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Modulation of energy homeostasis in maize and Arabidopsis to develop lines tolerant to drought, genotoxic and oxidative stresses

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TABLE OF CONTENTS

SCOPE AND AIMS

Plants growing in their natural environment are continuously exposed to biotic and abiotic stresses that adversely affect their growth and reproduction placing a severe threat to sustainable crop production in the changing climatic conditions. Abiotic stresses cause crop losses worldwide reducing average yield by more than 50% due to the high energy consumed to enhance the respiration rates resulting in excessive release of reactive oxygen species that cause the death of the plant cells and ultimately, of the whole plant. A biotechnological approach is taken to generate plants with reduced energy consumption under stress conditions and with improved yield stability particularly using a metabolic engineering strategy. In Arabidopsis and *Brassica napus*, metabolic engineering to broaden stress tolerance in plants by maintaining energy homeostasis under stress conditions was reported to enhance tolerance to drought, high light and heat stresses. When the stress-induced energy consumption was reduced by enhancing the energy use efficiency, the plants could overcome peak stresses or have the opportunity to adapt to moderate but persistent stresses (De Block et al., 2005).

Strong stresses in plants results in excessive production of reactive oxygen species that may lead to DNA damage which in turn activates a poly(ADP-ribose) polymerase (PARP). PARP uses high energy cost Nicotinamide Adenine Dinucleotide (NAD^+) as a substrate to synthesize polymers of ADP-Ribose important in triggering a DNA repair mechanism. PARP is one of the enzymes in the poly(ADP-ribosyl)ation metabolic pathway (PAR) which plays an important role in plant energy homeostasis and is also reported to regulate plant responses to abiotic and biotic stresses. Modulation of the level of an ADP-ribose specific Nudix hydrolase (NUDX), another PAR pathway protein, was reported to confer tolerance to oxidative stress in Arabidopsis through re-establishing the energy levels by supplying an ATP source thus regulating defense mechanism against oxidative DNA damage (Ishikawa et al., 2009).

The main aim of the study was to generate maize and Arabidopsis lines with an altered energy homeostasis to improve tolerance to drought, oxidative and genotoxic stresses through manipulation of the levels of PARP and NUDX. PARP gene activity in maize was knocked-out or downregulated via the CRISPR/CAS9 gene editing system and RNAi hairpin silencing approach respectively. Arabidopsis AtNUDX7 and its two maize homologues were overexpressed in B104 maize using *Brachypodium distachyon* pBDEF1α promoter and maize ubiquitin pZmUBIL promoter. In Arabidopsis, AtNUDX7 and its two maize homologues were overexpressed in Arabidopsis Col-0 wild type and in nudx7 SALK insertion mutant using either cauliflower mosaic virus p35S promoter,

Brachypodium distachyon pBDEF1α promoter or the maize ubiquitin pZmUBIL promoter. Maize and Arabidopsis transgenic lines showing different levels of expression of NUDX or PARP were selected for functional analysis. ZmNUDX Mutator transposon insertion lines and AtNUDX7 T-DNA insertion lines were obtained from the Maize genetics cooperation stock center and the Nottingham seed stock center respectively, characterised and used as control lines. Functional analysis of the generated maize and Arabidopsis lines was carried out using a hydroxyurea induced-DNA damage assay, a paraquat-induced oxidative stress assay and water deficit experiments in automated platforms.

SUMMARY

The main objective of this study was to generate maize and Arabidopsis lines with an altered energy homeostasis with an aim of improving tolerance to drought, oxidative and genotoxic stresses through manipulation of the levels of Poly(ADP-ribose) polymerase (PARP) and Nudix hydrolase proteins (NUDX) using genetic engineering approach. **Chapter 1** is a review of the poly(ADP-ribosyl)ation pathway (PAR) and its role in energy homeostasis and stress response. The PAR pathway proteins PARP, PARG and NUDX are described in details including their Arabidopsis and maize homologues, their protein domain structures, products, substrate and their roles in abiotic, biotic and genotoxic stresses. **Chapter 2** is a review paper that was published in the International Journal of Developmental Biology in September 2013. The principles and molecular tools of genetic transformation are described including shoot regeneration, gene delivery methods, commonly used selectable marker and reporter genes, different types of promoters and the vectors for higher plants transformation. In concluding, the Arabidopsis and maize transformation process at the VIB Center for Plant System Biology (PSB), Department of Plant Biotechnology and Bioinformatics, Ghent University is described. **Chapter 3** gives an overview of some of the genetic techniques used in modulating the Arabidopsis and maize poly(ADP-ribosyl)ation pathway proteins in our study. T-DNA insertional mutagenesis used in generating Arabidopsis mutants is described as well as transposon insertional mutagenesis for maize mutants. Uniform Mu transposon insertion collection is further elaborated giving its advantages over other maize mutant collections. We also detail the latest ground-breaking technology for genome editing using CRISPR/Cas9 that was used in altering ZmPARP catalytic domains. **Chapter 4** focuses on the maize PARP genes and the alteration of their gene expression using RNAi hairpin silencing and CRISPR/Cas9 gene editing system. Characterization and gene expression analysis of the transgenic PARP lines is described. We obtained lines showing 5-6 fold downregulation of the ZmPARP1 gene using RNAi hairpin silencing and more than 200bp deletion in ZmPARP2 catalytic domain on using the CRISPR gene editing system. The lines are analyzed for their genotoxic stress response using the hydroxyurea induced-DNA damage assay. The ZmPARP1 RNAi lines showed a trend of sensitivity to the DNA damage while mixed phenotypes for the ZmPARP2 CRISPR lines were observed, showing the importance of carrying out the assay using lines with stable and uniform CAS9-induced mutation. Evaluation of the lines for water deficit response was carried out together with maize NUDX gene, described in chapter 5 whereby the RNAi PARP lines showed a trend of sensitivity while the CRISPR lines showed a trend of tolerance to water deficit. **Chapter 5** makes a shift to the NUDX genes in which AtNUDX7 and its two maize homologues ZmNUDX2 and ZmNUDX8 were overexpressed using either maize ubiquitin or Brachypodium

pBDEF1α constitutive promoters in maize. In addition, a Mutator transposon insertion line in ZmNUDX8 gene was identified from the Uniform Mu collection. Lines with high, medium and low levels of overexpression were obtained and T3 lines with homozygous insertion of the transgene generated. A selected number of lines were analysed for their paraquat-induced oxidative stress response and water deficit stress responses in an automated platform at PSB-VIB-UGent. One AtNUDX7 OE event resulted in significant tolerance to water deficit stress in one experiment and a strong tendency to water deficit stress tolerance in a second experiment but the OE ZmNUDX events responded like wildtype to water deficit stress. The AtNUDX7 and ZmNUDX2 OE events resulted in sensitivity to paraquat induced-oxidative stress. In **Chapter 6** the AtNUDX7 and its two ZmNUDX homologues are overexpressed in Arabidopsis. The lines were analysed for their yield and yield related-parameters and also functional analysis was carried out for their oxidative and water deficit stress response. We make a first reporting of altered seed yield and yield related parameters in lines with modulated AtNUDX7 gene expression. Several lines overexpressing AtNUDX7 in Col-0 background showed a significant increase in total seed weight, seed number, seed size and mass in addition to early flowering time, reduced rosette leaf number and inflorescent height. Three AtNUDX7 overexpression Arabidopsis lines showed significant tolerance to paraquat induced oxidative stress. In addition an AtNUDX7 and two ZmNUDX Arabidopsis overexpression lines showed a trend of tolerance to mild drought stress in an automated platform at PSB-VIB-UGent. In **Chapter 7** the overall performance of the NUDX and PARP genes in maize and Arabidopsis observed in our study are discussed. Summary tables of the currently published morphological and stress phenotypes on different poly(ADP-ribosl)ation pathway genotypes and a summary table of the phenotypes we obtained in our study are shown. The future perspectives are discussed and an overview of the currently published enzymatic assays in Arabidopsis NUDX and PARP mutants shown.

LIST OF ABBREVIATIONS

ABA: Abscisic Acid

ADP: Adenosine Diphosphate

AMP: Adenosine Monophosphate

ATP: Adenosine Triphosphate

Bar: Bialaphos resistance gene

Cas9: CRISPR-associated protein 9

cDNA: complementary DNA

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

DNA: Deoxyribonucleic acid

EF1α: Elongation Factor 1 alpha

GUS: β-glucuronidase

gRNA: guide RNA

KO: Knockout

mRNA: Messenger RNA

Mu: Mutator

NAD: Nicotinamide Adenine Dinucleotide

NOS: Nopaline Synthase

NUDX: Nucleoside Diphosphate linked to a moiety X

OE: Overexpression

PAR: Poly(ADP-ribosyl)ation

PARP: Poly(ADP-ribose) Polymerase

PARG: Poly(ADP-ribose) Glycohydrolase

PCR: Polymerase Chain Reaction

PQ: Paraquat

PSB: Plant Systems Biology

QPCR: Quantitative PCR

RNA: Ribonucleic acid

RNAi: RNA interference

ROS: Reactive Oxygen Species

RT-PCR: Reverse transcription-polymerase chain reaction

T-DNA: Transfer DNA

UBIL: Maize Long Ubiquitin Promoter

CHAPTER 1

Poly(ADP-ribosyl)ation Pathway in Plant Energy Homeostasis and Stress Response

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Contribution: E.N wrote the chapter, M.V.L read and gave critical comments.

Abstract

Poly(ADP-ribosyl)ation (PAR) is a posttranslational protein modification process in which ADP-ribose subunits from NAD^+ are covalently attached to target proteins, a process mediated by the Poly(ADP-ribose) Polymerase enzyme. PAR activity can be reversed by poly(ADP-ribose) glycohydrolases generating free ADP-ribose molecules that can be degraded into easily recyclable nucleotides, AMP and ribose-5-phosphate, by an ADP-ribose specific Nudix hydrolase enzyme. The AMP produced can be utilised to replenish the ATP and NAD^+ leading to maintenance of cellular homeostasis. This pathway which has been broadly studied in animals is reported to play an important role in DNA repair, genotoxic stress response, chromatin structure, transcription regulation, apoptosis and cell cycle activities. PAR has been implicated in several plant physiological processes and described as an important regulatory mechanism modulating plant responses to abiotic and biotic stresses. This chapter gives an overview of the PAR pathway and its role in energy homeostasis, PAR proteins and their role in plant biotic and abiotic stress response.

1.1 Energy Homeostasis in plants

1.1.1 General background

Plants have to maintain high energy levels to grow and reproduce optimally. However, maintenance of this energy state is a daily challenge since in their natural environment plants need to cope regularly with multiple mild or severe stresses of biotic and abiotic nature consuming a lot of energy in the stress response mechanisms and in their struggle for survival. The decrease in energy content in a cell and in the whole plants can only be tolerated within a narrow range, further drop in the energy content below a certain level results in cellular damage which eventually becomes irreversible causing the death of the plant cell and ultimately the whole plant (De Block and Van Lijsebettens, 2011). High energy consumption enhances the rates of respiration resulting in the production of reactive oxygen species (Rizhsky et al., 2002; Tiwari et al., 2002). It has been shown that strong stresses in plants induce poly(ADP-ribosyl)ation activity causing a breakdown in the Nicotinamide Adenine Dinucleotide (NAD^+) pool and enhanced mitochondrial respiration (De Block et al., 2005). The enzyme responsible, Poly(ADP-ribose) Polymerase (PARP), is activated by DNA damage caused by the free radicals of reactive oxygen species (Vira´g and Szabo´, 2002). PARP uses NAD^+ as a substrate to synthesize polymers of ADP-Ribose on a range of nuclear enzymes. Stress induced depletion of NAD^+ results in a depletion of energy in turn, since ATP molecules are required to resynthesize the depleted NAD^+.

Many strategies to improve tolerance to stress have been developed in plants starting from conventional breeding methods to marker assisted breeding, giving desirable traits such as stress tolerance but they generally take a long time and are limited to the available germplasm. Genetic engineering overcomes fertilization restrictions within plant species and allows overproduction or reduction of specific proteins to improve plant performance in adverse environmental conditions and hence yield. Indeed, plants have been genetically modified to overproduce detoxification enzymes such as superoxide dismutases to scavenge reactive oxygen species, to accumulate osmoprotectants such as glycine betaine or proline in water deficit or salt stress condition, to overproduce abscisic acid, a plant hormone that regulates the adaptive response of plants to environmental stresses such as drought, salinity, and cold (Yang et al., 2010). In this work, we focus on the role of the PAR pathway in energy use efficiency and homeostasis, its role in stress reaction and its potential in generating stress tolerance in plants. Indeed, different reports have shown that when the stress-induced energy consumption is reduced by enhancing the energy use efficiency, the plant could overcome peak stresses or have the opportunity to adapt to moderate but persistent stress (Amor et al., 1998; De Block et al., 2005; Hayashi et al., 2005). De Block et al. (2005) showed

that down-regulation of PARP gene in Arabidopsis and *Brassica napus* by RNAi gene silencing resulted in plants with reduced NAD^+ depletion and ATP consumption and were tolerant to a broad range of abiotic stresses such as high light, drought, and heat. Therefore, breeding or genetic engineering for high energy-use efficiency under stress conditions is a valuable approach to enhance overall stress tolerance of crops. The higher energy-use efficiency avoids the need for a too intense mitochondrial respiration and consequently reduces the formation of reactive oxygen species. Since crop yields are frequently lowered by biotic and abiotic stresses, one of the most effective strategies to improve agricultural output is to breed or engineer plants tolerant of, or resistant to stress.

