003; Passardi *et al*., 2005). In an evolutionary context, it could be possible that ancestry peroxidase forms enabled to build up cell wall structures, which allowed plants to stand upright out of water and hence helped to adapt to terrestrial habitats (Passardi *et al*., 2005).

In this study the focus is on specific peroxidases. The haem containing, plasma membrane-bound class III peroxidases *zmprx01*, *zmprx66* and *Zmprx70* have to be further characterised. These peroxidases where discovered and first described by Angela Mika (Mika *et al*., 2008). Their membrane association was suggested because of a N-terminal signal peptide, which was predicted bioinformatically. The molecular masses were determined via non-reducing SDS-PAGE and gel filtration (*zmprx01* 138kDa, *zmprx66* 55kDa, *zmprx70* 57kDa). The PRX have already been analysed under different stress conditions. Maize seedling grew on hydroponics with different stress factors, elicitors and pathogens like H_2O_2, wounding, methyl jasmonate, salicylic acid, *Fusarium graminearum* extract, *Fusarium culmorum* extract, chitosan and cantharidin. The abundance and triggers for each peroxidase were evaluated on the proteomic level. For each peroxidase a different abundance could be monitored according to each trigger (Mika *et al*., 2010).

3.2 Respiratory burst oxidase homologue

In this investigation the maize respiratory burst oxidase homologs were analysed. The respiratory burst oxidase homolog (RBOH) is a NADPH-oxidase and plays a crucial role in ROS production. It is able to transfer electrons from cytosolic NADPH to apoplastic oxygen, which eventually leads to superoxide production (figure 4). The enzyme superoxide dismutase is then able to convert superoxide into hydrogen peroxide (Suzuki *et al*., 2011; Marino *et al*., 2012; Suzuki *et al*., 2012; Kadota *et al*., 2015). This type of enzyme is homolog to $gp91^{phox}$ subunit (haem binding subunit of the superoxide-generating NADPH oxidase) of mammal cells and to the NADPH oxidase genes (Lambeth, 2004; Glyan'ko & Ischenko, 2010). RBOH was found in tomato (Sagi *et al*., 2004), maize (Lin *et al*., 2009 B), *Arabidopsis* (Torres *et al*., 2002), tobacco (Yoshioka *et al*., 2003), wheat (Yamauchi *et al*., 2013) and other plant species (Branco-Price *et al*., 2005; Sagi & Fluhr, 2006).

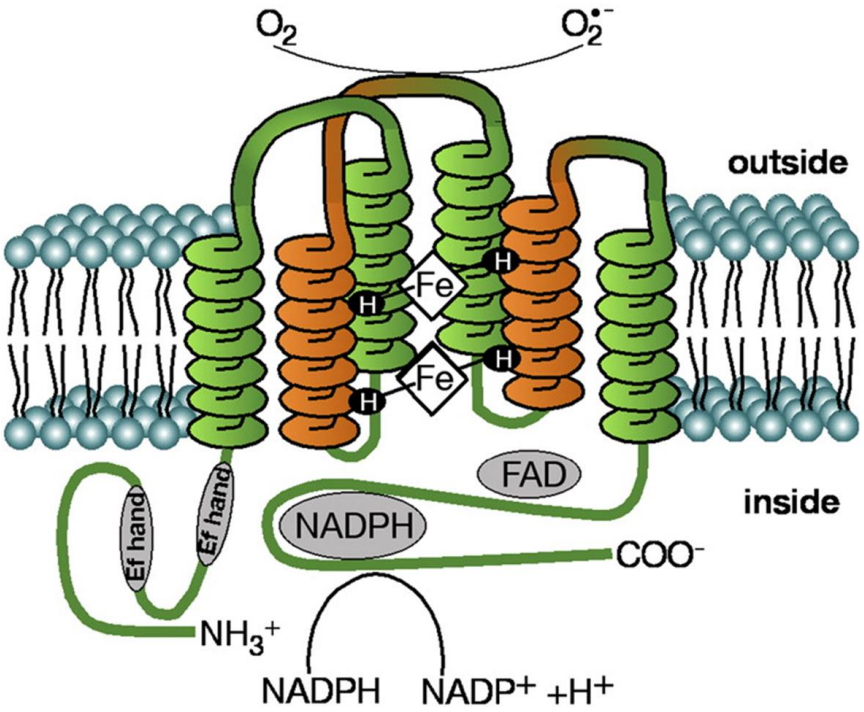


Figure 4: Scheme of respiratory burst oxidase homolog (Sagi & Fluhr, 2006). NH_3^+ is the N-terminus of the enzyme and COO^- is the C-terminus. The two N-terminal EF-hands are the calcium binding motif. The enzyme is associated with the plasma membrane by six transmembrane helices. Four histidine residues in helix three and helix five are binding sites for two haem iron atoms (Fe). The C-terminus includes an FAD co-factor and a NADPH substrate binding-site. When the enzyme is activated it transfers electrons from NADPH to FAD and across

the membrane via the haem irons. The electron reaches molecular oxygen on the apoplastic side, which is than processed to superoxide anions (Sagi & Fluhr, 2006; Glyan'ko & Ischenko, 2010; Lassègue *et al*., 2012).

RBOH is located in the plasma membrane (Simon-Plas *et al*., 2002). It uses cytoplasmic NADPH as electron donator. The electron is transferred through FAD and haem to the apoplast (figure 4). There it forms superoxide radicals by processing oxygen. RBOH is activated by Ca^{2+}. This is mediated by the N-terminal extension including EF-hand motifs (Sagi & Fluhr, 2001). RBOH is suggested to be involved in lipid rafts, this indicates coupling to other membrane components (Mongrad *et al*., 2004). In response to pathogens RBOH is known to be involved in ROS production (Lambeth, 2004; Torres *et al*., 1998). It was concluded that RBOH is involved in defensive responses, growth, development, biosynthesis of hormones, signal transduction and other processes (Lamb & Dixon, 1997; Babior *et al*., 2002; Sagi & Fluhr, 2006). Different studies were published proving that ROS produced by RBOH mediate multiple processes in plants. In *Arabidopsis AtrbohB* plays a role in seeds after ripening. It was observed that the messenger RNA (mRNA) was differentially spliced in dependence of the developmental stage of seeds. It was suggested that this is a mechanism for dormancy and after-ripening regulation (Müller *et al*., 2009). RBOH is involved in lignin production after cell wall damage. ROS produced by RBOH is necessary for a secondary RBOH-dependent oxidative burst and jasmonic acid accumulation. The resulting negative feedback loop alters the lignin production (Denness *et al*., 2011). RBOH is involved in signal transduction by generating an auto-propagating ROS wave. This wave is traveling through the apoplast quickly (8.4 cm/min). The ROS wave could be triggered by different abiotic stimuli (Miller *et al*., 2009; Suzuki *et al*., 2013; Mittler & Blumwald, 2015). Further it is involved in mechanosensing (Monhausen *et al*., 2009), programmed cell death (Torres *et al*., 2005), stomatal closure (Kwak *et al*., 2003) and pollen tube growth (Foreman *et al*., 2003; Potocký *et al*., 2007). Antisense against RBOH in *Lycopersicon esculentum* (L.) resulted in altered redox-related metabolism and induced multiple pleiotropic developmental effects. The systemic wound response was affected negatively (Sagi *et al*., 2004). In *Arabidopsis* ten RBOH (A-J) genes are discovered, as off yet. These different RBOH genes are differentially expressed and located in different tissues (Sagi & Fluhr, 2006). Until now there are four different RBOH known in

maize *rbohA*, *rbohB*, *rbohC* and *rbohD*. *rbohA* in maize is involved in root hair formation (Nestler *et al*., 2014) and various other processes as mentioned before. For *rbohB* two splice variants are known. In splice variant A there is an intron included, which is excluded in splice variant B. It is suggested to be involved in responses to environmental stress (Lin *et al*., 2009 A). For *rbohD* an involvement in signal transduction is known (Miller *et al*., 2009).

Until now some studies dealing with RBOH and maize are published (Lin *et al*., 2009 B; Yamauchi *et al*., 2011; Rahji *et al*., 2011). Further investigation is needed to clarify certain involvements in metabolic processes and stress inducement of RBOH.

3.3 Biotic and Abiotic Stress

It is well known, that PRX and RBOH are upregulated in the presence of severe stress like heavy metal, pollution, pathogens and wounding. Two kinds of responses are possible: passive through cell wall reinforcement (slow) or active through the production of ROS against pests (fast). This makes PRX and RBOH important for the elimination or the isolation of the conquering body (Passardi *et al*., 2005; Lecourieux *et al*., 2006). In this study cadmium, salicylic acid, sodium chloride, chitosan, waterlogging, hydrogen peroxide and wounding were used to further characterise the genes.

The heavy metal **cadmium** is a chemical element located in group twelve d-block of the periodic table. Within this group it is surrounded by the elements zinc, mercury and copernicium. In comparison to zinc cadmium is a non-essential element, which is toxic to life forms even in low concentration (Mengel *et al*., 2001; Ortega-Villasante *et al*., 2007; Järup & Akesson, 2009; Maruzeni *et al*., 2014; Nawrot *et al*., 2015). It mostly occurs in industrial areas and agricultural landscapes. Cadmium reduces plant growth and inhibits photosynthesis and is highly phototoxic. This element is very toxic for plants and affects cellular and molecular interactions. Cadmium has the ability to replace essential elements due to similar chemical properties (divalent ion). It inactivates and denatures biomolecules by binding their functional groups and increases the induction of reactive oxygen species, which affects the redox homeostasis (Hall, 2002; Bertin & Averbeck, 2006; Cuypers *et al*., 2010; Cuypers *et al*., 2012; Gallego *et al*., 2012). Cadmium can replace calcium in the cell wall (Webster & Gadd, 1996). The accumulation of ROS during

metal stress provokes severe damages in plants. The homeostasis of ROS acts as a signalling factor or as a damaging element (Grateo *et al*., 2005; Miller *et al*., 2008; Keunen *et al*., 2011). In barley it was observed that cadmium decreases the root growth, significantly. The higher the concentration the stronger is the decrease (Zelinová *et al*., 2013).

Salicylic acid (SA) is a monohydrobenzoic acid (figure 5). It acts as a phytohormone and is found in different plant tissues. Additionally, it is involved in pathogenic defence mechanisms, plant growth and development (Rivas-San Vincente & Plasencia, 2011; War *et al*., 2011). When plants are exposed to SA growth, nutrient uptake, water and stomatal regulations are affected (Hayat *et al*., 2010). Salicylic acid signalling inhibits apoplastic reactive oxygen species signalling (Xu & Brosché, 2014). SA has different functions in plants and could affect the plant in a positive and a negative way. Especially, it is important to analyse SA, because it inhibits apoplastic ROS signalling. There might be an involvement detectable and further parameters might be discovered.

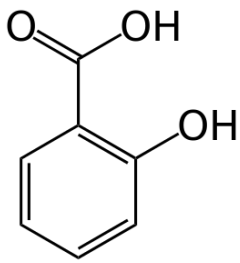


Figure 1: Structural formula of salicylic acid

Sodium chloride (salt) is an ionic compound. It mostly occurs in sea water. Furthermore ions of sodium and chloride are essential for plants. High concentrations of NaCl appear to be toxic. Salinisation gets more and more prominent in the environment due to more flooding events and parched water sources. These processes are affecting the metabolism of plants severely (Munns & Tester, 2008; Shavrukov, 2012; Deinlein *et al*., 2014). High concentration of salt causes osmotic stress leading eventually to oxidative stress. Since maize is known to be moderately sensitive to salt (Maas & Hoffman, 1977; Carpici *et al*., 2010), it is very necessary to know how the enzymes of interest are affected by salt. Although maize is moderate sensitive an alteration of the redox state still happens. Which enzymes might be involved in the rearrangement of the homeostasis? Up-regulation of PRX has been shown in the past (Liu & Li, 1991; Lin & Kao, 1999; Radic *et al*., 2006; Yang Hong & Kao, 2008).

Chitosan is a biopolymer, which could be used as an elicitor for pathogenic attack like fungi. It is gained from crustaceans (Hadwiger, 2013). PRX and RBOH are known to be

involved in plant defence mechanisms associated with microbe-associated molecular pattern (MAMP) triggered immunity (Torres *et al*., 2006; Kombrink *et al*., 2011). Chitosan does influence membrane depolarisation, oxidative burst, influx and efflux of ions as Ca^{2+}, DNA alteration, mRNA transcription, phytoalexins, lignification and callose deposition (Hadwiger, 2008; Hadwiger, 2013). If chitosan can influence membrane depolarisation or oxidative burst, reactive oxygen species have to be involved. It was published that ZmPrx66 was affected by chitosan at the protein level (Mika *et al*., 2010).

Global warming affects our environment by seasonal flooding. Under **waterlogged** conditions plants are able to form aerenchyma. Due to waterlogging in the rhizosphere plants are hindered to take up oxygen. To prevent a lack of oxygen in the rhizosphere plants are able to form aerenchyma (Jackson & Armstrong, 1999; Bailey-Serres & Voesenek, 2008; Colmer & Voesenek, 2009). Two different types of aerenchyma are known: lysigenous and schizogenous aerenchyma (Jackson & Armstrong, 1999; Videmsek *et al*., 2006). Regardless which type of aerenchyma is developed, ROS and ROS-specific enzymes are involved. For aerenchyma formation fully developed tissue needs to be rearranged. Peroxidases, especially membrane associated peroxidases, could be involved in this process. The lack of oxygen appears in two stages: hypoxia means a reduction of oxygen level below the optimum and the complete absence of oxygen is called anoxia. In a recent study ZmPrx66 was found in leaves under waterlogging conditions (Meisrimler *et al*., 2014).

Hydrogen peroxide belongs to the group of reactive oxygen species (ROS). It is a by-product formed during cell respiration. RBOH in combination with superoxide dismutase is involved in the production of hydrogen peroxide and acts as a substrate for peroxidases (Foyer *et al*., 1997; Neill *et al*., 2002). Hydrogen peroxide is involved in signal transduction pathways as a second messenger (Orozco-Cardenas & Ryan, 1999; Orozco-Cárdenas *et al*., 2001; Yang *et al*., 2013). Second messengers are able to trigger different processes, not triggered directly by the “first” messenger. Hydrogen peroxide treatment could give further information, if the enzymes are activatable by this second messenger. Especially for ROBH, which is involved in the production of hydrogen peroxide, it is necessary to know if there might be a positive feedback loop.

Herbivorous insects, mammals, microbial pathogens or environmental forces are **wounding** plants constantly. Wounding is distinguished in two different types; biotic and abiotic. Plants developed different mechanism to overcome this problem. Through wounding endogenous molecules are released that may act as Damage-Associated Molecular Patterns (DAMP). Plant immunity is triggered and wound responses like oxidative burst or the expression of defence-related genes take place (Reymond *et al*., 2000; Arimura *et al*., 2005; Savatin *et al*., 2014; Rehring *et al*., 2014). Oxidative bursts are ROS dependent. ROS producing enzymes, like RBOH and peroxidases might be upregulated by wounding.

By now lots of studies dealing with biotic and abiotic stress in association with PRX and RBOH have been published. Many studies on soluble PRX were published, whereas little is known about membrane-bound PRX. Co-regulation between RBOH and membrane-bound PRX, involvements in stress response or development remain unclear and need more investigation.

3.4 *Zea Mays* L.

Maize (*Zea mays* L.) belongs to the grass family (poaceae). It is one of the most cultivated crop in the world. The grain is used for animal feeding and it is one of the most important staple foods of human society. In the past decade it was used for the production of biofuels. Maize, originally derived from teosinte, has its origin in Central America (Linnaeus, 1753).

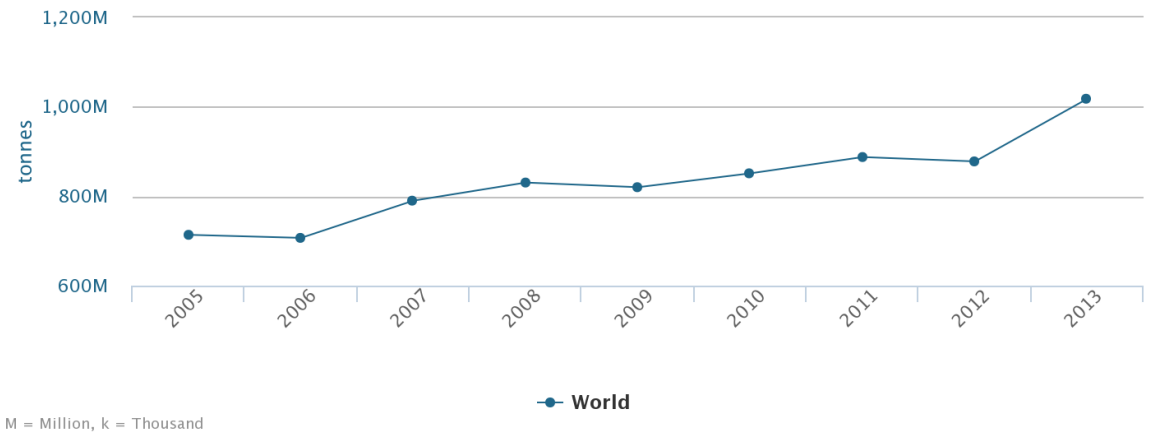


Figure 6: Average maize production in the world from 2005-2013. The sum of the production is indicated by tonnes (ordinate) per year (abscissa). (FAOSTAT, 2015)

With a production of 1,018.111 megatons it is the third most produce crop in the world after sugarcane and pumpkins for fodder. Since 2005 the production of maize increased with about 200 million tonnes until 2013 (FAOSTAT, 2015). By that the importance of maize as a staple food and a huge economic factor could be clarified, however, further investigation on maize is of certain relevance.

3.5 Ribonucleic acid interference (RNAi)

RNA interference (Fire *et al*., 1998), which is a mechanism in cells of mammals and plants, can be used for investigation on genes. In history a phenomenon was described as co-suppression after insertion of the transgenic construct in the genome (Jorgensen, 1990). The transcript of the gene could be confirmed but it was degraded soon by post-translational gene silencing. Accumulation of RNA was inhibited and the resulting protein could not be produced (Ingelbrecht *et al*., 1994; Cogoni & Macino, 2000).

RNAi, also known as post-translational gene-silencing, is a natural defence mechanism against double stranded RNA (dsRNA) of pathogenic viruses. It mediates the elimination of targeted mRNA. The occurrence of long dsRNA triggers the RNAi process. The long dsRNA is detected by a ribonuclease protein, called dicer, which binds to the endogenous dsRNA and cleaves it into small interfering RNA (siRNA). These siRNAs are about 20 nucleotides long and have a two nucleotide overhang at the 3’ end. The siRNA is able to form a ribonucleotide complex with the RNA induced silencing complex (RISC/argonaut protein/endonuclease) this protein includes slicer. The RISC mediates the unwinding of

the dsRNA to ssRNA. The complementary strand of the siRNA remains at the RISC while the passenger strand is degraded. RISC with the remaining complementary siRNA strand is able to bind to specific target mRNA in a sequence specific manner. The included slicer of the RISC cleaves the target mRNA in the middle of the complementary siRNA strand. The cleaved mRNA is degraded or acts as a template for the RNA-dependent RNA polymerase (RDRP). The cleaved mRNA, which is single stranded will be made double stranded through RDRP. Then the generated dsRNA will act as a trigger to undergo the RNAi process again. Until now it is not understood how the complementary mRNA is detected and found by the RISC.

Beside the RISC dependent RNAi reaction there is another RISC independent RNAi reaction known in plants. In that reaction the dsRNA is cleaved by dicer. Without the RISC complex the double stranded siRNA is unwound to ssRNA. These single stranded siRNA molecules bind to the target mRNA in a sequence specific manner, which is a trigger for RDRP. The mRNA is processed to a double strand and this double strand could be detected and cleaved by dicer again.

In both cases RISC dependent or independent reaction the translation of a specific mRNA is reduced. The resulting protein could not be produced. Using this mechanism a downregulation of a gene by interrupting the protein synthesis pathway could be achieved. The RNAi efficiency does vary from case to case (Eamans *et al*., 2008; Naqvi *et al*., 2009; Saurabh *et al*., 2014).

3.6 Aim of the study

Three class III PRX have been identified in highly enriched plasma membrane fractions of maize roots (Mika & Lüthje, 2003; Mika *et al*., 2008). By proteomic approaches functional analyses have been started (Mika *et al*., 2010). The new gained information suggested a function of membrane-bound peroxidases in oxidative stress and a tight, differential and overlapping regulation in order to interact with different stressors.

Both, RBOH and PRX may produce ROS at the plasma membrane/apoplast. ROS scavenging at the plasma membrane may be another function for membrane-bound PRX (Lüthje *et al*., 2011). Hence PRX and RBOH may act together in ROS signalling.

The aim of the present study is the functional analysis of plasma membrane-bound PRX. However, it is very difficult to identify the exact function(s) of plant peroxidases because of i) the huge amount of similar isoenzymes, ii) the broad substrate specificity, iii) the high number of possible functions and iv) the ability of other isoenzymes to compensate the absence of an enzyme in knock-out experiments (Hiraga *et al*., 2001). Additionally, unique PRX clusters exist in monocotyledons that are absent in dicotyledons (Duroux & Welinder, 2003) and real orthologues of *zmprx01* (pmPOX1), *zmprx66* (pmPOX3-2) and *zmprx70* (pmPOX2b) could not be identified in *Arabidopsis*.

Due to these reasons and former results the expression and regulation of *zmprx01*, *zmprx66*, *zmprx70* as well as the respiratory burst oxidase homologous (*rbohA*, *rbohB*, *rbohC*, *rbohD*) should be further investigated in maize. i) For more information about the distribution of the enzymes in roots a segment qPCR should be performed. ii) Especially, for cadmium short-term and long-term experiments should be performed to gain information about the velocity of the plant response. iii) The effects of the exposure to NaCl, salicylic acid, wounding, chitosan as a trigger dummy for pathogens and H_2O_2 should be investigated in conjunction to obtain information on a possible co-regulation of PRX and RBOH between 1 h and 4 h after stress treatment, i.e. after the oxidative burst and iv) the regulation of PRX and RBOH during waterlogging should be analysed by qPCR. v) For further characterisation of each membrane-bound PRX, RNAi mutants should be produced and gene silencing should be proved for at least one of the peroxidases.

4 Material and Method

4.1 Molecular biology

4.1.1 RNAi sequence investigation and production

For the planned RNAi experiment a suitable nucleotide sequence had to be investigated. Multiple sequence alignments were performed to gain information about the sequence similarities between *zmprx01*, *zmprx66* and *zmprx70*. The nucleotide alignments were performed with Clone manager (Sci-Ed Software, Morrisville, USA). With this software nucleotide alignments have been performed. Following settings were used:

- Alignment type: Global-Ref
    - Align all sequences against a reference sequence. Alignment spans entire length of sequences specified.
- Scoring matrix: Standard Linear
    - Mismatch penalty=2; Open Gap penalty=4;Extend Gap penalty=1

The reference of the PRX genes was taken from the NCBI data base. Simultaneously the sequences were newly sequenced from another maize strain (HiIIA x HiIIB) to detect differences between the reference sequence from the data base (B73) and the *de facto* used maize strain (HiIIA x HiIIB)(see 4.2.1). Only mRNA data was used to gain the RNAi sequences. The sequences of the three PRX showed high similarities (5.6); especially *zmprx66* and *zmprx70*. RNAi sequences were taken from a region of the RNA sequence with the lowest similarity rate to avoid unspecific priming within the RNAi process. Multiple sequence alignments were performed to proof the specificity of each RNAi sequence and a blast against the maize genome was performed to proof, whether there was no match with any other gene. The chosen RNAi sequence was than produced by Eurofins MWG operon (Ebersberg - Germany) on a synthetic way.

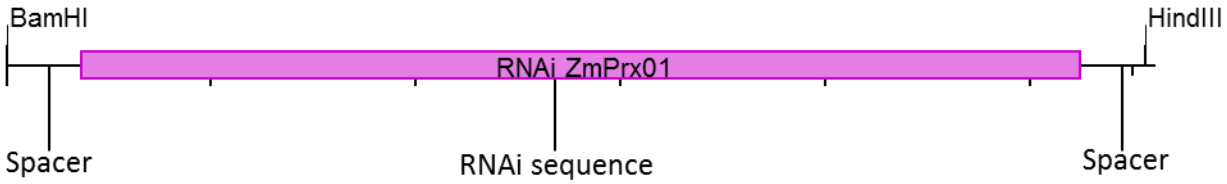


Figure 7: Schematic overview of the artificially produced RNAi insert for *zmprx01* including restriction sites. The RNAi triggering sequence (magenta) is flanked by two spacer including primer sites and two restriction sites.

This RNAi sequence is surrounded by a non-coding spacer and restriction sites *BamH*I and *Hind*III. Theses restriction sites where included to simplify the integration in the RNAi vector (4.1.2).

Table 1: M13 primers sequence and annealing temperature.

Name	Sequence	Annealing temperature [°C]	Location
M13 rev (-29)	CAG GAA ACA GCT ATG ACC	55	3'end of the artificial gene sequence
M13 uni (-21)	TGT AAA ACG ACG GCC AGT	55	5' end of the artificial gene sequence

Additionally, inside the non-coding spacer standard sequencing primer (table 1) sites were integrated. With these sites the detection of positive clones was enhanced. These primers are commonly used and standardised.

4.1.2 RNAi Construct

For further investigation of *zmprx01*, *zmprx66* and *zmprx70* RNAi was induced in maize. *Agrobacterium tumefaciens* mediated transformation (4.1.3) was performed. In this study a specific RNAi construct (P7i-Ubi-HSP-Cre-RNAi) was integrated within the maize genome. This construct includes an opposing dual promoters system (figure 8).

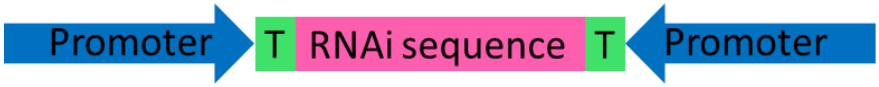
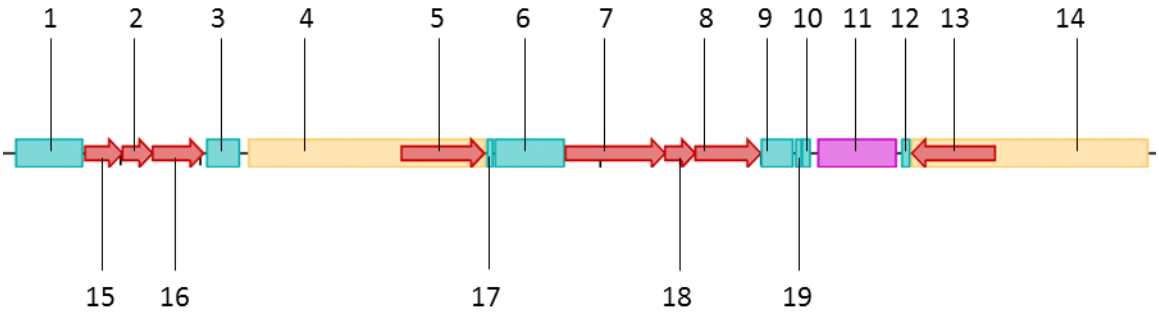


Figure 8: Simplified scheme of the opposing dual promoters system.

The RNAi triggering sequence was in between two promoters, which are controlled by a heat shock mediated cre/lox recombination system. The RNAi could actively be induced by a heat shock (42°C /5h).

Table 2: RNAi construct scheme.



No.	Abbreviation	Meaning	Properties
1	35S	Promoter from cauliflower mosaic virus	Start of transcription of the *bar* gene
2	Intron	*bar* intron	Avoids premature transcription in *Agrobacterium*
3	T35S	Terminator from cauliflower mosaic virus	End of transcription of the *bar* gene
4	Ubi int	*Ubiquitin* promoter of *solanum tuberosum* L.	Start of transcription of the RNAi triggering sequence
5	Intron	Intron from *ubiquitin* of *solanum tuberosum*	Enhancement of transcription output (forward)

6	HSP	Heat shock promoter of *glycine max* L.	Temperature dependent promoter (induced at 42 °C)
7	Cre1	Cre (causes recombination) gene artificial exon 1	Causing the recombination exclusion or inversion integration dependent
8	Cre2	*Cre* (causes recombination) gene artificial exon 2	Causing the recombination exclusion or inversion integration dependent
9	Tocs	terminator octopin synthase	Terminator for the HSP induced transcription
10	T35S	terminator from promoter from *cauliflower mosaic* virus	Terminator for the transcription of the RNAi triggering sequence
11	RNAi	RNAi sequence of interests (figure 29)	RNAi triggering sequence
12	T35S	terminator from promoter from *cauliflower mosaic* virus	Terminator for the transcription of the RNAi triggering sequence
13	Intron	Intron from *ubiquitin* of *solanum tuberosum*	Enhancement of transcription output
14	Ubi int	*Ubiquitin* promoter from *solanum tuberosum*	Start of transcription of the RNAi triggering sequence
15	Bar 1	Glufosinat resistance *bar* exon 1	Resistance for glufosinat treatment
16	Bar 2	Glufosinat resistance b*ar* exon 2	Resistance for glufosinat treatment
17	Lox A	recombination side A	"anker" for recombination
18	Intron	Intron from ubiquitin gene of *solanum tuberosum*	Here: avoids premature transcription in *Agrobacterium*
19	Lox B	recombination side B	"anker" for recombination

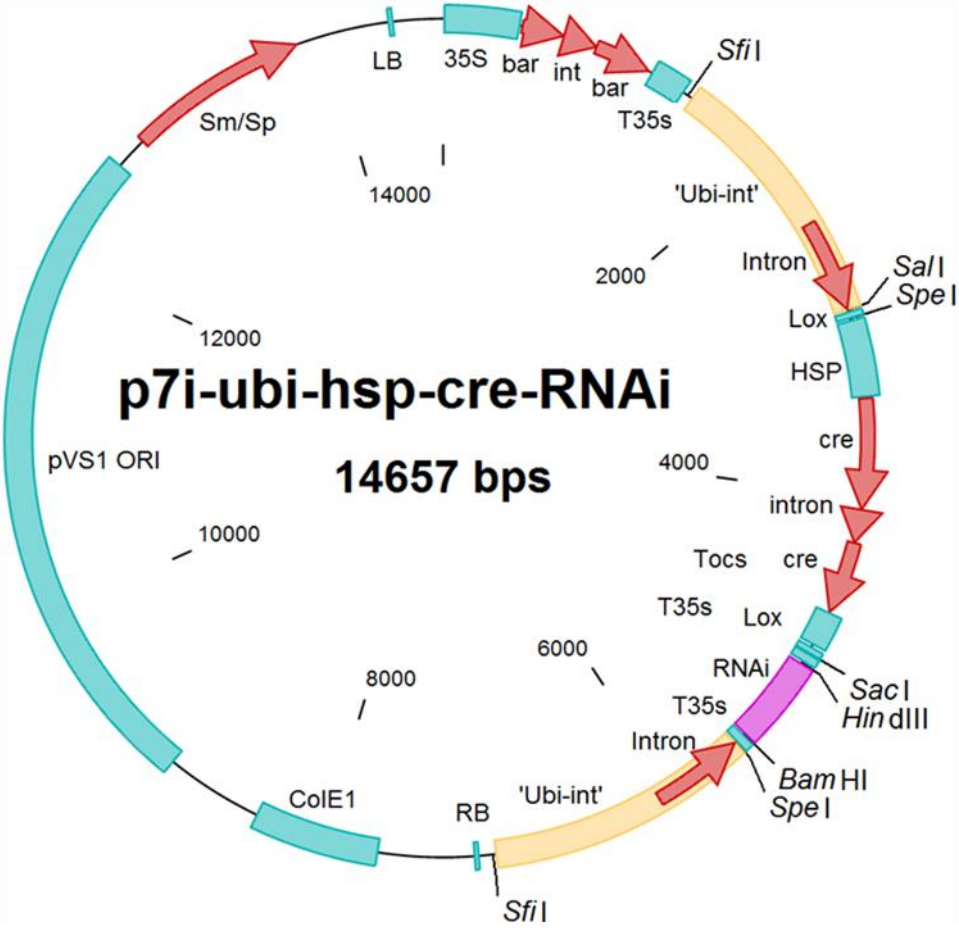


Figure 9: Complete inducible RNAi transformation vector with double opposing promoters. On the right side between left border (LB) and right border (RB) the RNAi construct (table 2) is located. This part was integrated into the genome of the transformed maize plants. On the left side ColE1 (origin of replication for *Escherichia coli*), the origin of replication for *Agrobacterium tumefaciens* (pVS1 ORI) and the resistance gene for the resistance for spectinomycin and streptomycin (Sm/Sp) is located. Essential restriction sites for engineering the vector are displayed. For further information about the construct please have a look at table 2.