1.1.2 Poly(ADP-ribosyl)ation Pathway

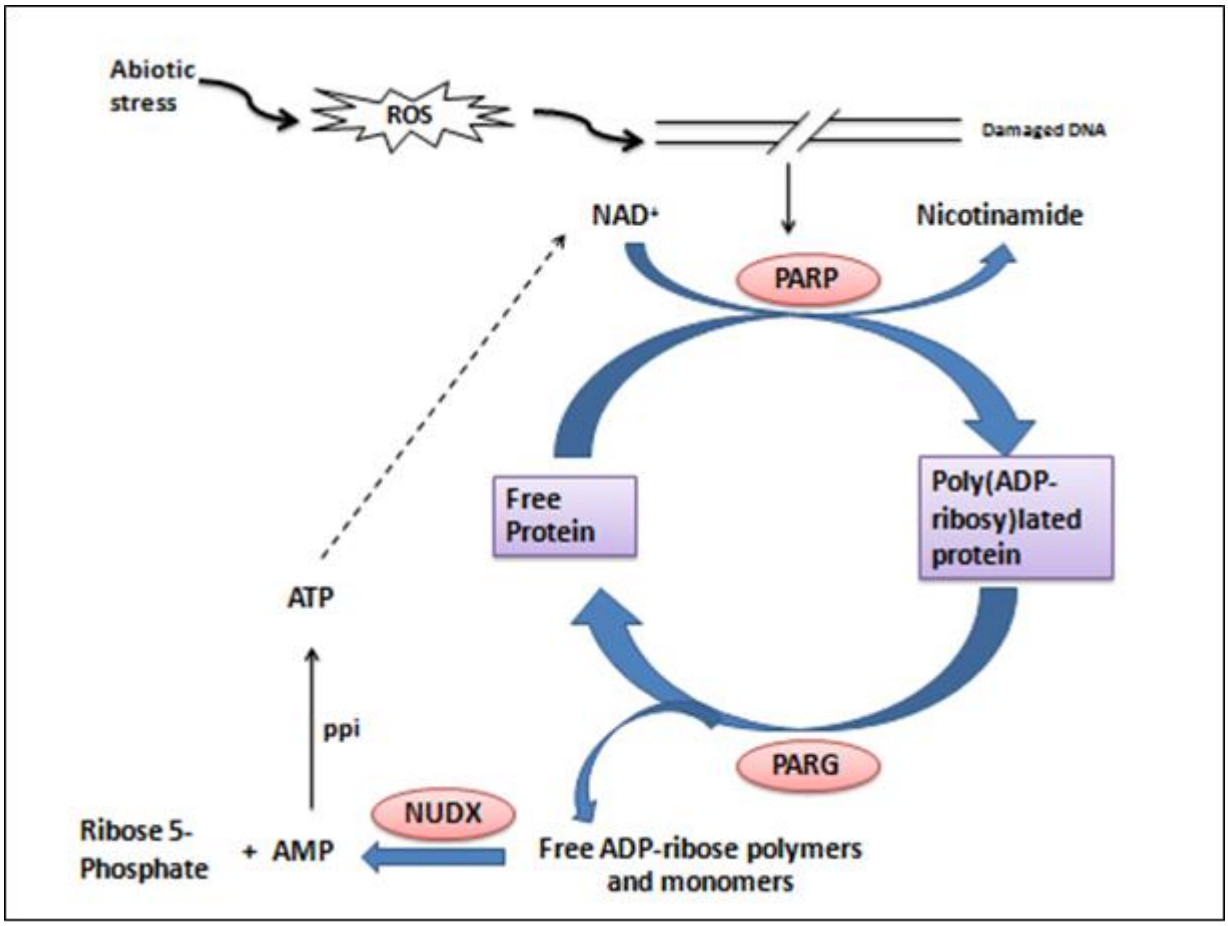

Fig. 1: Role of Poly(ADP-ribosyl)ation pathway in stress response and energy homeostasis. Reactive oxygen species (ROS) produced during abiotic stress may lead to a single or double stranded break in the DNA triggering PARP activity. PARP catalyses the formation of a poly(ADP-Ribose) chain on free proteins by sequential addition of ADP-ribose molecules from NAD^+. PARG catalyses the catabolism of the poly(ADP-Ribose) chain into free ADP-ribose monomers and polymers which are hydrolysed to AMP and Ribose 5-Phosphate by activity of ADP-Ribose specific NUDX enzyme. AMP is a ready precursor of ATP which can be used in replenishing the NAD^+ pool.

Poly(ADP-ribosyl)ation (PAR) is a posttranslational protein modification process mediated by the PARP enzyme that tags long-branched poly(ADP-ribose) polymers to nuclear target proteins such as

histone, transcription factors and most prominently on the PARP enzyme at DNA break sites through an auto-modification process to activate DNA repair processes. During stresses, there is a concomitant production of reactive oxygen species (ROS) which may lead to a single or double stranded break in the DNA strand which in turn activates the PARP enzyme. Activated PARP catalyzes the transfer of ADP-ribose moiety from the substrate nicotinamide adenine dinucleotide (NAD^+) to glutamic acid residue in the target acceptor protein followed by the further transfer of ADP-ribose monomers onto the newly formed adduct, forming the poly(ADP-ribose) chain. This modification can be reversed by poly(ADP-ribose) glycohydrolase (PARG) that hydrolyzes the poly(ADP-ribose) polymers to prevent excessive accumulation of poly(ADP-ribose), generating free ADP-ribose monomers and polymers. The free ADP-ribose is degraded into AMP and ribose-5-phosphate by an ADP-ribose specific Nudix hydrolase enzyme, also known as ADP-ribose pyrophosphatases (Kim et al., 2005; Briggs and Bent, 2011). Production of AMP through degradation of ADP-ribose generated by PARG has been reported to be an important pathway to re-establish utilizable energy units (Rossi et al., 2002). The ATP produced can be utilized to replenish the NAD^+ pool (see Fig 1. above).

In Arabidopsis, NAD^+ is produced through *De novo* synthesis from Aspartate or by recycling the NAD^+ metabolite, Nicotinamide, through the salvage pathway (Hashida et al 2009; De Block and Van Lijsebettens, 2011). NAD^+ acts as a substrate for (cyclic)ADP-ribose (cADPR) generation, a calcium mobilizing molecule important in abscisic acid (ABA) biosynthesis. NAD^+ is also an important substrate in cellular respiration pathways, redox reactions and production of $NADP^+$, a key molecule in photosynthesis and lipid biosynthesis (see Fig 2.).

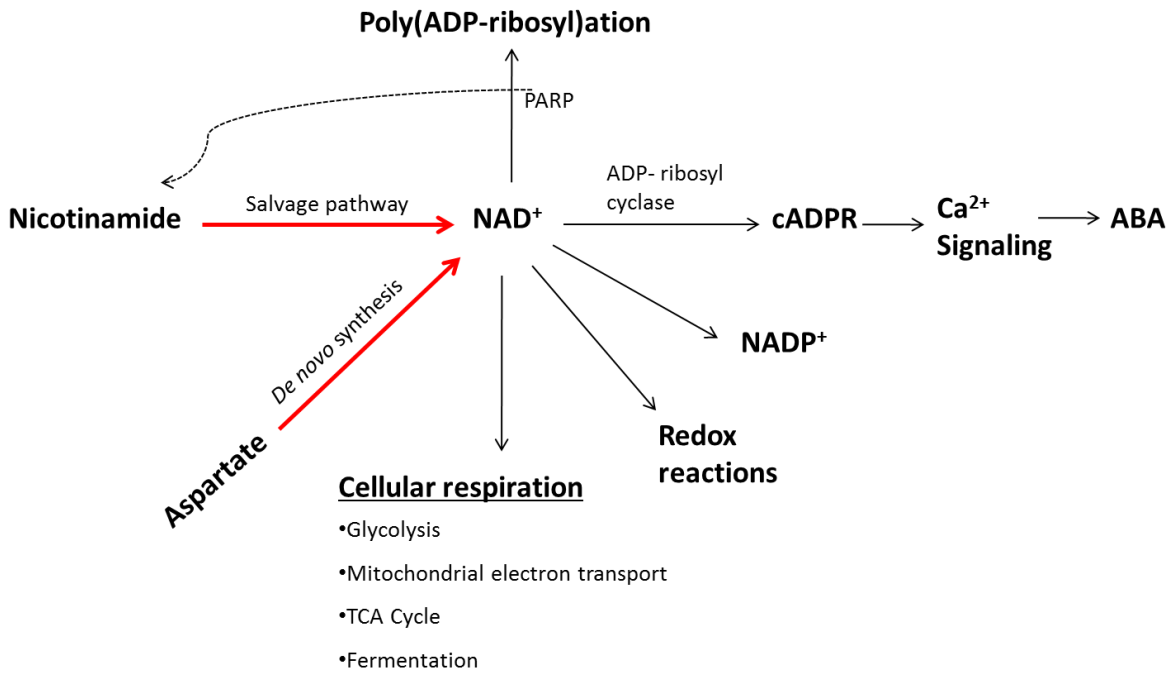

Fig. 2: Metabolism of Nicotinamide Adenine Dinucleotide (NAD^+) in plants. Biosynthesis through *De novo* or salvage pathways and utilisation through the Poly(ADP-ribosyl)ation, (cyclic)ADP-ribose (cADPR) and $NADP^+$ synthesis, redox reactions and cellular respiration (figure adapted from Vanderauwera et al., 2007)

PAR occurs in multicellular organisms including plants, animals and some lower unicellular eukaryotes, but is absent in prokaryotes and yeast. It is associated with DNA damage responses and programmed cell death. PAR plays an important role within the cellular response to genotoxic stress and modulates DNA synthesis and repair, maintenance of genomic stability, chromatin structure, transcription regulation, apoptosis, and cell cycle activities (D'Amours et al., 1999; Burkle, 2005; Kim et al., 2005). The extent of PAR is directly proportional to the severity of the stress and determines the type of cellular response, ranging from cellular defence under mild stress to DNA repair under moderate stress and to cell death under severe stress (Burkle et al., 2001). In the absence of DNA single and double strand breaks, poly(ADP-ribosyl)ation seems to be a very rare event in live cells, but it can increase over 100-fold upon DNA damage (Juarez-Salinas et al., 1979). More functions of the poly(ADP-ribosyl)ation pathway will be described in the topics below describing the respective gene families in PAR recycling.

1.2 Poly(ADP-ribose) Polymerase (PARP) Family

Poly(ADP-ribose) polymerases (PARPs) are a large family proteins displaying a conserved PARP catalytic domain. The enzymes catalyze the polymerization of ADP-ribose units from donor NAD^+ molecules onto target proteins, resulting in the attachment of linear or branched poly(ADP-ribose) polymers as described above. This enzymatic activity has been detected in eukaryotes ranging from

plants to mammals, but is absent in yeast (Rolli et al. 2000; Amé et al. 2004). The PARP protein family is involved in a diverse number of functions including DNA damage detection and repair, transcriptional regulation, intracellular trafficking, chromatin modification, mitotic apparatus formation, centrosome duplication telomere integrity, and cell death (Burkle, 2005). In humans, the PARP family members are encoded by 18 different genes (Amé et al., 2004).

Human PARP1, the most extensively studied, is a 113 kDa highly conserved and abundant nuclear enzyme. It is catalytically active as a dimer and is composed of three functional domains: an N-terminal DNA-binding domain containing two zinc-finger motifs essential for binding to single or double-stranded breaks with high affinity, a central auto-modification domain and a C-terminal catalytic domain, which is the most conserved region between PARP1 and PARP2. The catalytic domain contains a highly conserved block of 50 amino acids, referred to as the PARP signature. It sequentially transfers ADP ribose subunits from NAD^+ to protein acceptors, thereby forming the poly(ADP-ribose) chain (Rouleau et al 2010). PARP1 binds to a variety of DNA structures, including single- and double-strand breaks, crossovers, cruciforms, and supercoils, as well as some specific double-stranded sequences (Rolli et al. 2000). The activities and functions of the other PARP family members have not been studied to the same extent as PARP1.

PARP2 is a 62 kDa enzyme, also located in the nucleus, and is activated by DNA strand breaks. It is composed of an N-terminal DNA-binding domain without zinc-fingers and a C-terminal catalytic domain containing the PARP signature (Vira´g and Szabo´, 2002). PARP2 was discovered as a result of the presence of residual DNA-dependent PARP activity in embryonic fibroblasts derived from PARP1 deficient mice (Ame et al., 1999). PARP2 interacts with PARP1 sharing common proteins involved in the Single Strand Break Repair (SSBR) and Base Excision Repair (BER). In addition, both PARP1 and PARP2 localize to mitotic centromeres, the chromosomal regions where kinetochores form to capture microtubules from the mitotic spindle (Kim et al., 2005). PARP1 and PARP2 are so far the only enzymes with catalytic activity immediately stimulated by DNA strand breaks (Ame et al., 2004).

PARP3 is a 67KDa enzyme, identified as a core component of the centrosome and preferentially localised to the daughter centriole throughout the cell cycle (Augustin et al., 2003). Similar to PARP2, it contains a small 54 KDa N-terminal domain which is responsible for its centrosomal localisation. Augustin et al. (2003) showed that over-expression of PARP3 or its N-terminal domain does not influence centrosomal duplication or amplification but interferes with the G1/S cell cycle progression. In addition, PARP3 interacts with PARP1 during its localisation at the centrosome, suggesting a link between the DNA damage surveillance network and the mitotic fidelity checkpoint. It also catalyses the synthesis of poly(ADP-ribose) *in vitro* and in purified centrosome preparations.

PARP4, also known as VPARP (Vault PARP) is the largest of the family (192.6 KDa) since it was discovered in association with vault particles, cytoplasmic ribonucleoprotein, whose biological function is presently unknown but are proposed to be part of the nuclear pore complex and have also been implicated in multidrug resistance (Kickhoefer et al., 1999). PARP4 poly(ADP-ribosyl)ates the major vault protein within the vault particle and to a lesser extent itself. The N-terminal region of PARP4 contains a BRCT domain similar to the auto modification domain of PARP1, suggestive of a related function (Burkel, 2005).

Tankyrase1 (PARP5a) is a telomere-associated enzyme that binds to and poly(ADP-ribosylates) the telomeric-repeat binding factor 1 (TRF1), a negative regulator of telomerase (Smith et al., 1998) and also auto-poly(ADP-ribosylate) itself. It contains the smallest domain homologous to PARP1 that still displays PARP enzymatic activity. The C-terminus of tankyrase displays homology to the PARP1 catalytic region. Tankyrase activity does not depend on the presence of DNA strand breaks, since it lacks the DNA-binding domain, but seems to be regulated by the phosphorylation state of the protein. Over-expression of Tankyrase1 promotes ADP-ribosylation of TRF1, leading to its release from telomeres and to telomere elongation (Vira´g and Szabo´, 2002; Burkel, 2005).

Tankyrase2 (PARP5b) was originally described as a Golgi-associated protein (Chi and Lodish, 2000). It is an extranuclear PARP ubiquitously expressed in all tested tissues and its protein sequence displays 85% identity to Tankyrase1. The domain structures of the two proteins are strikingly similar except for the N-terminal region which lacks the HSP (Histidine-proline-serine rich) domain present in Tankyrase1. The two proteins also interact with the same set of proteins among which is TRF1 and probably mediate overlapping functions in telomere homeostasis, vesicle trafficking (glucose transport and insulin signalling), poly(ADP-ribosyl)ation of some of their interaction partners as well as themselves. However, Tankyrase2 displays preferential automodification activity and its over-expression caused rapid poly(ADP-ribosyl)ation-dependent cell death (Ame et al., 2004; Burkel, 2005).

In both maize and Arabidopsis, two PARP homologues were initially identified, one containing the classical Zn-finger DNA binding domain in the N-terminal region and is highly similar in its sequence and domain organization to the PARP1 enzyme from human (Chen et al., 1994; Lepiniec et al., 1995; Babiychuk et al., 1998). The second one is structurally a non-classical PARP protein lacking the characteristic N-terminal Zn-finger domain. The Arabidopsis genome encodes at least three putative PARPs [At2g31320 (PARP1), At4g02390 (PARP2), and At5g22470 (PARP3)]; Two *Zea mays* homologs of AtPARP2 have also been characterized: ZmPARP1 a 110-kDa Zn-finger poly(ADP-ribose) polymerase (ZAP) and ZmPARP2 a 73-kDa non-classical poly(ADP-ribose) polymerase (NAP)

(Babiychuk et al., 1998). These AtPARP1 and AtPARP2 are stress-inducible, structurally and functionally homologous to human PARP1 and 2 counterparts respectively. They are localized in the nucleus and are activated by DNA strand breaks (Babiychuk et al., 1998; Doucet-Chabeaud et al., 2001). AtPARP3 is expressed in seed tissue and was shown to be important for seed storage and viability (Rissel et al., 2013). In addition, it is reported that the catalytic domain of AtPARP3 has acquired changes that may interfere with its enzymatic activity (Lamb et al., 2012). Zhang et al. (2015) carried out a double and triple mutant analysis on the Arabidopsis PARPs and indicated that AtPARP1 and AtPARP2, but not AtPARP3, play a similar but not critical role in DNA repair in Arabidopsis seedlings. Fig. 3 shows a schematic representation of the PARP1 and PARP2 in Arabidopsis, maize and human as obtained from the NCBI's conserved domain database (Marchler-Bauer et al., 2015).

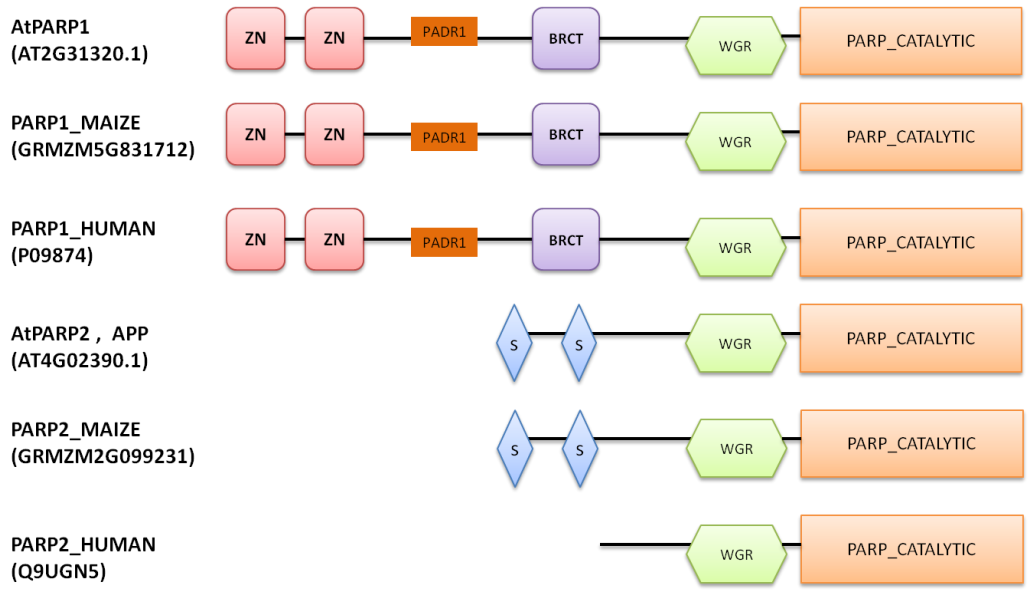

Fig. 3: Schematic domain architecture of the PARP 1 and 2 genes in Arabidopsis, Maize and Humans. The N-terminal DNA binding domain containing two Zinc finger motifs (ZN) in PARP 1 and SAP motifs in some PARP2 essential for DNA binding activity, PADR1 domain whose function is unknown, the Central auto- modification domain represented by the BRCT (Breast Cancer carboxy-terminal domain) and a C- terminus region containing the WGR domain proposed to function as a nucleic acid binding domain and the highly conserved PARP_Catalytic domain. (Figures adapted from NCBI's conserved domain database (Marchler-Bauer et al., 2015)).

Fig. 4 shows the close relationship of stress induced PARP1 and PARP2 proteins in Human, Arabidopsis and Maize in a phylogenetic tree generated using MEGA6 software (Tamura et al., 2013).

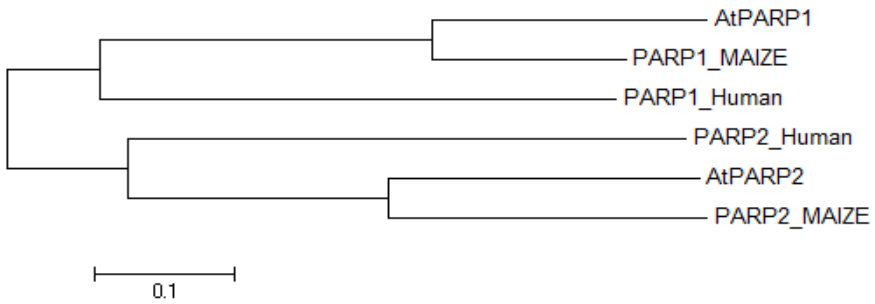

Fig. 4: Phylogenetic tree showing evolutionary relationship of Arabidopsis, maize and human PARP1 and PARP2 proteins. Figure generated using MEGA6 software (Tamura et al., 2013).

1.2.1 Role of plant PARP in stress response

In plants, PAR has been implicated in several physiological processes and described as an important regulatory mechanism modulating plant responses to abiotic and biotic stresses. Amor et al. (1998) showed the involvement of PARP enzyme in mild and severe oxidative stress by mediating DNA repair and programmed cell death (PCD) processes respectively in cultured soybean cells. The study showed that in soybean cells, PCD is preceded by a drop in cellular NAD^+ levels which indicates the activation of PARP. Application of PARP inhibitors, 3-aminobenzamide and nicotinamide, to soybean cells culture inhibited PCD induced by high dose of hydrogen peroxide. Additionally, transient overexpression of Arabidopsis PARP gene (app) in sense orientation in cultured soybean cells promoted DNA repair and inhibited cell death caused by mild oxidative stress but resulted in increased cell death upon severe oxidative stress. On the other hand overexpression of app gene in antisense orientation produced opposite effects: increased DNA nicks and inhibition of cell death at high but not mild doses of hydrogen peroxide. Doucet-Chabeaud et al. (2001) demonstrated that ionising radiation-induced DNA damage triggers rapid and massive accumulation of Arabidopsis AtPARP1 and AtPARP2 transcripts. After exposure to ionising radiation, the AtPARP1 transcripts first accumulated in all Arabidopsis plant organs followed by an increase in the AtPARP1 protein levels only in tissues that contain large amounts of rapidly dividing cells indicating a link between AtPARP1 activity and maintenance of DNA template integrity during replication. On the other hand, expression of AtPARP2 gene was induced by different environmental stresses such as severe water deficit (5-7 days without watering) and application of 50µM cadmium in the growing medium indicating an additional role for AtPARP2 independent of DNA damage. A recent study by Song et al. (2015) reported that in contrast to animals, Arabidopsis PARP2 rather than PARP1 plays a major role in DNA damage response induced by bleomycin, mitomycin C or gamma-radiation. In the study, more than 50% Arabidopsis *parp2* mutants failed to generate true leaves 14 days after germination on MS medium supplemented with 1.5µg/ml of bleomycin similar to *parp1parp2* double mutants whereas approximately 35% of Arabidopsis *parp1* mutants failed to generate true leaves under

similar bleomycin treatment. Upon 30µM mitomycin C treatment, parp2 mutants and parp1parp2 double mutant plants had significantly higher number of plants without true leaves 14 days after germination compared to wildtype whereas *parp1* mutants had moderately increased sensitivity to mitomycin C but not significant. A remarkable increase in amount of poly(ADP-ribosyl)ated proteins were detected in wildtype plants 20 to 60 minutes after 150Gy of gamma irradiation. The gamma-induced PARP activity was reduced in *parp1* mutants while a higher reduction was observed in parp2 single mutant and an even more complete reduction in gamma-induced PARP activity was observed in parp1parp2 double mutant (Song et al., 2015). De Block et al. (2005) also showed that stresses such as drought, high light and heat activate PARP causing NAD^+ breakdown and ATP consumption in plants and that when PARP activity is reduced by means of chemical inhibitors or by gene silencing, cell death is inhibited and plants become tolerant to a broad range of abiotic stresses. In Fig. 5, RNAi silencing of PARP genes through overexpression of dsRNA constructs containing 5'-end of the Arabidopsis AtPARP1 or AtPARP2 genes in *Brassica napus* and Arabidopsis thaliana enhanced the stress tolerance of the plants to drought and heat stresses. The study showed that the frequency with which stress tolerant lines were obtained indicated that downregulation of PARP2 was more effective that downregulation of PARP1. The group further indicated that Arabidopsis lines carrying a hairpin silencing construct targeting the highly conserved catalytic domain of AtPARP2 allowed silencing of both AtPARP1 and AtPARP2 and that the lines were tolerant to high light stress.

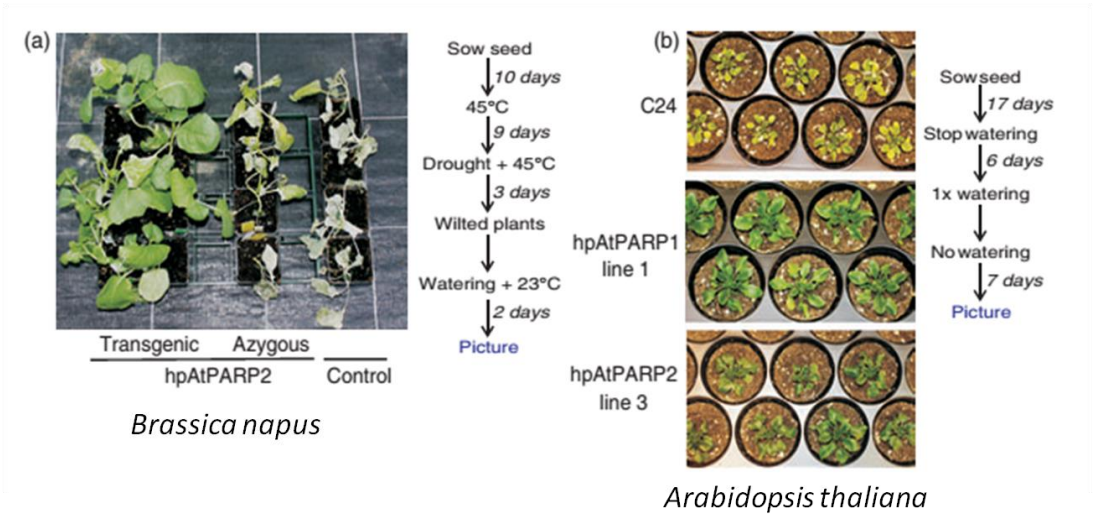

Fig. 5: Drought and heat stress tolerance in *Brassica* and *Arabidopsis* upon PARP downregulation. (**a**) Phenotypes of a control and a 3 transgenic: 1 azygous segregating *Brassica napus* hpAtParp2 line at the end of a stress experiment where heat and drought had been combined. (**b**) Phenotypes of *Arabidopsis thaliana* ecotype C24 lines at the end of a drought stress experiment (De Block et al., 2005).

Though the stress tolerance obtained was attributed solely to a maintained energy homeostasis due to reduced NAD^+ consumption, genome wide transcript analysis of stressed PARP2-deficient

transgenic Arabidopsis revealed the induction of specific ABA signalling pathways that might be steered through increased levels of the cyclic nucleotide cyclic ADP-ribose, facilitating the induction of a wide set of defense-related genes (Vanderauwera et al., 2007). Fig. 6 is a heatmap showing gene expression of Arabidopsis PARP and PARG genes under different perturbations (biotic, chemical, hormone, drought stress, genotoxic stress, osmotic stress and oxidative stress) which we selected from a wide microarray database in Genevestigator (Zimmermann et al., 2004). The figure was generated using Genesis software (Sturn et al., 2002) and the expression level ranges from -3.0 (light blue) to +3.0 fold expression (Red) compared to the expression in untreated controls.