Through the heat shock regime the HSP Promoter was activated that the recombinase (cre) could be produced. In the construct there were two lox sites located before the HSP promoter and the terminator for the cre. Through the cre and the lox sites the “HSP-Cre-cassette” was excluded from the construct. The first ubiquitin promoter moved to the RNAi sequence. Now the border which inhibited the transcription of the RNAi sequence was eliminated. The RNAi triggering sequence was now produced. For the proof of the insertion of the construct Southern blotting (4.1.8) was performed. To proof the

recombination of the construct inside the plants a specific screening PCR was performed (4.1.9). For the evaluation of the performance of the RNAi qPCR (4.1.10) was performed.

4.1.3 *Agrobacteria tumefaciens* mediated transformation

Agrobacterium tumefaciens has the ability to integrate DNA within the genome of a plant. This ability leads to the pAL4404 plasmid which contains *vir* genes which enables a t-DNA transfer. Through molecular biologic methods it is possible to modify the t-DNA or to substitute the t-DNA with a certain gene of interests. In this study this option was used to transform plants with a certain construct (4.1.2) to trigger a specific RNA interference for further investigation of the peroxidases *zmprx01*, *zmprx66* and *zmprx70*. For this the *Agrobacterium tumefaciens* strain LBA4404 (Ooms *et al*., 1982) was used. This binary vector system was firstly described in 1983 (Hoekema *et al*., 1983). For the transformation of maize a modified protocol from B.R. Frame (Frame *et al*., 2002) was used.

For the final production of the media plates including Phytagel® or Gelrite® a separate Phytagel® solution and Gelrite® solution was heated before adding it to the media. For the transformation a corn cob was used, which was pollinated 12 days before. After storage at 4°C for at least one night the embryos could be isolated under sterile conditions. The embryos were collected and stored in infection media until the isolation was finished. Then the embryos were rinsed twice with infection media. Now the embryos where gathered with the agrobacteria suspension while inverting 20 times. The embryos stayed in the suspension for 5 minutes. Next, the embryos were transferred and separated to co-cultivation media plates and incubated overnight in the dark at 21 °C. The next day the embryos were transferred to a fresh co-cultivation media plate in the opposite position and incubated overnight in the dark at 21 °C. After that the embryos were transferred to a resting media plate with the axis in contact to the media, for seven days at 28°C in the dark. Later the embryos began to develop callus, so the embryos needed to be transferred to selection media I plate and stored up to 21 days, at 28°C in the dark and on a selection media II plate for another 14 days. At last the embryos were transferred to a regeneration media plate in the light until the plants were tall enough for acclimatisation in the greenhouse. Information on media composition is located in the supplemental (table 17-19).

4.1.4 Transformation of *Agrobacterium tumefaciens*

Before an *Agrobacterium tumefaciens* mediated transformation could be performed the competent *Agrobacteria* cells need to be transformed. For this 1 µg of plasmid-DNA (4.1.2) was mixed with a competent *Agrobacteria* solution. The solution was kept on ice for five minutes. Afterwards the solution was kept in liquid nitrogen for another five minutes. Next, the solution was put on an incubator with 37°C, for 5 minutes. Then 1 mL of YEB-media (supplemental table 14) was added to the solution. An incubated up to 4 h at 28°C followed. For the selection of transgenic cells the solution was plated on a media containing the resistance marker (streptomycin) for two days.

4.1.5 RNA extraction

100 mg plant material in a 2 mL reaction tube was cooled down on -196 °C by liquid nitrogen and then fine grinded with two metal beads (calibre 4.5 mm) in a swing mill. RNA extraction was performed with peqGOLD Trifast (Peqlab Co., Erlangen, Germany) according to the producer's manual. Quality check of the RNA was performed by 1.5 % agarose gel electrophoresis with ethidium bromide staining and photometrical. RNA was photometrical quantified by a Nanodrop 2000 (thermo scientific, Waltham, Massachusetts, USA).

4.1.6 DNA extraction

To extract genomic DNA a modified protocol according to Pallota and colleagues (Pallotta *et al*., 2000) was performed.

400 mg plant material in a 2 mL reaction tube was cooled down on -196°C by liquid nitrogen and then fine grinded with two metal beads (calibre 4.5 mm) in a swing mill. The tissue powder was mixed with 800 µL (1 % N-Lauryl-Sarcosin; 100 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 100 mM NaCl). 800µL of phenol/chloroform/isoamyl alcohol (25:24:1) was added. The sample was than mixed for 2 minutes. Afterwards the tube was centrifuged for 5 minutes at 16,100 g. The supernatant was transferred to a new 2 mL reaction tube and was mixed with 80 µL NaOAc and 800 µL isopropanol for precipitation. The sample was again centrifuged for 10 minutes at 4°C. The supernatant was discarded and the pellet was rinsed with 1 mL 80 % ethanol, twice. Afterwards the pellet was dried on a heating block for 5 minutes at 55°C. The dry pellet was re-suspended in 100 µL R40

(40 µg/mL RNAse in TE (10:1, pH 8.0)) overnight at 4°C and incubated for 30 minutes at 37°C to eliminate RNA residues. DNA was photometrical quantified by a Nanodrop 2000 (thermo scientific, Waltham, Massachusetts, USA).

4.1.7 Agarose electrophoresis

For quality check and to separate a mixed population of DNA or RNA an agarose gel electrophoresis was performed.

Table 3: List of required components for agarose gel electrophoresis (ts = thermo scientific, Waltham, Massachusetts, USA).

TBE-buffer: 45 mM Tris/HCl, 45 mM boric acid, 1 mM EDTA pH 8,0
Agarose "electrophorese grade" (DNA cloning service, Hamburg, Germany)
10 mg / ml EtBr
6 x DNA loading Dye (ts)
2 x RNA loading Dye (ts)
Dig-labeled Marker VII (Roche, Mannheim, Germany)
100 bp DNA Ladder (ts)
1kb DNA Ladder (ts)

For quality check of gDNA, specific DNA fragments and RNA different variants of agarose electrophoresis was performed. For the distribution of DNA fragments for Southern blot analysis gels with an amount of 0.8 % agarose were made. Electrophoresis ran for 4 h at 80V/100mA. For Southern blot analysis a specific DIG labelled marker was used. The same procedure was performed for the quality check of gDNA without a DNA ladder. All DNA samples were prepared with a DNA loading dye. For the electrophoresis of DNA fragments the concentration of agarose was increased according to the size of the DNA fragment of interests. For a quality check of RNA the samples were prepared with a specific RNA loading dye. The agarose concentration was 1.5 %. To all gels ethidium bromide was added. Gels were observed under UV-radiation.

4.1.8 Southern blot

For the proof of certain DNA of interests Southern blotting was performed (Sambrook *et al.*, 1989).

Before Southern blotting was performed agarose gel electrophoresis was performed (4.1.7). To prepare the gel for the blotting procedure after agarose gel electrophoresis the gel was rinsed with 0.25 M HCl for 5 minutes and afterwards swivelled in denaturing buffer for 30 minutes followed by 2 x 15 minutes rinsing in neutralisation buffer. The gel was kept in 10 x SSC (standard saline citrate) until Southern blot set up.

The gel was now ready to set up the Southern blot.

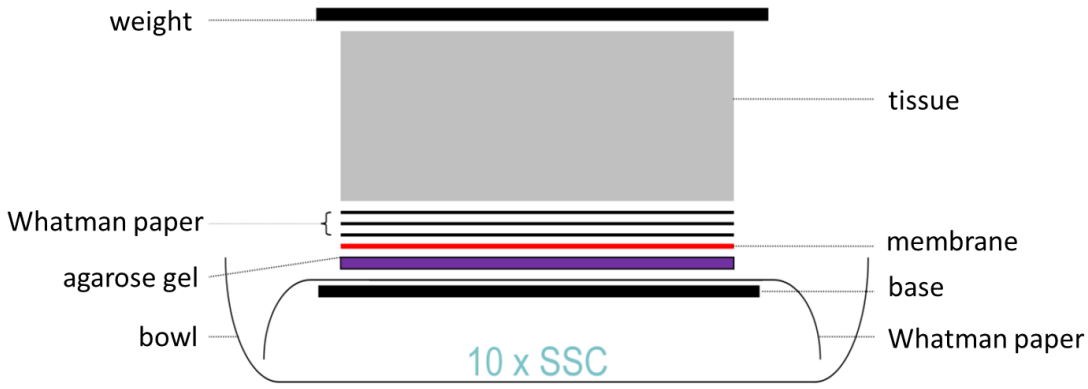


Figure 10: Schematic Southern blot set-up.

Blotting was performed overnight. After blotting the membrane was cross linked with 1200 joule (Stratagene UV-Stratalinker 2400). The membrane was now hybridised with a specific, DNA sequence of interest matching, dig-labelled probe. After hybridisation the membrane was washed 2 x 15 minutes in washing solution at 65°C. Afterwards the membrane was blocked by B2 buffer for 30 minutes. Next the membrane was incubated in B2-buffer + antibody for 30 minutes. Later the membrane was rinsed with washing buffer (4 x 15 minutes). Subsequent it was equilibrated in B3 buffer. To trigger the chemiluminescence B3-buffer + CSPD (chemiluminescence substrate for alkaline phosphatase detection) was added. The membrane was covered with Clingfilm. Together with an autographic film the membrane was stored in a hybridisation cassette in the dark for 90 minutes at 37 °C. Afterwards the film could be developed. Alternatively, the

membrane could be evaluated by an observation chamber (LAS 3000 imager, Fujifilm, Minato, Tokyo, Japan). For Southern blot reagents see supplemental (table 15).

4.1.9 Polymerase chain reaction (PCR)

The polymerase chain reaction is a method to amplify a certain DNA sequence out of a template. This reaction is basing on the ability of DNA-polymerase to duplicate DNA. Through an enzymatic reaction nucleotide sequences between two oligonucleotides could be amplified. cDNA was checked via PCR with intron spanning primers (4.1.14 O,P) to detect contamination with gDNA. Screening of the transgenic plants was performed via PCR to proof the selection by BASTA® and the recombination event (4.1.14 U-Z). To perform PCR the polymerase "Dream taq" (thermo scientific, Waltham, Massachusetts, USA) was used according to the manufacturer's recommendation. Customized primers (4.1.14) were used. The PCR-program was in dependence of the primers properties and the length of the amplicon.

4.1.10 Quantitative real time PCR

Quantitative real time PCR is a method to quantify DNA fragments during a running PCR (4.1.9). This method was used for gene expression analysis under certain conditions and for the distribution of the transcript of each gene.

For each gene to be analysed specific primer fulfilling the properties for qPCR were designed (4.1.14 A-N, Q-T) on the basis of sequences from the NCBI database (Properties: Annealing temperature 60 °C; amplicon in the range of 100 – 130 bp; GC content about 50%). Two independent reference genes (4.1.14 Q-T) were used for the allocation.

0.05 µg of cDNA (4.1.11) was used for each reaction. A SYBR green mix QuantiTect SYBR® Green PCR Kit (Qiagen Co., Hilden, Germany) including dNTPs, polymerase, SYBR green was used according to the producer's manual. The qPCR program was: initial: 95°C, 1 min; 40 cycles with 95°C for 10 s and 60°C for 30 s. At last a heating gradient was performed from 60°C to 95°C. This was made for the proof of the amplicons. False priming could be detected this way. The gained data out of the qPCR was allocated according to Pfaffl method (Pfaffl, 2001). The results were than further allocated to the final value in percentages. In case of significant differences a *t-test* was performed.

4.1.11 cDNA synthesis

cDNA synthesis was performed with QuantiTect Reverse Transcription Kit (Qiagen Co., Hilden, Germany) according to the producer's manual. This kit eliminates contamination of genomic DNA in one step. A quality check of the cDNA was performed by standard PCR (4.1.9) with intron spanning primers (4.1.14 O and P).

4.1.12 PCR screening for recombination of the RNAi construct

The RNAi construct (4.1.2) had the ability for a heat induced recombination. After this recombination the RNAi process could start correctly. To get information about an acceptable recombination a PCR based screening method was developed.

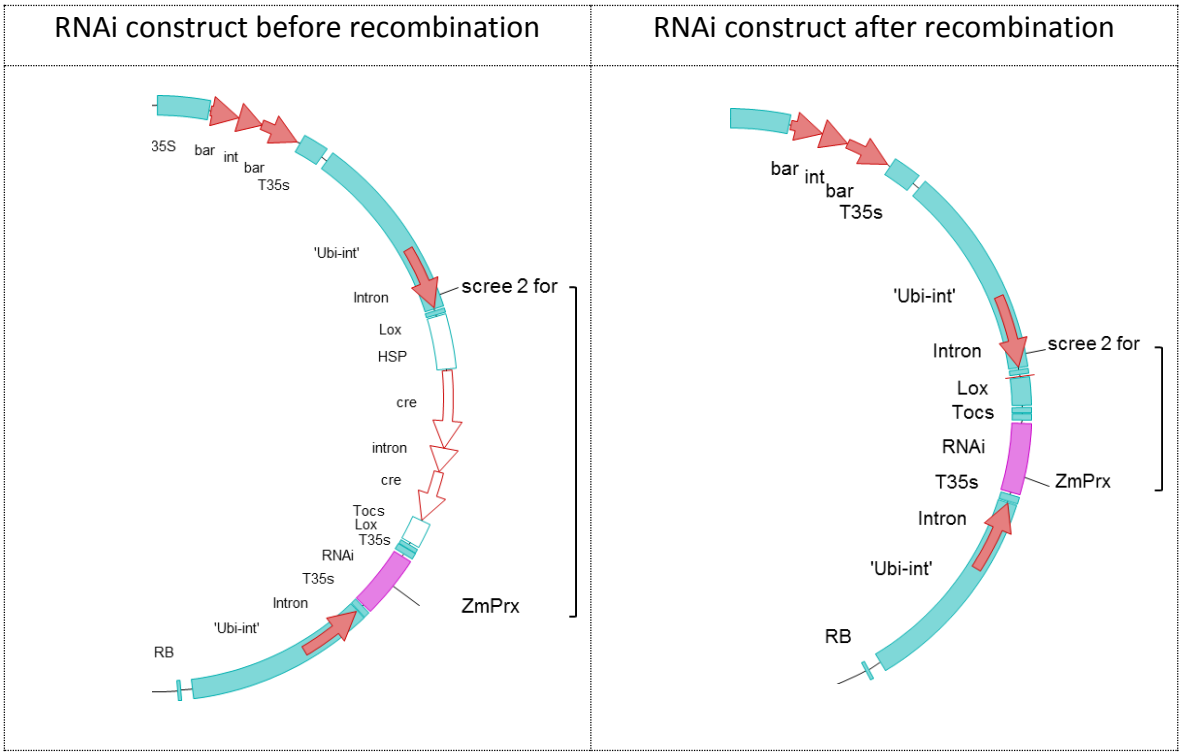


Figure 11: Scheme of the RNAi construct before and after recombination including primer sizes (ZmPrx= PRX specific screening primer site; scree 2 for Ubi-int specific primer site). The white filled objects indicate the recombination cassette (left) which is excluded (right) after heat shock.

With this method it was possible to detect a recombination event specific for each PRX. One primer was PRX specific and was located inside the RNAi sequence. The other primer was located at the promoter region of the RNAi sequence in 5' direction. In detail it was located inside the Ubi-intron sequence of that promoter construct. The used primers are gained from different other project to develop a PCR based screening. Preliminary work is

not mentioned inside this document. The screening primers for each PRX (mentioned in table 4 U – Z) were checked with the screening primer Scree 2 for (TTT AGC CCT GCC TTC ATA CG / annealing temperature: 60°C) in both directions (upstream and downstream the RNAi construct). This was necessary because of the second Ubi-Intron promoter inside the construct carrying the same primer binding site for further information please look at 5.6.2. To perform the final procedure of the screening gDNA was extracted from leaves (4.1.6). PCR (4.1.9) was performed and the final result was gained by agarose gel electrophoresis (4.1.7).

4.1.13 Reference genes for qPCR

Reliable reference genes are one of the most important things for real time qPCR. Especially when genes are analysed under specific stress conditions it is necessary to find stabile genes which are not affected by the treatment in comparison to the control. In this thesis reference genes needed to be found for cadmium treatments. For maize there are no studies about cadmium and real time qPCR. In the literature information for *Arabidopsis thaliana* was found (figure 12). Remans *et al.* 2008 tested ten different genes for its stability to cadmium treatments in *Arabidopsis* (Remans *et al*., 2008). This publication was a hint for later experiments on maize.

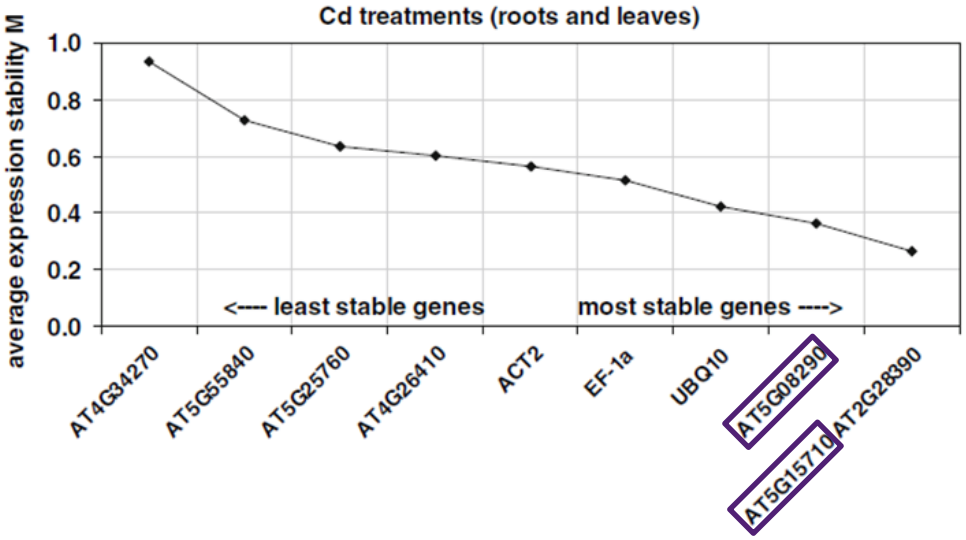


Figure 12: Selected genes tested as reference genes for *Arabidopsis thaliana* under cadmium treatment (taken from Remans *et al.,* 2008). In the diagram the genes are located on the abscissa in a gradient. On the left side the least stable genes and on the right side are the most stable genes are located. The ordinate shows the average expression stability [M]. The curve

gives detailed information about the stability of each gene. The marked gene IDs were further analysed for maize experiment usage.

Based on the publication of Remans *et al*. (2008) the coding sequence of the genes AT5G08290.1 and At5G15710 were blasted (NCBI/ blastn/ organism: Zea mays (taxid:4577)/ more dissimilar sequences (discontiguous megablast)) against the database. In *Arabidopsis* the gene ID AT5G08290.1 stands for yellow-leaf-specific gene 8, YLS8. In maize EU959841.1, Zea mays clone 220137 mitosis protein dim1 mRNA was found with a query coverage of 100%, E-value of 4e-129 and 84 % sequence identity and similarities of 69% mRNA / 57% Protein sequence. For ID At5G15710 Zm_BFb0082E15 (NM_001138607.1) was found. Query coverage was 44 % with 73 % identity. Match: 46% mRNA / 16% Protein sequence. Another reference gene was found independently. EF-TuM Zea mays elongation factor thermo unstable mitochondrial (AF264877.1) was used.

The gained data was an input for an applied check if the genes are stable or not. For this the experimental set-up mentioned in 4.2.4 was used. The whole procedure including qPCR was performed. Additionally a concentration test was performed based on the standard primer concentration of 0.25 µM for each primer to find the most efficient primer ratio. This was made by a serial dilution with the steps 1; 1:2; 1:4; 1:8; 1:16 and 1:32.

After this procedure and after evaluation the genes AF264877.1 and EU959841.1 were declared as acceptable reference genes. The primers (table 4 A-T) were used for all real time qPCR analyses. Standard “housekeeping” genes like actin and ubiquitin were tested as well but failed due to the cadmium treatment.

4.1.14 Primer list

Table 4: List of gene specific primers. The primers A – N and Q - R were developed and used for real time qPCR. These primers fulfil the properties for qPCR (4.1.10). The primers Q – R were used for amplification of amplicons of references genes. The primers O and P were used for the proof of cDNA not to have a contamination with genomic DNA. These primers are intron spanning. In case of a contamination with genomic DNA an additional band will appear in the agarose gel electrophoresis including an intron of the glyceraldehyde-3-phosphate gene. The primers U-Z were used for a specific proof after the transformation. Through these primers the endogenous genes were amplified as well but an additional band due to the RNAi sequence would be present if the tested plant was transgenic. DNA sequencing would give additional confirmation. In this table only gene specific primers are mentioned. Primers which are specialised for specific DNA sequences which are not gene derived or complement for parts in the transformation vector are shown in each concerning chapter.

	Gene	ID	Chromo-some	Orientation	Primer name	Sequence	Temp. anneal
A	*zmprx01*	542029	3	3'-5'	zmprx01 rev	TTC GTG CTT GTG TTC CAG AC	60 °C
B				5' - 3'	zmprx01 for	ACT TGT TCA AGG CCA AGG AG	60°C
C	*zmprx66*	100101534	2	3'-5'	zmprx66 rev	CGA AGG CGG AGT TGA TGT TG	60°C
D				5' - 3'	zmprx66 for	CGA CAT GGT TGC ACT CTC AG	60°C
E	*zmprx70*	542571	1	3'-5'	zmprx70 rev	TTC GGA TTA GCG GTC TGC TC	60°C
F				5' - 3'	zmprx70 for	CCA CCT CCA TGA CTG CTT TG	60°C
G	*rbohA*	778438	3	5' - 3'	Rboh A for	ATA ACT TCG GCA CCA GGC GAT	60°C

	Gene	ID	Chromo-some	Orientation	Primer name	Sequence	Temp. anneal
H				3'-5'	Rboh A rev	TAC TTG TGC CTG GCA AGC CTT	60°C
I	*rbohB*	100037794	3	5' - 3'	Rboh B for	CTC CCA ATA TGC CGT AAC AC	60°C
J				3'-5'	Rboh B rev	CCT GCA TGG AGG ATT ATA CC	60°C
K	*rbohC*	100101532	6	5' - 3'	Rboh C for	CTT CTT CGA GCA GAC GAA AC	60°C
L				3'-5'	Rboh C rev	GTG GCA CCA ATA CCT AAT CG	60°C
M	*rbohD*	100136880	4	5' - 3'	Rboh D for	TGC CTA CTT CTA CTG GGT GAC	60°C
N				3'-5'	Rboh D rev	AGT TGT GCA GCT CGA TGA C	60°C
O	*glyceraldehyde-3-phosphate*	542583	4	5' - 3'	gap1	AGG GTG GTG CCA AGA AGG TTG	60°C
P				3'-5'	gap2	GTA GCC CCA CTC GTT GTC GTA	60°C
Q	mitosis protein *dim1*	100282486	1	5' - 3'	D2 for	GTC TGG TGA TTG CTC CAA AG	60°C
R				3'-5'	D2 rev	AAC TGT CCG TGT AAA CAT CC	60°C
S	*translational elongation factor EF-TuM*	AF264877.1	1	5' - 3'	Ef for	CGC AGT TGA TGA GTA CAT CC	60°C
T				3'-5'	Ef Rev	AAC ACG CCC AGT AAC AAC AG	60°C

	Gene	ID	Chromo-some	Orientation	Primer name	Sequence	Temp. anneal
U	*zmprx01*	542029	3	5' - 3'	Pox 1 scree for	AGT TCT ACC GTT GTA AAA CGA CGG CCA GTG	60°C
V				3'-5'	Pox 1 scree rev	CGC CGC GAA TTT CTC CTT CCA CAG CGT CTC	60°C
W	*zmprx66*	542571	1	5' - 3'	Pox 2 scree for	CAG ACC GCT AAT CCG AAC GTC GGC TCC ATC	60°C
X				3'-5'	Pox 2 scree rev	CAC AGG AAA CAG CTA TGA CCC GCC GCG AAG	60°C
Y	*zmprx70*	100101534	2	5' - 3'	Pox 3 scree for	GTT GTC GTG AAC AGC ATC AAG GCG CAG GTG	60°C
Z				3'-5'	Pox 3 scree rev	TGT CTG GCC TGG GAA TGA AGC GGT AGA GTC	60°C

4.2 Horticulture experimental set-up

4.2.1 Maize strains

A) *Zea mays* L. vr. Gelber Badischer Landmais (Saaten Union, Hannover, Germany)

This strain was used for experiments without genetic engineering. It was chosen for this study because earlier studies used this strain (Mika & Lüthje, 2003; Mika *et al.*, 2008). Another fact, dove breeders are feeding with this strain. Therefor it will be available for a long time.

B) HiIIA x HiIIB

For the transformation the HiIIA and HiIIB maize strains were used (4.1.1). These two strains were crossed and the resulting embryos were used for the transformation (4.1.3). Under certain conditions this strain is able to generate embryogenic callus cultures. HiIIA and HiIIB are partially inbred lines. They resulted out of a crossing of the maize strains A188 and B73 (Armstrong *et al.*, 1991).

4.2.2 Plant breeding greenhouse

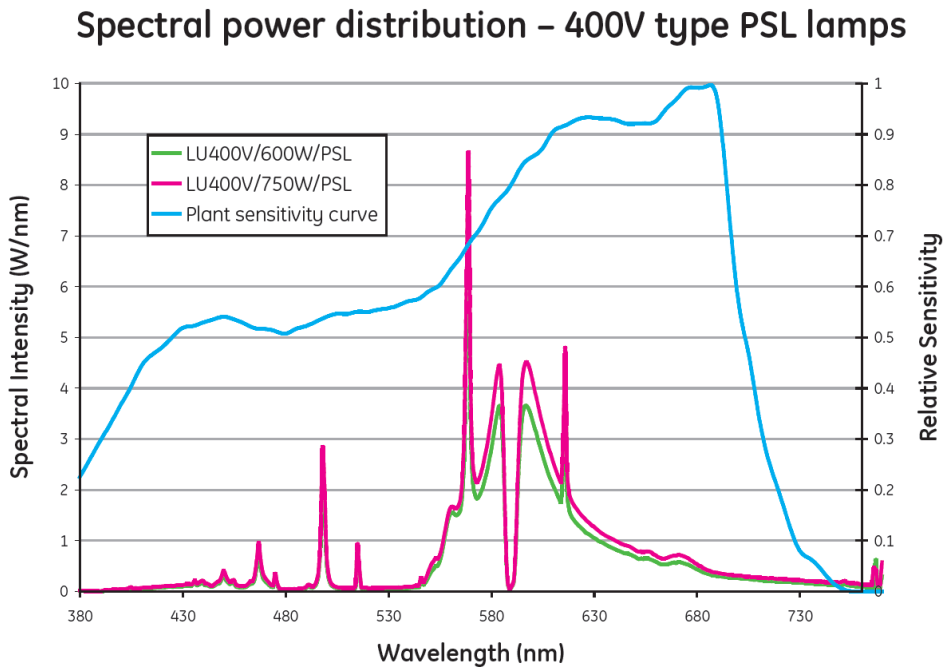


Figure 13: Properties of the used light source Lucalox™PSL (gelightning.com). The spectral intensity is indicated by watt per nanometre (ordinate 1) per wavelength in nanometre (abscissa). The second ordinate indicates the relative sensitivity of plants. The plant sensitivity

per wavelength is shown (blue curve) and the spectral intensity of the bulb models LU400V/600W/PSL (green curve) and LU400V/750W/PSL are shown. These bulbs are optimised for plant breeding in greenhouses.

In the greenhouse the plants grew under certain light conditions: long day conditions (16h light) by a maximum photon flux density of about 600 kLuxh /d. The bulb model LU400/600W/PSL was used.

After the transformation and regeneration in petri dishes the plants were cultivated in the greenhouse in substrate GS 90 (Einheitserdewerk Uetersen Werner Tantau GmbH & Co. KG, Uetersen, Germany). For selection of transgenic plants, they were treated with BASTA® (Bayer CropScience, Monheim, Germany).

4.2.3 Plant breeding hydroponics

Maize caryopses were swelled under water for 2 h and afterwards germinated on water soaked filter paper at 28°C in the dark for three days. The seedlings were then cultivated in 9 L hydroponics with a nutrition solution (Hoagland solution) (KNO_3 5.25 mM; $Ca(NO_3)_2$ x 4 H_2O 7.75 mM; $MgSO_4$ 7 H_2O 4.06 mM; KH_2PO_4 1 mM; Fe(III)-EDTA 100 µM; H_3BO_3 46 µM; $MnSO_4$ Hydrate 9.18 µM; $ZnSO_4$ 7 H_2O 5.4 µM; $CuSO_4$ 7 H_2O 9 µM; Na_2MoO 2 H_2O 2 µM). The pH-value was adjusted to pH 5.5 by KOH. The hydroponics were cultivated in a climate chamber (18 days under long-day conditions (16h) with 22°C at day and 18°C at night and a relative humidity of 70%. Photosynthetic photon flux density was 140 µmol m^{-2} s^{-1}.)

4.2.4 Long-term cadmium exposure experiment

For long term cadmium exposure the three days old etiolated maize (4.2.1 A) seedlings were cultivated hydroponically in a climate chamber for 18 days (under long-day conditions (16h) with 22°C at day and 18°C at night and a relative humidity of 70%. Photosynthetic photon flux density was 140 µmol m^{-2} s^{-1}). The nutrition solution (4.2.3) was renewed every seven days. For the cadmium treatment a $Cd(NO_3)_2$ $4H_2O$ solution was added to a final concentration of 15 µM. Hydroponics with and without cadmium were analysed in parallel. Sampling was performed at day one, three, five, ten and 18. Each sample was collected into a 2 mL reaction tube containing two metal beads (calibre 4.5 mm). The samples were cooled down in liquid nitrogen (-196°C) immediately and then

stored at -80°C. RNA was extracted (4.1.5) and cDNA was made (4.1.11). qPCR was performed for each sample (4.1.10).

4.2.5 Short-term cadmium exposure experiment

To perform short term cadmium stress a $Cd(NO_3)_2$ $4H_2O$ solution was added to a final concentration of 15 µM. The nutrition solution (4.2.3) was mixed by a magnetic stirrer. Sampling was performed 30 s after mixing at 0 min, 10 min, 15 min, 30 min, 45 min and 60 min. Control plants experienced the same treatment, but without cadmium. Each sample was collected into a 2 mL reaction tube containing two metal beads (calibre 4.5 mm). The samples were cooled down in liquid nitrogen (-196°C) immediately and then stored at -80°C. The experimental set-up was according to 4.2.3. RNA was extracted (4.1.5) and cDNA was made (4.1.11). qPCR was performed for each sample (4.1.10).

4.2.6 Proof of element-dependent impact of cadmium

The plants were cultivated with $CdCl_2$ (15µM) instead of $Cd(NO_3)_2$ in the same experimental set-up (4.2.4), to have the proof that the effect of cadmium nitrate is due to cadmium and not to nitrate.

4.2.7 Stress profiling experiment

To identify triggers for an altered expression of named PRX and RBOH a stress profiling was performed. The experimental set-up was according to 4.2.3. For this experiment different abiotic stress factors were injected to the nutrition media (4.2.3). The expression was analysed under the impact of chitosan (20mg/L) for 4 h, H_2O_2 (2mM) for 1 h, NaCl (200mM) for 2 h, salicylic acid (0,5mM) for 1 h and wounding for 1 h. A control was performed in parallel. To perform wounding the roots were cut into 2 cm long pieces which stayed in the nutrition media in a 2 mL reaction tube for 1 h. Each sample was collected into a 2 mL reaction tube containing two metal beads (calibre 4.5 mm), after the treatment. The samples were cooled down in liquid nitrogen (-196°C) immediately and then stored at -80°C. RNA was extracted (4.1.5) and cDNA was made (4.1.11). qPCR was performed for each sample (4.1.10).

4.2.8 Distribution analyses via qPCR

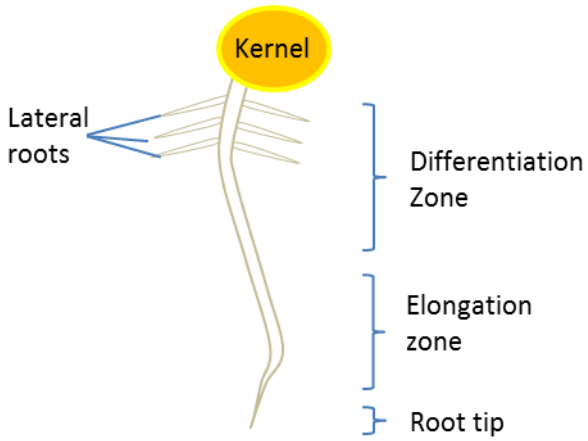


Figure 2: Scheme of selected root segments.

To investigate the distribution of *zmprx01*, *zmprx66*, *zmprx70*, *rbohA*, *rbohB*, *rbohC* and *rbohD* in roots etiolated maize seedlings were cultivated in hydroponics (4.2.3) for three days. Four different sections of the roots were harvested tip, elongation zone, lateral roots and differentiation zone. Each sample was collected into a 2 mL reaction tube containing two metal beads (calibre 4.5 mm). The samples were cooled down in liquid nitrogen (-196°C) immediately and then stored at -80°C. RNA was extracted (4.1.5) and cDNA was made (4.1.11). qPCR was performed for each sample (4.1.10).

4.2.9 Waterlogging experiment

To gain information if there is an implication between waterlogging and the genes of interests a waterlogging experiment was performed (Meisrimler *et al.*, 2014). For this the maize strain "Gelber Badischer Landmais", Saatenunion, Germany (4.2.1 A) was used. The plants were cultivated in containers in the greenhouse for 28 days in substrate GS90, at 28°C during the day and 16-18°C at night. The photon flux density was 1000 µmol/m^2*s with a variation of 50 µmol/m^2*s. Waterlogging was performed at day 29. The containers were constantly flooded, 15 cm above the soil surface for three days. The conditions like temperature, pH value and oxygen concentration were monitored. Sampling was performed at 4 h, 28 h (anoxia) and 52 h (hypoxia) during waterlogging at the same daytime. Control plants were cultivated in parallel without waterlogging and sampling was performed in the same manner. For sampling the oldest and the youngest leaf was sampled. Each sample was collected into a 2 mL reaction tube containing two metal beads (calibre 4.5 mm). The samples were cooled down in liquid nitrogen (-196°C) immediately and then stored at -80°C. RNA was extracted (4.1.5) and cDNA was made (4.1.11). qPCR was performed for each sample (4.1.10).

4.2.10 Cultivation of transgenic plants for flash test screening

The transgenic plants (generation T2) were cultivated in petri dishes on water soaked filter paper at 28°C in the dark for up to five days. If the seeds did not develop enough root tissue the cultivation time was prolonged. Up to two centimetre of the main root was harvested and used for the screening. RNA was extracted (4.1.5) and qPCR was performed (4.1.10) to gain information about the performance of the RNAi (RNAi efficiency). For this specific primers (4.1.14 A-F) were used. Each peroxidase had its own RNAi construct. Therefore transgenic plants with the RNAi construct for *zmprx01* could be used as a similar treated "wild type" individual for the screening of the other peroxidases due to its high specificity, e.g. in a transgenic plant carrying a RNAi construct for *zmprx01* a plant carrying a RNAi construct for *zmprx66* should not give any implications in the screening for the efficiency of *zmprx01*. This technique was used because non treated individuals who did not ran the transformation process could have a different gene expression in comparison of the transformed individual. The gained qPCR data was allocated and gave a hint about the RNAi efficiency.

5 Results

5.1 Distribution of *zmprx01*; *66*; *70* via qPCR in maize roots

To discover the root compartments with the highest amount of the genes of interest different segments of the root were analysed by qPCR.

For this the roots of the five days old maize seedlings were separated into the main parts, root tip, elongation zone, differentiation zone and lateral roots (figure 15 D). Each part was independently analysed via qPCR for each gene. The gained data was brought in relation, relatively. The following results show the distribution of the RNA of *zmprx01*, *zmprx66* and *zmprx70* inside the root.

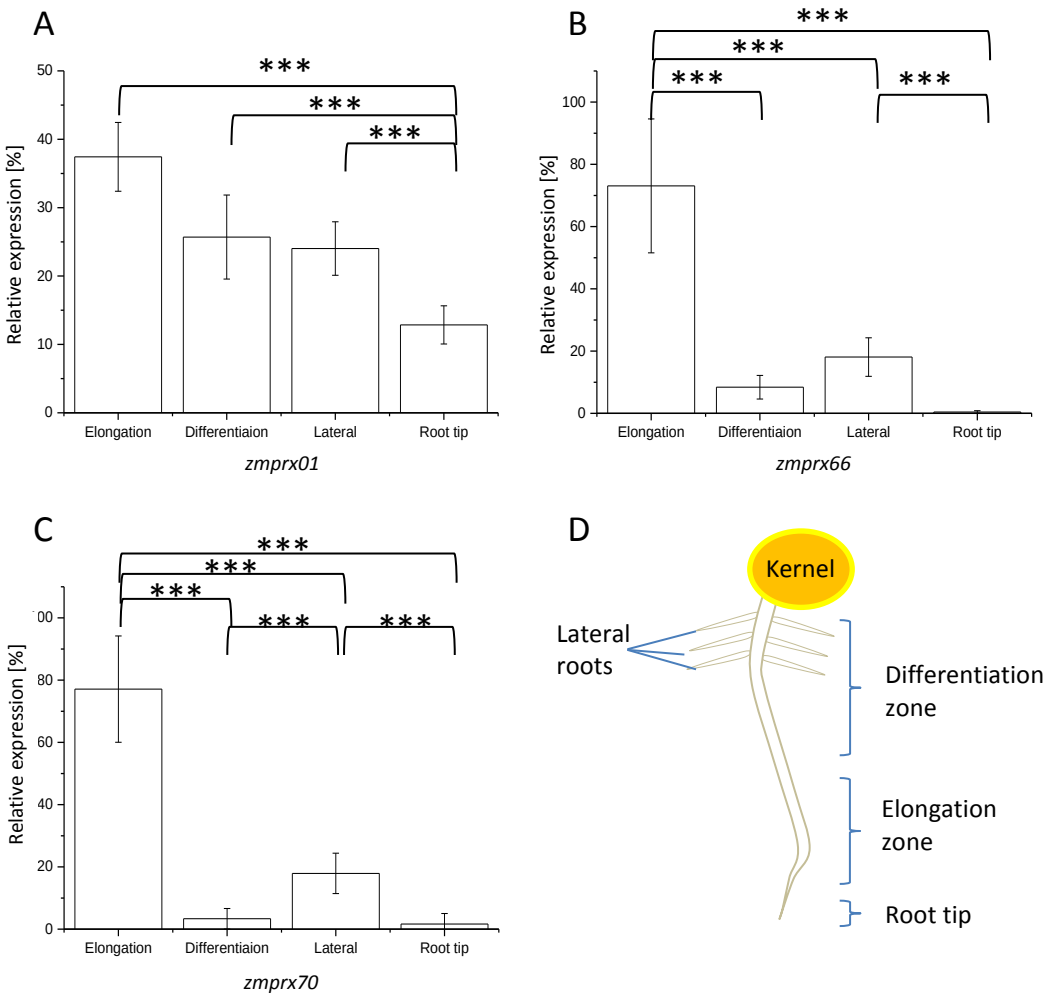


Figure 15: Distribution of PRX via qPCR A) *zmprx01*; B) zmprx*66*; C) *zmprx70*; D) overview root segments. The data were allocated to the control and are shown in percentage. In these

graphics (A-C) the relative expression in percentage (ordinate) and each root segment (abscissa) are shown. For each value standard deviation and significances are shown (*<0.05; **<0.005; *<0.001). n≈16**

In all analysed root compartments each peroxidase could be detected, except *zmprx66* which was almost not detected in the root tip (figure 15B). *zmprx01* (figure 15A) was mostly expressed in the elongation zone and less expressed in root tips. The expression value in the elongation zone was three times higher than in root tips. This difference was highly significant. The value of the differentiation zone and the lateral roots around 25 % was similar.

With an expression value about 70 % *zmprx66* (figure 15B) was with distance mostly expressed in the elongation zone and significantly higher in comparison to the other root compartments. In lateral roots the value was with around 20 % twice as high as in the differentiation zone.

zmprx70 (figure 15C) had a similar expression profile in comparison to the other PRX. The expression value was with distance the highest in the elongation zone. With a similar tendency to *zmprx66* the value for the root tip was the lowest closely followed by the differentiation zone. With 20 % the expression value was almost five times higher in the lateral root in comparison to the differentiation zone but at all much lower in comparison the elongation zone with about 75 %.

For the three analysed PRX two different profiles could be detected. *zmprx66* and *zmprx70* seem to have similar properties due to their strong appearance in the elongation zone. *zmprx01* showed a different profile. The value for the elongation zone was still the highest but not with the same distance as for *zmprx66* and *zmprx70*. Here a more homologues profile was observed.

5.2 Distribution of *rbohA*; *B*; *C*; *D* via qPCR

To discover the root compartments with the highest amount of the genes of interest different segments of the root were analysed by qPCR.

For this the roots of the five days old maize seedlings were distinguished in the main parts, root tip, elongation zone, differentiation zone and lateral roots (figure 16D). The

following results show the distribution of the mRNA of *rbohA*, *rbohB*, *rbohC* and *rbohD* inside the root.

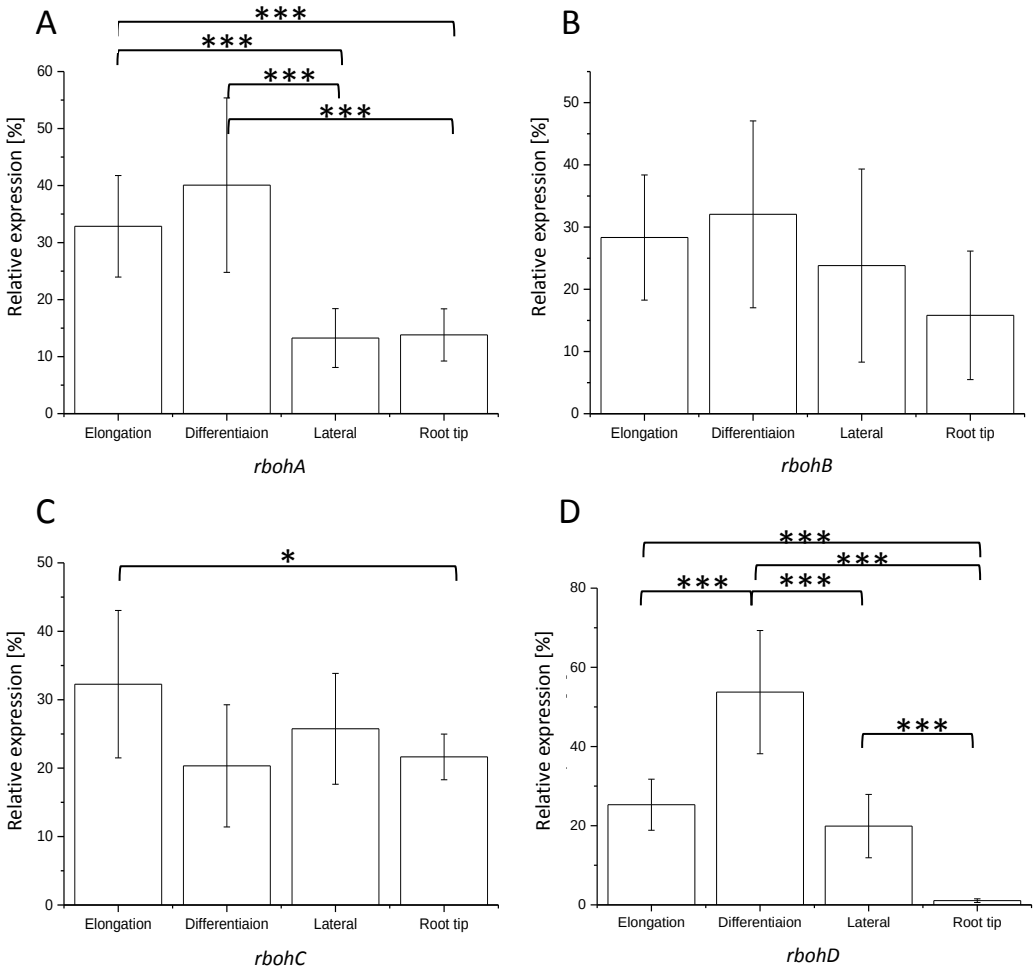


Figure 16: Distribution of A) *rbohA*; B) *rbohB*; C) *rbohC*; D) *rbohD* via qPCR in maize roots. The data were allocated to the control and are shown in percentage. In this graphic the relative expression in percentage (ordinate) and each root segment (abscissa) are shown. For each value standard deviation and significances are shown (*<0.05; **<0.005; *<0.001). n≈16**

For each gene a certain profile was detected. Each gene has its own expression profile within the root in the aspect of distribution. The expression of *rbohA* (figure 16 A) was the highest in the differentiation zone and the lowest in lateral roots and in the root tip. In the elongation zone the expression was twice as high as in lateral roots or in the root tip.

rbohB (figure 16 B) shows a more bilateral distribution of the values. The standard deviation was very high although the same amount of replicates and samples as for the other genes was used. Here, it could be deviated that the expression was the lowest in

the root tip with about 17 % and the highest in the differentiation zone with about 32 %. The standard deviation was too high to take this data as stable. No significances could be calculated.

For *rbohC* (figure 16 C) the expression was distributed in a more homologues way between the analysed root parts. The standard deviation was quite high, that the accurate interpretation of the data was not possible although the same samples were used for all analyses. It was possible to deviate a tendency. The highest expression could be detected in the elongation zone and the lowest in the differentiation zone. A low significance could be detected between the root tip and the elongation zone.

The expression of *rbohD* (figure 16 D) was significantly the highest in the differentiation zone with more than 50 %. In contrast, there was almost no expression detectable in the root tip. In the elongation zone and in the lateral roots with values between 20 – 25 % the expression was quite low in comparison to the differentiation zone. This result was highly significant at all.

For the four analysed *rboh* a relatable profile could be detected. Each of the *rboh* has its own unique expression profile. This leads to different abilities each of the *rboh* might have. For *rbohD* (figure 16 D) there was no expression detectable in the root tip. That means that there was almost no RNA of interest inside that tissue. In comparison, the highest expression was found in the differentiation zone. In difference, in the lateral roots the expression was less than a half high as in the differentiation zone. *rbohA* (figure 16 A) was mostly expressed in the differentiation zone and in the elongation zone.

The data gained from this experiment gave further information about the distribution of each enzyme on the transcription level.

5.3 Cadmium stress

For further investigation of the genes of interests the plants were cultivated in hydroponics (4.2.3). The plants were cultivated for up to 18 days in a nutrition media including cadmium. After certain time morphological differences could be monitored in comparison to the non-treated plants (figure 17).

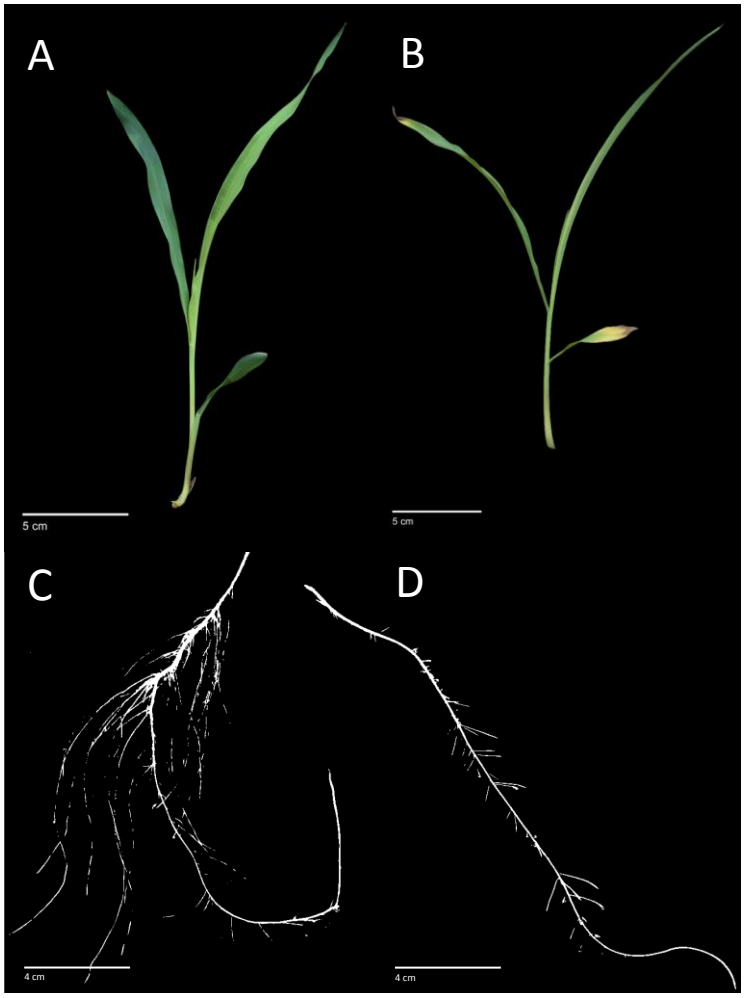


Figure 17: Cultivated maize plants grown in hydroponics with and without a cadmium [$Cd(NO)_3 \cdot 4H_2O$] concentration of 15 µM. A) Shoot of control plant; B) Shoot of cadmium stressed plant; C) Root of control plant; D) Root of cadmium stressed plant. Morphological differences are displayed.

The cadmium treated plants showed morphological changes in root and shoot. The development was decelerated in comparison to the control plants. The leaf tips showed necrosis and chlorosis. The root was affected as well. Lateral roots were significantly shorter than the lateral roots of the control. The root surface was reduced, thus the plant

was restricted in nutrient uptake. Cadmium had a severe impact on maize roots and shoots.

To proof that the morphological changes were caused by cadmium and not by nitrate another experiment using cadmium chloride was done. The morphological changes were the same, after the same period of time.

5.3.1 Cadmium measurements

The cadmium concentration within the hydroponics was measured. 10 mL of the solution were sampled from fresh and used (seven days old) hydroponics. This was performed to get information about how the cadmium concentration varies. The measurements were performed externally by Eurofins WEJ Contaminants.

Table 5: Overview of cadmium measurements

variant		[mg/L]
treatment	day zero	186.102
	day seven	182.799
control A	day zero	0.00126
	day seven	0.298
control B	day zero	0.00014
	day seven	0.00303

By the measurements the concentration of cadmium could be monitored. For the treatment the changes in the cadmium concentration are not significant. In the controls low concentration could be detected due to a normal pollution (acceptable level) of the water source. The table shows results of preliminary work. It was observed that the amount of cadmium was higher after 7 days in the control. This could be explained by the pots used were not clean. Residues of cadmium could solute into the nutrition solution. For the following experiments always the same pots for control and treatment were used. The accumulation of cadmium in the control is still under the critical value for drinking water. The permitted cadmium concentration in drinking water is less than 0.005 mg/L (ifau.org, 2015).

5.3.2 Short-term cadmium exposure

To analyse the implication of cadmium to the expression of *zmprx01*, *zmprx66* and *zmprx70* three days old etiolated maize seedlings were exposed to a nutrition solution including cadmium nitrate in a final concentration of 15 µM (4.2.5). To detect the short term impact, the plants were cultivated on the contaminated solution for 60 min and sampling was performed at time: 0 min, 10 min, 15 min, 30 min, 45 min and 60 min. A control experiment was performed in parallel without cadmium.

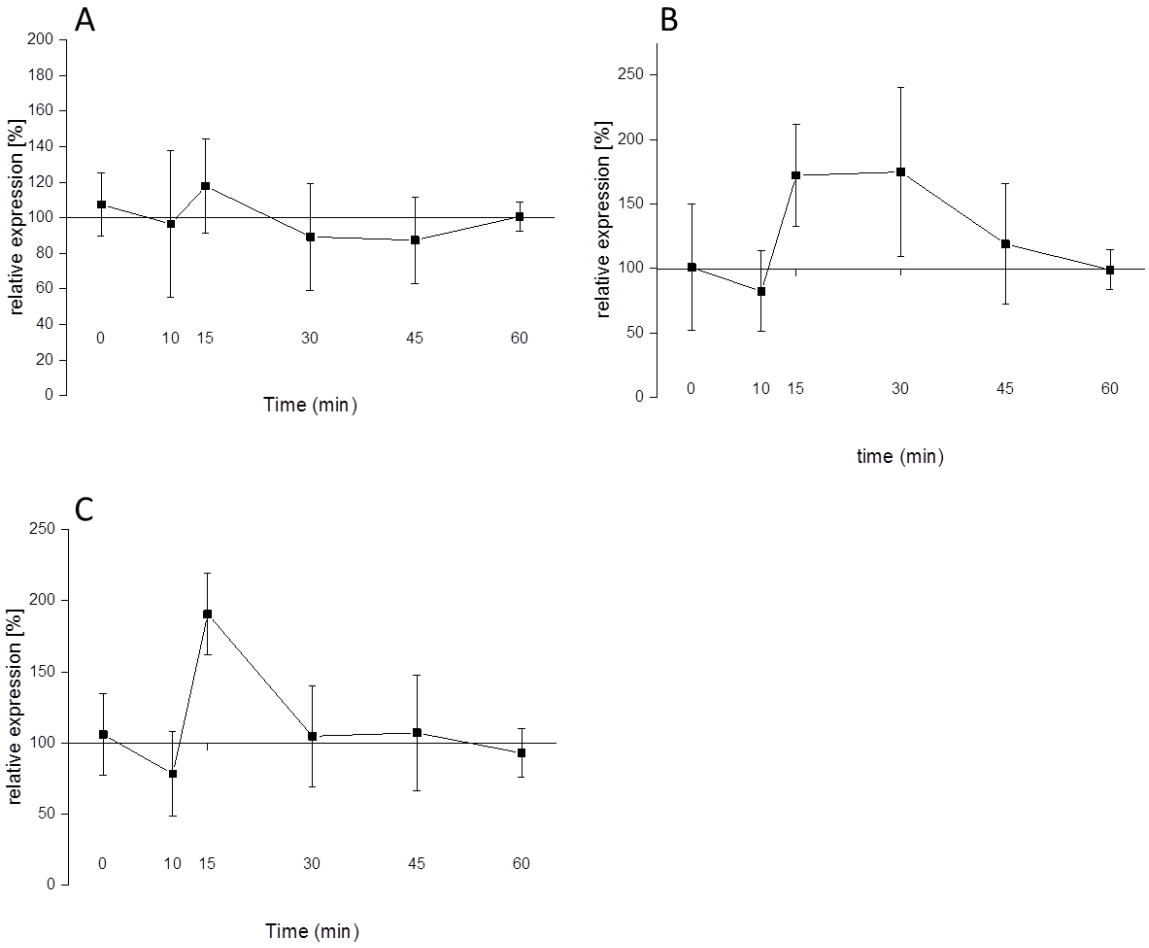


Figure 18: Expression profile of the membrane-bound PRX A) *zmprx01*, B) *zmprx66* and C) *zmprx70* in maize roots at short term cadmium exposure. The graphic shows the allocated expression in comparison to the control in percentage. 100 % in this case was the mean value of the expression of the control plants. Expression values are indicated in percentage and standard deviations are shown, n ≈ 18.

The short term cadmium exposure experiment showed which of the three peroxidases analysed was responding quickly to cadmium stress. *zmprx01* responded to cadmium

treatment with weak changes in the expression within 60 min. After 15 min the expression increased up to 117 % and then decreased down to 89 % after 30 min and 87 % after 45 min in comparison to the control. The expression value normalises back to 100 % after 60 min.

In contrast, the expression profile of *zmprx66* decreased down to 78 % after 10 min and then the expression increased to a peak with 190 % after 15 min. After that the expression decreased back to nearly 100 %.

For *zmprx70* the expression decreased at first to 82 % and then increased to 172 %. Until 30 min the expression stayed on that increased level and decreased after 45 min to 60 min back to 100 %. Both, *zmprx66* and *zmprx70* showed a quick response to cadmium stress within 15 min. In this experimental set-up *zmprx01* showed a weak response to cadmium treatment, in comparison.

5.3.3 Long-term cadmium exposure

For detection of a long-term impact of cadmium the plants were cultivated on previously named hydroponics (4.2.4) for 18 days. Sampling was performed at the day one, three, five, 10 and 18. A control experiment was performed in parallel without cadmium.

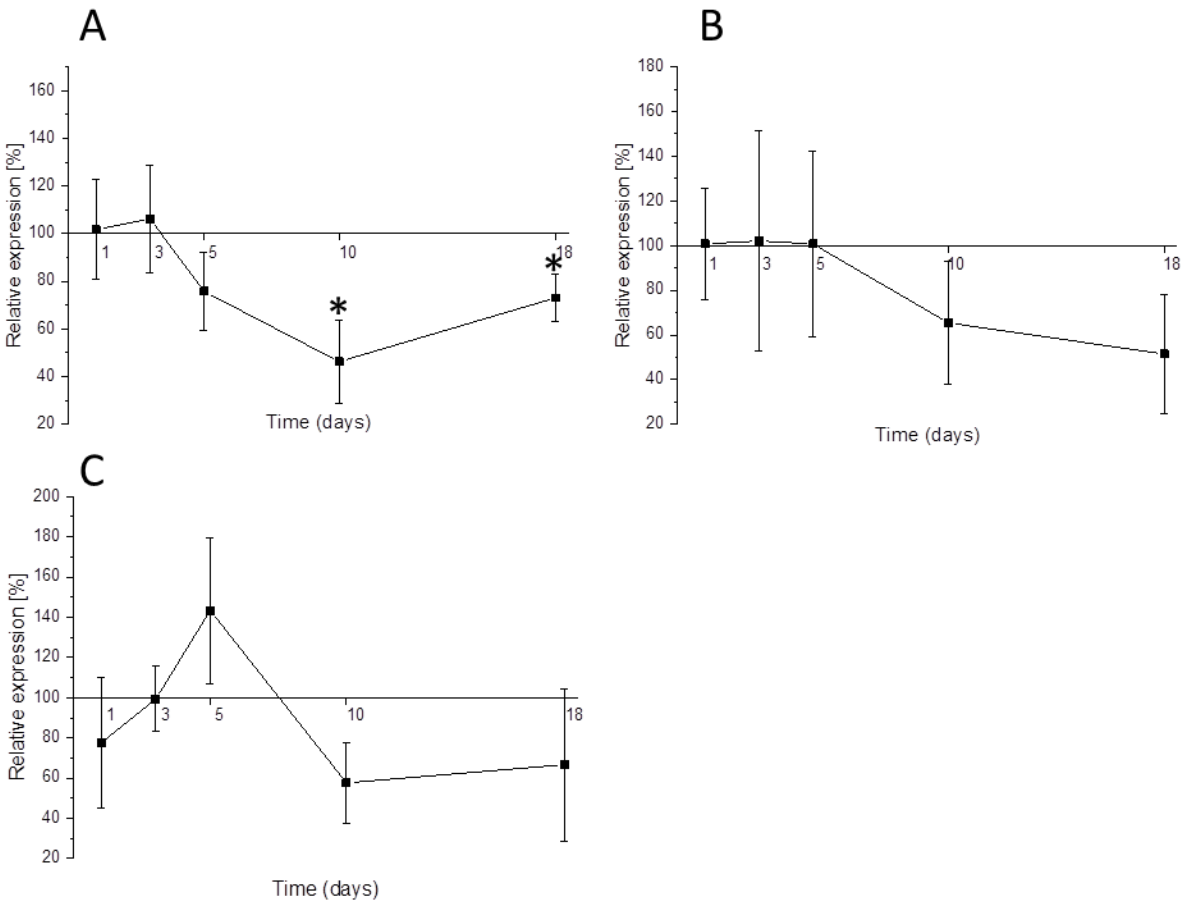


Figure 19: Expression profile of the membrane-bound peroxidases A) *zmprx01*, B) *zmprx66* and C) *zmprx70* in maize roots at long-term cadmium exposure. Analyses of the transcription level of *zmprx01*, *zmprx66* and *zmprx70* via qPCR. The graphic shows the allocated expression in comparison to the control in percentage. 100 % in this case was the mean value of the expression of the control plants. For each value standard deviation and significances are shown (*<0.05; **<0.005; *<0.001), n ≈ 10.**

The expression of each peroxidase was decreasing after ten days of cadmium exposure. For *zmprx01* a decrease of the expression was measured after five days. At day ten the lowest expression for *zmprx01* was measured. The value decreased down to 46 % in comparison to the control. An increase of the expression up to 73 % could be detected

after 18 days in comparison to the control. Within five to 18 days the expression level was below the expression level of the control plants.

For *zmprx66* a continuous decrease of the expression was detected within five to 18 days. The expression decreased down to 65 % at day ten and finally decreased down to 51 % in comparison to the control, at day 18. Within day one to day three no impact in comparison to the control could be detected.

zmprx70 showed a different expression level due to cadmium exposure in comparison to *zmprx01* and *zmprx66*. At day one the expression was decreased down about 80 %. At day three the expression level increased up to 100 % (same value as the control plants). On the fifth day of cadmium exposure the expression increased up to 140 % in comparison to the control and then decreased down to 57 % at day ten. Until day 18 the expression stayed that downregulated (66 %). This gained data proofs an implication between cadmium and *zmprx01*, *zmprx66* and *zmprx70* during a long-term exposure.

5.4 Stress profiling

To identify triggers for a reinforced or weakened expression of named *zmprx* and *rboh* and to detect a certain correlation between mRNA and protein abundance a stress profiling experiment was performed according to Mika *et al.,* 2010. For this experiment (4.2.7) different abiotic stress factors were injected to the nutrition media. The expression was analysed under the impact of chitosan for 4 h, H_2O_2 for 1h, NaCl for 2h, salicylic acid for 1h and wounding for 1h. Additionally, it could be observed if the genes are co-regulated.

Table 6: Allocated results of the stress profiling of *zmprx01*, *zmprx66* and *zmprx70* in percentage. For each value standard deviation (±) and significances are shown (*<0.05; **<0.005; *<0.001). Significant up-regulations and down-regulations are marked with an arrow, n ≈ 15.**

Treatment	Duration (h)	Relative expression (%)		
		zmprx01	*zmprx66*	*zmprx70*
Chitosan	4	66.0 ±19.6*** ↓	70.0 ±10.4* ↓	81.0 ±35.7
H_2O_2	1	70.0 ±14.0** ↓	57.6 ±12.5** ↓	68.2 ±26.6
NaCl	2	92.1 ±16.0	85.6 ±30.5	85.6 ±30.1
Salicylic acid	1	91.1 ±17.8	81.9 ±14.0	181.3 ±41.8*** ↑
Wounding	1	104.8 ±26.5	108.6 ±30.7	119.1 ±43.1

Table 7: Allocated results of the stress profiling of *rbohA*, *rbohB*, *rbohC* and *rbohD* in percentage. For each value standard deviation (±) and significances are shown (*<0.05; **<0.005; *<0.001). Significant up-regulations and down-regulations are marked with an arrow, n ≈ 15.**

Treatment	Duration (h)	Relative expression (%)			
		rbohA	*rbohB*	*rbohC*	*rbohD*
Chitosan	4	69.0 ±19.9** ↓	94.4 ±36.8	101.5 ±23.1	73.5 ±18.33** ↓
H_2O_2	1	90.3 ±16.1	95.0 ±20.7	184.4 ±28.9*** ↑	165.56 ±33.5*** ↑
NaCl	2	66.15 ±10.9*** ↓	142 ±51.3	137.1 ±36.7* ↑	111.9 ±24.8
Salicylic acid	1	94.2 ±20.7	90.5 ±27.2	111.1 ±27.9	90.8 ±16.7
Wounding	1	41.5 ±9.0*** ↓	61.21 ±19.1**↓	100.1 ±30.1	632.0 ±134.8*** ↑

With this experimental set-up it was possible to discover more about the replication of each gene. The shown data in table 6 and table 7 were gained from qPCR analyses. All the

data were allocated with the control experiment which was performed in parallel. Table 6 and table 7 show the mean values and the standard deviation in percentage as well as certain significances. A value of 100 indicates no difference between control and treatment. A down-regulation of the expression is shown by a value lower than 100 and an up-regulation is shown by values higher than 100.

Chitosan in all cases except for *rbohC* seems to down-regulate the expression, after 4 h. The PRX were affected. *zmprx01* was highly significant decreased down to 66 %, like *zmprx66* which was downregulated to 70 %, significantly. *zmprx70* was 19 % decreased in comparison to the control. *rbohA* (69 %) and *rbohD* (73.5 %) were affected in the same manner. The expression was decreased. For *rbohB* (94.4 %) and *rbohC* (101.5 %) there was no significant change of the expression measureable.

H_2O_2 caused a decrease of the expression for the *zmprx*. *zmprx01* was significantly decreased in its expression down to 70 %, as well as *zmprx66* which was downregulated to 57.6 %. The expression of *zmprx70* was downregulated to 68.2 % (not significant). For *rbohA* (90.3 %) and *rbohB* (95 %) a slightly, insignificant down-regulation of the expression was measurable. For *rbohC* (184.4 %) and *rbohD* (165.56 %) a highly significant up-regulation of the expression was triggered.

NaCl lowered the expression of *zmprx66* and *zmprx70* in the same way down to 85.6 %. The expression of *zmprx01* was like the other *zmprx* insignificantly downregulated to 92.1 %. *rbohA* decreased down to 66.15 %, highly significant, while the others increased. The amount of the transcript of *rbohB* and *rbohC* increased up to 42 and 37 %, significantly. *rbohD* slightly increased about 10 %, insignificantly.

Salicylic acid enhanced the expression of *zmprx70* on a final value of 181.3 %, significantly, while *zmprx01* (91.1 %) and *zmprx66* (81.9 %) were downregulated. All tested *rboh* appeared to be not highly affected by salicylic acid, after 1 h. The transcription values have a range about 20 % ranging from 90 % to 111 %.