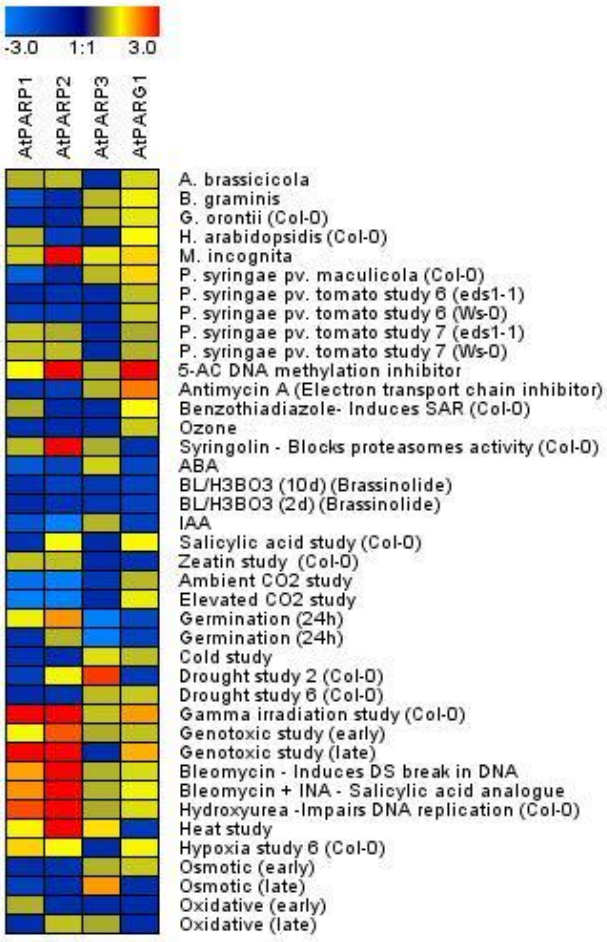

Fig. 6: Heatmap showing the gene expression pattern of Arabidopsis PARP1, 2 & 3 and PARG1 genes under biotic, chemical, hormonal, drought, genotoxic, osmotic and oxidative stress perturbations using the Genevestigator microarray data analysis software (Zimmermann et al., 2004). Fold expression level ranges from -3.0 (light blue) to +3.0 (Red) compared to the expression in untreated controls (Sturn et al., 2002)

Plant PARP has also been implicated in biotic stress response; inhibition of PARP in Arabidopsis triggers certain innate immune responses (such as callose deposition, lignin deposition, pigment accumulation and phenylalanine ammonia lyase activity) on treatment with microbe-associated molecular patterns (MAMPs) such as flg22 and elf18 (Adams-Phillips et al., 2010). In a recent

reporting, Feng et al. (2015) showed that AtPARP1 and AtPARP2 positively regulate immune gene activation and plant resistance to virulent bacterial pathogen infection whereas Arabidopsis parg1 mutant displayed enhanced immune gene activation and immunity to pathogen infection thus indicating that the reversible posttranslational PARylation process mediated by AtPARPs and AtPARGs plays a crucial role in mounting successful innate immune responses upon MAMPs perception in Arabidopsis. Song et al. (2015) also showed that AtPARP2 makes a greater contribution than AtPARP1 to plant immune responses including restriction of pathogenic *Pseudomonas syringae pv*. tomato growth and reduction of pathogen induced DNA damage. The versatile function of PARP in plant growth is shown whereby chemical downregulation of PARP activity using 3-Methoxybenzamide (3MB) reduced the accumulation of stress protective agents such as anthocyanin and ascorbate under stress conditions which was correlated with enhanced biomass production and growth of Arabidopsis plants (Schulz et al., 2012) and later in a non-stress condition where similar inhibition of PARP using 3MB led to enhanced growth of *Arabidopsis thaliana* through increased cell number in the leaves indicating a regulatory function of PARP within cell growth and potentially development (Schulz et al., 2014).

1.3 Poly(ADP-ribose) glycohydrolase (PARG) Family

Poly(ADP-ribose) glycohydrolase (PARG) is an enzyme in the poly(ADP-ribosyl)ation pathway that hydrolyzes the glycosidic linkages between the ADP-ribose units of Poly(ADP-ribose) chain producing free ADP-ribose monomers and polymers (Davidovic et al., 2001). It is an important player in Poly(ADP-ribose) homeostasis, possessing both endoglycosidic and exoglycosidic activity and being the only enzyme known to catalyse the hydrolysis of ADP-ribose polymers preventing their excessive accumulation. This protein was first reported by Miwa and Sugimura in 1971, it has been identified in mammalian cells, flies, worms and plants. It has a catalytic domain, putative regulatory domain, nuclear localization signal and a nuclear export signal (Davidovic et al., 2001). Only the catalytic domain shows a high level of homology across species, the arrangement of the other motifs and domains within the protein varies from species to species (Amé et al. 2000).

In contrast to PARPs, only one gene for PARG has been detected in mammals and insects. In humans, the hPARG gene is expressed in 3 different isoforms encoded by the same open reading frame and targeted either to the nucleus or cytoplasm (Meyer-Ficca et al., 2004). Over-expression studies revealed that the largest isoform of hPARG is targeted to the nucleus while the two smaller isoforms show mostly cytoplasmic localization. In Arabidopsis however, two adjacent PARG genes resulting from gene duplication have been identified. Multiple PARG genes have also been predicted in several plants including rice, poplar, tomato and maize (Briggs and Bent, 2011). PARG has not been

vastly characterised like its counterpart PARP and much of its function remains unknown possibly because of its low abundance in cells and extreme sensitivity to proteases making it difficult to study (Bonicalzi et al., 2005). However few functions have recently been reported both in animals and plants.

In animals PARG plays an important role in embryonic development (Hanai et al., 2004), cell death (Erdelyi et al., 2009) and DNA repair (Fisher et al., 2007; Fujihara et al., 2009). Drosophila mutants lacking a conserved catalytic domain of PARG exhibited lethality in the larval stages at the normal development temperature of 25°C while those that developed to adult stage at elevated temperature of 29°C showed progressive neurodegeneration with reduced locomotor activity and a short lifespan (Hanai et al., 2004). Using cell lines with stable silencing of PARG and PARP, Erdelyi et al. (2009) demonstrated PARG similar to PARP1 serves as an apoptosis to necrosis switch in severe oxidative stress. Additionally, Fisher et al. (2007) identified PARG as a critical component of single-strand break repair that accelerates the repair process in concert with PARP1. This role was further elucidated by functional inhibition of PARG which showed enhanced lethality of mouse cell lines exposed to DNA damaging agent (Fujihara et al., 2009).

There are two adjacent genes encoding PARG in the Arabidopsis genome (TEJ / AtPARG1 At2g31870; AtPARG2, At2g31865) as well as a pseudogene At2g31860 (Doucet-Chabeaud et al., 2001; Panda et al., 2002). AtPARG1 has been shown to play a role in regulating circadian rhythms; parg1 mutant plant has increased leaf movement, early flowering under both short and long days and lengthened the period of all known circadian clock-controlled gene expression (Panda et al., 2002). A few recent studies indicate that PARG, just like PARP play a role in plant biotic and abiotic stress responses. The heatmap in Fig. 6 above shows induction of AtPARG1 upon pathogenic attacks, chemical and abiotic stress such as drought, osmotic and genotoxic stresses. AtPARG2 was significantly upregulated in multiple resistance-avirulent gene interactions between Arabidopsis and *Pseudomonas syringae pv tomato* DC3000 (Adams-Phillips et al., 2008). In addition, a robust up-regulation of AtPARG2 gene expression was observed upon infection with *Botrytis cinerea* (Adams-Phillips et al., 2010). Further functional analysis indicates that *parg1* mutant plant is sensitive to microbe-associated molecular pattern (MAMP) elf18 and plants exhibit exaggerated seedling growth inhibition. The same group further reported that both Arabidopsis *parg1* and *parg2* knockout plants show accelerated the onset of disease symptoms upon infection with *Botrytis cinerea* (Adams-Phillips et al., 2010). These observations were also reported by Feng et al. (2015) who carried out a genetic screen and identified elevated immune gene expression upon multiple MAMP and pathogen treatment in Arabidopsis parg1 mutant. Oxidative stress induced through application of paraquat in Arabidopsis

resulted in upregulation of AtPARG2 expression (Ogawa et al., 2009). Li et al. (2011) described the role of PARG in drought, osmotic and oxidative stress tolerance in Arabidopsis indicating that *parg1* mutant plants showed reduced tolerance to drought (withholding water), osmotic (mannitol treatment), and oxidative stress (methyl viologen treatment). The plants had an increased level of cell damage under osmotic and oxidative stress and reduced survival under drought stress when compared with the wild type plants. Additionally, the germination rates of the *parg1* mutant plants were reduced upon osmotic or oxidative stress compared with wildtype seeds indicating PARG's important role in abiotic stress response in plants. Upon extensive biochemical assays Feng et al. (2015) concluded that AtPARG1 and not AtPARG2 possess detectable poly (ADP-ribose) glycohydrolase activity *in vitro* and *in vivo.* Another recent study on DNA repair of AtPARPs and AtPARGs, based on their mutant phenotypes under genotoxic stress, indicated that AtPARG1 is the key factor promoting cell survival among the enzymes regulating poly(ADP-ribosyl)ation. In the reporting, mutation of PARG1 resulted in increased DNA damage level and enhanced cell death in plants after bleomycin treatment indicating that PARG1 modulates the DNA repair process by preventing over-induction of DNA repair genes (Zhang et al., 2015).

1.4 The Nudix hydrolase family

Nudix hydrolases are a widely distributed family of protein present in viruses, archaea, bacteria and eukaryotes and characterized by a conserved Nudix motif GX5EX7REVXEEXGU, where U represents a bulky hydrophobic amino acid such as Ile, Leu, or Val and X is any amino acid (Bessman et al., 1996). Almost all of the major substrates for these enzymes are nucleoside diphosphates linked to a moiety, X, hence the acronym "Nudix". These substrates include: dinucleoside polyphosphates, ADP-ribose, NADH, nucleotide sugars, or ribo- and deoxyribonucleoside triphosphates, coenzymeA, mRNA cap and FAD (Bessman et al., 1996; Dunn et al., 1999; Ogawa et al., 2005, 2008). Accumulation of these substrates may be toxic to the cell thus their intracellular levels need to be precisely regulated. Bessman et al. (1996) suggested the role of Nudix hydrolases in sanitizing or modulating the accumulation of these metabolites.

The earliest characterized Nudix hydrolase is the MutT protein of Escherichia coli which preferentially hydrolyses 8-oxo-deoxyguanosine 5' -triphosphate (8-oxo-dGTP), a mutagenic nucleotide formed by activity of reactive oxygen species on free guanine molecule, thereby preventing misincorporation of 8-oxo-GTP into DNA. 8-oxo-dGTP can pair with cytosine and adenine nucleotide with almost equal efficiency resulting in a spontaneous transverse mutation in the DNA (Maki and Sekiguchi, 1992). The E. coli mutT gene (Treffers et al., 1954) codes for a protein nucleoside triphosphate pyrophosphohydrolase (MutT) that specifically degrades 8-oxo-dGTP to 8-

oxo-dGMP preventing misincorporation of 8-oxo-GTP into DNA during DNA synthesis (Maki and Sekiguchi, 1992). This early study led to the name of enzymes bearing a conserved MutT signature sequence to be called “MutT family”. Bessman et al. (1996) proposed a change of the family name to “Nudix hydrolase “ because the initial classification was misleading since many of the proteins in the family are not directly involved in preventing mutations nor do they catalyze the nucleoside triphosphate pyrophosphohydrolysis reaction originally described for MutT. In addition, all the enzymes characterized from that family hydrolysed a nucleoside diphosphate linked to a moiety X. The acronym of the family name was thus modified to NUDT and later to NUDX and the MutT proteins are then a subgroup in the Nudix hydrolase family. Studies of the Nudix hydrolase family have been carried out in *E. coli, S. cerevisiae*, mouse and humans (McLennan et al., 2006). Table 1 summarises the plant species in which NUDX genes have been reported and the particular number of NUDXs in each species. Nudix hydrolases from *Arabidopsis thaliana* were used as a reference in this work.

Table 1: Plants species and NUDX gene number currently reported in literature

Plant	**No of NUDX genes**	**Reference**
Arabidopsis (*Arabidopsis thaliana*)	28	Yoshimura and Shigeoka, 2015
Rice (*Oryza sativa*)	20	Yoshimura and Shigeoka, 2015
Tomato (*Solanum lycopersicum*)	32	Yoshimura and Shigeoka, 2015
Poplar plant (*Populus trichocarpa*)	53	Kraszewska, 2008
Grape vine (*Vitis vinifera*)	30	Kraszewska, 2008
Chrysanthemum (*Chrysanthemum lavandulifolium*)	8	Huang et al.,2012
Barley (*Hordeum vulgare*)	14	Tanaka et al., 2014

1.4.1 Arabidopsis Nudix hydrolases

The first plant Nudix protein was isolated from *Lupinus angustifolius* as a diadenosine tetraphosphate (Ap_4A) hydrolase (Maksel et al., 1998). So far however, Nudix hydrolases from Arabidopsis thaliana are the most substantially characterized in higher plants. Ogawa et al. (2005) reported that 24 Nudix hydrolase genes exist in Arabidopsis thaliana and their proteins can be classified into three types based on their subcellular localization: the cytosol (AtNUDX1 to -11), mitochondria (AtNUDX12 to -18), and chloroplast (AtNUDX19 to -24). Later, Mun˜oz et al. (2006) reported seven additional genes encoding Nudix hydrolases in the Arabidopsis genome: AtDCP2 which was characterised as a novel Nudix hydrolase having mRNA-decapping activity (Gunawardana et al., 2008), AtNUDX25 which showed hydrolysis activity toward Ap_4A; AtNUDX26 and AtNUDX27 whose proteins were predicted to be located in chloroplasts (Yoshimura et al., 2007) and three other genes (At2g04440, At3g02780, and At5g16440) in which the Nudix motif was hardly conserved in

their respective proteins. Yoshimura and Shigeoka (2015) sort to clarify the differences in the number of NUDX genes in Arabidopsis, he indicated that three genes (At2g04440, At3g02780, and At5g16440) lacked the Nudix motif and also renamed AtNUDX26 to 28 enzymes concluding that Arabidopsis has 28 Nudix hydrolase proteins. Table 2 gives a summary of the AtNUDX genes subcellular localisation and preferential recombinant protein substrate that defines their subfamily (Yoshimura and Shigeoka, 2015).