Wounding did show a totally different profile. *rbohD* was 6-fold intensified up to an expression of about 632 %, highly significant. That was the highest amount in the whole experiment. *rbohC* (100.1 %) was not really affected and *rbohA* and *rbohB* were

downregulated. Especially the transcription of *rbohA* decreased down to 41.5 %, significantly, which was the lowest value in the whole experiment. *rbohB* (61.21 %) was significantly downregulated by wounding. *zmprx01* (104.8 %) and *zmprx66* (108.6 %) seemed not to be affected by wounding. The transcription for *zmprx70* was upregulated to 119 %, insignificantly.

In summary *zmprx01* was mostly affected by chitosan. *zmprx66* was mostly affected by H_2O_2 and *zmprx70* was mostly affected by salicylic acid. At all, every analysed *zmprx* was negatively affected in its transcription, except *zmprx70* under treatment of salicylic acid and by wounding, possibly. In contrast, the RBOH showed a more heterologous profile. It was remarkable that *rbohA* was mostly downregulated under wound stress and *rbohD* was more than six times intensified under wound stress. *rbohA* was additionally downregulated by chitosan and NaCl, significantly. *rbohB* was downregulated by wounding and *rbohC* was upregulated by H_2O_2 and NaCl, significantly. Besides wounding, *rbohD* was significantly upregulated by H_2O_2 and downregulated by chitosan. Definite co-regulations between respiratory burst oxidase homolog and peroxidases could not be observed. Possible co-regulation could be suggested for *zmrpx1* and *rbohA* under chitosan treatment. It was obvious that *zmprx1* and *zmprx66* were downregulated by H_2O_2, while *rbohC* and *rbohD* were significantly upregulated. At the end every analysed gene showed a unique profile, in this experiment.

5.5 Waterlogging

For further characterisation of the *zmprx* and the *rboh* in the aspect of flooding a waterlogging experiment was performed (4.2.9). Only data for *zmprx01*, *rbohA*, *rbohB*, *rbohC*, and *rbohD* are presented. Preliminary work showed that *zmprx66* and *zmprx70* were not sufficient transcribed in leaves of control plants, at that developmental stage. No mRNA of *zmprx66* and *zmprx70* could be detected via qPCR. The following figures show the results for each gene.

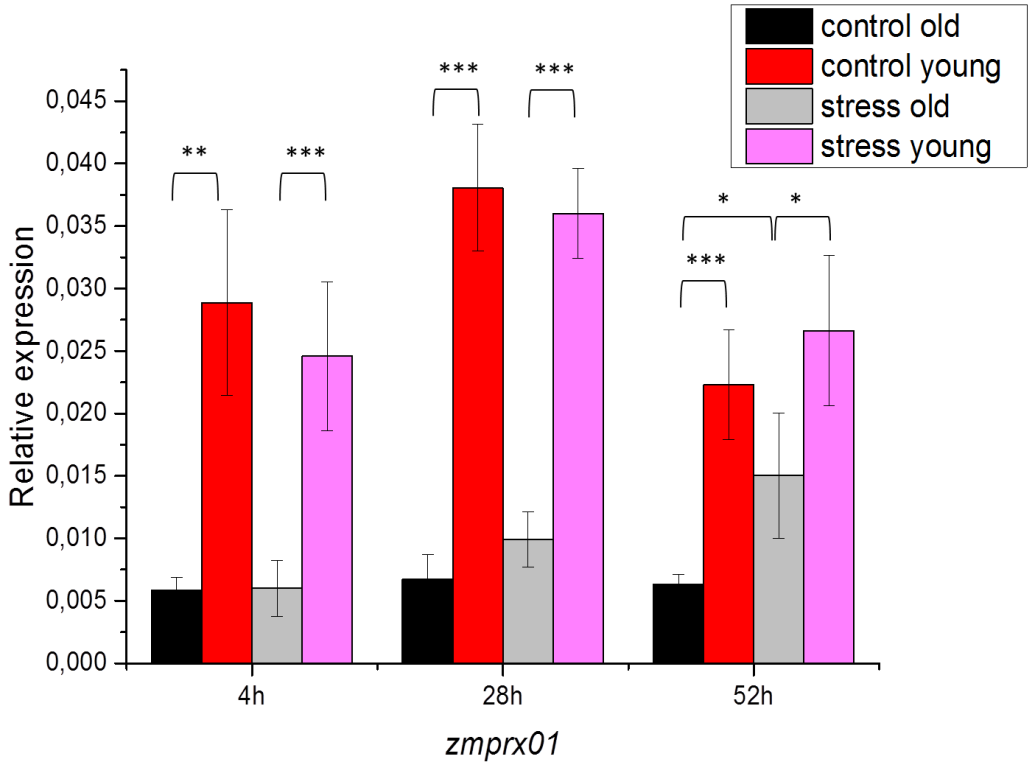


Figure 20: Relative expression of *zmprx01* after 4 h, 28 h and 52 h of waterlogging. In this graphic the relative expression (ordinate) and each time of sampling (abscissa) are shown. For each value standard deviation and significances are shown (*<0.05; **<0.005; *<0.001), n ≈ 10.**

After 4 h of waterlogging the first sampling was performed. On the first look it was obvious that the old leaves underlie the young leaves in the expression of *zmprx01* in general. This difference was significant, at all points of time. For stressed old leaves a continuous increase of the expression of *zmprx01* could be detected. The expression was twice as high as after 4 h and the control. During the time of waterlogging for younger

leaves (stress and control) an increase of the expression could be detected after 28 h. After 52 h the values decreased down to about the same value as after 4 h. *zmprx01* seems to be affected by waterlogging. Especially in older leaves this implication was obvious.

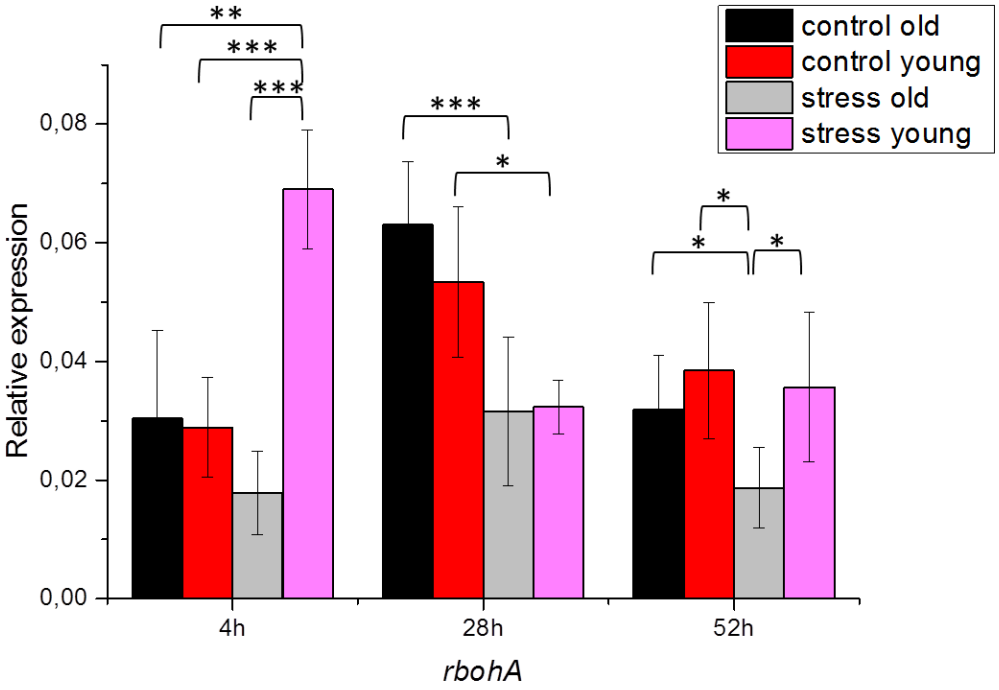


Figure 21: Relative expression of *rbohA* after 4 h, 28 h and 52 h of waterlogging. In this graphic the relative expression (ordinate) and each time of sampling (abscissa) are shown. For each value standard deviation and significances are shown (*<0.05; **<0.005; *<0.001), n ≈ 10.**

For *rbohA* a very high impact could be detected after 4 h of waterlogging. In the young stressed leaves the expression of *rbohA* was almost three times higher than in the other variants. This difference is significant to all other values after 4 h for *rbohA*. In the control the values are in the same range. The value for stressed old leaves was the lowest, even lower than the control. *rbohA* seems to be influenced by waterlogging, quickly. In old leaves the expression decreased and in young leaves the expression increased, significantly.

After 28 h the expression profile changed completely. The values for the control passed the values for the stressed plants. In comparison a significant decrease for the young stressed leaves of about 50 % could be detected. The value for stressed old leaves

increased up to the same level as the young stressed leaves. For both, control young and control old leaves, an increase of the expression could be detected, but more in the old leaves. After 52 h the expression profile changed again. The expression in the control leaves decreased almost 50 % for the old leaves and 20 % for the young leaves. The value for the stressed young leaves stayed in the same range as after 28 h. The expression for the stressed old leaves decrease back on the same level as after 4 h. It seems that especially in young leaves *rbohA* was showing a response to waterlogging even after 4 h.

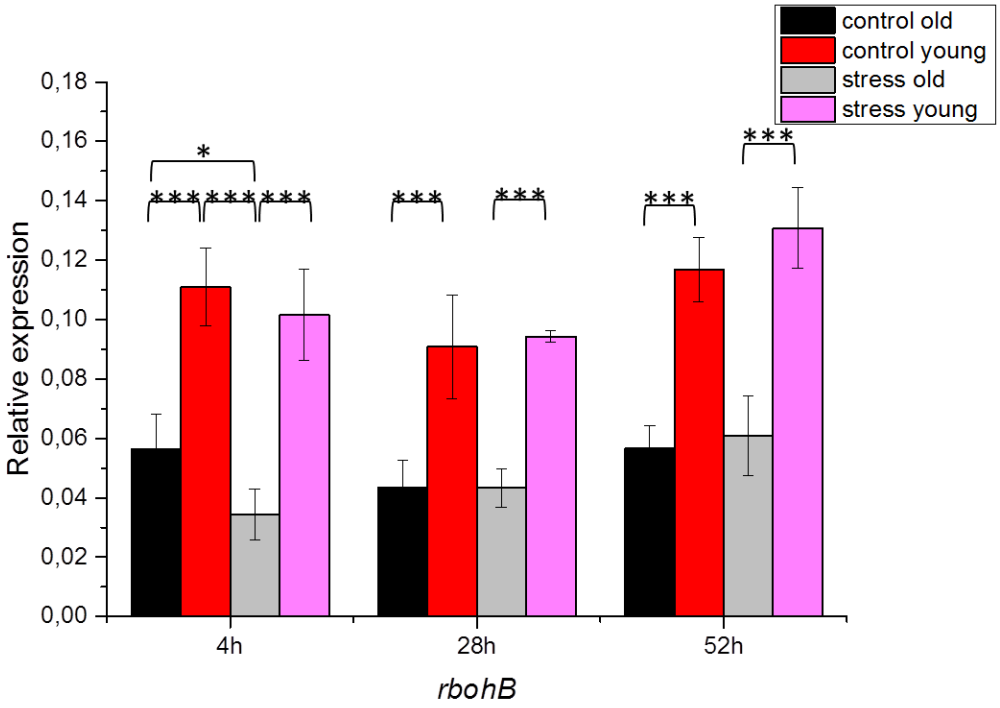


Figure 22: Relative expression of *rbohB* after 4 h, 28 h and 52 h of waterlogging. In this graphic the relative expression (ordinate) and each time point of sampling (abscissa) are shown. For each value standard deviation and significances are shown (*<0.05; **<0.005; *<0.001), n ≈ 10.**

For *rbohB* the expression profile reminds of *zmprx01*. At every time the expression in the younger leaves was round about two-fold higher than in the old leaves. The value for stressed old leaves increased until 52 h, continuously. All other values seem to be alleviated after 28 h. After 52 h the values increased slightly. After 4 h the expression was downregulated in stressed old leaves in comparison to the control. Through the whole experiment the expression values stayed nearly at the same range and relation to each

other except for stressed old leaves. At all a significant difference between young leaves and old leaves could be detected. Especially in older leaves this implication was obvious.

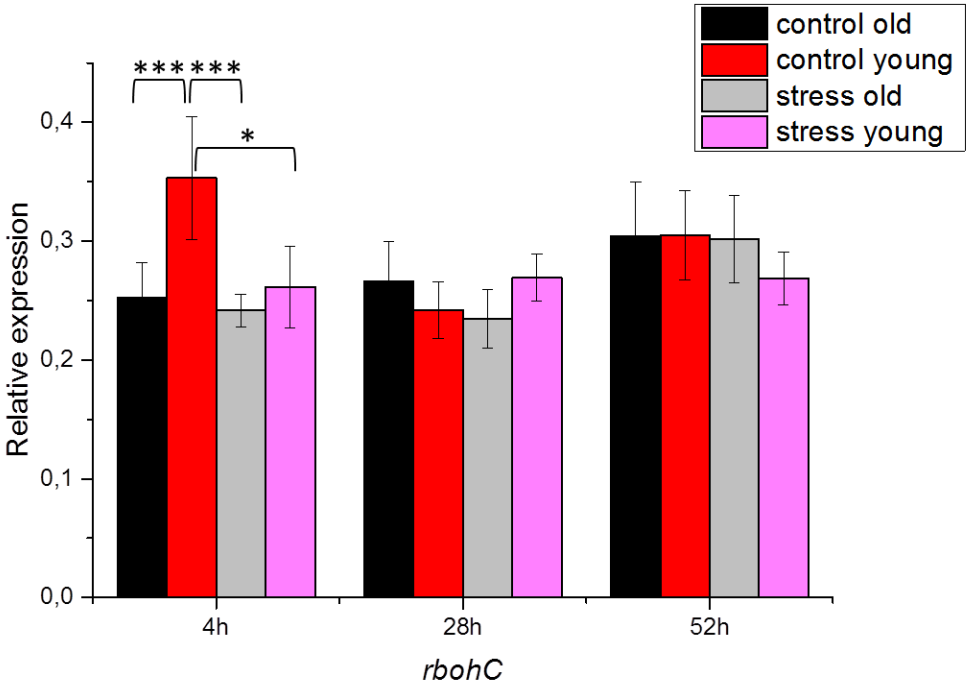


Figure 23: Relative expression of *rbohC* after 4 h, 28 h and 52 h of waterlogging. In this graphic the relative expression (ordinate) and each time of sampling (abscissa) are shown. For each value standard deviation and significances are shown (*<0.05; **<0.005; *<0.001), n ≈ 10.**

In the waterlogging experiments there was almost no implication with *rbohC* detected. All the values stayed in the same range independent of age, duration of waterlogging, control or stressed plants. Until 52 h the expression for all variants increased slightly. Only for control young leaves, there was a significant higher expression detected in comparison to the other variants, after 4 h of waterlogging.

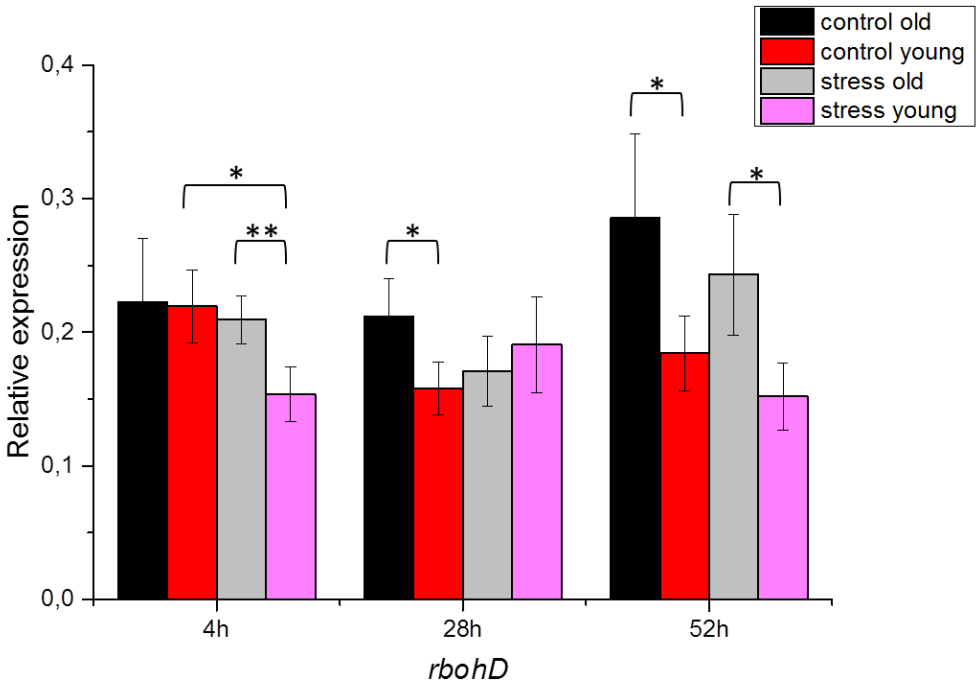


Figure 24: Relative expression of *rbohD* after 4 h, 28 h and 52 h of waterlogging. In this graphic the relative expression (ordinate) and each time point of sampling (abscissa) are shown. For each value standard deviation and significances are shown (*<0.05; **<0.005; *<0.001), n ≈ 10.**

In comparison to the other *rboh*, for *rbohD* a diverse expression profile was detected. After 4 h control old, control young and stressed old leaves have an expression value in the same range. Stressed young leaves have an approximately 20 % decreased value in comparison. The value for control old leaves stayed constant including 28 h. After 52 h the value increased 20 % and was significant higher than in young leaves (control and stress) and even higher than for stressed old leaves. In the control young leaves the expression decreased about 15 % after 28 h and slightly increased approximately 5 % after 52 h. For the stressed old leaves the value decreased almost 10 % after 28 h and increased up to 20 % after 52 h. At all it seems to be a tendency for an up-regulation of *rbohD* in older leaves during the time of the experiment in control and stressed plants, but the expression in the stressed leaves underlies the expression of the control. For stressed young leaves the value was lower in the beginning of sampling. After 28 h it increased up to 10 % and falls back on the same value as before at 4 h. *rbohD* seems not to be highly affected by waterlogging. Only a significant lack of expression after 4 h in

young leaves and the tendency that the expression was slightly weaker in the stressed leaves could be detected.

In summery different expression profiles could be detected for each gene of interest. There was a difference between old and young leaves of control and stressed plants. The time of submergence had an impact as well. With this experimental set-up it could be shown that the expression of *zmprx01*, *rbohA*, *rbohB*, *rbohC*, *rbohD* was affected by waterlogging. Especially for *zmprx01* and *rbohB* a tendency to be more expressed in younger leaves could be monitored. For *zmprx01* it could be detected that in older leaves the expression increased during a long-term waterlogging. *rbohC* seems not to be affected through this experimental set-up. For *rbohA* a quick response to waterlogging was detected. Proceeding the waterlogging the expression went back to the value of the control.

5.6 RNAi sequence investigation

To perform RNAi it was necessary to find a unique nucleotide sequences to guarantee a specific reaction. Unspecific sequences could affect not just the gene of interest but an unknown number of other genes. One important approach was the alignments of reference sequences and putative RNAi sequences. The following results show the pure nucleotide sequence of each RNAi construct, its position on the RNA / coding sequence and the similarities between the *zmprx* and the RNAi sequences.

Figure 25: Alignment of *zmprx01* reference (Ref *zmprx01*) and the RNAi triggering sequence (RNAi *zmprx01* including the C-terminal and N-terminal extension) (4.1.2). The effective RNAi sequence is shown in the red frame. Similarities are marked in green.

The RNAi sequence for *zmprx01* is located near the 3' (three prime) end of the coding sequence. There is one single nucleotide polymorphism at RNAi position 447. This is

because the reference was from B73 and the RNAi sequence is based on the HiIIA background. Similarities are marked in green. The discontinuous matches are cause by the included spacer and restriction sites. Due to the lower homology to the other two peroxidases (*zmprx66* and *zmprx70*) it was possible to select a continuous sequence. A blast of this RNAi sequence did not show any similarities that could be able to cause false priming within the RNAi process with other unwanted genes. The pure RNAi sequence was 487 bp long.

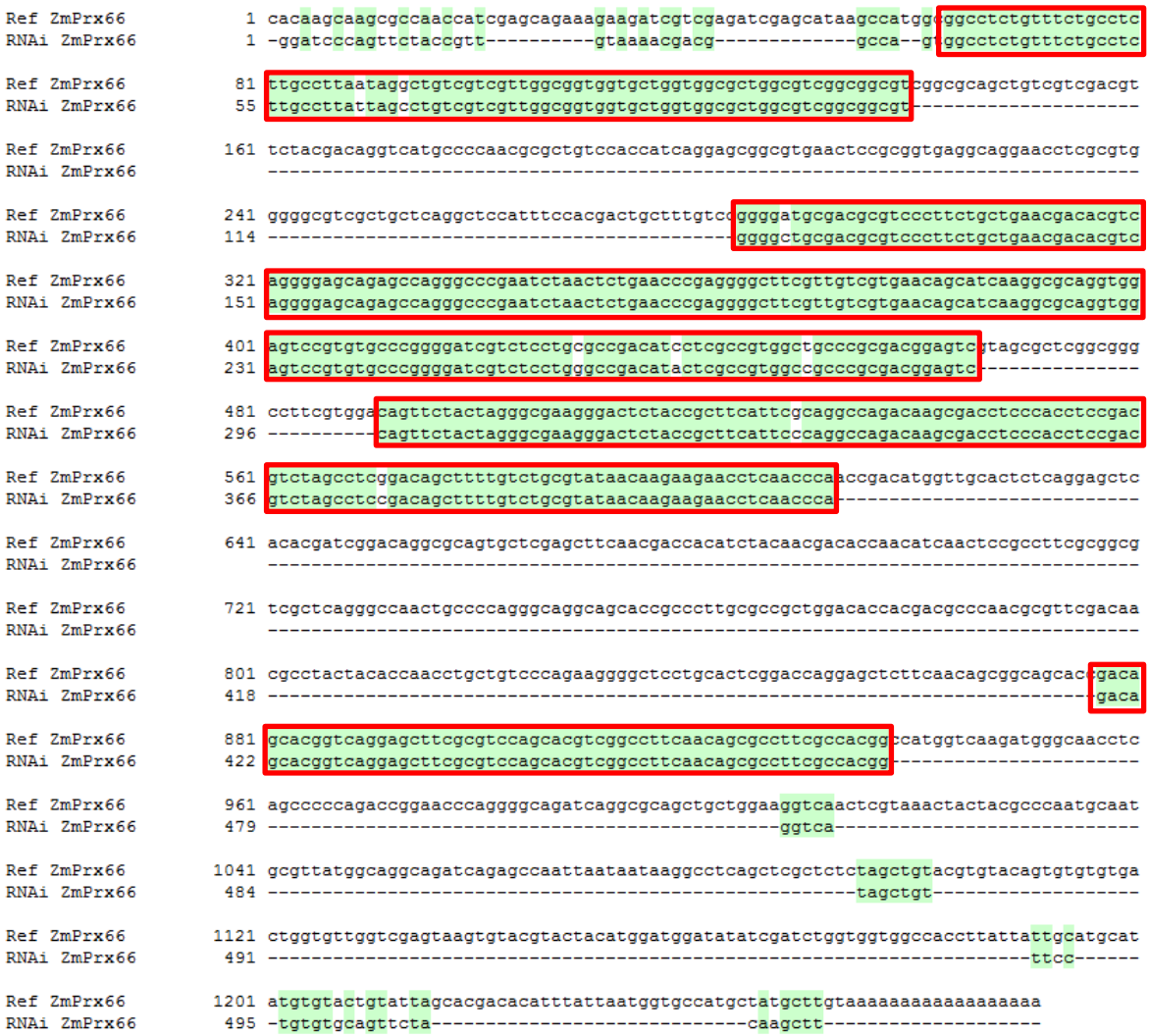


Figure 26: Alignment of *zmprx66* reference (Ref *zmprx66*) and the RNAi triggering sequence (RNAi *zmprx66* including the N-terminal and C-terminal extension) (4.1.2). The effective RNAi sequence is shown in the red frame. Similarities are marked in green.

In figure 26 the pure RNAi sequence of *zmprx66* with its extensions is shown. The sequence similarities are marked in green. The discontinuous matches are cause by the

included spacer and restriction sites. *zmprx66* has a very high homology to *zmprx70*. Therefor a continuous RNAi sequence could not be selected. Different parts of the RNAi construct are distributed along the whole RNA sequence and mostly located at the 5' end of the mRNA. Within the RNAi sequence are six single nucleotide polymorphisms (SNP) at RNAi sequence nucleotide position 64 bp, 68 bp, 119 bp, 260 bp, 269 bp, 281 bp, 334 bp and 377 bp. These SNPs appeared due to the differences of the reference gene of B73 and the sequence of HiIIA x HiIIB. These SNPs are insignificant and did not cause any side effects. A blast of this RNAi sequence did not show any similarities to other genes that could be able to cause false priming within the RNAi process with unwanted genes. The pure RNAi sequence was 439 bp long. The sequence parts were ligated in the order shown in figure 26.

Figure 27: Alignment of the reference sequence of *zmprx70* (ref ZmPrx70) and the RNAi triggering sequence (RNAi ZmPrx70 including N-terminal and C-terminal extension). The effective RNAi sequence is shwon in the red frame. Similarities are marked in green.

The RNAi sequence of *zmprx70* was built with four parts of the RNA sequence. These parts are distributed along the whole reference. It was necessary to select the parts of the sequence, because of the very low sequence similarities with *zmprx66* (figure 28). In this RNAi sequence no SNPs could be found. The similarity of the reference gained from the maize strain B73 are in these parts 100 % homologous with HiIIA x HiIIB. A blast of this RNAi sequence did not show any similarities to other genes that could be able to cause false priming within the RNAi process with unwanted genes. The pure RNAi sequence was 357 bp long. The sequence parts were ligated in the order shown in figure 27.

Figure 28: Sequence alignment of *zmprx70* and *zmprx66*. Similarities are marked in green.

In figure 28 the alignment of *zmprx70* and *zmprx66* (RNA) is shown. A very high sequence homology of 62 % and 829 matches could be detected. Especially in the middle (*zmprx66*) the sequence homology is very dense with only single SNPs in between. The homology is weaker at the 3’ end and the 5’ end.

Figure 29: Multiple sequence alignment of the three RNAi constructs (RNAi *zmprx01*, *zmprx66* and *zmprx70*). The similarities at the 5 prime and 3 prime ends (blue frame) are due to the extensions including the same spacer and restriction sites for easier cloning. Similarities are marked in green.

The multiple sequence alignment of the RNAi sequence of *zmprx01*, *zmprx66* and *zmprx70* (figure 29) showed no significant homologies between the two spacer regions. This alignment was the additional proof of the specificity of each *zmprx* to the nucleotide BLASTs (mentioned before). The total sequence similarities in the blue boxes are due to the same spacers and restriction sites.

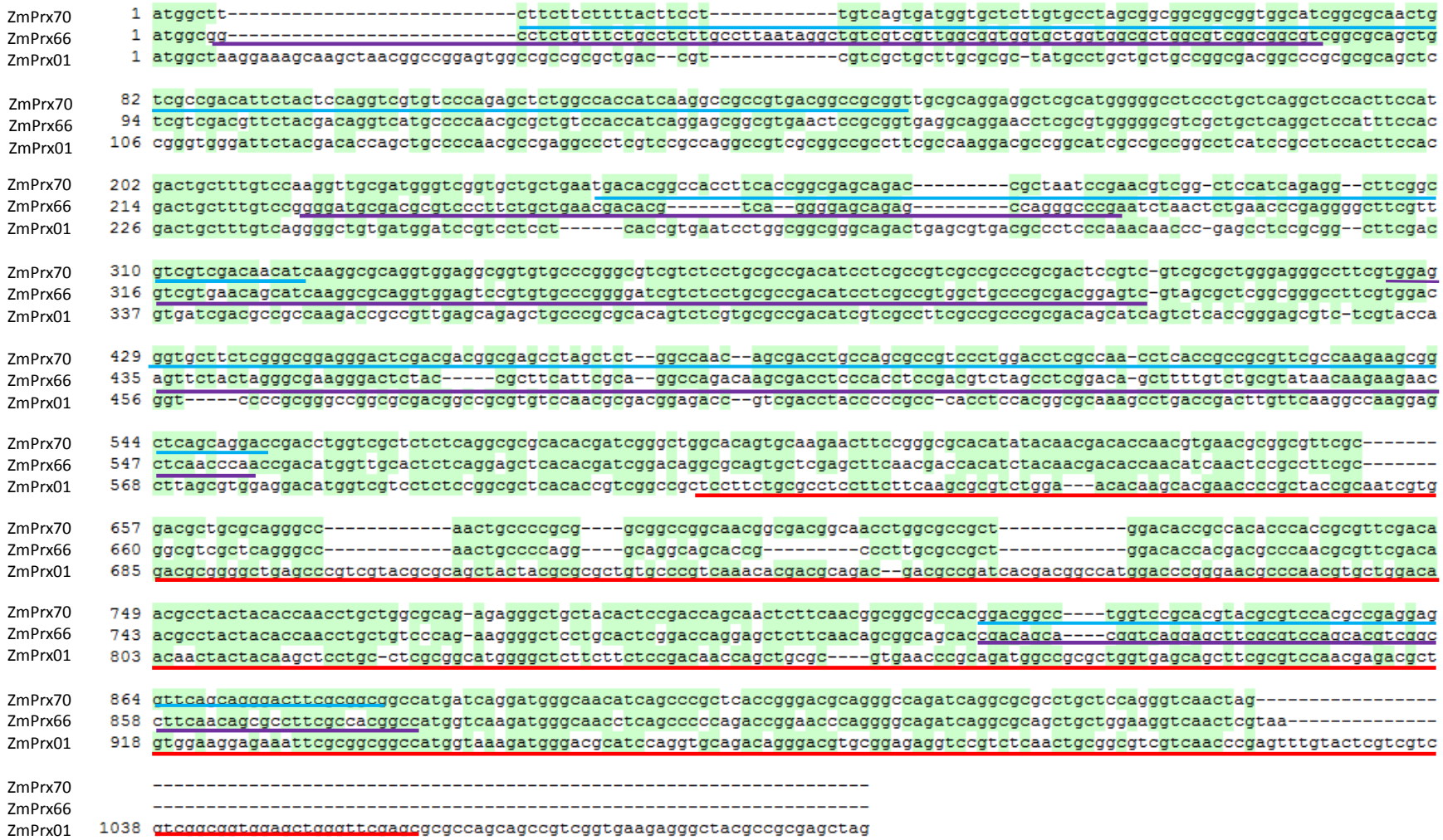


Figure 30: Multiple sequence alignment of the coding sequence of *zmprx01*, *zmprx66* and *zmprx70*. Similarities are marked in green. For each *zmprx* the RNAi triggering sequence is highlighted by an underline (*zmprx01* - red; *zmprx66* - violet; *zmprx70* - light blue).

In figure 30 a multiple sequence alignment of the coding sequence of *zmprx01*, *zmprx66* and *zmprx70* is shown. For each *zmprx* the RNAi triggering sequence is highlighted by an underline (*zmprx01* - red; *zmprx66* - violet; *zmprx70* - light blue). Through this alignment the differences between the RNAi sequences is clearly visible. Especially for *zmprx70* and *zmprx66* (high sequence similarity / figure 28) it was necessary to create RNAi sequences with no similarities affecting both genes with one RNAi construct. figure 30 shows the differences between the specificities between the RNAi sequences.

5.6.1 Transgenic screening

Southern blot analysis

For the proof of transgenic plants Southern blot analysis was performed. The following figure is showing a Southern blot analysis to check multiple integration events (clonally lines and amount of integrations).

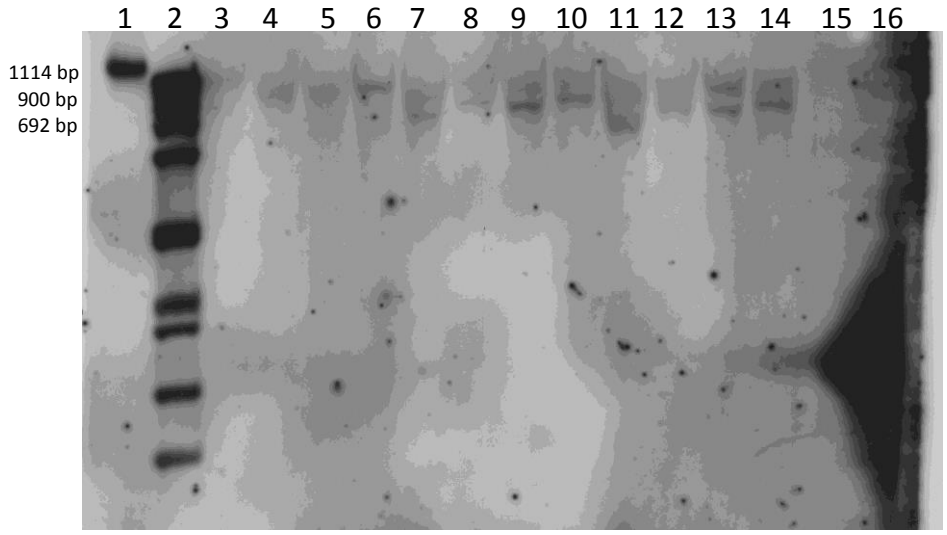


Figure 31: Exemplary Southern blot analysis of putative transgenic plants.

Table 8: Sample scheme of Southern blot analysis (Figure 31).

Lane	1	2	3	4	5	6	7	8
Sample	**Positive Control**	Dig Labelled DNA Ladder	2b1	2b2	2b3	2b3b	2b4	2b5
Lane	**9**	10	11	12	13	14	15	16
Sample	**2b5x**	2b6	2b7	2b8	2b9	2b10	--	Negative control

In figure 31 the results of a Southern blot analysis of putative transgenic plants are shown. The used probe was *bar*-specific. The extracted genomic DNA was incubated with the restriction enzyme *BamH*I. Inside the transgenic DNA fragment there was only one restriction site for *BamH*I. The enzyme cut inside the transgenic DNA fragment and outside in the genomic DNA. Due to the fact, that single integration events of transgenic

DNA sequences have unique localisations the restriction result of different integration events could vary. With this method clonally plants could be identified as well as the amount of integration events could be detected. The example in figure 31 shows these results. Samples of putative transgenic plants carrying the RNAi construct for *zmprx70* did pass through this procedure. In no sample the same restriction polymorphism was visible. All detected bands are on different heights meaning they have different sizes. In this case no clonal plants could be detected. In lane 7 and 13 two plants could be detected that have at least two integrations of the same transgenic construct. The positive control shows if the reaction did work and the negative control (no signal is desired) shows if the reaction was specific to the known transgenic DNA sequence. This method is one possibility to get information about whether a plant is transgenic or not, how many copies of the transgenic DNA sequence are included and if plants are clonal.

5.6.2 PCR screening for transgenic plants and recombination proof

Screening for transgenic plants via PCR

For an additional proof and later for a cheaper and faster way to proof the positive integration of the RNAi sequence a PCR screening for transgenic plants was developed. The following figure shows the result of this procedure for *zmprx66*.

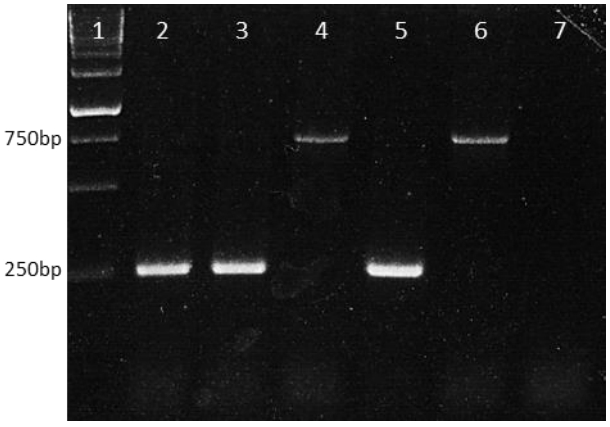


Figure 32: Agarose gel of PCR screening for transgenic plants. Agarose concentration was 2.3 % / 120 Volt for 3 h.

Table 9: scheme of the samples in Figure 32.

Lane	1	2	3	4	5	6	7
Sample	DNA ladder	Mix1	Mix2	D1	D2	Wild type	H_2O

The agarose gel shown in figure 32 gives information about the integration of the RNAi sequence for *zmprx66*. The sample definition is shown in table 9. Sample two and three were mixtures of transgenic gDNA including the RNAi sequence and wild type gDNA. D1 was a gDNA sample which was gained from a putative transgenic plant. Preliminary check (among other things by BASTA® selection) of D1 showed that it was not transgenic, while D2 was proven to be transgenic. Track six was gDNA from a wild type individual. Track seven was the contamination check. This PCR reaction was not contaminated with anything, which could affect the reaction. The water control showed no band. For all samples the same primer combination was used (Material and Methods: table 4 Y and Z).

For the mixed samples (Mix1 and Mix2) a specific band at 250 bp could be detected. This was the expected band size for the amplicon gained from the RNAi sequence. There was no band at 750 bp which was the expected size for the genomic *zmprx66* gene sequence amplicon. In D1 there was a band at 750 bp as well as in wild type. D2 showed a band at 250 bp. Through this test it was now clear which of the plants are carrying the RNAi sequence for *zmprx66*. Track two, three and five were transgenic and carry the RNAi sequence for *zmprx66*. D1 and wild type showed a band at 750 bp which was the proof that there was no transgenic RNAi sequence inside. The screening PCR was able to discriminate between transgenic RNAi and genomic gene sequences. In Mix1 and Mix2 as well in all other transgenic individuals there was a genomic PRX gene sequence by nature. In this screening only the transgenic derived amplicon was preferred in the reaction.

Recombination check via PCR

To check if the recombination of the RNAi construct was performed correctly a specific PCR procedure was developed to proof the recombination. The following figure shows the final result of this procedure for *zmprx66*.

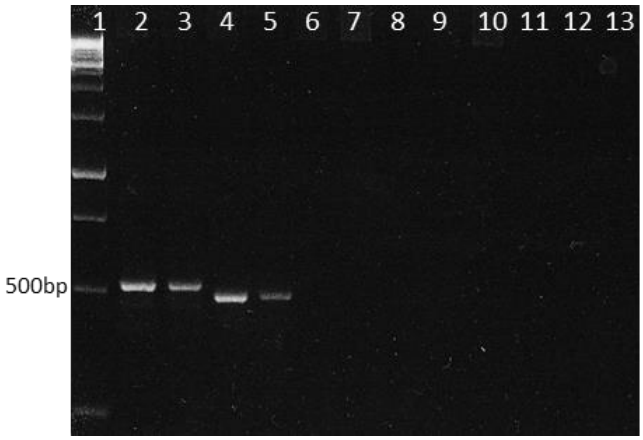


Figure 33: Agarose gel of PCR screening for recombination events. Agarose concentration was 2.3 % / 120 Volt for 3 h.

Table 10: scheme of the samples in Figure 33.

Lane	1	2	3	4	5	6	7	8
Sample	**DNA ladder**	HS 3-2 (*zmprx66*) 1/8 (A)	HS 3-2 (*zmprx66*) 8/7 (A)	HS 3-2 (*zmprx66*) 1/8 (B)	HS 3-2 (*zmprx66*) 8/7 (B)	WT (A)	T1 ZmPrx01 (A)	cDNA ZmPrx66 (A)
Lane	**9**	10	11	12	13			
Sample	**WT (B)**	T1 ZmPrx01 (B)	cDNA ZmPrx66 (B)	H_2O (A)	H_2O (B)			

The samples which were used for the screening are named in table 10. Different primer combinations were tested. Combination A (A) was “Scree 2 for” and “Pox 3-2 for” and combination B (B) was “Scree 2 for” and “Pox 3-2 rev” (see Material and Methods table 4). Combination A was meant to produce an amplicon which goes upstream the RNAi vector and combination B was meant to produce an amplicon downstream the RNAi

vector. The amplicon for A was expected to have a size about 505 bp after recombination and for B about 496 bp on basis of a *zmprx66* RNAi carrying transgenic DNA anyway.

Sample two and three are gDNA from two different transgenic plants. The plants were not clonal. They carried the RNAi construct for *zmprx66*. In track two to five the expected band could be detected. Sample six was wild type gDNA. T1 ZmPrx01 was genomic DNA from a transgenic plant carrying the RNAi construct for *zmprx01*. This sample was taken to check for the specificity of the primer combinations. cDNA 3-2 was cDNA from a transgenic plants carrying the RNAi construct for *zmprx66*. Track 12 and 13 were the water controls without a template to proof for contaminations. The sample six to eleven did not show any band for both primer combinations. The primers were designed for transgenic sequences especially for *zmprx66* (RNAi sequence). Because of that cDNA could not give a signal. The sequence of the Ubi-int promoter was not transcripted to mRNA. The binding site for the primer “Scree 2 for” is not to find in cDNA.

With this procedure it could be proved that the plants with the names HS 3-2 1/8 and HS 3-2 8/7 are carrying the RNAi construct for *zmprx66*. The recombination could be proved as well. For high throughput application the results of this procedure were checked and confirmed by DNA sequencing.

5.6.3 RNAi Plants

For the characterisation of *zmprx01*, *zmprx66* and *zmprx70* RNAi was induced in before transformed plants (4.1.3). The transformed plants were than cultivated in the greenhouse. Before that the new seeds were subjected to a heat shock regime (4.1.2). This next generation of plants (T1) was cultivated in the greenhouse. For the proof of the working RNAi a screening flash test was established (4.2.10). With this test transgenic plants with an efficient RNAi could be selected. figure 34 shows heat shocked and non-heat shocked transgenic and wild type plants including the RNAi vector triggering the *zmprx66* RNAi.

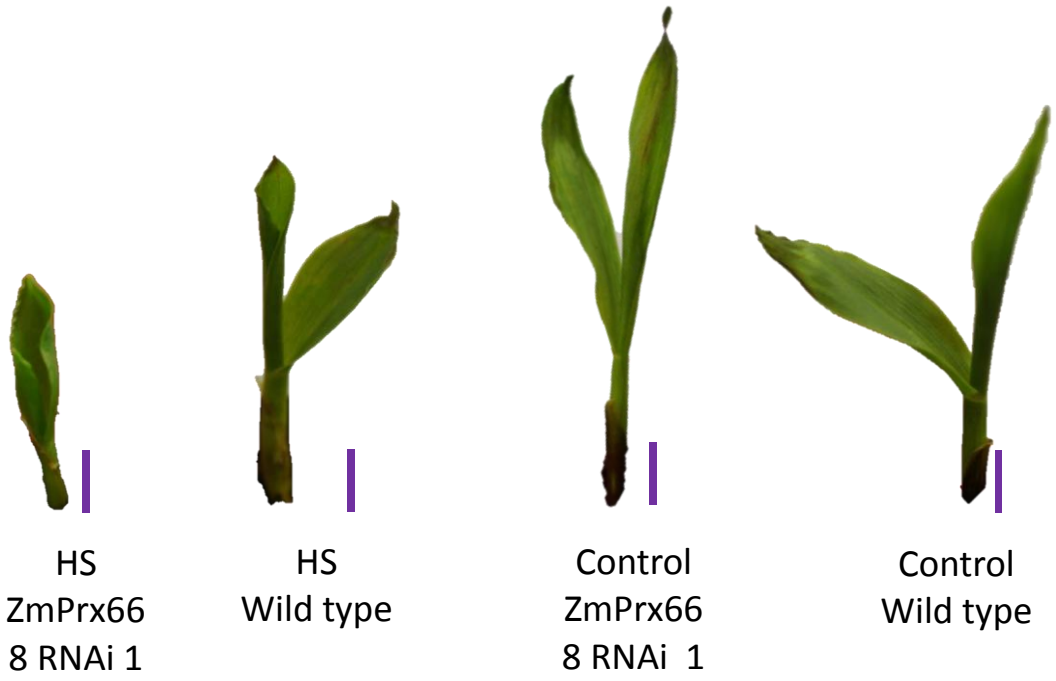


Figure 34: Phenotype screening of heat shock activated RNAi maize plants (ten days old) for *zmprx66*. The plants do show different growth sizes. The difference between the transgenic plants was much more intensive in comparison to the control plants, HS = heat shock; scale bar = 1 cm.

The heat shock treatment was necessary for the activation of the inducible RNAi transformation vector with double opposing promoters (4.1.2). It was inducible through a heat shock promoter of *Glycine max* (L.) Merr. combined with a circulation recombination

system (Cre/Lox) (3.1.2). The promoters for the RNAi sequence are intron enhanced for a higher expression level. These selected plants have been analysed via the RNAi screening flash test. The RNA was extracted (4.1.5) from roots and qPCR 4.1.10) was performed (figure 35).

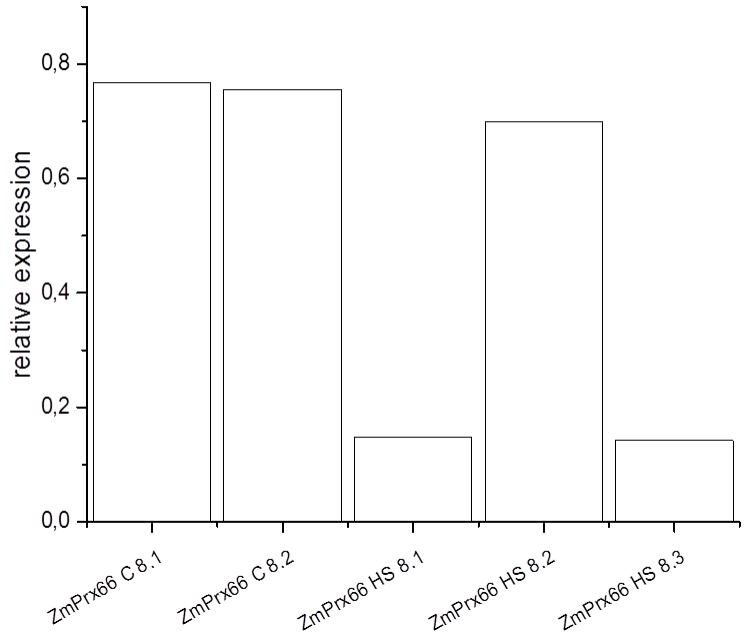


Figure 35: RNAi screening flash test. Preliminary work for the RNAi screening flash test. The ordinate shows the relative expression and the abscissa shows each tested individual. "C" stands for control (no heat shock treatment); HS stands for heat shock treatment. The last two numbers are consecutive ID numbers.

Real-time qPCR was performed for several individuals to check if the RNAi level could be measured and therefore individuals could be selected. ZmPrx66 C 8.1 and ZmPrx66 C 8.2 was used as a reference. These plants have not been heat shock treated. The expression of *zmprx66* was meant to be 100 %. This test showed that the heat shock regime wasn't working in every individual (figure 35). For ZmPrx66 HS 8.2 no reduction of the expression of *zmprx66* could be detected. But for ZmPrx66 HS 8.1 and ZmPrx66 HS 8.3 a reduction of the expression of *zmprx66* up to 80 % could be detected.

The gained data showed that the heat shock induced RNAi works and that the RNAi could be detected via real-time qPCR. For this procedure it was not necessary to analyse a wild

type individual. The same transgenic individuals for the RNAi for *zmprx66* have been analysed for the other PRX of interest. In this study the same level of expression for *zmprx01* and *zmprx70* was detected in HS and non-HS plants as for non-HS plants for *zmprx66*. So the other PRX could be used as a reference.

This part of the project was severely affected by the closing of the greenhouse due to dilapidated conditions and to an intensive pest infestation. Most gained seeds were highly contaminated by mould and aphids. This caused contamination inside the kernel. Usual procedures to decontaminate the kernels failed. The breeding of generation T2 had a failure quota of about 70 to 100 %. The project of the analysis of *zmprx01*, *zmprx66* and *zmprx70* via RNAi was indefinitely aborted. Until now there are more than 130 new developed maize strains not suitable analysed, yet.

6 Discussion

The aim of this thesis was to get new insights about the genes *zmprx01*, *zmprx66*, *zmprx70*, *rbohA*, *rbohB*, *rbohC* and *rbohD*. The studies are linked to different horticultural experiments and to gene expression analyses. This thesis could be separated into three main parts distribution in roots, stress factors and RNAi.

6.1 Distribution

For the distribution of each gene inside the root the expression value was detected via real time qPCR. The root was separated into four parts root tip, elongation zone, differentiation zone and lateral roots. For *zmprx01* (figure 15A) the highest expression was detected in the elongation zone as well as for *zmprx66* and *zmprx70*. For *zmprx66* and *zmprx70* the value for the elongation zone was the highest with distance with more than 70 %. In leaves of tall fescue PRX activity was higher in the elongation zone (MacAdam *et al*., 1992). PRX are involved in cell growth (Zheng & van Huystee, 1992) and therefor a high abundance in the elongation zone is in accordance with this data. In pea roots it was investigated that ionically bound peroxidases with an iso-electric point of 9.34 and 9.5 were induced with root growth and the activity of covalently bound peroxidases were more related to the formation of the cell wall in non-elongating tissue (Kukavica *et al*., 2012).

Another reason, in the elongation zone cells grow due to a water pressure (turgor) which is stretching the cells. This stretching goes in hand with cell wall loosening and wall deposition (McCann *et al*., 2001). In comparison to that, inside the root tip the expression of *zmprx66* and *zmprx70* is very low. In leaves of tall fescue the PRX activity lowers distally (MacAdam *et al*., 1992). It could be concluded that *zmprx66* and *zmprx70* are not active inside the root tip, under the conditions investigated in the present study. In the lateral roots *zmprx66* and *zmprx70* have an expression value about 20 %. This could be declared as lateral roots have an elongation zone as well. It could be possible, that the results may differ if the lateral roots would be treated like the main root by separating them into the three main parts. A lateral root has the same segments than the main root has.

Although the same samples were used for all genes for the distribution project *zmprx01* showed a different expression profile. The expression was more or less consistent distributed inside the root. Still inside the elongation zone the expression was the highest and inside the root tip the expression value was the lowest. The values for differentiation zone and lateral roots were kind of equal. In comparison to the other PRX *zmprx01* seems to be involved in the differentiation of the root cells after elongation. The value for the lateral roots could be the same as for *zmprx70* and *zmprx66*. The value ranges between the value for the root tip and the elongation zone.

In accordance with additional data of the eFP Browser (figure 37) *zmprx01* was found mostly in the primary root and less in the root tip, fourth internode and the base of stage two leaves (Winter *et al*., 2007; Sekhon *et al*., 2011). The enzyme was discovered in the plasma membrane fraction of maize roots (Mika *et al*., 2008). In this thesis only weak expression could be detected in leaves in 28 days old plants. This might be a hint that zmprx01 is root specific. The major abundance of *zmprx01* in roots was again confirmed by a recent publication (Wang *et al*., 2015) and in accordance with the eFP browser.

zmprx66 was expressed in roots and especially in the primary root six days after sowing. The expression decreased by proceeding development (figure 38) (Winter *et al*., 2007; Sekhon *et al*., 2011). The eFP browser only displays root data until eight days after sowing. In this thesis it could be proved that *zmprx66* was expressed in roots until the 18th day after sowing (plants grew in hydroponics) (figure 19). The data from the eFP browser fits to the results gained in the preliminary work for the waterlogging experiment. mRNA of *zmprx66* was not detectable in leaves. *zmprx66* seems to be root specific. It was found in the membrane fraction of maize roots (Mika *et al*., 2008). The specificity to be expressed in roots was recently confirmed (Wang *et al*., 2015). Although mRNA of *zmprx66* was not found in leaves the protein was found in leaves, at that developmental stage (Meisrimler *et al*., 2014). The publication was about soluble class III peroxidases. It was suggested that this discovery might be due to a contamination or to disbanding of the protein under specific conditions from the plasma membrane. Anyway the protein was found in that tissue. By that it could be concluded that *zmprx66* is not root specific and might be induced under stress conditions in leaves.

zmprx70 was expressed in roots, only and mostly in the primary root 6 days after sowing. The expression decreases by proceeding development (figure 39) (Winter *et al*., 2007; Sekhon *et al*., 2011). The eFP browser only displays root data until eight days after sowing. In this study it could be proved that *zmprx70* was expressed in roots until the 18th day after sowing, at least (plants grew in hydroponics) (figure 19). Just like for *zmprx66* it was not possible to detect the mRNA of z*mprx70* in the preliminary work for the waterlogging experiment. In both, stressed and control plants, no mRNA of z*mprx70* could be detected in leaves. *zmprx70* seems to be root specific. This statement is correlating with the observations of other studies (Mika *et al*., 2008; Wang *et al*., 2015).

The similarities between *zmprx66* und *zmprx70* could be traced back on the high sequence homology (shown in 5.6). Therefore these two PRX could be involved in the same processes. By recent sequence analysis of the three membrane-bound peroxidases analysed in this thesis it was published that they were the same genes, respectively (so called gene duplications). They are located on different chromosomes (Wang *et al*., 2015). Numerous PRX were found in all plants for example more than 119 in *Zea mays* (excluding iso-enzymes), 93 in poplar, in *Arabidopsis thaliana* 73 and in rice 138. It could be supposed that the PRX family expanded more in monocotyledons than in eudicotyledons. It was said that gene duplication is one of the major forces in genetic systems and the evolution of genomes (Moore & Purugganan, 2003; Wang *et al*., 2015).

In comparison to the peroxidases *rbohA*, *rbohB*, *rbohC* and *rbohD* showed a different expression pattern. *rbohA* was mostly expressed in the differentiation zone and in the elongation zone. It was higher expressed in root tips in comparison to PRX and the expression in the lateral roots was on the same value as for the root tip. This gene was expressed in every tested root segment with at least 10%. In turn, *rbohA* was higher expressed in meiotic tassels, anthers and immature leaves. Additionally it was found in the innermost husk (Winter *et al*., 2007; Sekhon *et al*., 2011). These compartments are all in a developing stage. In general *rbohA* was found in almost all compartments (figure 40). In the differentiation zone were *rbohA* was mostly found. Lots of processes are active to achieve a specific development of each cell. Due to that *rbohA* could be responsible for cell stiffening or for the exclusion of cells to develop side roots. In anthers the production

of pollen is with distance the most frequent process including plenty of different genes (Ma *et al*., 2008). The activity inside the anthers fits to the activity in the meiotic tassels. In this study the focus was not on pollen but especially the pollen tube which develops after the contact with the stigma need a specific enzyme activity because of its extreme elongation (Bedinger, 1992). In that elongation *rbohA* could be involved as well.

Just like *rbohA*, *rbohB* was expressed in every tested root segment but predominantly in the differentiation zone. It was highly expressed and found in meiotic tassels, in the eighth leaf in stage V9 and in developing seeds 16 days after pollination (Winter *et al*., 2007; Sekhon *et al*., 2011). In comparison to *rbohA* there was no high expression in the anthers. That might be a hint that *rbohB* is involved in different processes. Especially inside the seed development (mentioned before).

For *rbohC* another expression pattern was detected. It was expressed in every root segment in a range of 20 % to 32 %. The value for root tips was the highest in comparison to PRX and the remaining RBOH. *rbohC* was expressed in the root tip, remarkably. The root tip including the root meristem is very active in developing cells. *rbohC* was additionally found in silks, innermost husks and meiotic tassels (Winter *et al*., 2007; Sekhon *et al*., 2011). Silks and tassels are the female and male inflorescence, respectively. These tissues are responsible for the reproduction. *rbohC* might be involved in important processes including the development of inflorescences. The result for *rbohC* in this experiment and especially the steady distribution fits to the data which is shown in the eFP browser (figure 42). *rbohC* seems to be involved in every tissue and developmental stage but predominately in inflorescences and not in roots (in accordance to the eFP browser).

For *rbohD* a more distinct expression pattern was observed. This gene was mostly expressed in the differentiation zone. In the root tip almost no expression was found in comparison. The expression in the elongation zone and in lateral roots was more than 50 % lower than in the differentiation zone. The eFP browser for *rbohD* (figure 43) showed that the expression in the root was low in comparison to the expression in the anthers and in the pericarp (Winter *et al*., 2007; Sekhon *et al*., 2011). In general it could be found in almost every part of the plant and in every developmental stage. Just like for

rbohA the expression of *rbohD* was the highest in the anthers. The anthers are responsible for the pollen production. Due to its abundance *rbohD* seems to be involved in this process. As described before for *rbohA* the production of pollen is with distance the most frequent process including plenty of different genes inside the anthers (Ma *et al*., 2008). Another property of *rbohD* is about wounding which will be discussed later.

rbohA and *rbohD* had the tendency to be mostly expressed in the differentiation zone. The differentiation zone is the part of the root where root hair development happens. RBOH are involved in root hair development (Nestler *et al*., 2014). This could be a hint that in detail *rbohA* and *rbohD* are directly involved in root hair development. In *Arabidopsis AtrobhC* was detected to be a part of the mechanism controlling cell expansion (Foreman *et al*., 2003). A high amino acid sequence homology between *rbohA* and *AtrbohC* (query coverage 97 %) is strengthening this suggestion.

In *Arabidopsis* it was investigated that *atrboh* are differentially expressed in different tissues. But in *Arabidopsis* more RBOH genes were found than in maize. *AtrbohD* and *atrbohF* were expressed in all tissues. In roots *AtrbohA; B; C; D; E; F; G* and *I* were found and *AtrobhH* and *AtrbohJ* were specifically found in a pollen specific manner (Sagi & Fluhr, 2006).

Further experiments would be the confirmation of these results by proteomic studies. Through this it could be discovered if the distribution and the activity / appearance of the enzyme correlates with the distribution of the mRNA. Especially in case of *zmprx66*, this could give a hint whether the mRNA or the Protein is mobile inside the organism and not resident at its point of transcription or translation. On one hand, it is possible that the transcription of mRNA takes place in one place but the translation happens in another place. On the other hand, a translated protein can move intercellular to the point where it is “needed”. This long-distance transport of protein or mRNA through the phloem is known in plants (Walz *et al*., 2002; Kehr & Buhtz, 2008; Kehr, 2009; Notaguchi, 2015). Active PRX have been found in phloem sap of cucumber and pumpkin. It was suggested that these enzymes are important for avoiding damage to essential components of the sieve elements due to oxidative stress (Walz *et al*., 2002). In this case these PRX might have been soluble.

The experiment gave a strong hint about the distribution of the appearance of the mRNA of the RBOH and PRX genes, in maize roots. Furthermore it was possible to even conclude information about the properties of each gene due to the distribution inside the root.

6.2 Cadmium stress

Cadmium is one of the most prominent heavy metals in the environment, which is toxic to flora and fauna on the same way and even in low concentrations (Maruzeni *et al*., 2014; Nawrot *et al*., 2015). Cadmium is a toxic element which could not be metabolised by yet known organisms. It especially appears in areas which are highly used for industrial and agronomic purposes (Vangronsveld & Clijsters, 1994; Chary *et al*., 2008). In cells cadmium is known to trigger oxidative stress (Bertin & Averbeck, 2006; Cuypers *et al*., 2010). In plants cadmium reduces the growth and is able to inhibit photosynthesis (Gallego *et al*., 2012). Plants are able to accumulate cadmium in the roots and are able to move it into the shoot (Clemens, 2006; DalCorso *et al*., 2008). Different weed species like *Cichorium intybus* (L.) or *Polygonum thunbergii* (Mill.) can accumulate high cadmium concentrations in the shoot. Most *asteraceae* species accumulate a higher cadmium concentration in the shoot than in roots. *Gramineae* species have the reverse tendency (Abe *et al*., 2008).

In this experiment the expression of *zmprx01*, *zmprx66* and *zmprx70* under cadmium treatment was determined by qPCR. The plants were exposed to cadmium for a short-term and a long-term trial. Morphological changes could be observed (figure 17). The lateral roots were severely shortened. The development was extremely decelerated. On the shoot senescence could be observed. At the leaf tips necrosis was detected as well. These were typical morphological changes triggered by a high cadmium concentration (Lux *et al*., 2010). In contrast it was observed that near the root apex the root hair development was increased (Seregin & Ivanov, 2001). Similar observations were made for *Sorghum bicolor* (Kuriakose & Prasad, 2008) and *Raphanus sativus* (Durcekova *et al*., 2007) suggesting that these cells were accelerated in their maturation. In this experiment the increase or accelerated root hair development was not observed. The deformation was more leading to reduced nutrient uptake. Through the decreased root growth the nutrient uptake was negatively affected.

By the short-term experiment (figure 18) it could be observed if *zmprx01*, *zmprx66* and *zmprx70* are affected by cadmium. The expression was altered for all genes by cadmium, but mostly for *zmprx66* and *zmprx70*. These two PRX genes showed a high sequence similarity (figure 28). Therefor it could be suggested that these two PRX genes are more or less involved in the same processes. The expression of *zmprx01* altered insignificantly in comparison to *zmprx66* and *zmprx70*. Especially, after 15 minutes it was detected that the expression of *zmprx66* and *zmprx70* increased more than 50 %. In the observed time scale this even was the peak of the expression of these two peroxidases. The expression decreased after 30 minutes back to normal level. This observation leads to the suggestion that *zmprx66* and *zmprx70* are involved in a quick response to cadmium exposure. Cadmium has the property that allows replacing of divalent ions like calcium and copper. The cadmium uptake is enabled through calcium channels (Hall, 2002). By that it could be concluded that the cadmium uptake and the resulting response of the organism was detectable after 15 minutes. The minimum threshold was passed and the oxidative stress (Bertin & Averbeck, 2006; Cuypers *et al*., 2010) triggered the increase of peroxidases. For further information it would be important to know how the amount of the enzymes differs to the expression value. The protein abundance is the key for a better understanding of the relationship between expression, translation and activity of these PRX. If the mRNA stability is taken into account it could be supposed that this quick response belongs to the first defence processes against oxidative stress to keep the ROS homeostasis balanced.

Additionally a long-term cadmium exposure experiment was performed (figure 19). Through the short-term experiment we know that *zmprx66* and *zmprx70* are involved in quick defence mechanisms. 15 minutes of cadmium exposure resulted in a peak of expression and then the expression went back to normal level. If this was taken into account it is not surprising that the expression of *zmprx01* and *zmprx66* were more or less on the same expression value as in the last breakpoint of the short-term experiment. After three days the expression of *zmprx01* decreased, significantly. After ten days the lowest level in the whole set-up with a decrease of about 50 % was measured. After this inflexion the expression value went back to normal level as far as the set-up was able to display the expression until day 18. The short-term measurements showed no impact for

zmprx01 but after 10 days of cadmium exposure the expression was decreased by more than 50 %. This could be a hint that a cadmium concentration of about 15µM represses the expression of *zmprx01*. By that it could be supposed that *zmprx01* is not involved in the defence of the ROS homeostasis in presence of cadmium treatment. The decrease of *zmprx01* could be a hint that it is more involved in the production of ROS than in ROS scavenging. It is known that peroxidases are able to undergo two different cycles the peroxidative cycle and the hydroxylic cycle (Passardi *et al*., 2005).

6.3 Stress profiling

To get more insights about *zmprx01*, *zmprx66*, *zmprx70*, *rbohA*, *rbohB*, *rbohC* and *rbohD* stress profiling experiments were performed. Different stress factors were used in different concentrations and incubation times (4.2.7). All these triggers are known to trigger ROS production and therefor RBOH or PRX activity or expression. In case of PRX, this experiment was performed in accordance to Mika *et al*. (2010) to analyse a possible correlation between protein abundance and mRNA. A potential co-regulation of *rboh* expression was proofed by the experiments.

The gained data were evaluated and significant effects could be detected. For each enzyme a unique expression pattern was observed. For the peroxidases the results could be discussed under consideration with the results of Mika *et al.* (2010). The expression data of this study appeared to be in contrast to the data of Mika *et al*. (2010). They did the studies by a proteomic approach and not on a transcriptional level. Beside RBOH data the qPCR data of PRX will be discussed under consideration with the spot intensities / protein abundance of solubilisates from washed plasma membrane separated by 2D-PAGE of Mika *et al*., 2010.

In case of **salicylic acid**, for *zmprx01* the expression decreased (table 6) and the spot intensity increased with about 247 %. The expression of *zmprx66* decreased and the spot intensity was increased up to 422 %, significantly. By salicylic acid treatment the expression of *zmprx70* was intensified. A similar result was gained in the studies of Mika *et al* (2010). The protein abundance was more than eight-times intensified in comparison to the mRNA level which was only 80 % higher compared to the control. The spot

intensity was not correlating with the expression. Even a decrease of expression was observed while protein abundance was increased.

The expression of *rbohA*, *rbohB*, *rbohC* and *rbohD* was not affected significantly by salicylic acid, after 1 h. Salicylic acid is involved as an important signalling component for plant defence signalling pathways. It is able to mediate the phenylpropanoid pathway and plays an important role in defence against pathogens (insects and fungi) (Maffei *et al*., 2007) and abiotic stress. If plants are exposed to salicylic acid exogenously it influences physiology, antioxidative enzyme activity, molecular biological and biochemical processes (Rivas-San Vincente & Plasencia, 2011). It is involved in its own signalling pathway and in other plant resistance processes via cross-talk (War *et al*., 2011). Under stress salicylic acid affects plant growth by nutrient uptake, water and stomatal regulations and the photosynthesis (Hayat *et al*., 2010). With this in mind it could be concluded that PRX at this time are not positively affected. Under consideration of the cadmium data (5.1/5.2) the expression peak could have been over, already. A short term analyses could give more information. By the proteomic data it is obvious that PRX are affected in a positive way. The insignificant change of the expression of RBOH at this time might indicate no effects by salicylic acid but as for the PRX it would be very interesting to know the protein abundance at this time.

Chitosan was used to simulate a pathogenic activity like fungi. By that it could be possible to check if the genes could be associated with the MAMP-triggered immunity or to another immunisation process (figure 36). Chitosan is a biopolymer which is based on chitin. It is derived from the exoskeleton of crustacean (Hadwiger, 2013). This biopolymer is able to influence membrane depolarisation, oxidative burst, influx and efflux of ions such as Ca^{2+}, DNA alteration, mRNA transcription, phytoalexins, lignification and callose deposition (Hadwiger, 2008; Hadwiger, 2013). By that oligochitosan is able to trigger cellular changes (Yin *et al*., 2010).

zmprx01 revealed a decrease under chitosan treatment on the mRNA abundance in contrast to an increase about 36 % for the spot intensity. Chitosan treatment for 4 h decreased the expression of *zmprx66* while the spot intensity increased about 881 % in comparison to the control. That means that the amount of this enzyme was enlarged

more than eight times due to the presence of chitosan. For *zmprx70* the expression level under chitosan treatment was decreased, insignificantly and the protein level was increased, significantly. In this case the same conclusions could be made as for SA. At this time (after 4 h) the expression was not or negatively affected, although the protein abundance was high.