Table 2: Arabidopsis AtNUDX subfamilies and subcellular localisation (Yoshimura and Shigeoka, 2015)

Preferential recombinant protein substrate (Subfamily)	**Subcellular localization**	**AtNUDX member**
8-oxo-(d)GTP	Cytosol	AtNUDX1
ADP-ribose/NAD(P)H	Cytosol	AtNUDX2,6,7 and 10
	Mitochondrion	AtNUDX14
	Chloroplast	AtNUDX19
GDP-mannose	Cytosol	AtNUDX9
CoA	Cytosol	AtNUDX11
	Mitochondrion	AtNUDX15
	Mitochondrion or Peroxisome	AtNUDX15a
ApnA/ppGpp	Mitochondrion	AtNUDX13
	Cytosol	AtNUDX25
	Chloroplast	AtNUDX26 and 27
Thiamin diphosphate	Chloroplast	AtNUDX20
FAD	Chloroplast	AtNUDX23
mRNA cap	Cytosol	AtDCP2
Not identified	Cytosol	AtNUDX3, 4, 5 and 8
	Mitochondrion	AtNUDX12, 16, 17 and 18
	Chloroplast	AtNUDX21, 22 and 24

1.4.2 Cytosolic Nudix hydrolases

Cytosolic Nudix hydrolases are Arabidopsis Nudix hydrolases targeted to the cytosol and include AtNUDX1 to 11, 25 and AtDCP2. Ogawa et al. (2005) reported that AtNUDX1 is a functional homologue of E. coli MutT and is involved in prevention of spontaneous mutation. The group showed that AtNUDT1 protein has the ability to hydrolyze 8-oxo-dGTP with a high affinity and completely reduce the increased frequency of spontaneous mutations in the Escherichia coli mutT strain. Further studies indicate that AtNUDX1 plays an important role in protection against oxidative DNA and RNA damage in plant cells through sanitization of their precursor pool in the cytosol of Arabidopsis cell since the levels of 8-oxo-dGTP in knock out AtNUDX1 increased significantly compared with those in wildtype plants under normal and oxidative stress conditions (Yoshimura et

al., 2007). However, Kraszewska (2008) indicated that AtNUDX1 mutant plants did not exhibit any noticeable changes in their phenotype both under normal or stressful condition, hence it remains to be shown whether NUDX1 mutation have any physiological effects in Arabidopsis plants. Zhang et al. (2013) demonstrated that AtNUDX5 promoter is pathogen responsive activated by both avirulent and virulent Pseudomonas syringae pv. tomato strains while Ogawa et al. (2005) showed that AtNUDX11 had specific activity towards coenzyme A. AtNUDX2, 6, 7, and 10 have been shown to have pyrophosphohydrolase activity toward both ADP-Ribose and NADH (Ogawa et al., 2005). In Fig. 7, the relationship of AtNUDX2, 6, 7 and 10 and their maize homologues (ZmNUDX2, ZmNUDX8 and ACN26985) is shown in a phylogenetic tree generated using MEGA6 software (Tamura et al., 2013).

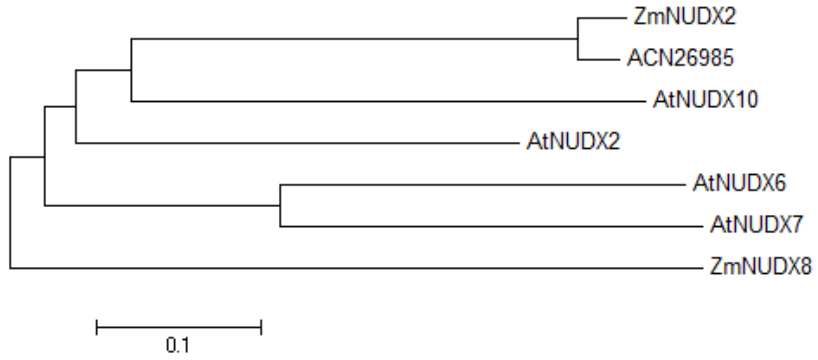

Fig. 7: Phylogenetic tree showing evolutionary relationship of ADP-ribose specific AtNUDX proteins with their maize homologues. Figure generated using MEGA6 software (Tamura et al., 2013).

The free ADP-ribose, produced during the reverse degradation of protein bound mono- or poly-(ADP ribose), is highly reactive and can mono-(ADP-ribosyl)ate proteins non-enzymatically thereby altering or eliminating their function. Thus, ADP-ribose phyrophosphohydrolase activity of the NUDX proteins is very important in regulating the levels of free ADP-ribose and maintaining protein integrity in the cell. In addition, NADH pyrophosphatase activity is reported to be involved in regulation of the cellular NADH/NAD ratio which is important in maintaining the balance between the anabolic and catabolic pathways in the cell (Frick et al., 2015). Ogawa et al. (2009) showed that overexpression of AtNUDX2 encoding ADP-ribose pyrophosphatase, confers enhanced tolerance of oxidative stress on Arabidopsis plants which results from maintenance of NAD^+ and ATP levels by nucleotide recycling from free ADP-ribose molecules under stress conditions. In addition Ishikawa et al., 2010 showed that AtNUDX6 is a modulator of NADH rather than ADP-Rib metabolism and it significantly impacts the plant immune response as a positive regulator of NPR1-dependent salicylic acid signalling pathways.

AtNUDX7, an ADP-Ribose/NADH pyrophosphohydrolase has been extensively studied and identified as a multiple stress induced gene. It has been shown to have impacts on both biotic and abiotic

stress (oxidative stress) responses (Bartsch et al., 2006; Jambunathan and Mahalingam, 2006; Ge et al., 2007; Adams-Phillips et al., 2008; Ishikawa et al., 2009; Jambunathan et al., 2010). AtNUDX7 showed preferential activity for ADP-ribose and NADH when expressed in E. coli cells (Ge et al., 2007). It has been proposed as the predominant NADH and ADP-Ribose Pyrophosphatase in Arabidopsis cells (Ishikawa et al., 2009). AtNUDX7 has also been shown to play a role in seed germination (Zeng et al., 2014).

1.4.3 Role of cytosolic Nudix hydrolases in stress response

Just like PARP and PARG, a number of ADP-ribose/NADH pyrophosphohydrolase specific NUDX genes have been reported to play a vital role in biotic and abiotic stress response. Regulation of ADP-ribose and/or NADH levels through the hydrolysis activity of AtNUDX2, 6 and 7 have been shown to contribute to the modulation of defence responses to both biotic and abiotic stresses. The heatmap in Fig. 8 shows the gene expression of AtNUDX2, 6, 7 and 10 under different perturbation (biotic, chemical, hormone, drought stress, genotoxic stress, osmotic stress and oxidative stress) which we selected from a wide microarray database in Genevestigator (Zimmermann et al., 2004). The figure was generated using Genesis software (Sturn et al., 2002) and the expression level ranges from -3.0 (light blue) to +3.0 fold expression (Red) compared to the expression in untreated controls. AtNUDX6 and 7 are the most highly upregulated under pathogenic attacks, chemical and hormonal treatments, drought and genotoxic stresses.

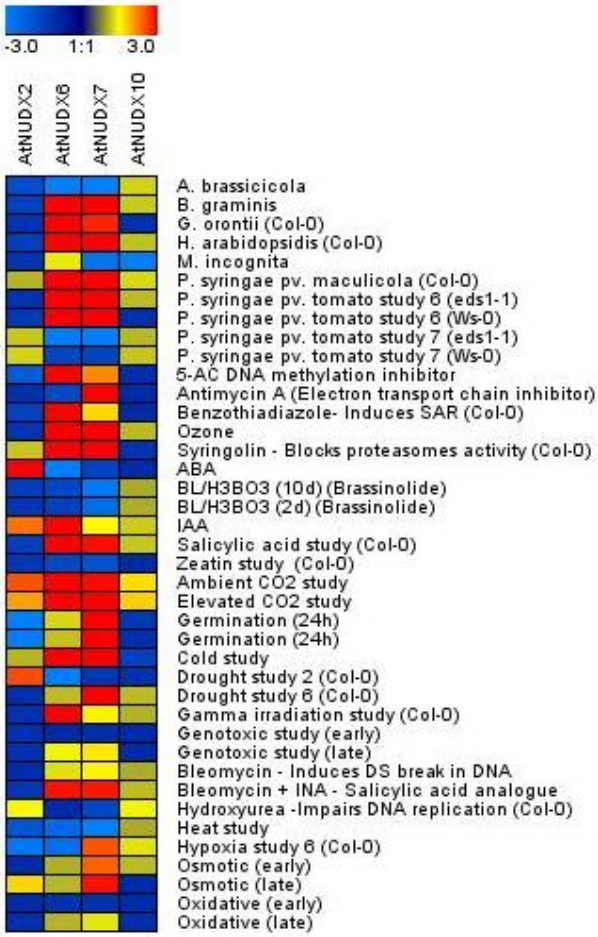

Fig. 8: Heatmap showing the gene expression pattern of ADP-ribose specific Arabidopsis NUDX genes under biotic, chemical, hormonal, drought, genotoxic, osmotic and oxidative stress pertabations using Genevestigator microarray data analysis software (Zimmermann et al., 2004). Fold expression level ranges from -3.0 (light blue) to +3.0 (Red) compared to the expression in untreated controls (Sturn et al., 2002).

Overexpression of AtNUDX2 conferred Arabidopsis plants with enhanced tolerance to oxidative stress due to maintenance of NAD^+ and ATP levels by nucleotide recycling from free ADP-ribose molecules (Ogawa et al., 2009). In the study, overexpression of AtNUDX2 protein resulted in 1.2 to 2 fold higher ADP-ribose pyrophosphatase activity than in control plants. Also, the overexpression AtNUDX2 lines showed significant suppression of root growth inhibition as opposed to control plants whose root growth was severely inhibited upon 0.3µM and 3µM paraquat treatment. However, only a slight reduction in ADP-ribose pyrophosphatase activity was observed in RNAi-AtNUDX2 plants and there was no significant difference in the degree of tolerance to oxidative stress caused by paraquat between the control plants and RNAi-AtNUDX2 plants. The endogenous expression of AtNUDX2 protein was noted to be low and mentioned that it may not contribute substantially to cellular defense systems in nature even under stressful conditions (Ogawa et al., 2009). In a different study, Arabidopsis plants having high expression levels of AtNUDX7 showed enhanced tolerance to paraquat induced oxidative stress while KO-AtNUDX7 plants had a decreased tolerance as assessed by the leaf phenotype, survival rates and chlorophyll content as shown in Fig. 9. In this study, the

ADP-ribose pyrophosphatase activity was increased by 1.2 to 2.5 fold in OE AtNUDX7 lines and reduced significantly to 76.9% in mutant KO-*nudx7* plant compared to the control plants under normal conditions. The amount of poly(ADP-ribose) in the control plants was increased under 3µM paraquat treatment. In OE AtNUDX7 and KO-*nudx7*, the amount of poly(ADP-ribose) was considerably larger or smaller, respectively, compared to the control plants both under normal and 3µM paraquat treatment indicating a positive correlation between the AtNUDX7 expression levels and the levels of PAR reaction. In addition, depletion of NAD^+ and ATP resulting from the activation of the PAR reaction under oxidative stress was completely suppressed in the overexpression AtNUDX7 plants while accumulation of NAD^+ and ATP was observed in the KO-AtNUDX7 plants (Ishikawa et al., 2009). Experimental studies showed that the AtNUDX7 transcript levels were rapidly and transiently induced during both biotic stresses imposed by avirulent pathogens and abiotic stresses like ozone and osmoticum (Jambunathan and Mahalingam, 2006). AtNUDX7 gene has also been found in many stress-responsive Arabidopsis cDNA libraries (Jambunathan and Mahalingam, 2006).