Chitosan did affect *rbohA* and *rbohD* significantly. The expression was decreased. *rbohB* was decreased, insignificantly and *rbohC* was not affected, after 4 h of exposure. But at all it seems that all RBOH were not involved in chitosan triggered processes by an increase of expression, after 4 h. It is not clear if the decrease of the expression could be necessary to trigger any defence mechanisms. Until now there is not enough information available dealing with co-regulation in this content for further suggestions. Therefor it would be necessary to know what happens when *rbohA* and *rbohD* were downregulated, in maize. For example, in tobacco *NtrbohD* was detected to be involved in the production of active oxygen species, such as superoxide and hydrogen peroxide. This was concluded by antisense RNA application (Simon-Plas *et al*., 2002).

Here again the same suggestions as for salicylic acid treatment could be made. The expression peak might be over and proteomic investigation would help to clarify this. By expression data the conclusion will be totally different to the conclusion made under consideration of protein abundance. A short-term trial for the expression analyses (like for cadmium) would give further insights for an expected expression peak. By that mRNA stability or protein activity could be further investigated.

Chitosan can be identified by receptors in plants which trigger specific pathogen defence mechanisms. Fungi and insects present chitin. The sudden response to an infiltrating pathogen is called microbe-associated molecular pattern triggered immunity (MAMP-trigger immunity). Specific plant receptors are able to detect MAMP. A specific defence process will be released. Other pathogens developed an effector with the ability to block the receptors responsible for the MAMP-triggered immunity. This process is called effector-triggered susceptibility. In that scenario no immunity would be triggered and the pathogen could be successful. To get rid of that problem plants derived an effector receptor which is able to detect the MAMP blocking effector. After detection of the

effector the defence process starts just like for the MAMP-triggered immunity. This process is the so called effector triggered immunity (figure 36) (Kombrink *et al*., 2011).

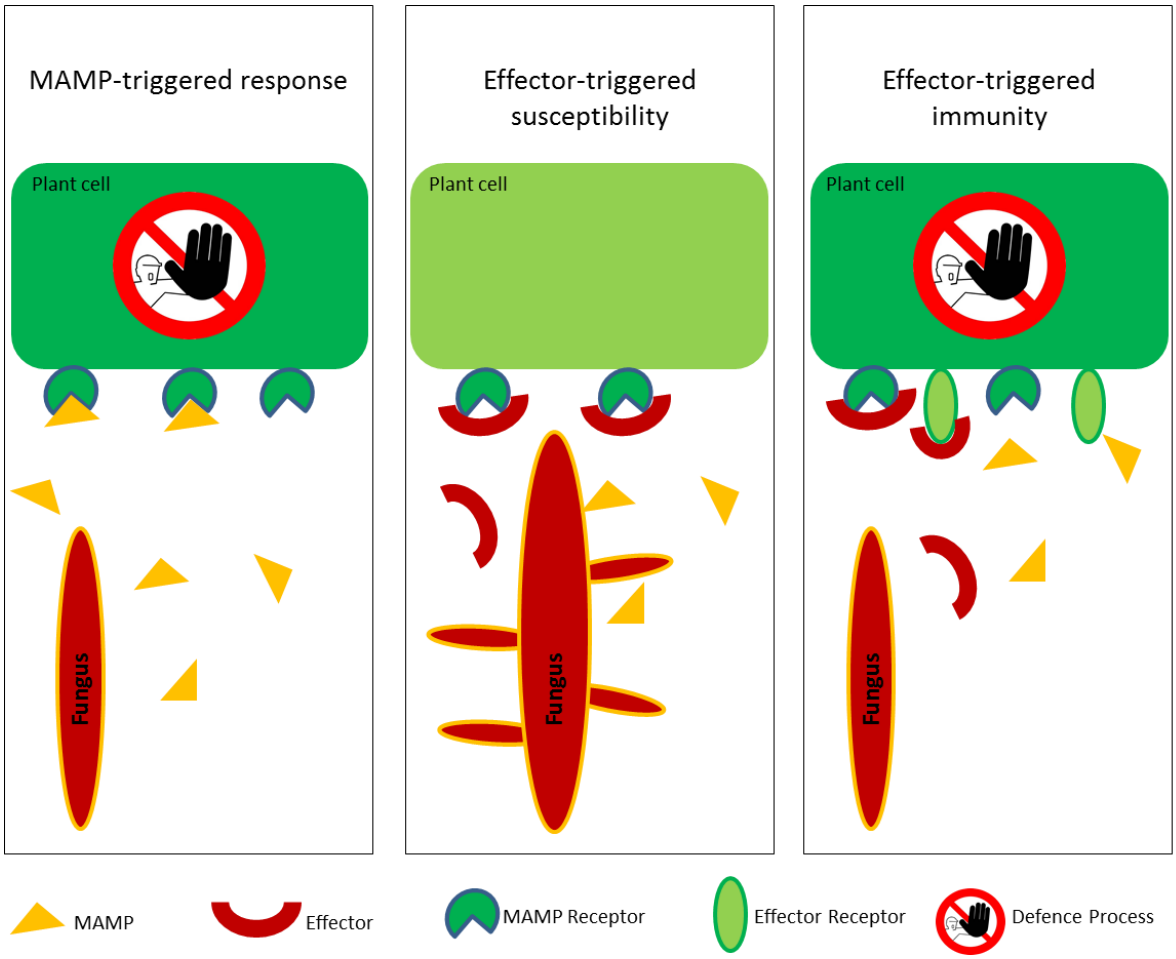


Figure 36 Scheme of plant defence and pathogen evolution (based on Kombrink. *et al*, 2011)

The MAMP happens in a sudden after pathogenic attack. Genes that might be involved in MAMP associated defence could be upregulated earlier. In another study chitosan was used for plant defence for table grapes, strawberries and sweet cherries. It was very effective in inducing resistance responses in host tissues and reduced growth of decay causing fungi. This double effect makes chitosan a useful compound in plant defence (Romanazzi, 2010). By 4 h chitosan treatment the expression of PRX and RBOH was clarified. It seems that there is no correlation between RBOH and PRX. For the PRX there is was not correlation between protein abundance / activity and mRNA abundance detected. It needs to be clarified if the protein activity can be linked to the expression and if the quick oxidative burst (Wojtaszek, 1997) is RBOH derived. For *zmprx66* the

expression was very controversial to the protein activity. It could be suggested that *zmprx66* might be involved in cell wall stiffening (Cordoba-Pedregosa *et al*., 1996) or in the protection of the membrane.

Hydrogen peroxide or H_2O_2 belongs to the group of reactive oxygen species and is toxic. This molecule could act as a substrate for peroxidases and catalases. It is a signalling molecule (Foyer *et al*., 1997; Neill *et al*., 2002).

After damage of the cell or in case of biotic and abiotic stress H_2O_2 could be accumulated (Sandalio & Romero-Puertas, 2015). It does act as a second messenger for the induction of defence genes for example in tomato in response to wounding (Orozco-Cárdenas *et al*., 2001) and is generated systemically (Orozco-Cardenas & Ryan, 1999). These properties and the substrate specificity of PRX make H_2O_2 an interesting stress factor.

For H_2O_2 a decrease of the expression of about 30 % could be detected for *zmprx01*, while on the proteomic aspect a slight increase was monitored. H_2O_2 did almost halve the expression of *zmprx66* while the spot intensity decreased as well down to 47 %. This was consistent with the expression value. For *zmprx70* the expression level by H_2O_2 treatment was decreased, insignificantly, while the protein level was increased, significantly. H_2O_2 which is a member of the reactive oxygen species group had different impacts on *rbohA*, *rbohB*, *rbohC* and *rbohD*. The expression of *rbohA* and *rbohB* was decreased, insignificantly. H_2O_2 did significantly increase the expression of *rbohC* and *rbohD*, after 1 h. In case of *zmprx01* and *zmprx70,* how could the decrease on mRNA abundance and an increase in protein abundance be explained? A decrease means a value lower compared the control. Just as mentioned before it is possible that the expression peak had been already. A decrease in protein abundance might be delayed in comparison to a sudden decrease of expression. For PRX again a controversy was detected. For rbohC and rbohD a very clear affinity to H_2O_2 could be detected. H_2O_2 and peroxidases can produce O_2 radicals (Passardi *et al*., 2005; Lüthje *et al*., 2013). O_2 radicals can be produced by RBOH (Sagi & Fluhr, 2006; Glyan'ko & Ischenko, 2010; Lassègue *et al*., 2012). So there might be a co-regulation between PRX and RBOH.

Plants are sessile and by that they are exposed to mechanical damage. This damage/**wounding** could be caused by the environment, mammals, insects and pathogens like fungi, bacteria. Typical defence mechanisms after pathogenic attack are MAMP (figure 36) (Kombrink *et al*., 2011) and damage-associated molecular patterns (DAMPs) (Akira *et al*., 2006). Theses mechanisms are similar to that against mechanical damage even the characteristics of the triggers are different.

After wounding the response occurs nearby the injury site. This so called local response is the beginning of a larger defence process. Later it moves to non-injured cells and tissues (systemic response) (Farmer & Ryan, 1992). The signal of an injury is typically transported extracellular (Roberts, 1992) and genes for general stress responses are induced quickly (Reymond *et al*., 2000; Delessert *et al*., 2004). H_2O_2 is induced by wounding through RBOH and PRX (Minibayeva *et al*., 2015).

In this study, for *zmprx01* there was no significant change observed whether in the expression analysis than in the spot intensity. For wounding a weak increase of *zmprx66* was detected but the spot intensity was decreased. The expression of *zmprx70* was upregulated by wounding, while the protein amount was decreased (about 50 %), significantly. And again there are controversies in mRNA and protein abundance. But this time the other way around. mRNA abundance was higher than protein abundance. This might be due to the time of sampling. It might be that the transcription just started and the translation did not start, yet.

rbohC was not affected by wounding. The expression of *rbohA* and *rbohB* was decreased, significantly. *rbohD* was more than six-fold intensified. This could be used as an evindence that *rbohD* is involved in wound response, after 1 h. This data was gained in the same year (2013) *rbohD* (*AtrbohD*) was found in *Arabidopsis* to be involved in plant protection (Suzuki *et al*., 2013). In detail it is involved in the generation of a systemic auto-propagating wave of ROS. This wave is traveling inside the apoplast quickly (8.4cm/min). By that *AtrbohD* is a very important participant in the signal transduction after mechanical damage in *Arabidopsis* (Suzuki *et al*., 2013; Mittler & Blumwald, 2015). Before that *AtrbohD* was detected to be responsible for ROI (reactive oxygen intermediates) in interaction with avirulent pathogens in *Arabidopsis* (Torres *et al*., 2002).

The wounding set-up gave new insights. Especially for *rbohD,* which was extremely intensified new suggestions could be made. Under consideration of Suzuki *et al*. (2013) and Mittler & Blumwald (2015) the specificity to wounding of *rbohD* could be concluded.

NaCl as a stress factor was used for the expression analysis assay, only. In this case PRX results could not be discussed under consideration of protein data. NaCl was chosen, because salinisation meanwhile gets more and more prominent in the environment due to huge flooding or shrivelled water sources (Munns & Tester, 2008; Deinlein *et al*., 2014). Salt stress could severely affect the development of the root and by that the whole plant. High salinity could cause plasmolysis of cells and therefore cause dehydration of the plant (Shavrukov, 2012). This osmotic stress is also the beginning of drought stress. Drought stress is involved in the regulation of the stomatal movement and in increasing cellular concentrations of osmolytes (James *et al*., 2006). So how are PRX and RBOH affected by osmotic stress caused by NaCl?

zmprx01 seemed not be severely affected by salt application. For *zmprx01* insignificant chances could be detected. By NaCl treatment the expression decreased for *zmprx66* and the expression of *zmprx70*, insignificantly.

NaCl treatment did decrease the expression of *rbohA*, significantly. After one step salt exposure and 2 h of incubation *rbohB*, *rbohC* and *rbohD* were positively affected. By sudden osmotic stress triggered by salt (James *et al*., 2006) it could be concluded that these enzymes might be involved in the prevention of the osmotic stress and thereby to hinder a loss of water. Salt stress can cause an elevated H_2O_2 production in plants (Demidchick *et al*., 1998). This elevated production can lead to accumulation that causes oxidative stress, which damages biological membranes (Vranova *et al*., 2002). Under salt stress, sodium ions accumulate in plants, which competitively inhibits potassium ion uptake. This leads to a lack of potassium ions in the cytoplasm, which causes metabolic disorders in plants (Zhu, 2007). Potassium ions are transported across the plasma membrane into or out of roots through potassium channels or transporters in plants. In this context it was concluded that homeostasis of sodium and potassium ions is important to survive in saline environments (Shabala & Cuin, 2007). By that is of published that

AtrbohD and *AtrbohF* are involved in regulating inward potassium channels under normal and salt stress conditions (Ma *et al*., 2012).

Superior salt concentration in the soil could alter the expression of genes and triggers ROS production. But there are different opinions on salt stress applications. Two different stress applications are published, yet. Single step salt application (salt shock) and multiple salt step application (salt stress) in a gradient to simulate a gradual salinity increase to a final concentration (Shavrukov, 2012). As mentioned before salinity could cause osmotic stress. Plants exposed to a high salt concentration appear to have an osmotic shock. It was concluded that by an exposure to 200mM NaCl causes an osmotic shock in clover (Abogadallah, 2010). Due to the current opinions it was concluded that in this experiment salt shock was performed. *Zea Mays* L. was confirmed to be moderate sensitive to salt stress (Maas & Hoffman, 1977; Carpici *et al*., 2010). Maize is able to accumulate Na^+ in the shoot, excessively. *Sorghum* species were detected to exclude Na^+ uptake from shoots (Niu *et al*., 2012). Under consideration of salt shock an impact was not detected after 2h, but *rbohB*, *rbohC* and *rbohD* were affected.

In summary, for *zmprx01* it was remarkable that all expression values decreased after this stress treatment except for wounding. For chitosan, H_2O_2 and salicylic acid the values for the spot intensity was increased in comparison to the control. *zmprx66* showed a totally contentious expression pattern in comparison to the spot intensity. In a recent publication (Wang *et al*., 2015) several peroxidases of maize were analysed. Among others the expression of *zmprx66* and *zmprx70* (in Wang *et al*., 2015: ZmPrx75 and ZmPrx26) was analysed by treatment with H_2O_2 (10mM), salicylic acid (10mM) and NaCl (20mM). In this publication, three weeks old maize seedlings were treated with these stress triggers by spraying a solution on the leaves. The roots were sampled after 3 h, 6 h and 12 h. In this publication the incubation time was longer than in this study. For example, Wang *et al*., 2015 detected a 4-fold increase of the expression of *zmprx70* after 3 h, a decrease back to normal after 6h and a 3-fold increase after 12 h. For salicylic acid a decrease about 60 % of the expression after 3 h, a 2-fold increase (in comparison to the control [200%]) after 6 h and a decrease just like at 3 h, was detected after 12 h. A treatment with NaCl caused an intensive decrease to almost no expression after 6 h and

an increase of the expression after 12 h but still significantly lower in comparison to the control. Only for NaCl the gained value of this study could fit to the published data of Wang *et al.,* 2015. The values for all other samples were completely different to the data shown in this study. Anyway the experimental set-up was totally different. The concentration of the stress triggers was lower, the incubation time was prolonged and the stressors were applied by spraying them on the leaves. These might be reasons why the results are so different.

In comparison to the gained data from Mika *et al*. 2010 it will be essential to perform the stress profiling again but with a time trail in sampling. By that it would be possible to find the peak of expression. That could give a proof for mRNA stability or enzyme accumulation. On one hand it could be discussed whether the assays were suitable applications for that analyses, but on the other hand it could be discussed if the time of sampling was appropriate. In fact it was not wanted to gain correlating data but the differences are very high. One reason could be that mRNA is transcribed in a specific manner to compromise the new situation caused by stress. The transcription amount is not linked to the translation amount. The mRNA stability could differ from case to case. It was investigated that *zmprx01*, *zmprx66* and *zmprx70* have certain cis-elements (Mika *et al*., 2008). That means that under certain stress conditions the transcription could be more effective by a specific trigger.

A more stable mRNA could be translated more times. The mRNA could be more stable to ensure a prolonged duration of translation for highly expressed genes (Russel & Klausner, 1997). Are we dealing with highly expressed genes? In comparison to that mRNA which was transcribed after internal or external stimuli might have a short half-life (Guhaniyogi & Brewer, 2001). It even could be that the translation is not affected but the transcription. Another reason could be the stability of the enzymes. Due to a prolonged life time the enzyme could accumulate and by that the increase of the spot intensity could be explained. An alternative reason could be that the transcription peak was already over. In other experiments a quick response to cadmium stress was detected (5.3.2) increasing the mRNA amount. The expression lowers after a certain peak. Proteomic studies for further insights weren't performed, yet. By now it could be

concluded that *zmprx01*, *zmprx66* and *zmprx70* are affected by the applied stress triggers in different patterns (Mika *et al*., 2010; Wang *et al*., 2015).

In case of RBOH no co-regulation could be observed by this set-up. There was no trigger that did increase the expression of all RBOH at once. No interaction could be discovered, but definite triggers could be detected by this set-up. Significant up-regulations have been discovered for *rbohC* through H_2O_2 and NaCl and for *rbohD* through H_2O_2 and wounding. Significant down-regulations could be discovered for *rbohA* through chitosan, NaCl and wounding, for *rbohB* through wounding and for *rbohD* through chitosan. Co-regulation between PRX and RBOH could not be suspended, completely. Under certain condition there might be a co-regulation between *zmprx01* and *rbohA*. Until now this hypothesis need to be further investigated. However, for the physiological function on any gene the product in its active form is needed.

6.4 Waterlogging

The waterlogging experiment was performed to investigate the involvement of *zmprx01*, *zmprx66 zmprx70*, *rbohA*, *rbohB*, *rbohC* and *rbohD* in waterlogging response. As a seasonal environmental factor flooding affects the development of woody plants, negatively (Kozlowski, 1997). Maize is known to developed aerenchyma under specific circumstances and is resistant to waterlogging (Colmer & Voesenek, 2009). The aerenchyma formation takes place by a specific programmed cell death in cortical cells in roots. Aerenchymas are waterlogging derived channels inside the root which ensures the transport of gases (Jackson & Armstrong, 1999; Colmer, 2003; Evans, 2003). Anyway waterlogging has different impacts on the plant. Height and crop yield of maize were decreased after waterlogging (Ren *et al*., 2014; Meisrimler *et al*., 2014). In this experiment long-term waterlogging was performed to analyse the impact of the two different stages of the lack of oxygen, hypoxia and anoxia. Hypoxia means a reduction of oxygen level below the optimum and the complete absence of oxygen is called anoxia. It occurs in soils which are exposed to long-term waterlogging.

The qPCR analyses were performed in accordance to a certain study (Meisrimler *et al*., 2014). The shoot diameter significantly increased after 52h of waterlogging and the chlorophyll a/b ratio decreased in comparison to the control (Meisrimler *et al*., 2014) a

similar result was detected in *Chrysanthemum morifolium* (Zhang *et al*., 2009). In case of qPCR studies only leaf material was used for the analyses. Preliminary work showed that *zmprx66* and *zmprx70* were not expressed in leaves of control plants. This result goes along with the information gained from the eFP browser (figure 38 and figure 39).

In the present study it was observed that *zmprx01* was mostly expressed in young leaves. There were no significant changes between waterlogged plants and the control. After 52h the expression in stressed old leaves was upregulated in comparison to the control. That could be a hint that *zmprx01* is involved in the response to waterlogging in leaves. Older leaves of maize are closer to the rhizosphere and therefore nearby the stress application. Through the simultaneously decreasing chlorophyll content it could be concluded that *zmprx01* might be involved in the senescence process.

Peroxidases in general are involved in leaf development and in leaf senescence. In 1979 already, it was concluded that PRX activity was high after sowing (2-5 days) and decreased with increasing development. After zenith of the development the peroxidase activity increased again (Patra & Mishra, 1979). In consideration of the decreasing chlorophyll content (Meisrimler *et al*., 2014) it could be proved that the senescence was accelerated by waterlogging. In rice and *Ramonda serbica* it was observed that PRX are active in the senescence process in leaves (Kar & Mishra, 1976; Veljovic-Jovanovic *et al*., 2006).

rbohA in stressed young leaves was upregulated in comparison to the control, significantly. The gene was detected to be highly expressed in immature leaves (figure 40) (Winter *et al*., 2007; Sekhon *et al*., 2011). After 28 h the expression decreased in stressed young leaves and in the control the expression was higher. At the end by reaching the anoxia level the expression of *rbohA* was decreased. After 4 h it was assumed that this short period time of waterlogging did not really affect the expression. But the significant change of the expression under waterlogging could be a hint for a short-term response. By microarray technology it was detected that *rbohA* was downregulated during lysigenous aerenchyma formation in maize roots (Rahji *et al*., 2011).

Not depending on treatment or time *rbohB* was significantly higher expressed in young leaves. Through waterlogging only insignificant changes could be detected (figure 22). The expression was not affected by waterlogging by this experimental set-up. Under consideration with the eFP browser (figure 41) it could be confirmed that the expression was higher in immature leaves. There was no effect on the expression detectable so it could be concluded that in leaves *rbohB* is not affected by waterlogging.

rbohC was more expressed in immature leaves (figure 42). By the expression analysis in leaves while waterlogging no significant changes could be detected. The only change of the expression was measured in the control in young leaves. The expression was about 20 % higher than the mean of all other samples after 4 h. If this was taken into account it could be supposed that by waterlogging the expression of *rbohC* was decreased. Even the difference between “control young” and “stress young” was significant. Further conclusions could not be made based on that fact. In general it seems that *rbohC* was not affected by waterlogging through this experimental set-up.

In comparison to *rbohC* the expression of *rbohD* was more divers. The expression in young leaves decreased already after 4 h, while the expression did not change for the other samples at that time. The expression in young leaves stayed on that decreased level, while the expression in old leaves increased significantly after 52h. *rbohD* which was highly affected by wounding stress was not altered by waterlogging significantly. There were some changes detected which might be due to waterlogging.

This experimental set-up was focused on leaves. It gave new insights about the expression of PRX and RBOH in leaves under the lack of oxygen in the rhizosphere. It was observed that the expression of *zmprx01* and *rbohA* was affected by waterlogging. Additionally, a development dependant distribution was observed for *zmprx01* and *rbohB*. Predominately, these genes are expressed in young leaves. Because of the physiology of maize leaves are directly related to the basal stem at the developmental stage used in this experimental set-up. Leaves are affected by waterlogging and following processes due to the lack of oxygen.

On the other hand waterlogging has an impact on the rhizosphere, firstly. Through water the roots are separated from oxygen. Plants like maize are able to form aerenchyma (Jackson & Armstrong, 1999; Colmer, 2003; Evans, 2003; Yamauchi *et al*., 2011). Two different types of aerenchyma are known: schizogenous aerenchyma which is formed by differential cell expansion and not by cell death and lysigenous aerenchyma which is formed by cell death and lysis of cortical cells in roots. This was detected in cereals like maize, barley, rice and wheat (Jackson & Armstrong, 1999; Evans, 2003; Seago JR *et al*., 2005). In maize lysigenous aerenchyma can be induced by waterlogging or a lack of nutrients (Drew *et al*., 1979). In recent studies the expression of different genes affected by waterlogging was analysed. It could be concluded that RBOH genes were upregulated (*rbohD*) or downregulated (*rbohA*) by waterlogging in stellar cells and cortical cells in maize (Rahji *et al*., 2011). RBOH is known to be involved in the production of ROS (Glyan'ko & Ischenko, 2010) which is necessary for programmed cell death to develop lysigenous aerenchyma. Until now it has become clear, that RBOH are involved in the development by aerenchyma in association with ROS scavenging related metallothionein (Yamauchi *et al*., 2011). In cucumber roots RBOH and PRX were upregulated by waterlogging (Qi *et al*., 2012). RBOH has an important role in defence processes, programmed cell death, ROS-mediated signalling and development in *Arabidopsis* (Torres *et al*., 2002; Foreman *et al*., 2003). Especially programmed cell death is an important factor for the development of aerenchyma.

6.5 RNAi

It is known that the suppression or knock-out of specific peroxidases alters different mechanisms in the plant. The knock-down of an ascorbate peroxidase (Apx1) in *Arabidopsis* resulted in an accumulation of hydrogen peroxide and by that growth and development was suppressed, stomatal response was altered and induction of heat shock proteins during light stress was augmented (Pnueli *et al*., 2003). In rice the loss of function of *osapx2* affected growth and development of rice seedlings, causing semi-dwarf seedlings, yellow-green leaves, leaf lesions mimic and seed sterility (Zhang *et al*., 2013). On the other hand it was observed that in rice mutants double silenced for cytosolic APXs (APx1/2s) the expression of other peroxidases was increased. The mutants

were able to cope with abiotic stress like salt, heat, high light and methyl viologen, similar to wild type plants (Bonifacio *et al*., 2011). The same observation was made for double knock-down rice plants for *osapx7* and *osapx8*. The phenotype was not altered but the proteins were differentially expressed. Exposure to high light and methyl viologen resulted in an altered metabolism. The lack of *osapx7* and *osapx8* triggered an overexpression of other antioxidant enzymes to respond to applied stress factors (Caverzan *et al*., 2014).

To trigger RNAi for *zmprx01*, *zmprx66* and *zmprx70* the opposing dual-promoter system was used (Schmidt *et al*., 2012). The RNAi was triggered by heat through a heat shock promoter of *Glycine max*. An inducible RNAi construct was selected to prevent eventual lethality due to the lack of one of the peroxidases. By that it could be proved that the transformation itself did not affect the plant. It was supposed that the knock-down of *zmprx01*, *zmprx66* and *zmprx70* alters the response to different stress factors. Located at the plasma membrane (Mika *et al*., 2008) these PRX are the last defence barrier along with other enzymes before the cytoplasm. This location leads to an involvement of the peroxidases in defence mechanisms. The analysis of RNAi plants (*zmprx66*) revealed that the growth and the development were decelerated after heat shock activation of the RNAi. But the heat shock regime itself did affect the development as well. In young seedlings this was observed (figure 34). Anyway a significant difference could be observed. Control plants (exposed to heat and not) and non-heat shock treated transgenic plants reached the two leaf stage earlier. The RNAi efficiency was proved by PCR. The screening is shown in figure 35. For the knock-down strain ZmPrx66 HS 8.1 (which was observed to be delayed in development) a RNAi efficiency of 80 % was achieved. By ratings of more than 130 adult plants (Supplemental table 18 and table 19) it could be observed that the growth of the transgenic plants (RNAi ZmPrx01 and RNAi ZmPrx70) was reduced (see supplemental figure 44). This phenomenon could be described by the influence of BASTA as a selection marker. It was investigated that herbicides can affect the plant development (Black *et al*., 1996; Sanyal & Shrestha, 2008).

The opposing dual-promoters system (Schmidt *et al*., 2012; Hinze & Becker , 2012) was a suitable tool for the investigation of further insights of the membrane bound peroxidases.

6.6 Conclusions

In this study many new insights could be gained for *zmprx01*, *zmprx66 zmprx70*, *rbohA*, *B, C, D*. It could be concluded that the three peroxidases are mostly expressed in the elongation zone of the root. The feature to be mostly expressed in roots was confirmed by this investigation, by the observations of Mika *et al*. (2008; 2010), by the waterlogging experiment, where the preliminary work showed no expression for *zmprx66* and *zmprx70* in leaves (under normal conditions), and by the eFP browser (Winter *et al*., 2007; Sekhon *et al*., 2011). For the peroxidases, it could further be said that SA was increasing the expression of *zmprx70*, which leads to an involvement of *zmprx70* in defence mechanisms, in association with wounding. All other peroxidases were downregulated by the applied stressors at the time of sampling. For *zmprx01* it was observed that it is significantly higher expressed in immature leaves. In mature leaves the expression was more than 80 % lower. By that it could be concluded that *zmprx01* is involved in developmental process in leaves. It was even affected by waterlogging. In mature leaves the amount of mRNA of *zmprx01* was significantly higher in comparison to the control. *zmprx66* and *zmprx70* are not expressed in leaves after 28 days. But ZmPrx66 was found in leaves after 2D-PAGE analysis (Meisrimler *et al*. 2014). *zmprx01* was not observed to be affected by cadmium. *zmprx66* and *zmprx70* were significantly affected by cadmium. The expression increased significantly after 15 minutes after exposure. By that result it could be concluded that *zmprx66* and *zmprx70* are involved in quick response defence mechanisms. After ten days of exposure to cadmium all PRXs were downregulated. A suitable set-up for a reliable expression analysis of cadmium treated plants and by other abiotic and biotic factors was established.

For the further investigation via RNAi it could be concluded that the double-opposing promoter system was a suitable application. It could be observed that a down-regulation of *zmprx66* decelerated the development of the plant.

The four RBOH could be further characterised through this study. *rbohA* was mostly expressed in the differentiation zone of the root. *rbohB* and *rbohC* were distributed equally. With the tendency that *rbohC* was more expressed in the elongation zone and *rbohD* was mostly expressed in the differentiation zone. By that each RBOH has its own

expression pattern, which leads to different tasks. The stress profiling clarified the different involvements of each RBOH. *rbohC* and *D* were upregulated by H_2O_2, significantly. RBOH are suggested to be involved in a positive feedback loop involving H_2O_2, ZmMPK5 in ABA signalling (Lin *et al*., 2009 B). Salt exposure triggered *rbohB* and *rbohC*. For SA no significant alterations could be observed and wounding increased the expression of *rbohD*. This could be explained in the involvement of *rbohD* in the signal transduction after mechanical damage. Additionally, it was observed that the occurrence of RBOH were different in the plant. *rbohB* was significantly higher expressed in immature leaves. *rbohA* was affected by waterlogging.

This investigation was started to further clarify the involvements of the plasma membrane-bound peroxidases *zmprx01, zmprx66, zmprx70* and the respiratory burst oxidase homologs *rbohA*, *rbohB*, *rbohC* and *rbohD*. For each enzyme specific new insights were gained. The impact of cadmium, waterlogging, salicylic acid, hydrogen peroxide, chitosan as an elicitor for pathogenic attacks, salt to analyse the osmotic impacts and wounding for mechanical damage was further investigated. The distribution inside the root could be clarified for seven genes. It is now clear in which compartment of the root, which gene is predominantly expressed. This will be an additional input for further studies dealing with the localisation of each enzyme and its functions.

The RNAi part (not finished, yet) gave essential new insights for possible necessities of *zmprx66*. By this study specific applications and methods had been established in the laboratory. Through this investigation it is now possible to screen for positive transformation events, for RNAi activity and its efficiency, by different PCR methods. The now standardised method for expression analyses makes it possible to easily and quickly analyse problematic genes like PRX and RBOH. A pool of reference genes for special applications was applied.

Stress profiling could be very “stressful” due to the stressors impact on the whole metabolism of a plant. Specific reference gens for maize application had been discovered that cope the stress triggers used in this study. A standardised rating was established to collect eight different parameters of plants. Through that morphological changes could be detected.

6.7 Future Prospects

The gained conclusions of this study will be a suitable input for new projects on the characterisation of membrane-bound peroxidases and RBOH in maize. It is important to find out how the expression of RBOH would differ under cadmium treatment. Preliminary work has already been made by Kerstin Wöltje (Masterthesis 2012).

The stress profiling gave new insights about the correlations between mRNA and protein abundance. It is now clear which gene is triggered by the stress factors applied. The next step will be to perform a short-term and long-term trial with selected stressors. Is there a quick response as well, for the PRX? Additionally, proteomic studies should complete the experiments. Furthermore it is necessary to have a look if the expression of the genes differs under different salt application set-up. It was concluded that there are two different ways to apply salt to plants for a stress profiling. Sudden salt exposure in superior concentrations triggers different mechanism in plants in comparison to a smooth increase of the salt concentration in the hydroponic.

Until now there are many RNAi plants not yet analysed. If it is possible to grow them without any pathogenic attack it could be clarified which genes are substituting the missing PRX. A proteomic approach and a transcriptomic approach could give further insights. By that it could be concluded if there are other membrane-bound PRX which were not yet discovered. The same experiments could be made for the RBOH. The waterlogging experiment should be repeated with root material. It is now clear that all the analysed genes are expressed in the root. Waterlogging affects the rhizosphere at first. If it is possible a transcriptome analysis should be performed. This would give many more information about the expression of the genes under stress at ones. With all these suggestions it would be possible to clarify the mystery of the PRX and RBOH in the whole metabolism, in more details.

7 Literature

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8 Supplemental

8.1 Maize transformation (nutrient media)

In the following tables (table 11 - 13) the components and compositions for all nutrient media for maize transformation are listed.