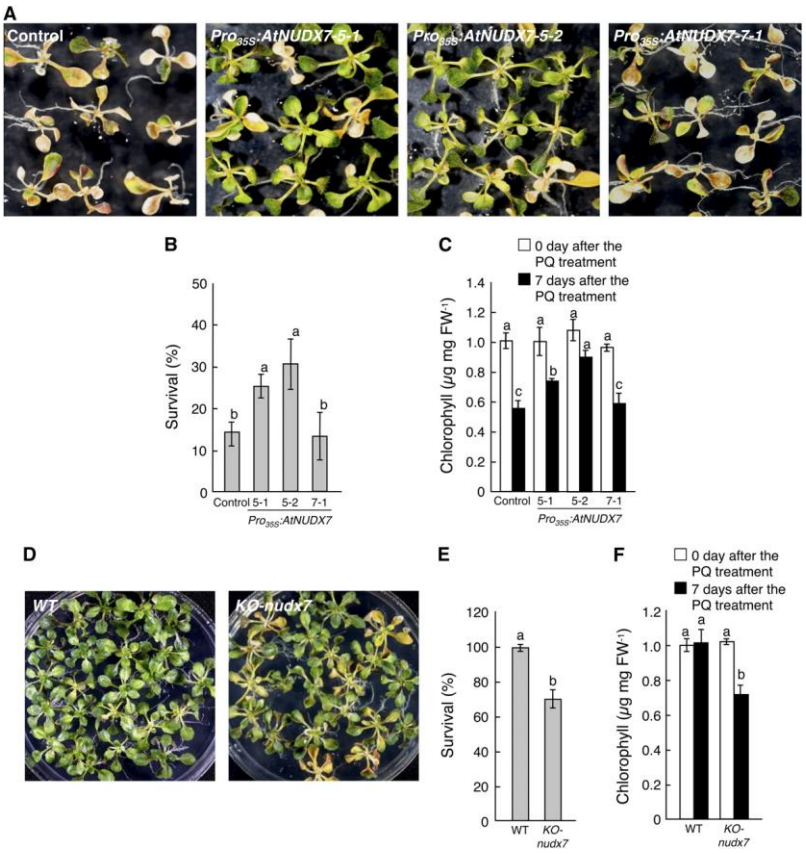

Fig. 9: Effects of overexpression or disruption of AtNUDX7 on oxidative stress tolerance. **A**, Phenotypes of the control and Pro_{35S}:AtNUDX7 plants after oxidative stress caused by PQ (paraquat) treatment. Seven-day-old seedlings were grown on MS medium containing 3 µM PQ for 7 days under long-day conditions. The seedlings were grown then on MS medium without PQ for an additional 7 days. **B**, Survival rates of the control and Pro_{35S}:AtNUDX7 plants under the PQ treatment. **C**, Chlorophyll contents of the control and Pro_{35S}:AtNUDX7 plants under normal conditions and oxidative stress. Data are means +/-SD for three individual experiments (n

= 3). Different letters indicate significant differences (P < 0.05). **D**, Phenotypes of the wild-type (WT) and KO-nudx7 plants after oxidative stress caused by PQ treatment. Two-week-old Arabidopsis plants were grown on MS medium containing 2 µM PQ for 7 days under long-day conditions. The plants were grown then on MS medium without PQ for an additional 7 days. **E**, Survival rates of wild-type and KO-nudx7 plants under PQ treatment **F**, Chlorophyll contents of wild-type and KO-nudx7 plants under normal conditions and oxidative stress. Data are means +/- SD for three individual experiments (n = 3). Different letters indicate significant differences (P < 0.05). FW, Fresh weight (Ishikawa et al., 2009)

In a molecular characterisation of barley (*Hordeum vulgare*) Nudix hydrolases, Tanaka et al. (2015) reported that seven HvNUDXs (HvNUDX1, 2, 6, 7, 11, 12, and 13) were up-regulated significantly under drought stress (dehydration on paper towel) and four HvNUDXs (HvNUDX6, 7, 12, and 14) up-regulated significantly UV-C stresses, respectively. HvNUDX1, the only one classified into ADP-ribose pyrophosphohydrolase subfamily gene of which the amino acid sequence shows 55% identity with that of AtNUDX2, was up-regulated 1.32-fold after 24 h of drought stress by dehydration on paper towel (Tanaka et al., 2015). Similarly, characterisation of *Chrysanthemum lavandulifoliua* Nudix hydrolases showed that ClNUDX1, 2, 3, 7 and 8 were up-regulated under various abiotic stress treatments (Salt, drought, cold and heat). ClNUDX2 the only ADP-ribose pyrophosphohydrolase so far among *Chrysanthemum lavandulifoliua* Nudix hydrolases, was upregulated by drought and salinity stresses (Huang et al., 2012).

The role of Nudix hydrolases in biotic stress response has been reported predominantly in AtNUDX7. Ge et al. (2008) identified AtNUDX7 as one of the early pathogen responsive genes that showed significant accumulation of its transcript within 30 minutes of infection by Pseudomonas syringae. AtNUDX7 was found to be a negative regulator of the basal defense response, and its loss of function mutation, KO-AtNUDX7, results in enhanced resistance to infection by *Pseudomonas syringae*. The KO-AtNUDX7 mutation does not cause a strong constitutive disease resistance phenotype, but it leads to a heightened defense response, including accelerated activation of defense-related genes that can be triggered by pathogenic and non-pathogenic microorganisms. The KO-AtNUDX7 mutation enhances two distinct defense response pathways: one independent of and the other dependent on NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) and Salicylic Acid (SA) accumulation (Ge et al., 2007). AtNUDX7 has also been defined as a negative regulator of salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) signalling required for basal resistance to invasive pathogens (Bartsch et al., 2006). Ishikawa et al. (2010) reported that AtNUDX6 significantly impacts the plant immune response as a positive regulator of NPR1-dependent SA signalling pathways. The group showed that expression of SA-induced genes which depend on NPR1, a key component required for pathogen resistance, was significantly suppressed in the KO-AtNUDX6 plants and enhanced in overexpression AtNUDX6 plants, under the treatment with SA. Expression of thioredoxin h5, which catalyzes SA-induced NPR1 activation, was suppressed and accelerated in the

KO-AtNUDX6 and overexpression AtNUDX6 plants, respectively (Ishikawa et al., 2010). In a recent study, AtNUDX7 is reported to be transcriptionally regulated and that the AtNUDX7 promoter is rapidly inducible by ozone and pathogens. The study further showed that an ethylene response motif called the GCC box is present in the AtNDUX7 promoter and plays a role in the ozone response of Arabidopsis ecotypes (Muthuramalingam et al., 2015). Additionally, another study showed that Atnudx7 mutant, which has both constitutively expressed NPR1- dependent and NPR1-independent SA signalling pathways, was tolerant to salt and oxidative stress unlike npr1-5, which lacks NPR1-dependent SA signalling thus showing the role of endogenous salicylic acid signalling cascades in plant responses to salt and oxidative stresses (Jayakannan et al., 2015).

1.5 Perspectives

Studies in Arabidopsis provide evidence of the involvement of plant PAR proteins in oxidative, drought, osmotic, genotoxic stress responses and in plant immune responses against pathogenic infection (Amor et al., 1998; Doucet-Chabeaud et al., 2001; De Block et al., 2005; Vanderauwera et al., 2007; Ogawa et al., 2009; Ishikawa et al., 2009; Adams-Phillips et al., 2010; Li et al., 2011; Feng et al., 2015; Song et al., 2015; Zhang et al., 2015). This milestone has mainly been achieved through biotechnological studies that involve modulating PAR genes by upregulation or downregulation of their expression level or complete gene knock-outs. There are still a number of outstanding questions in this subject which can be addressed via the biotechnological approach for instance, in each PAR pathway gene type only one or two representative family members have been reported to respond to biotic or abiotic stresses, are there similar responses in the other genes of the same family? How does modulation of one PAR pathway gene such as PARP affect PARG and NUDX gene expression and resultant response to biotic and abiotic stresses or vice versa? Similary only few reports in other plants apart from Arabidopsis have been reported so far. Transgenic or gene editing research offers a great potential to explore this field by enabling expression of Arabidopsis PAR genes with known functionality in other crops of interest, screening for Arabidopsis PAR gene homologous in these crops and modulating their function or carrying out a gene expression profiling through microarray or RNA-seq upon stress perturbation. In this work, we attempt to address some of these questions by modulating PARP gene expression in maize, introducing AtNUDX7 gene in maize and overexpressing AtNUDX7 maize homologues in maize and in Arabidopsis.

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CHAPTER 2

Higher plant transformation: principles and molecular tools

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Abstract

Genetic transformation that is part of the toolbox for the study of living organisms had been reported in higher plants only 30 years ago, boosting basic plant biology research, generating superior crops, and leading to the new discipline of plant biotechnology. Here, we review its principles and the molecular tools. In vitro regeneration through somatic embryogenesis or organogenesis, are discussed because they are prerequisites for the subsequent Agrobacterium tumefaciens-mediated transferred (T)-DNA or direct DNA transfer methods to generate transgenic plants. Important molecular components of the T-DNA are examined, such as selectable marker genes that allow the selection of transformed cells in tissue cultures and are used to follow the gene of interest in the next generations, and reporter genes that have been developed to visualize promoter activities, protein localizations, and protein-protein interactions. Genes of interest are assembled with promoters and termination signals in Escherichia coli by means of GATEWAY-derived binary vectors that represent the current versatile cloning tools. Finally, future promising developments in transgene technology are discussed.

KEY WORDS: *Agrobacterium tumefaciens*, T-DNA, transgene, plant biotechnology, plant transformation, somatic embryogenesis, organogenesis

Shoot regeneration in tissue culture

Genetic transformation usually involves DNA delivery to explants and subsequent tissue culture in which transformed cells are selected and induced either to form transgenic callus, shoots, and roots or somatic embryos. Hence, the tissue culture-induced regeneration capacity of a plant genotype is crucial for a successful genetic transformation. Indeed, recalcitrance to *in vitro* regeneration prevents genetic transformation in a large number of plant species or varieties. The *in vitro* shoot regeneration capacity has a genetic basis because it can be introgressed from a highly regenerative into a recalcitrant genotype (Koornneef *et al*., 1993; Anami *et al*., 2010). Therefore, identification of genes promoting or inhibiting the tissue culture-induced regeneration capacity will help to broaden the range of plant species for genetic transformation. Tissue culture regeneration occurs through organogenesis or somatic embryogenesis, which are discussed below and are schematically presented in Fig. 1.

Somatic embryogenesis

Somatic embryos develop from undifferentiated somatic cells in cultures, are morphologically and developmentally very similar to zygotic embryos that are formed upon fertilization, surrounded by maternal tissue. Somatic embryogenesis was first demonstrated in embryogenic carrot (*Daucus carota*) liquid cultures in 1958 that differentiated into somatic embryos at high frequency when diluted, sieved for the embryogenic subpopulation, and transferred to a medium lacking the synthetic hormone 2,4-dichloro-phenoxyacetic acid (2,4-D) (Steward *et al*., 1958) (Fig. 1A). This embryogenic suspension culture system of carrot has been used as a model to study the regulatory genetic program of plant zygotic embryogenesis (Zimmerman, 1993). Indeed, all stages of the embryo formation, such as globular, heart, and torpedo, can be distinguished in the embryogenic cultures and, upon removal of the exogenous auxin, the induction of the gene expression programs is comparable to that of the zygotic embryogenesis (Borkird *et al*., 1986). The somatic embryogenesis receptor-like kinase SERK1 marks somatic plant cells that are competent to form somatic embryos in the carrot cultures (Schmidt *et al*., 1997). Endogenous abscisic acid signaling is essential for carrot cells to acquire the embryogenic competence and in zygotic embryogenesis (Parcy *et al*., 1994; Kikuchi *et al*., 2006). Transcription factors expressed during embryogenesis, such as the ABSCISIC ACID-INSENSITIVE3 (ABI3), LEAFY COTYLEDON1 (LEC1), FUSCA3 (FUS3), are specific markers for embryogenesis that play a crucial role in the embryogenic process as demonstrated by knockout or overexpression lines (Lotan *et al*., 1998).

Today, many plant species of agronomic and horticultural importance are regenerated in tissue cultures through somatic embryogenesis, among which the cereals (Shrawat and Lörz, 2006). Somatic embryogenesis in crop plants requires high concentrations of auxin, 2,4-D or dicamba to promote the vegetative-to-embryogenic transition in a small subset of cultured cells that are then committed to form somatic embryos. The somatic embryo is a bipolar structure with closed radicle in contrast to the monopolar shoot structure originating through organogenesis. The embryo arises from a single cell and has no vascular connection with the maternal callus tissue or the cultured explant. Hence, somatic embryos are clonal unlike shoots regenerated by organogenesis that usually arise from several individual cells and might be chimearic, i.e. consisting of a mixture of independently transformed cells. Furthermore, induction of somatic embryogenesis requires a single hormonal signal to trigger a bipolar structure capable of forming a complete plant consisting of root and shoot and resembling a "seedling" (Fig. 1B). In organogenesis, shoots induced from organogenic callus are removed from the callus to form roots on hormone-free or "rooting" medium (Fig. 1C).

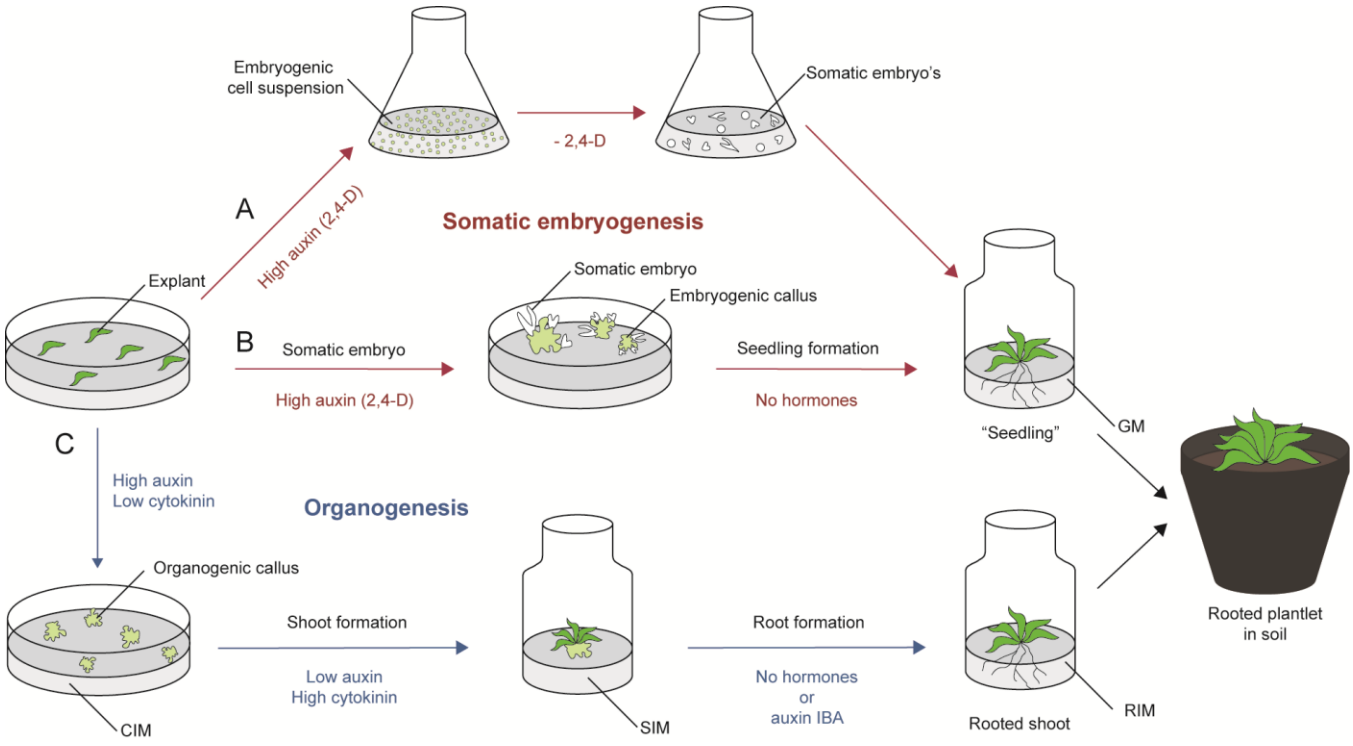

Fig. 1. Somatic embryogenesis versus shoot regeneration. (A) *Somatic embryogenesis through embryogenic cell suspension cultures*. **(B)** *Somatic embryogenesis through embryogenic callus cultures*. **(C)** *Shoot regeneration* through organogenesis