Table 11: Basic component-solutions for the production of nutrient stock media solutions.

10x Murashige-Skoog Marcoelements	
NH_4NO_3	0.206 M
KNO_3	0.18 M
KH_2PO_4	0.012 M
$MgSO_4 \times 7H_2O$	1.5 M
$CaCl_2 \times 2H_2O$	0.029 M
→ autoclaved	

1000x Murashige-Skoog Microelements	
$MnSO_4 \times H_2O$	0.099 M
$ZnSO_4 \times 7H_2O$	0.034 M
H_3BO_3	100.2 µM
KJ	0.005 M
$Na_2MoO_4 \times 2H_2O$	0.001 M
$CoCl_2 \times 6H_2O$	10 µM
$CuSO_4 \times 5H_2O$	10 µM
→ sterile filtrated;	

1000x Murashige-Skoog Vitamins	
Glycine	0.02 M
Thiamin-HCl	0.002 M
Pyridoxine-HCl	2.4 mM
Niacin	0.004 M
→ sterile filtrated	

myo-Inositol-solution	
Myo-Inositol	0.277 M

500x NaFe-EDTA-solution	
NaFe-EDTA	0.02 M

10x N6 Macroelements	
$(NH_4)_2SO_4$	0.35 M
KNO_3	0.279 M
KH_2PO_4	0.029 M
$MgSO_4 \times 7H_2O$	0.007 M
$CaCl_2 \times 2H_2O$	0.011 M
→ autoclaved	

1000x N6 Microelements	
$MnSO_4 \times H_2O$	0.022 M
$ZnSO_4 \times 7H_2O$	5.2 mM
H_3BO_3	2.5 mM
KJ	0.004 M
→ sterile filtrated	

1000x N6 Vitamins	
Glycine	0.02 M
Thiamin-HCl	0.002 M
Pyridoxine-HCl	2.4 mM
Niacin	0.004 M
→ sterile filtrated	

Table 12: Composition of nutrient media stock solutions for the production of nutrient media plates.

Infections media	
10x N6 Macroelements	1x
1000x N6 microelements	1x
N6 Vitamins	1x
500x NaFeEDTA	1x
L-Proline	0.006 M
Sucrose	0.2 M
Glucose	0.2 M
→ sterile filtrated	pH5.8

Co-cultivations media	
10x N6 Macroelements	1x
1000x N6 microelements	1x
N6 Vitamins	1x
500x NaFeEDTA	1x
L-Proline	0.006 M
Sucrose	0.08 M
→ sterile filtrated	pH5.8

Resting media	
10x N6 Macroelements	1x
1000x N6 microelements	1x
N6 Vitamins	1x
500x NaFeEDTA	1x
L-Proline	0.006 M
Sucrose	0.08 M
MES	2.5 mM
→ sterile filtrated	pH 5.8

Regeneration media	
10x MS Macroelements	1x
1000x MS Microelements	1x
500 x NaFe-EDTA	1x
1000x MS Vitamins	1x
Myo-Inositol solution	55 µM
Sucrose	0.08 M
→ sterile filtrated	pH5.8

Table 13: Reagents for nutrient media plates for *Agrobacteria tumefaciens* mediated transformation of *Zea mays.*

Infection media	
Infections media	1x
H_2O sterile	1x
Acetosyringone	100 µM

Co-cultivation media	
Co-cultivation media	1x
$AgNO_3$	7.5 mM
Acetosyringone	100 µM
L-Cysteine	2.5 µM
2,4 D	10 µM
Gelrite™	0.3 %

Resting media	
Resting media	1x
$AgNO_3$	5 µM
Cefotaxim	220 µM
Vancomycin	70 µM
2,4 D	6.5 µM
Phytagel™	0.3 %

Selection media I	
2x Resting media	1x
$AgNO_3$	5 µM
Cefotaxim	220 µM
Vancomycin	70 µM
2,4 D	6.5 µM
Glufosinate	10 µM
Phytagel™	0.3 %

Selection media II	
2x Resting media	1x
$AgNO_3$	5 µM
Cefotaxim	220 µM
Vancomycin	70 µM
Glufosinate	34 µM
Phytagel™	0.3%

Regeneration media	
2x Regeneration media	1x
0.6% Gelrite™ heated	0.3 %
Glufosinate	16 µM

Table 14: Ingredients for one litre of YEB-media for *Agrobacteria* cultivation.

YEB-Media	
Bacto-Pepton	5g
Yeast-extract	1g
Beef-extract	5g
Sucrose	5g
$MgSO_4$ x 7 H_2O	493mg
pH 7,2	

Table 15: Southern blot reagents.

HCl	0.25 M HCl
Denaturing buffer	1.5 M NaCl 0.5 M NaOH
Neutralisation buffer	1.5 M NaCl 0.5 M Tris 1 mM EDTA pH 7.2
20x SSC	3 M NaCl 0.3 M Na-Citrat
Wash solution	0.5 M SSC 0.1 % SDS
Washing buffer	1 x B1-buffer 0.4 % TWEEN 20
10x B1-buffer	100 mM Maleinacid 150 mM NaCl pH 7.5 (NaOH)
B2-buffer:	800 mL 1 x B1-buffer + 8 g Blocking Reagent (Blocking Reagent, Roche, Mannheim, Germany)
B2-buffer + Antibody (AB)	21.5 µL AB in 200 mL B2-buffer (Anti-Dioxigenin-AP, FAB fragments, Roche Mannheim, Germany)
B3-buffer	100 mM Tris-HCl, pH 9.5 100 mM NaCl 50 mM $MgCl_2$
B3-buffer + CSPD-Substrate	15 mL B3-buffer 75µL CSPD (1:200) 37.5 µL CSPD (1:400) (CSPD, Roche, Mannheim, Germany)

8.2 Overview of expression of *zmprx01*, *66*, *70* and *rbohA*, *B*, *C*, *D*

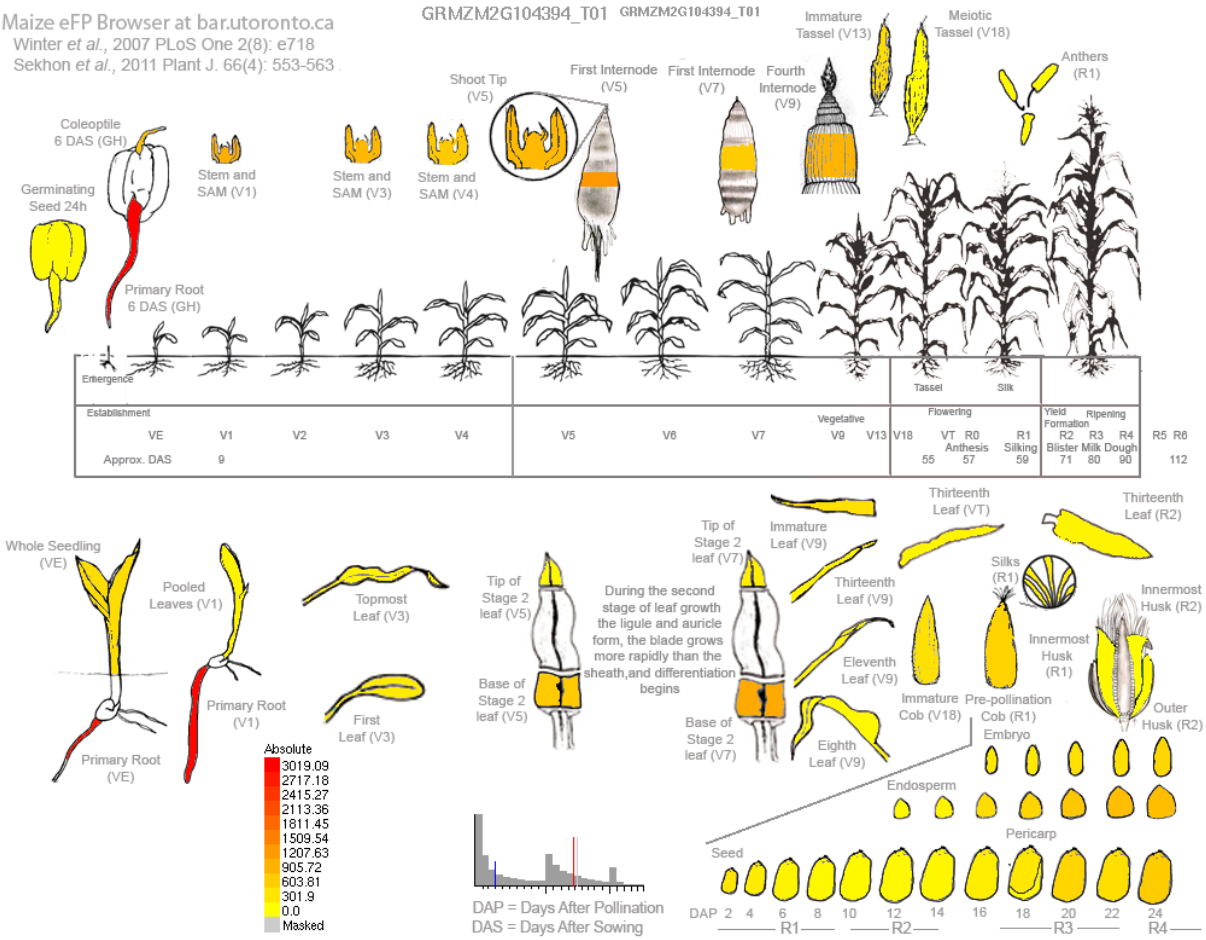

Figure 37: Maize eFP Browser for *zmprx01*. The eFP Browser shows the location of the expression of *zmprx01* in dependence of developmental stage. The data is gained from different works. Short the expression intensity is marked in a gradient (red – the most, yellow – no expression detected). *zmprx01* was mostly expressed in the roots of immature individuals. It was found in shoot tips as well as in the first and fourth internode. In immature tassels it could be detected but not in meiotic tassels. It was not found in anthers. *zmprx01* could be detected immature leaves. In the base of stage 2 leaf V5 and V7 it could be detected. In seeds it could be detected after two days after pollination with a decrease after eight days and a second increase after 16 days. In the endosperm which could be analysed after 12 days it could be detected after 16 days in a low level but with an increasing tendency. At all, this gene was expressed in roots and leaves mostly in developing tissue.

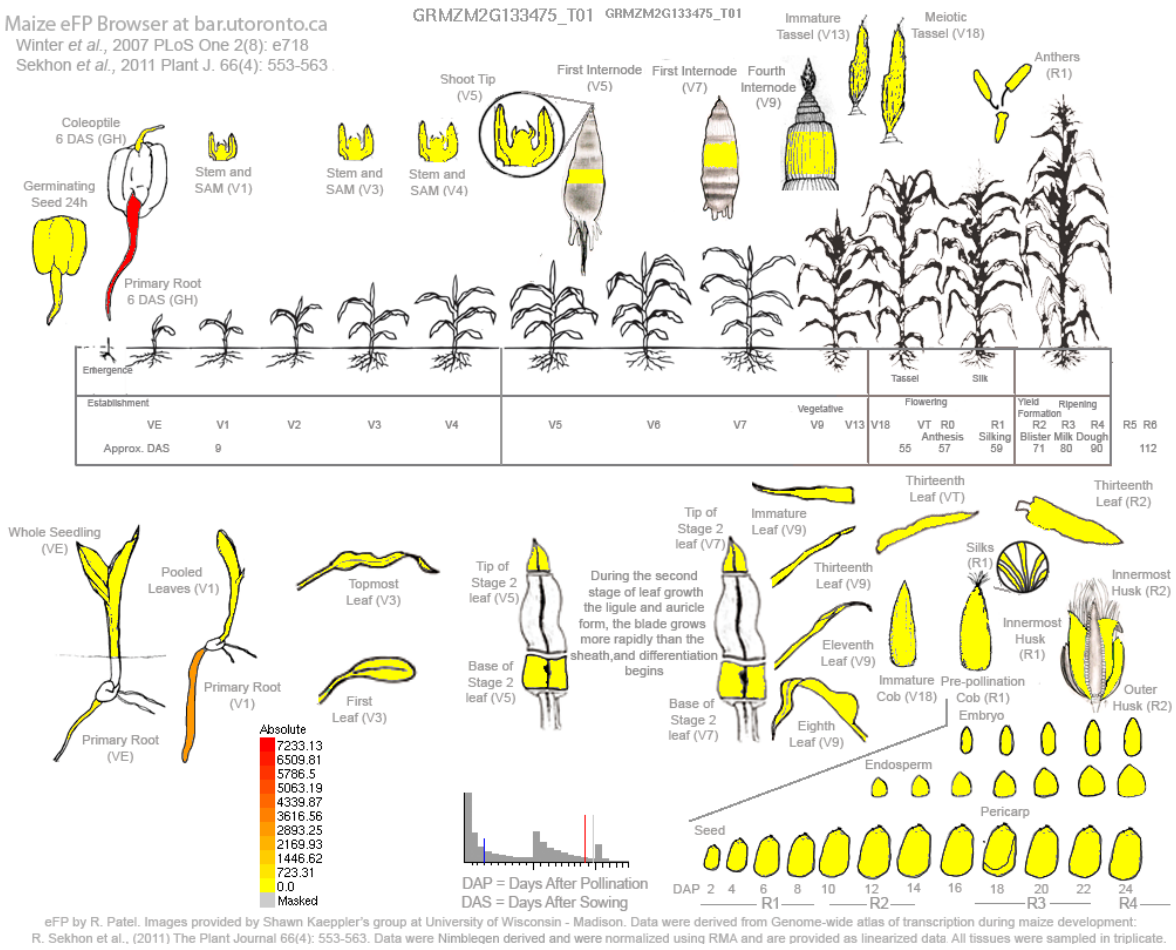

Figure 38: Maize eFP Browser for *zmprx66*. The eFP Browser shows the location of the expression of *zmprx66* in dependence of developmental stage. The data is gained from different works. Short the expression intensity is marked in a gradient (red – the most, yellow – no expression detected). *zmprx66* was detected in the primary root especially six days after sowing. In all other compartments and tissues tested no expression of *zmprx66* could be detected.

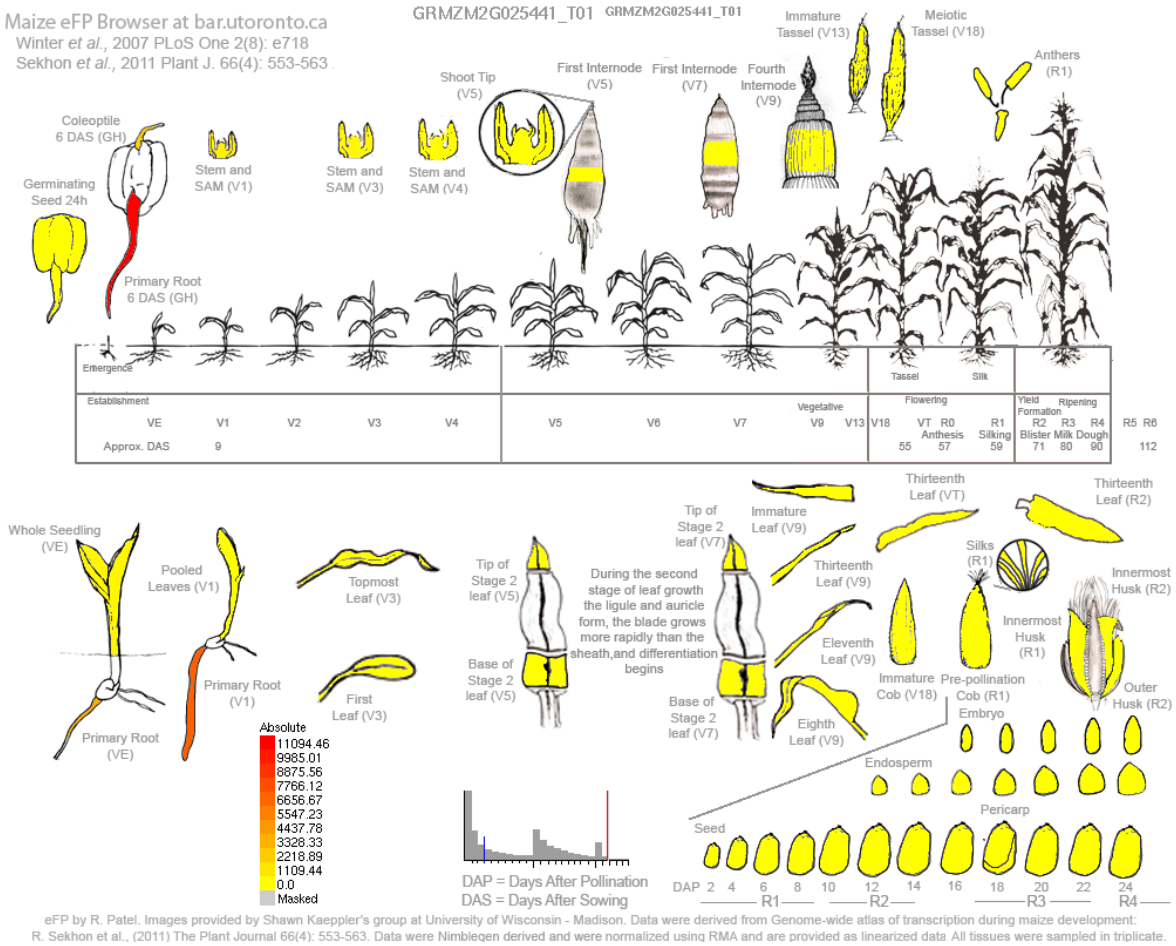

Figure 39: Maize eFP Browser for *zmprx70*. The eFP Browser shows the location of *zmprx70* in dependence of developmental stage of the expression. The data is gained from different works. Short the expression intensity is marked in a gradient (red – the most, yellow – no expression detected). The expression profile of *zmprx70* is similar to *zmprx66*. It was detected in the primary root especially six days after sowing. In all other compartments and tissues tested no expression of *zmprx70* could be detected.

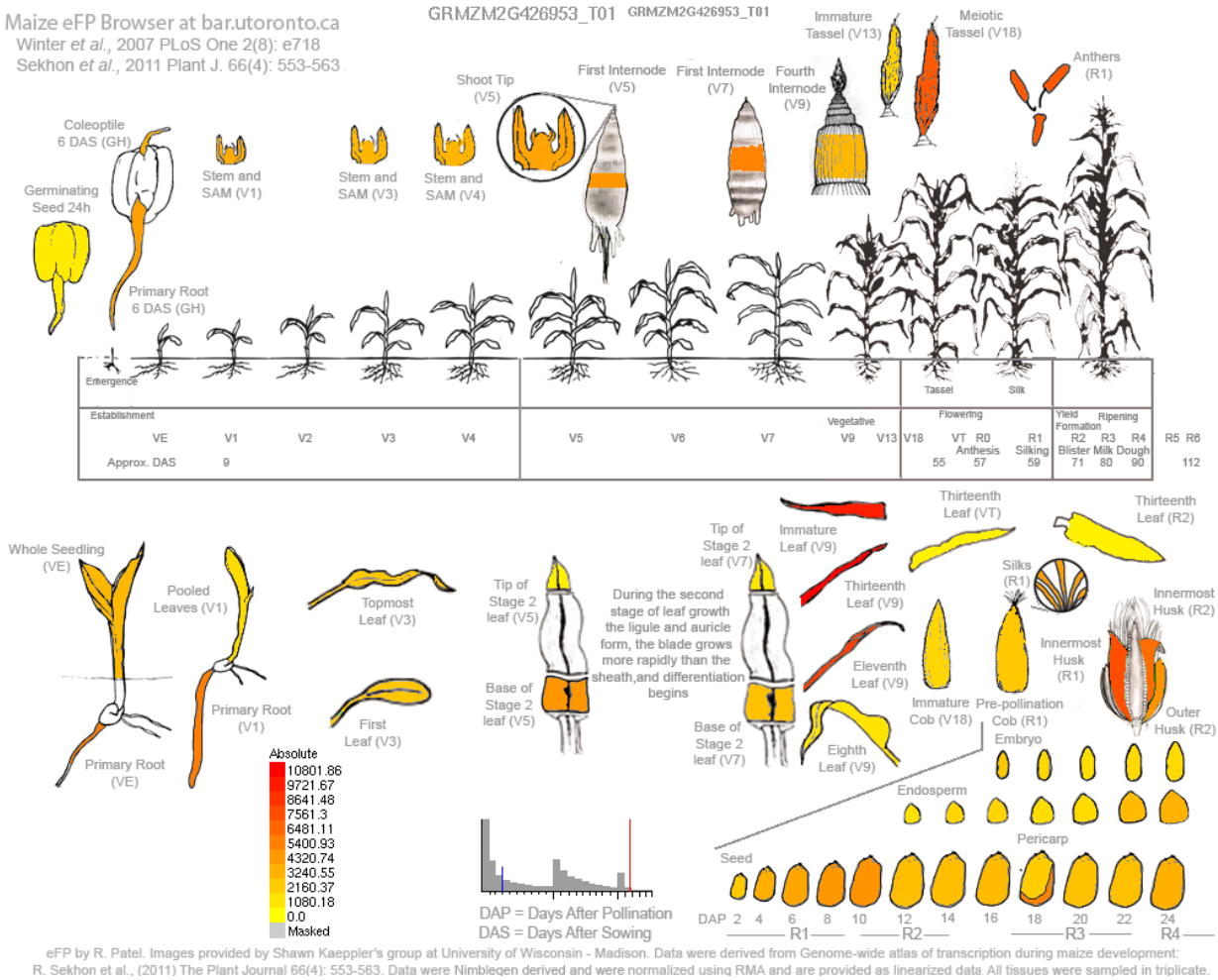

Figure 40: Maize eFP Browser for *rbohA*. The eFP Browser shows the location of the expression of *rbohA* in dependence of developmental stage. The data is gained from different works. Short the expression intensity is marked in a gradient (red – the most, yellow – no expression detected). *rbohA* was expressed in roots and leaves. Mostly it was expressed in meiotic tassels, anthers and immature leaves. Additionally it was highly expressed in the thirteenth leaf V9 and eleventh leaf V9 and as well in the innermost husk (R2). It was expressed in all different tested developmental stages of the seed after pollination. It was found in the pericarp. In the embryo and endosperm and in pre-pollinated cob (R1) it could be detected. *rbohA* was found in the shoot tip and internodes. This gene was found in almost every tissue but not in germinating seeds within 24 h as well thirteenth leaf (VT) and thirteenth leaf (R2) it was not detected.

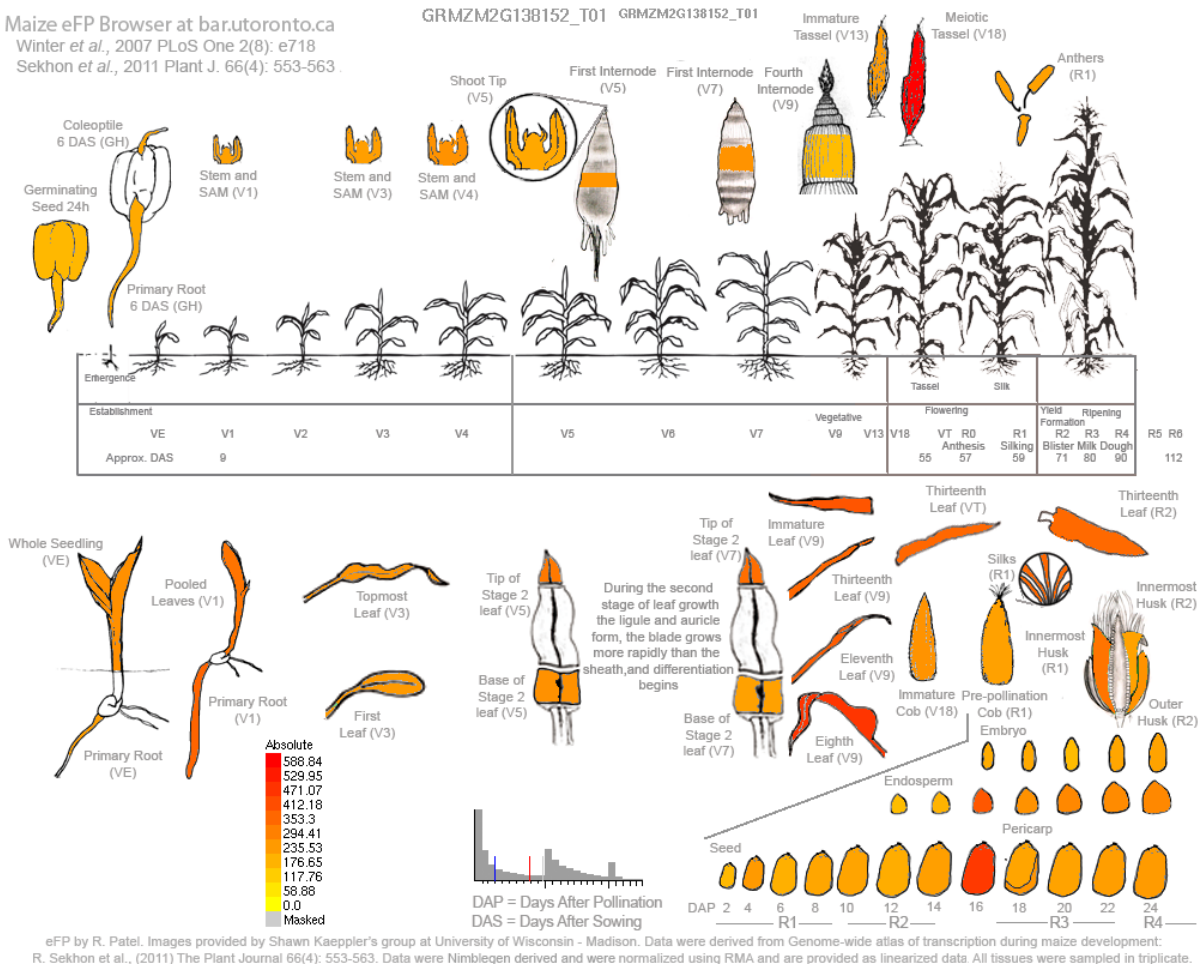

Figure 41: Maize eFP Browser for *rbohB*. The eFP Browser shows the location of the expression of *rbohB* in dependence of developmental stage. The data is gained from different works. Short the expression intensity is marked in a gradient (red – the most, yellow – no expression detected). *rbohB* was expressed in all tissue tested and mostly it was medium to high expressed. Mostly *rbohB* was expressed in meiotic tassels and in the eighth leaf (V9). Remarkably, it was found in the seeds and endosperm in all tested stages but with a significant increase after 16 days (peak) of pollination.

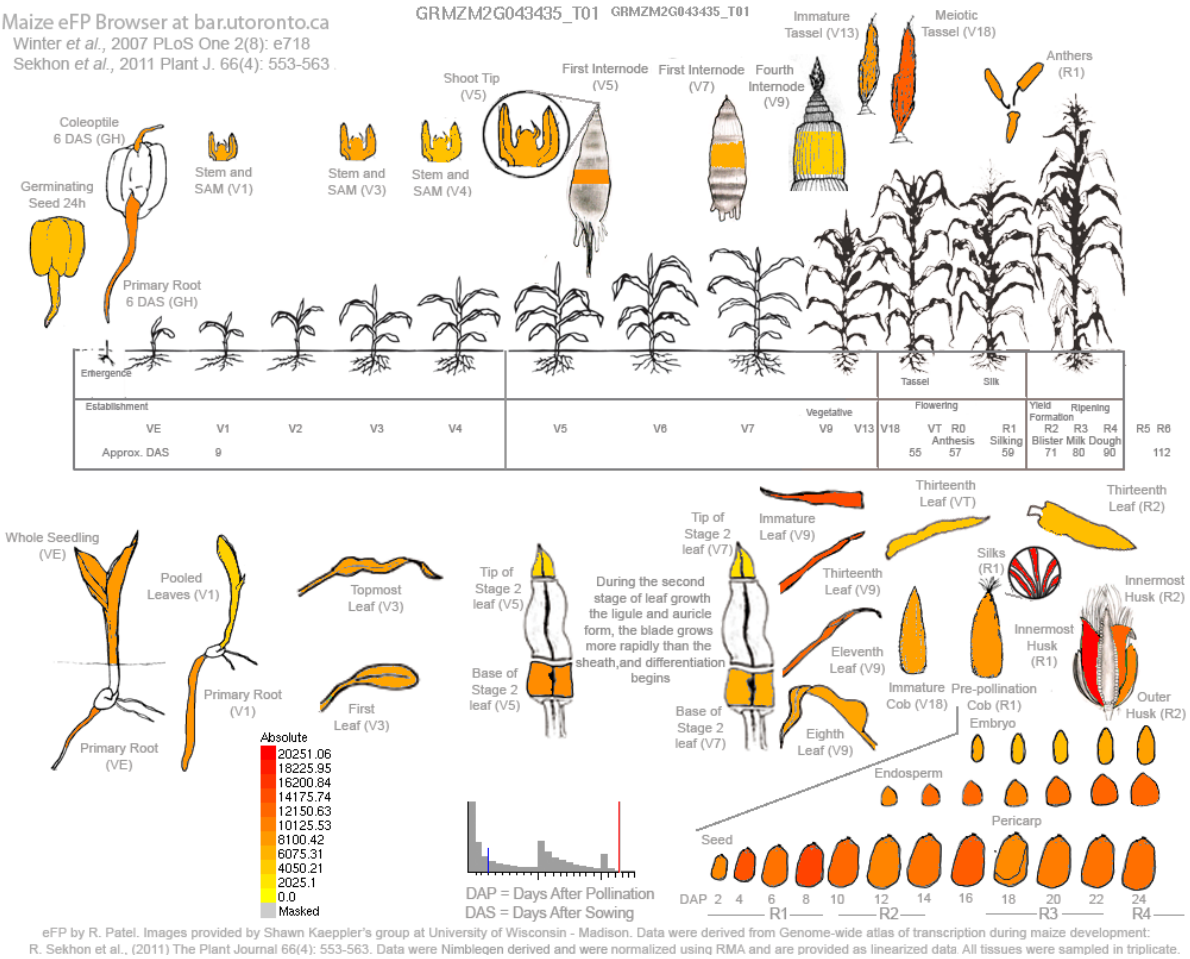

Figure 42: Maize eFP Browser for *rbohC*. The eFP Browser shows the location of the expression of *rbohC* in dependence of developmental stage. The data is gained from different works. Short the expression intensity is marked in a gradient (red – the most, yellow – no expression detected). *rbohC* was found in every tissue tested. It was medium expressed in roots. Mostly expressed it was in seeds and the regenerative organs like silks and meiotic tassels. It was highly expressed in the innermost husk (R1). In immature leaves (V9) and in the thirteenth leaf (V9) it was expressed highly.

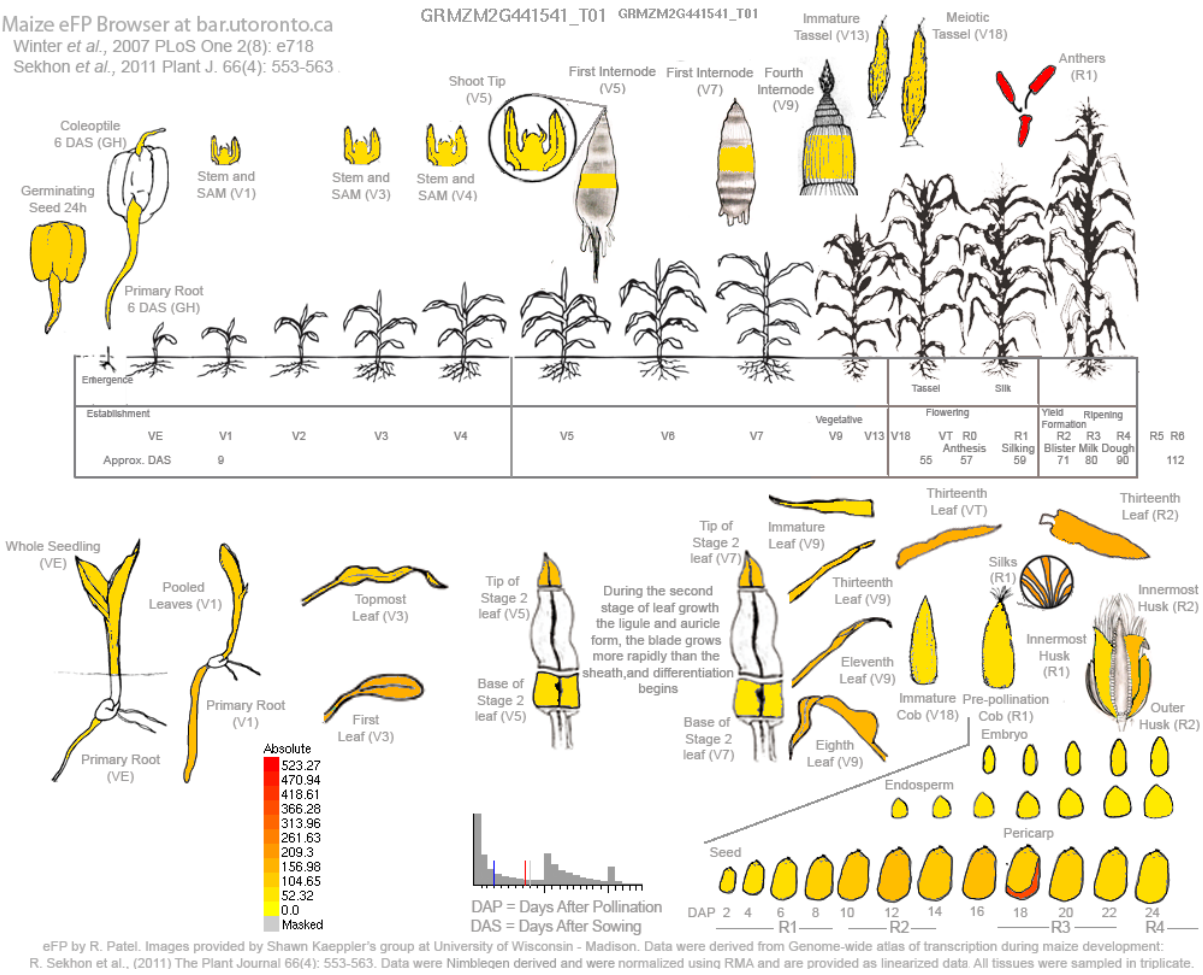

Figure 43: Maize eFP Browser for *rbohD*. The eFP Browser shows the location of the expression of *rbohD* in dependence of developmental stage. The data is gained from different works. Short the expression intensity is marked in a gradient (red – the most, yellow – no expression detected). In comparison to the other Rboh, *rbohD* is very low expressed in all tissues tested. With distance it was highly expressed in the anthers and in the pericarp. In all other tissue it was weakly expressed. At all it is expressed in roots and leaves.

8.3 Height measurements

In the following figure the height measurements of transgenic plants T1 generation after heat shock for RNAi *zmprx01*, RNAi *zmprx70* and wild type as control are displayed.

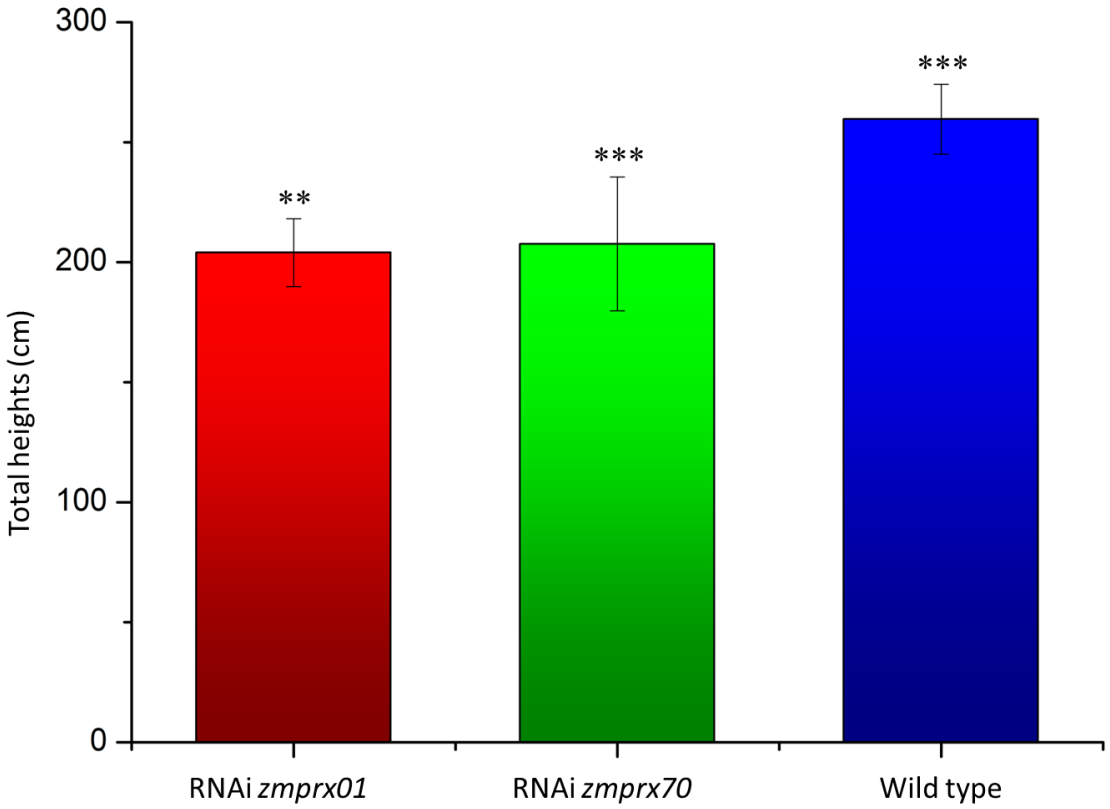

Figure 44: Rating of transgenic plants (T1 generation after heat shock/RNAi *zmprx01*, RNAi*zmprx70* and wild type as control). Heights were measured of adult plants. For each value standard deviation and significances are shown (*<0.05; **<0.005; *<0.001). For the complete rating data for T1 generation after heat shock see table 18 (page 138).**

8.4 Overview of new produced maize strains

Table 16: Overview of all maize strains generated in this project (T0 generation). X=ok / ¤=self-pollinated / ¬= cross-pollinated / A=with HilIA pollen /slashed = not available anymore.

	Plant ID	Basta test 1, 2, 3	pollinated	Material sampled DNA RNA	gDNA	PCR	Southern blot (single Cut BamHI /amount of integrations [abbreviation])	RNA	cDNA	Seed stock	Heat shocked	info
~~1~~	~~pmpox 1 RNAi -1~~	~~x~~	~~A~~	~~xx~~	~~x~~	~~x~~	~~1[SB3]~~	~~x~~	~~x~~	~~no seeds~~		~~no seeds~~
2	1 2	x	¤	xx	x	x	1[SB3]	x	x	x	x	
~~3~~	~~1 3~~	~~x~~		~~xx~~	~~x~~	~~x~~	~~0[SB13]~~	~~x~~	~~x~~			
~~4~~	~~1 4~~	~~x~~	~~¤~~	~~xx~~	~~x~~	~~x~~	~~1[SB13]~~	~~x~~	~~x~~	~~x~~	~~x~~	~~empty~~
5	1 5	x	¤	xx	x	x	1[SB13]	x	x	x	x	less
6	1 6	x	xA	xx	x	x	2[SB3]	x		x		
7	1 7	x	xA	xx	x	x	1[SB3]	x		x	x	
8	1 8	x	+A	xx	x	x	2[SB3]	x		x		
9	1 9	x	xA	xx	x	x	1[SB3]	x		x	x	
10	1 10	x	¤	xx	x	x	1[SB3]	x		x		
11	1 11	x	¤	xx	x	x	3[SB3]	x		x		
12	1 12	x	xA	xx	x	x	3[SB3]	x		x		

	Plant ID	Basta test 1, 2, 3	pollinated	Material sampled DNA RNA	gDNA	PCR	Southern blot (single Cut BamHI /amount of integrations [abbreviation])	RNA	cDNA	Seed stock	Heat shocked	info
13	1 13	x	xA	xx	x	x	1[SB4]	x		x		
14	1 14	x	(A)	xx	x	x	0[SB13]	x		x		
~~15~~	~~1 15~~	~~x~~		~~xx~~	~~x~~	~~x~~	~~1[SB4]~~	~~x~~				~~no seeds~~
16	1 16	x	¤	xx	x	x	2[SB4]	x		x		
17	1 17	x	¤	xx	x	x	1[SB4]	x		x		
18	1 18	x	¤	xx	x	x	0[SB13]	x		x		
~~19~~	~~1 19~~	~~x~~		~~xx~~	~~x~~		~~2[SB13]~~					
~~20~~	~~1 20~~	~~x~~		~~xx~~	~~x~~		~~1[SB13]~~					~~no seeds~~
21	1 21	x	'+A	xx	x		1[SB4]			x		
~~22~~	~~1 22~~	~~x~~	~~'+A~~	~~xx~~	~~x~~		~~1[SB4]~~			~~x~~		
23	1 23	x	¤	xx	x		2[SB13]			x		
~~24~~	~~1 24~~	~~x~~		~~xx~~	~~x~~		~~1[SB13]~~					
25	1 25	x	¤	xx	x		2[SB5]			x		
26	1 26	x	¤	xx	x		1[SB5]			x		
27	1 27	x	'+A	xx	x		0[SB13]			x		
28	1 28	x	?A	xx	x		0[SB13]			x		

	Plant ID	Basta test 1, 2, 3	pollinated	Material sampled DNA RNA	gDNA	PCR	Southern blot (single Cut BamHI /amount of integrations [abbreviation])	RNA	cDNA	Seed stock	Heat shocked	info
29	1 29	x	xA	xx	x		1[SB5]			x		
30	1 30	x	¤	xx	x		1[SB5]			x		
31	1 31	x	¤	xx	x		1[SB5]			x		
32	1 32	x	'+A	xx	x		1[SB5]			x		
33	1 33	x	'+A	xx	x		1[SB5]			x		
34	1 34	x	xA	xx	x		3[SB5]			x		
35	1 35	x	'+A	xx	x		1[SB5]			x		
36	1 36	x	'+A	xx	x		1[SB5]			x		
37	1 37	x	'+A	xx	x		1[SB6]			x		
38	1 38	x	¤	xx	x		0[SB13]			x		
39	1 39	x	¤	xx	x		[SB6]			x		
40	1 40	x	¤	xx	x		[SB6]			x		
41	1 41	x	¤	xx	x		[SB6]			x		
42	1 42	x	¤	xx	x		[SB6]			x		
~~43~~	~~1 43~~	~~x~~	¤	~~xx~~	~~x~~		~~[SB6]~~					
44	1 44	x	¤	xx	x		1[SB6]			x	x	

	Plant ID	Basta test 1, 2, 3	pollinated	Material sampled DNA RNA	gDNA	PCR	Southern blot (single Cut BamHI /amount of integrations [abbreviation])	RNA	cDNA	Seed stock	Heat shocked	info
45	1 45	x	¤	xx	x		1[SB6]			x	x	
46	1 46	x	¤	xx	x		[SB6]			x		
47	1 47	x	¤	xx	x		1[SB6]			x	x	
48	1 48	x	A	xx	x		1[SB6]			x	x	
49	1 49	x	'+A	xx	x		[SB7]			x		
50	1 50	x	¤	xx	x		[SB7]			x		
51	1 51	x	'+A	xx	x		[SB7]			x		
52	1 52	x	¤	xx	x		2[SB7]			x		less
53	1 53	x	¤	xx	x		1[SB7]			x		
54	1 54	x	B	xx	x		1[SB7]			x		
55	1 55	x	'+A	xx	x		1[SB7]			x		
56	1 56	x	¤	xx	x		[SB7]			x		
57	1 57	x	¤	xx	x		1[SB7]			x		
~~58~~	~~1 58~~	~~x~~		~~xx~~	~~x~~		~~1[SB7]~~					~~no seeds~~
59	1 59	x	'+A	xx	x		[SB7]			x		
60	1 60	x	'+A	xx	x		1[SB7]			x		

	Plant ID	Basta test 1, 2, 3	pollinated	Material sampled DNA RNA	gDNA	PCR	Southern blot (single Cut BamHI /amount of integrations [abbreviation])	RNA	cDNA	Seed stock	Heat shocked	info
61	1 61	x	'+A	xx	x		[SB8]			x		
62	1 62	x	A	xx	x		[SB8]			x		less
63	1 63	x	¤	xx	x		[SB8]			x		
~~64~~	~~1 64~~	~~x~~		~~xx~~	~~x~~		~~[SB8]~~					
~~65~~	~~1 65~~	~~x~~		~~xx~~	~~x~~		~~[SB8]~~					~~no seeds~~
66	1 66	x	¤	xx	x		[SB8]			x		
~~67~~	~~1 67~~	~~x~~		~~xx~~	~~x~~		~~[SB8]~~					~~no seeds~~
68	1 68	x	B	xx	x		[SB8]			x		
69	1 69	x	¤	xx	x		[SB8]			x		
70	1 70	x	¤	xx	x		[SB8]			x		
71	1 71	x	¤	xx	x		[SB8]			x		
72	1 72	x	A	xx	x		[SB8]			x		
~~73~~	~~1 73~~	~~x~~		~~xx~~	~~x~~		~~[SB9]~~					~~no seeds~~
74	1 74	x	A	xx	x		[SB9]			x		less
~~75~~	~~1 75~~	~~x~~		~~xx~~	~~x~~		~~1[SB9]~~					~~no seeds~~
~~76~~	~~1 76~~	~~x~~		~~xx~~	~~x~~		~~[SB9]~~					~~no seeds~~

	Plant ID	Basta test 1, 2, 3	pollinated	Material sampled DNA RNA	gDNA	PCR	Southern blot (single Cut BamHI /amount of integrations [abbreviation])	RNA	cDNA	Seed stock	Heat shocked	info
77	1 77	x	¤	xx	x		[SB9]			x		
~~78~~	~~1 78~~	~~x~~		~~xx~~	~~x~~		~~[SB9]~~					~~no seeds~~
79	1 79	x	¤	xx	x		1[SB9]			x		
80	1 80	x	A	xx	x		[SB9]			x		
81	1 81	x	¤	xx	x		[SB9]			x		
~~82~~	~~1 82~~	~~x~~		~~xx~~	~~x~~		~~[SB9]~~					~~no seeds~~
83	1 83	x	¤	xx	x		1[SB9]			x		
84	1 84	x	¤'+A	xx	x		1[SB9]					
85	1 85	x	¤'+A	xx	x		[SB10]			x		
~~86~~	~~1 86~~	~~x~~		~~xx~~	~~x~~		~~[SB10]~~					~~no seeds~~
87	1 87	x	¤	xx	x		[SB10]			x		
88	1 88	x	¤	xx	x		[SB10]			x		
89	1 89	x	¤	xx	x		1[SB10]			x		
90	1 90	x	¤	xx	x		1[SB10]			x		clonal
91	1 91	x	¤	xx	x		1[SB10]			x		
~~92~~	~~1 92~~	~~x~~		~~xx~~	~~x~~		~~[SB10]~~					~~no seeds~~

	Plant ID	Basta test 1, 2, 3	pollinated	Material sampled DNA RNA	gDNA	PCR	Southern blot (single Cut BamHI /amount of integrations [abbreviation])	RNA	cDNA	Seed stock	Heat shocked	info
93	1 93	x	¤	xx	x		1[SB10]			x		
94	1 94	x	A	xx	x		[SB10]			x		
95	HiIIAxPox1-15									x		
96	pmpoxRNAi 2b-1	x	¤	xx	x	x	1[SB1]	x	x	xx	xx	
97	2b-2	x	A	xx	x	x	2[SB1]	x	x	x	x	
98	2b-3	x	¤	xx	x	x	0[SB1]	x	x	x	x	
99	2b-3b	x	A	xx	x	x	1[SB1]	x	x	x	x	
100	2b-4	x	¤	xx	x	x	2[SB1]	x	x	x	x	less
101	2b-5	x	A	xx	x	x	1[SB1]	x	x	x	x	
102	2b-5x	x	A	xx	x	x	2[SB1]	x	x	x	x	
103	2b-6	x	¤ ¬	xx	x	x	1[SB1]	x	x	xx	xx	
104	2b-7	x	B	xx	x	x	1[SB1]	x	x	x	xx	2b7 x A empty
105	2b-8	x	A	xx	x	x	1[SB1]	x	x	xx	xx	
~~106~~	~~2b-9~~	~~x~~	~~A~~	~~xx~~	~~x~~	~~x~~	~~2[SB1]~~	~~x~~	~~x~~			
~~107~~	~~2b-10~~	~~x~~	~~A~~	~~xx~~	~~x~~	~~x~~	~~1[SB1]~~	~~x~~	~~x~~	~~x~~	~~x~~	~~empty~~

	Plant ID	Basta test 1, 2, 3	pollinated	Material sampled DNA RNA	gDNA	PCR	Southern blot (single Cut BamHI /amount of integrations [abbreviation])	RNA	cDNA	Seed stock	Heat shocked	info
108	2b-11	x	¤	xx	x	x	1[SB12]	x	x	x	x	
109	2b-12	x	¤ A							xx	xx	
~~110~~	~~2b-13~~	~~x~~	~~A~~	~~xx~~	~~x~~	~~x~~	~~1[SB12]~~	~~x~~	~~x~~			
111	2b-13x	x	A	xx	x	x	1[SB12]	x	x	x	x	clonal
~~112~~	~~2b-13x2~~	~~x~~	~~¤~~	~~xx~~	~~x~~	~~x~~	~~1[SB12]~~	~~x~~	~~x~~	~~less~~	~~x~~	
~~113~~	~~2b-14~~	~~x~~	~~A~~	~~xx~~	~~x~~	~~x~~	~~1[SB12]~~	~~x~~	~~x~~	~~x~~	~~x~~	~~less~~
114	2b-15	x	A	xx	x	x	1[SB12]	x	x	xx	xx	
115	2b-16	x	xA	xx	x	x	2[SB12]	x	x	xx	xx	clonal
116	2b-17	x	A		x	x	2[SB12]	x	x	x	x	
117	2b-18	x	A	xx	x	x	1[SB12]	x	x	x	x	
~~118~~	~~2b-19~~	~~x~~	~~A~~	~~xx~~	~~x~~	~~x~~	~~1-2[SB12]~~	~~x~~	~~x~~			
119	2b-20	x	A	xx	x	x	1[SB12]	x	x	x	x	
~~120~~	~~2b-21~~	~~x~~		~~xx~~	~~x~~	~~x~~	~~1[SB12]~~	~~x~~	~~x~~			
121	PmPOX RNAi 3-2 1	x	¬ auf hi A	xx	x	x	[SB10]	x		x		
~~122~~	~~3-2 2~~	~~x~~		~~xx~~	~~x~~	~~x~~	~~1[SB10]~~	~~x~~				~~no seeds~~

	Plant ID	Basta test 1, 2, 3	pollinated	Material sampled DNA RNA	gDNA	PCR	Southern blot (single Cut BamHI /amount of integrations [abbreviation])	RNA	cDNA	Seed stock	Heat shocked	info
123	3-2 3	x	x A	xx	x		[SB11]			x		
124	3-2 4	x	¤	xx	x		1[SB11]			x		
125	3-2 5	x	¤	xx	x		[SB11]			x		
126	3-2 6	x	x A	xx	x		[SB11]			x		
127	3-2 7	x	'+A	xx	x		[SB11]			x		clonal to 8
128	3-2 8	x	¤	xx	x		[SB11]			x		clonal to 7
129	3-2 9	x	'+A	xx	x		[SB11]			x		
130	3-2 10a	x	¤	xx	x		[SB11]			x		
131	3-2 10b	x	¤									~~no seeds~~
~~132~~	~~3-2 11~~	~~x~~		~~xx~~	~~x~~		~~[SB11]~~					~~no seeds~~
133	3-2 12	x	A	xx	x		[SB11]			x		
134	3-2 13	x	¤	xx	x		[SB11]			x		
~~135~~	~~3-2 14~~	~~x~~		~~xx~~	~~x~~		~~1[SB11]~~					~~no seeds~~
136	3-2 15	x	¤							x		less
137	HiIIA x 3-2 10	x								x		
138	HiIIA x 3-2 2	x								x		

	Plant ID	Basta test 1, 2, 3	pollinated	Material sampled DNA RNA	gDNA	PCR	Southern blot (single Cut BamHI /amount of integrations [abbreviation])	RNA	cDNA	Seed stock	Heat shocked	info
139	HiIIA x 3-2 1	x								x		

Table 17: Documentation of all plants generated in this project (T1 generation). X=ok / ¤=self-pollinated.

Seed ID	Basta test 1, 2, 3	pollinated	Material sampled DNA / RNA
2b 1 1	xxx	¤	xx
2b 1 2	xxx		xx
2b 1 3	xxx		xx
2b 1 4	xxx		xx
2b 10 x A	xxx		xx
2b 11 ¤ 1	xxx		xx
2b 11 ¤ 2	xxx		xx
2b 11 ¤ 3	xxx		xx
2b 11 ¤ 4	xxx		xx
2b 12 ¤ 1	xxx		xx
2b 12 ¤ 3	xxx		xx
2b 12 ¤ 4	xxx	¤	xx
2b 12 ¤ 5	xxx	¤	xx
2b 12 ¤ 7	xxx		xx
2b 12 ¤ 8	xxx	¤	xx
2b 12 ¤ 9	xxx		xx
2b 12 ¤ t1 2	xxx		xx
2b 12 6	xxx		xx
2b 13 x x A 1	xxx		xx
2b 13 x x A 2	xxx	¤	xx
2b 13 x x A 3	xxx	¤	xx
2b 15 1	xxx		xx
2b 15 2	xxx		xx
2b 15 3	xxx	¤	xx
2b 15 4	xxx	¤	xx
2b 15 5	xxx		xx
2b 15 x A 1	xxx	¤	xx
2b 15 x A 2	xxx		xx
2b 15 x A 3	xxx		xx
2b 15 x A 4	xxx	¤	xx
2b 15 x A 5	xxx		xx
2b 17 1	xxx	¤	xx
2b 17 3	xxx	¤	xx
2b 17 4	xxx	¤	xx
2b 17 5	xxx		xx
2b 2 1	xxx	¤	xx
2b 2 2	xxx		xx
2b 2 3	xxx		xx
2b 2 4	xxx		xx
2b 2 5	xxx		xx
2b 20 1	xxx	¤	xx
2b 20 2	xxx		xx
2b 5	xxx	¤	xx
2b 5 x x A 1	xxx		xx
2b 5 x x A 2	xxx	¤	xx
2b 6 1	xxx	¤	xx
2b 6 3	xxx	¤	xx
2b 6 4	xxx	¤	xx
2b 7 x B 1	xxx	¤	xx
2b 7 x B 2	xxx	¤	xx
2b 7 x B 3	xxx	¤	xx
2b 7 x B 4	xxx	¤	xx
2b 8 1	xxx	¤	xx
2b 8 2	xxx	¤	xx
2b 8 3	xxx	¤	xx
2b 8 4	xxx	¤	xx
A x 2b 18 1	xxx	¤	xx

Seed ID	Basta test 1, 2, 3	pollinated	Material sampled DNA / RNA
A x 2b 6 2	xxx		xx
A x 2b 8 1	xxx	¤	xx
A x 2b 8 2	xxx	¤	xx
A x 2b 8 3	xxx	¤	xx
A x 2b 8 4	xxx	¤	xx
1 5 3		¤	
1 5 ¤ 3	x		xx
1 5 ¤ 2	x		xx
1 5 1	x		xx

8.5 Ratings

Table 18: Complete plant rating may, 18th – 19th 2011 T1 generation after heat shock.

	Plant ID	height [cm]	Amount nodes	Internode size [cm]	Amount cob	stamina (more - less)	Male infertility	Distinctive feature	Amount leaves
1	2b 1 1	210	9	20	1	o	no	no	9
2	2b 1 2	230	12	19	2	o	no	no	12
3	2b 1 3	213	11	22	1	o	no	no	11
4	2b 1 4	224	10	20	1	o	no	no	10
5	2b 10 x A	225	12	19	1	o	no	no	12
6	2b 11 ¤ 1	241	12	20	1	o	no	no	12
7	2b 11 ¤ 2	193	10	24	1	o	no	no	10
8	2b 11 ¤ 3	177	12	14	1	o	no	no	12
9	2b 11 ¤ 4	240	12	19	1	o	no	no	12
10	2b 12 ¤ 1	205	12	14	1	o	no	no	12
11	2b 12 ¤ 3	228	13	17	1	o	no	no	13
12	2b 12 ¤ 4	230	12	18	1	o	no	no	12
13	2b 12 ¤ 5	209	13	18	1	o	no	no	13
14	2b 12 ¤ 7	148	10	16	0	o	no	no	10
15	2b 12 ¤ 8	162	11	16	1	less	no	no	11
16	2b 12 ¤ 9	193	12	14	1	o	no	no	12
17	2b 12 ¤ t1 2	155	14	10	0	o	no	Twisted leaves	14
18	2b 12 6	174	14	15	1	less	no	no	14
19	2b 13 x x A 1	241	12	21	1	o	no	no	12
20	2b 13 x x A 2	254	17	17	1	o	no	no	17
21	2b 13 x x A 3	235	14	18	1	less	no	no	14
22	2b 15 1	194	9	24	1	o	no	no	9
23	2b 15 2	180	12	18	1	o	no	no	12
24	2b 15 3	215	13	18	1	o	no	no	13

	Plant ID	height [cm]	Amount nodes	Internode size [cm]	Amount cob	stamina (more - less)	Male infertility	Distinctive feature	Amount leaves
25	2b 15 4	222	12	18	1	o	no	no	12
26	2b 15 5	222	11	17	1	o	no	no	11
27	2b 15 x A 1	159	11	19	2	no Pollen	yes	no	11
28	2b 15 x A 2	228	13	20	1	less	no	no	13
29	2b 15 x A 3	219	14	16	1	o	no	no	14
30	2b 15 x A 4	194	12	20	1	less	no	no	12
31	2b 15 x A 5	225	14	11	1	o	no	no	14
32	2b 17 1	225	15	15	1	o	no	no	15
33	2b 17 3	245	12	20	1	o	no	no	12
34	2b 17 4	226	14	19	1	o	no	no	14
35	2b 17 5	219	10	18	1.5	o	no	adventive Cob through leaf	10
36	2b 2 1	220	15	15	1	less	no	no	15
37	2b 2 2	219.5	14	16.5	1	less	no	no	14
38	2b 2 3	126	9	16	1	very less	no	no	9
39	2b 2 4	233	13	20	1	less	no	no	13
40	2b 2 5	210	11	22	1	o	no	no	11
41	2b 20 1	240	15	16	1	o	no	no	15
42	2b 20 2	270	16	18	1	o	no	no	16
43	2b 5	147	9	14	1	o	no	no	9
44	2b 5 x x A 1	232	12	23	1	o	no	no	12
45	2b 5 x x A 2	261	11	19	1	o	no	no	11
46	2b 6 1	262	13	19	1	o	no	no	12
47	2b 6 3	166	13	12	1	o	no	no	13
48	2b 6 4	263	12	24	1	o	no	no	12
49	2b 7 x B 1	202	11	17	1	o	no	no	11
50	2b 7 x B 2	180	11	16	1	less	no	Small stamina	10
51	2b 7 x B 3	210	8	19	1	o	no	no	8
52	2b 7 x B 4	208	11	20.5	1	less	no	no	11
53	2b 8 1	211	11	22	2	o	no	no	11
54	2b 8 2	236	14	20	2	less	no	no	14
55	2b 8 3	254	13	21	1	o	no	no	13
56	2b 8 4	215	11	16	1	o	no	no	11
57	A x 2b 18 1	152	8	18	1	less	no	deformed stamina	8
58	A x 2b 6 2	230	12	17	1	o	no	no	12
59	A x 2b 8 1	210	13	15	1	o	no	Stamina shortened	13
60	A x 2b 8 2	224	12	18	1	o	no	First leaf shortened	13
61	A x 2b 8 3	200	10	20	1	o	no	Stamina shortened	10
62	A x 2b 8 4	200	15	15	2	less	no	Stamina shortened	15
63	1 5 ¤ 3	189	8	14.5	1	o	no	no	8
64	1 5 ¤ 2	200	10	19	1	less	no	no	10

	Plant ID	height [cm]	Amount nodes	Internode size [cm]	Amount cob	stamina (more - less)	Male infertility	Distinctive feature	Amount leaves
65	1 5 1	223	13	18	1	o	no	Delayed in development / more vital but same age as the others	13
66	WT	255	13	22	1	less	no	no	13
67	WT	286	14	24	2	o	no	no	14
68	WT	250	13	20	1	o	no	no	13
69	WT	263	11	21	1	o	no	no	11
70	WT	244	11	20	1	less	no	no	11
71	WT	232	13	17	1	less	no	no	13

Table 19: Complete plant rating may, 23th 2013 T1 generation after heat shock.

	Plant ID	height[cm]	amount leaves	size internode [cm]	amount cob	Stamina (more - less)	male infertility	Distinctive feature	stem calibre [cm]
1	HS WT 4	200	11	14	1	less	-	-	1
2	HS 3-2 7 1	215	14	15.5	1	normal	-	-	2
3	HS 1 66 3	215	14	13	1	less	-	-	1.6
4	K 3-2 7 1	219	10	15.5	1	more	-	-	1.6
5	HS 3-2 1 8	162	10	13	1	normal	-	-	1.4
6	HS 3-2 8 3	232	12	15	1	normal	-	-	1.9
7	K 1 6 2	249	14	14	3	normal	-	-	1.4
8	HS 3-2 8 2	260	10	16	1	normal	-	-	1.3
9	HS 3-2 7 2	210	12	14.5	1	more	-	-	1.7
10	HS WT 1	233	10	15	1	normal	-	-	1.7
11	K 3-2 7 2	185	10	13	1	normal	-	chlorothic	1.5
12	1 45 HS 3	162	11	13	1	less	-	-	1.4
13	T1 1 53 1	187	12	11	1	less	-	-	1
14	1 45 hs 1	220	11	12	1	less	-	-	1.1
15	3-2 4 3	264	13	14	1	normal	-	-	1.3
16	2b 13x hs2	195	11	12	1	less	-	-	0.8
17	1 44 HS 6	227	13	12	1	normal	-	-	1.3
18	2b 13x hs 1	182	12	8	-	less	-	-	0.9
19	1 9 1	257	13	11	1	normal	-	-	1.3
20	1 47 hs5	227	12	12	1	normal	-	-	1.2
21	3-2 10a 6	224	10	15	1	normal	-	-	1
22	3-2 10a 5	194	11	10	-	normal	-	-	1.1
23	1 47 5	220	10	12	-	less	-	-	1.1
24	1 45 hs5	209	11	11.5	-	normal	-	-	1.2
25	2b 1 hs 1	262	9	14	1	normal	-	-	1.2
26	2b 1 hs 4	215	10	13	1	normal	-	-	1.3

	Plant ID	height[cm]	amount leaves	size internode [cm]	amount cob	Stamina (more - less)	male infertility	Distinctive feature	stem calibre [cm]
27	2b 13x hs3	265	10	14	1	less	-	-	1.2
28	1 44 hs 1	265	13	15	2	normal	-	-	1.6
29	2b 6 hs 4	177	11	11	-	-	positive	-	1.1
30	2b 11 hs 2	159	9	11	1	-	positive	-	0.7
31	1 45 hs 2	126	10	10	-	-	positive	-	1
32	2b 11 hs 5	136	11	7.5	-	less	-	-	1
33	T1 1 53 2	138	9	11.3	-	less	-	-	0.7
34	3-2 4 hs 4	235	12	14	2	normal	-	-	2
35	WT	243	11	16	1	normal	-	-	1.3
36	WT	234	10	14.5	1	less	-	-	1.2
37	WT	257	12	13.5	1	more	-	-	1.5
38	3-2 10a hs 2	188	11	12.5	1	normal	-	-	1.8
39	3-2 4 hs 3	222	12	12.5	1	normal	-	-	1.5
40	3-2 4 hs 2	260	12	15	1	normal	-	-	1.7
41	1 44 hs 4	49	11	3.5	1	-	positive	-	0.6
42	WT	280	12	13	1	less	-	-	0.9
43	WT HS	248	12	15	1	less	-	-	1.1
44	1 7 2	232	9	13	1	normal	-	-	1.1
45	1 7 2 b	228	12	11	1	less	-	-	1.1
46	1 7 1	234	12	12	1	less	-	-	1.2
47	1 47 2	244	13	12	1	less	-	-	1.1
48	1 47 6	234	13	15	1	normal	-	-	1.2
49	3-2 10a 4	240	10	14	1	less	-	-	1.4
50	1 44 hs 3	257	13	14	1	less	-	-	1.1
51	3-2 10a 3	280	13	16	1	normal	-	-	1.3
52	3-2 10a 2	232	10	13	1	normal	-	-	1.3
53	1 47 hs 4	265	12	17	1	less	-	-	1.5
54	3-2 10a 8	230	10	14	-	normal	-	-	2
55	WT HS	250	10	15	1	less	-	-	0.9
56	Ax2b 18 1	247	10	10	1	-	positive	-	0.8
57	1 44 1	257	12	14	1	less	-	-	1.3
58	1 44 3	243	13	13	1	normal	-	-	1.4
59	3-2 4 2	238	11	14	1	normal	-	-	1.5
60	1 44 hs 2	209	11	12.5	1	less	-	-	1.3
61	1 47 hs 3	248	11	13	2	normal	-	-	1.1
62	1 47 hs 1	290	12	16	1	normal	-	-	1.3
63	3-2 10a 9	220	10	13	1	normal	-	-	1.2
64	1 47 3	247	10	16	1	more	-	-	1.3
65	3-2 5 2	241	10	14	1	more	-	-	1.6
66	3-2 5 1	247	10	16	1	normal	-	-	1.2
67	3-2 10a 1	247	12	15	1	less	-	-	1.6

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Eidesstattliche Versicherung
Declaration on oath

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and help.

François Clement Perrineau