Organogenesis

According to the "classical theory", *in vitro* organogenesis refers to organ formation of *de novo* origin, involving phytohormone perception, dedifferentiation of differentiated cells into callus, acquisition of organogenic competence, re-entry of quiescent cells into the cell cycle, and organization of cell division to form specific organ primordia and meristems (Sugiyama. 1999). The pioneering work of Skoog and Miller (1957) and Christianson and Warnick (1983) established that auxin and cytokinin are the predominant growth regulators of tissue culture organogenesis. Shoot regeneration in tissue cultures usually requires a two-step hormone treatment (Fig. 1C). A high auxin/cytokinin ratio in the medium induces organogenic callus from an explant and, subsequently, a high cytokinin/auxin ratio induces shoot formation. Such shoots originate typically from monopolar and callus-derived organ primordia and develop into shoots with leaves that form roots upon transfer to medium without hormones or containing the "rooting" auxin isobutyric acid. Organogenic callus cultures with subsequent shoot organogenesis have been established in many species by means of numerous types of explants, including tobacco (*Nicotiana tabacum* L.) protoplasts (Nagy and Maliga, 1976), *Arabidopsis thaliana* root explants (Valvekens *et al*., 1988) and leaf explants in various species. A number of gene regulatory circuits important for dedifferentiation, redifferentiation, and adventitious meristem organization during *in vitro* regeneration of plants have been identified of which their components might be developed into new tools to improve the plant regeneration efficiency (Duclercq *et al*., 2011). Organogenic callus induction on auxin-containing medium corresponds with the competence (cell dedifferentiation) acquisition by which tissues respond to hormonal signals and the upregulation of AUXIN/INDOLE-3-ACETIC ACID genes. Shoot induction on cytokinin-containing medium coincides with the upregulation of the shoot apical meristem genes *WUSCHEL* (*WUS*) and *CLAVATA3* (*CLV3*) (Che *et al*., 2006; Bao *et al*., 2009), which might be applied in the future to enhance *in vitro* propagation as demonstrated by the overexpression of the *SHOOTMERISTEMLESS* (*STM*) and *ZWILLE2* (*ZLL2*) genes in *Brassica napus* (canola) and *Arabidopsis* plants (Elhiti and Stasolla, 2012; Chatfield *et al*., 2013).

In tomato (*Solanum lycopersicum*) and *Brassica rapa* (turnip), in which organogenic callus induction and plant regeneration are quantitatively controlled by several genes (Trujillo-Moya and Gisbert, 2012; Seo *et al*., 2013), candidate genes in the genetic quantitative trait locus (QTL) intervals were *APETALA2* (*AP2*)-containing ethylene response factors (ERFs), homologous to the *Arabidopsis ENHANCER OF SHOOT REGENERATION1* (*ESR1*) that is a well-characterized gene for shoot regeneration (Banno *et al*., 2001), MADS box genes related to adventitious shoot regeneration

(Prakash and Kumar, 2002), and serine-threonine kinases of which the tomato shoot kinase1 LESK1 is an *in vitro* organogenesis competence marker (Torelli *et al*., 2004).

Recent literature on shoot regeneration in *Arabidopsis* shows the need to revisit the concept of cell dedifferentiation upon organogenic callus formation (Sugimoto *et al*., 2011). Shoots originate from organogenic callus derived from specific pericycle-like cells surrounding the vasculature in roots, hypocotyls, or cotyledon explants (Che *et al*., 2007; Atta *et al*., 2009). The transcript profile of organogenic callus is strikingly similar to that of lateral roots (Che *et al*., 2006), indicating that shoot regeneration occurs via a "root developmental pathway" (Sugimoto *et al*., 2010). As pericycle cells are the progenitors of lateral roots in primary roots, they might be considered as "adult meristem cells" that differentiate into organogenic callus from which shoots develop upon hormonal stimuli in tissue cultures (Sugimoto *et al*., 2011).

Inherent to tissue culture procedures is the so-called somaclonal variation that refers to mutations, chromosome rearrangements and multiplication in some of the regenerated shoots, hinting at induction of stress reactions and accumulation of genetic aberrations during the dedifferentiation/redifferentiation processes (Jiang *et al.*, 2011). In order to restrict somaclonal variation, tissue culture duration should be kept as short as possible, tissue culture-related "stress" should be lowered by the addition of antioxidantia and organic buffer to the medium and lowering light intensity, and minimal concentrations of selectable agents and hormones should be used.

Techniques for higher plant transformation

The role of transgenes in higher plants can be studied through both transient and stable transformation methods. Stable transformants are generated by means of *Agrobacterium* infection or particle bombardment and contain a stably integrated transgene in their plant genome that segregates as a Mendelian trait and is inherited in subsequent generations (Fig. 2). In contrast, transient transformants are obtained by biolistic treatment or agroinfection of explants, such as epidermis of onion (*Allium cepa*) or protoplats by which gene construct expression, subcellular localization, or protein targeting can be tested without integrating the transgene into the genome and circumventing *in vitro* shoot regeneration procedures (Sheen, 2001). Agroinfiltration of *Nicotiana benthamiana* leaves is the preferred method to investigate *in-vivo* protein-protein interactions by means of fluorescence resonance energy transfer and bimolecular fluorescence complementation technology in which proteins are tagged with a fluorescent protein and their interaction is visualized by confocal microscopy (Yang *et al*., 2000; Boruc *et al*., 2010). Recently, transient transformation assays have been developed in cereals, such as rice (*Oryza sativa*) and

maize (*Zea mays*). A high fluorescent marker gene expression has been demonstrated in bombarded leaf explants of maize that consisted of the basal-most 3 cm above the ligule of an approximately 50-cm growing adult leaf using different fluorescent protein tags and correct localization to the endoplasmic reticulum, the Golgi apparatus, and the plasma membrane was demonstrated (Kirienko *et al*., 2012).

In stable transformants, transgenes are studied at the genetic, morphological, physiological, cell biological, and biochemical levels to gain insight into their function and might be the basis for translational or biotechnological research. Below, *Agrobacterium*-mediated transformation and direct gene transfer are discussed.

Agrobacterium-*mediated transformation*

Agrobacterium-mediated transformation exploits the bacterium as the biological vehicle to transfer gene(s) of interest into the plant cell. The basic biology related to *Agrobacterium tumefaciens*-mediated genetic transformation involves a number of proteins derived from both the plant host and the bacterial pathogen (Gelvin, 2012). Naturally, the bacterium induces crown gall tumors on various plant species, including many agronomically important crops. During its infection, *Agrobacterium* replicates a single-stranded copy of the bacterial transferred (T)-DNA that is located on the tumor-inducing (Ti) plasmid and transfers it into the plant host cell where it subsequently integrates into the host genome. The wild-type T-DNA encodes several genes involved in auxin and cytokinin biosynthesis that are expressed in the infected plant cells, with cell proliferation and tumor formation as a consequence. With the help of other T-DNA-encoded genes, the tumors synthesize and secrete opines, amino acid derivatives that can be metabolized mainly by *Agrobacterium*. This unique infection strategy allows *Agrobacterium* to hijack the host cell machinery and turn it into its own “food factory”. Although *Agrobacterium* mainly infects dicotyledonous plants in nature (De Cleene and De Ley, 1976), it can genetically transform virtually any eukaryotic species under laboratory conditions and has become the transformation vehicle of choice for the genetic manipulation of most plant species (Tzfira and Citovsky, 2006).

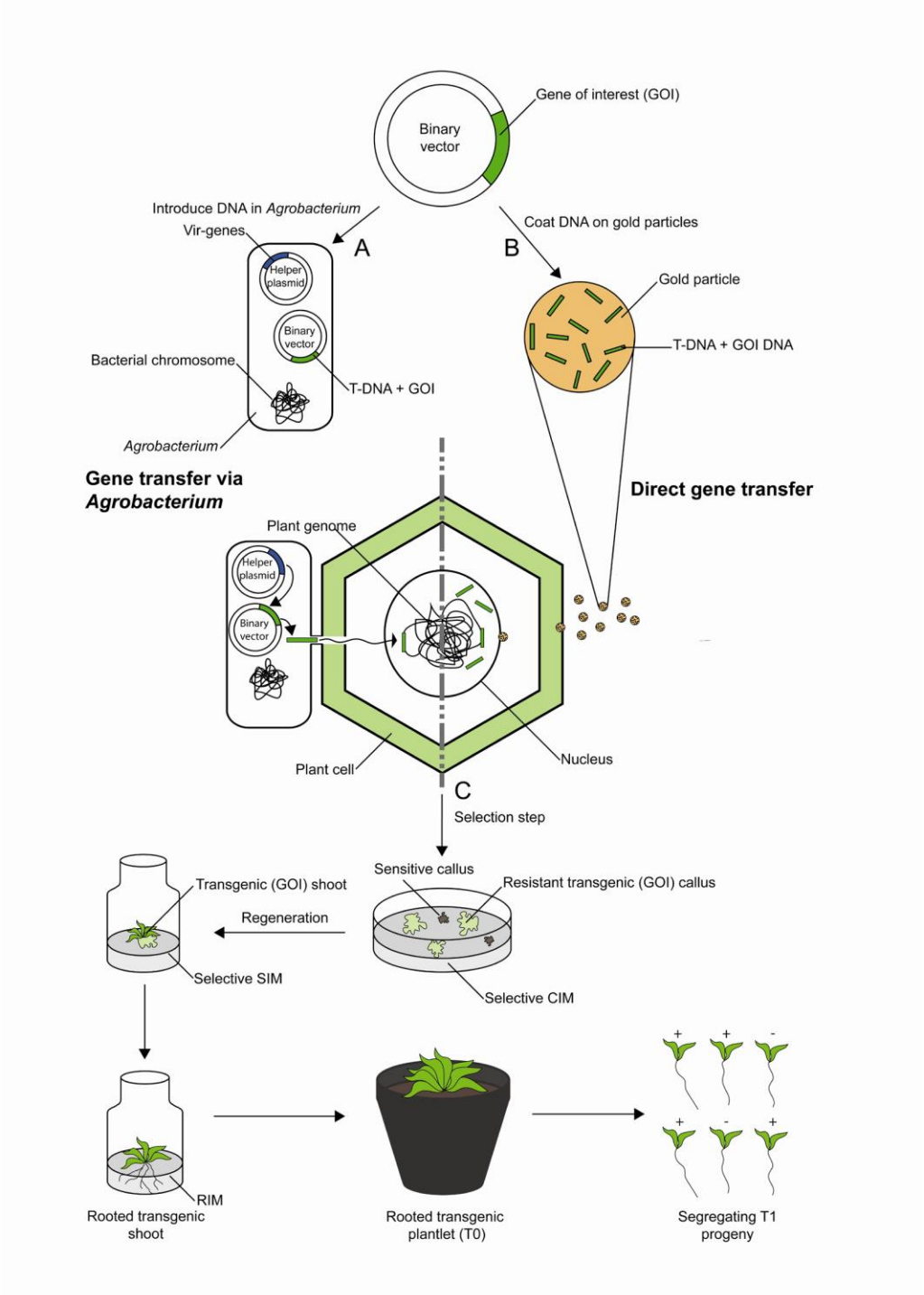

Fig. 2. Techniques for genetic transformation. (A) Agrobacterium-*mediated T-DNA transfer*. **(B)** *Direct DNA transfer through particle bombardment or biolistics.* **(C)** *Regeneration and selection of transformed callus and shoots.*

The T-DNA was adapted for genetic engineering by removing the hormone and opine biosynthesis genes and by replacing them with a selectable marker gene and one or more genes of interest, flanked by the T-DNA left and right border sequences that delineate the transferred DNA.

The T-DNAs are engineered in *Escherichia coli* on a binary vector that is introduced into an *Agrobacterium* strain containing a T-DNA-less, so-called "helper", Ti plasmid harboring the *vir* functions that are required for the bacterium-host recognition, the T-DNA replication in the bacterium, and the T-DNA transfer to and integration into the plant genome (Fig. 2A). Agrobacteria containing the engineered vectors are usually co-cultivated with specific explants from which transgenic shoots or somatic embryos are regenerated and selected in tissue cultures (Fig. 2C). In *Arabidopsis*, floral buds can be submerged in a liquid *Agrobacterium* culture and transgenic plants can be selected from the seeds (Clough and Bent, 1998), a method that avoids tissue culture procedures.

Upon *Agrobacterium*-mediated transformation, usually intact, single, or tandemly arranged T-DNA copies in one or two loci are stably integrated into AT-rich regions of the nuclear plant genome with minimal rearrangement of the target site (Gheysen *et al*., 1991). T-DNAs truncated at their left border do occur upon *Agrobacterium* transformation at low frequency as well as integration of vector backbone DNA (Tzfira and Citovsky, 2006). The selectable marker and the gene of interest, located on the T-DNA, are inherited in subsequent generations and segregate according to Mendelian genetics (De Block *et al*., 1984; Horsch *et al*., 1984). *Agrobacterium*-mediated transformation is the method of choice for overexpression or down-regulation of genes of interest in functional basic research or for generation and commercialization of superior crops thanks to its rather precise replication through the T-DNA border sequences upon infection and the low-copy T-DNA insertion into the genome. The T-DNA has been used as mutagen itself or to introduce transposons for mutagenization in several plant species, resulting in mutagenized collections that are invaluable resources for gene identification and functional analysis (Alonso *et al*., 2003).

Direct gene transfer

In nature, the host range of plant species and genotypes that are competent for *Agrobacterium* infection is limited, indeed, competence is determined by bacterial and plant host genes the study of which might help to enlarge the natural *Agrobacterium* host range. To overcome competence barriers, "direct" gene transfer methods have been developed in which genes of interest are delivered directly into regenerable plant tissues. Gene transfer by high-velocity microprojectiles (biolistic or particle bombardment) is widely used and has enlarged the range of species and genotypes for genetic engineering (O'Kennedy *et al*., 2011) (Fig. 2B). Metal particles coated with naked plasmid DNA containing the gene of interest are transported to the plant cells by means of an electric discharge or in a pressurized helium stream. Nearly all of the physical and

chemical parameters (rupture pressure, DNA concentration, particle travel distances, and vacuum degree) can be adjusted to different tissues and species. One of the first successes was the transfer of foreign genes into intact maize cells of a Black Mexican Sweet cultivar suspension culture (Klein *et al*., 1988). Particle bombardment is frequently used for the transformation of cereals with immature embryos as explants (Shrawat and Lörz, 2006) and is the only effective method to transform chloroplasts in plants.

However high-copy numbers and extensive rearrangements of the foreign DNA have frequently been reported in plants stably transformed with direct gene transfer methods. The integration of too many copies of the same gene within the genome normally results in gene silencing. In addition, sequences of the introduced gene have been found to be truncated, making the transgene analysis difficult and undesirable. Only DNA fragments of less than 10 kb in size can be transferred by the biolistic technology because large fragments get destroyed during the bombardment or adhere poorly to the metal particle, with messy DNA integration events as a consequence (Shou *et al*., 2004).

Selectable marker genes

Selectable marker genes are used to identify and select cells that have incorporated the T-DNA with the marker gene and the gene of interest upon genetic transformation of explants as well as to monitor and select the transformed individuals in subsequent generations. Upon infection or biolistic treatment of an explant, only a very small proportion of the cells is transformed; thus, the probability of recovering transformed lines without a selection system is very low. The most frequently used selectable markers include antibiotic resistance genes such as neomycin and hygromycin phosphotransferases, herbicide resistance genes such as phosphinothricin *N*-acetyltransferase and aceto lactate synthase, and metabolism-related genes such as phosphomannose isomerase that have been adopted for widespread use because of their efficiency and general applicability to a wide range of species and tissue culture systems (Table 1). Additional selectable markers related to metabolism such as xylose isomerase, trehalose-6P-synthase, and protoporphyrinogen oxidase are less frequently used and have been discussed before (Miki and McHugh, 2004). To function in a variety of cell types, selectable marker genes are constructed as chimeric genes, including regulatory sequences that ensure constitutive expression throughout the plant (Miki and McHugh, 2004).

Table 1: Frequently used selectable marker and reporter genes in plant transgenic research

Gene	Substrate and Enzyme	Origin	Effect	Reference
NPT II	Neomycin, kanamycin, geneticin (G418) and paramomycin neomycin phosphotransferases II	*Escherichia coli*	Inactivates a number of aminoglycoside antibiotics by phosphorylation	Fraley *et al*. (1983)
HPT	Hygromycin and hygromycin phosphotransferase	*Escherichia coli*	Inhibitor of protein synthesis	Waldron *et al*. (1985)
BAR, PAT	Phosphinothricin (PPT) and phosphinothricin acetyltransferase	*Streptomyces hygroscopicus, Streptomyces viridochromogenes*	Acetylation of PPT a competitive inhibitor of glutamine synthetase	De Block *et al*. (1989), Wohlleben *et al*. (1988)
ALS	Sulfonylureas and imidazolinones and acetolactate synthase	Mutated form: *Arabidopsis thaliana, Oryza sativa, Zea mays, Malus domestica*	Mutant ALS enzymes insensitive to herbicides	Olszewski *et al*. (1988)
manA	D-mannose and phosphomannose isomerase	*Escherichia coli*	Mannose used as carbon source	Joersbo *et al*. (1998)
EGFP	None and enhanced green fluorescent protein	*Victoria aequorea* (modified from GFP)	Fluorescence	Yang *et al*. (1996)
GUS	β-glucuronides and β-glucuronidase	*Escherichia coli*	Hydrolyzation of β-glucuronides	Jefferson *et al*. (1987)
LUC	Luciferin and luciferase	*Photinus pyralis*	Oxidative decarboxylation of luciferin	Ow *et al*. (1986)
R & Cl	None, R and Cl Anthocyanin transcriptional regulators	*Zea mays*	Anthocyanin accumulation in cell vacuoles	Ludwig *et al*. (1990); Lloyd *et al*. (1992)

Neomycin phosphotransferase II gene

Neomycin phospotransferase II (NPTII), also known as bacterial amino-glycoside 3'-phosphotransferease II (APH [3']), is an enzyme encoded by the *nptII* or *neo* gene that had been isolated from the *E. coli* transposon Tn*5*. It confers resistance to its host cells against a wide range of amino-glycoside antibiotics, including kanamycin, neomycin, geneticin (G418), and paramomycin, by catalyzing the phosphorylation of the 3'-hydroxyl group of the amino-hexose portion of these aminoglycosides. In this manner, NPTII detoxifies the antibiotic, allowing transformed *nptII*-

expressing plants to tolerate certain concentrations of the aminoglycoside antibiotics, unlike the nontransformed plants that undergo bleaching and growth inhibition at similar concentrations. The *nptII* gene has been used as a selection marker for vectors in prokaryotic and eukaryotic cells. It is the most widely used selectable marker system for generating transgenic plants, especially in dicotyledonous plants, such as *Arabidopsis* (Miki and McHugh, 2004). For constitutive expression in plants, the coding region of the *nptII* gene has been fused to the 5'- and 3'-regulatory sequences of the *Agrobacterium* T-DNA gene nopaline synthase (*nos*). This gene construct has been shown to be efficient in selection of tobacco cells on kanamycin or G418 (Herrera-Estrella *et al*., 1983). Transformants sensitive and resistant to kanamycin and their progenies can be distinguished through different techniques, including: seed germination assays on kanamycin-containing medium carried out to follow the *nptII* gene segregation in the progeny of primary transformants (De Block *et al*., 1984) and callus induction tests (Van Lijsebettens *et al*., 1991). Enzymatic *in vitro* assays that can detect the NPTII protein quantitatively or semiquantitatively are based on the transfer of the ^{32}P-labeled γ-phosphate group from ATP to kanamycin (Reiss *et al*., 1984), whereas an enzyme-linked immunoabsorbent assay has been developed as well (Nagel *et al*., 1992).

Hygromycin phosphotransferase gene

The hygromycin phosphotransferase (*hpt*) gene, also designated *aphIV*, was isolated from *E. coli* and encoded the enzyme hygromycin B phosphotransferase that confers resistance to the antibiotic hygromycin B (Waldron *et al*., 1985), which is an aminocyclitol antibiotic that inhibits protein synthesis with a broad spectrum activity against prokaryotes and eukaryotes. The *hpt* gene had been modified for expression in plants cells by developing a chimeric gene consisting of the nopaline synthase regulatory elements and the *E. coli*-derived *hpt* gene (van den Elzen *et al*., 1985). Hygromycin is more toxic in plants than kanamycin; hence, the *hpt* selectable marker is used when *nptII* is not effective. Hygromycin resistance in transformed plants can be checked in several ways, including callus induction tests (Van Lijsebettens *et al*., 1991), seed germination assays that can be used to score the segregation of the *hpt* gene in the progenies of the transgenic plants, and enzymatic assays (Datta *et al*., 1990).

Bialaphos-resistant and phosphinothricin* N-*acetyltransferase genes

The bialaphos-resistant (*bar*) gene, isolated from *Streptomyces hygroscopicus* (Murakami *et al*., 1986) and the *pat* gene, isolated from *Streptomyces viridiochromogenes* (Wohlleben *et al*., 1988), are 87 % homologous and code for the enzyme phosphinothricin *N*-acetyltransferase (PAT) that catalyzes the conversion of phosphinothricin (PPT) into a nontoxic acetylated form (Thompson

et al., 1987). PPT, also designated glufosinate ammonium, is an active ingredient of several broad-spectrum herbicide formulations, such as Basta™, Ignite™, and Liberty™ (BayerCrop Science) is an L-glutamic acid analog and a competitive inhibitor of glutamine synthetase, the only enzyme that can catalyze ammonium assimilation into glutamic acid in plants. Inhibition of glutamine synthetase results in the rapid accumulation of ammonia and, eventually, plant cell death. The PAT enzyme catalyzes the acetylation of the free amino group of PPT, making it unable to bind to and inactivate glutamine synthetase. To engineer herbicide resistance in plants, the *bar* gene was placed under the control of a *35S* cauliflower mosaic virus (CaMV) promoter and transgenic tobacco plants were resistant to high doses of PPT and bialaphos (De Block *et al*., 1987). Today, it is frequently used in cereals, such as maize, wheat (*Triticum aestivum*), rice, and barley (*Hordeum vulgare*) (Shrawat and Lörz, 2006). Several assays have been developed for the *bar*/*pat* marker gene activity in transgenic progenies, such as germination on PPT-containing medium, spraying plants, or painting leaves with the herbicide. Upon PPT treatment, ammonium accumulation in the medium can be determined with a colorimetric assay, specifically of nontransgenic seedlings that cannot assimilate ammonium due to glutamine synthase inhibition as opposed to transgenic seedlings that do assimilate ammonium (De Block *et al*., 1995). PAT proteins can be detected simply and quickly with the PAT assay kit (AgraStrip®LL Strip test kit; Romer Labs®, Union, MO, USA) that is based on a double-antibody sandwich principle.

Acetolactate synthase gene mutants

Acetolatate synthase (ALS) is the first common enzyme in the biosynthetic pathway of the branched-chain amino acids isoleucine, leucine, and valine. The plant *ALS* gene is a target for several classes of herbicides, including sulfonylurea, imidazolinone, and pyrimidinyl carboxyl. Naturally occurring mutations in *ALS* confer herbicide resistance to a number of plant species (Chang and Duggleby, 1998). Single or double amino acid substitutions can make ALS herbicide resistant and have been utilized as effective selectable markers in homologous or heterologous plant species. For instance, the *Arabidopsis als* mutant was applied in the selection of transgenic tobacco plants growing in a sulfonylurea herbicide-containing culture (Olszewski *et al*., 1988). Different point mutations in the *ALS* gene that alter conserved amino acids have been isolated in rice and introduced as selectable makers into rice, wheat, and soybean (*Glycine max*) transformations (Rosellini, 2011). The maize *als* mutant very efficiently selected transgenic maize in embryogenic cultures (Fromm *et al*., 1990); similarly, the apple (*Malus domestica*) *als* mutants generated by site-specific mutagenesis were used successfully as selectable markers in tobacco and apple transformation (Yao *et al*., 2013). The use of mutant plant *ALS* genes as selectable markers for

transgenic plants is gaining importance because they occur in all plants and, thus, are expected to be publicly acceptable, not prompting food safety concerns as in the case of bacteria-derived selection genes.

Phosphomannose isomerase gene

The phosphomannose isomerase gene (*pmi*) gene isolated from *E. coli* (also designated *manA* gene) encodes the enzyme phosphomannose isomerase (PMI) that catalyzes the reversible interconversion of mannose-6-phosphate and fructose-6-phosphate. Mannose-6-phosphate is obtained from mannose through a hexokinase phosphorylation activity; unlike fructose-6-phosphate, most plants cannot use it as a natural carbon source. Transgenic PMI-producing plants have a metabolic advantage over the nontransformed plants, because they are able to utilize mannose as a carbon source by converting mannose-6-phosphate to fructose-6-phosphate and can survive on a mannose-containing medium as the only or major carbon source. In nontransformed plants, mannose-6-phosphate accumulation inhibits glycolysis and leads to an arrest in cell growth and development. Although PMI is widely distributed in nature and found across kingdoms, it is absent in many plants, with the exception of soybean and other leguminous plants (Goldsworthy and Street, 1965). The PMI selection system employing the *E. coli manA* gene under the control of the 35S promoter has been reported to be very efficient. Indeed, transformation frequencies in sugar beet (*Beta vulgaris*) increased 10-fold when compared to the kanamycin selection (Joersbo *et al*., 1998). Similarly, the *E. coli manA* gene under the control of the maize ubiquitin promoter was reported to perform well in dicotyledonous and monocotyledonous plants (Reed *et al*., 2001). *PMI* expression assays have been applied to identify transgenic events and to sort them through segregating populations, such as a modified chlorophenol red assay (Kramer *et al*., 1993), in which the medium changed from red to yellow in tissues that could not metabolize mannose, and a seedling germination assay, in which the germination of nontransgenic seedings was completely inhibited when mannose was introduced into the medium (Reed *et al*., 2001).

Reporter genes

Reporter genes, also called screenable or scorable markers, are genes that code for proteins that can be detected directly or catalyze specific reactions with easily detectable products. They are particularly useful for the analysis of promoter activity, protein localization, and/or interaction studies. An ideal genetic reporter system should be *in situ* detectable, sensitive, quantitative, rapid, reproducible, safe, and with low or without endogenous background activity. The β-glucuronidase

(GUS), luciferase (LUC), enhanced green fluorescent protein (EGFP) and anthocyanin are the most commonly used reporter genes in plant research (Table 1).

Green fluorescent protein

The green fluorescent protein (GFP) is a photoprotein cloned from the jelly fish *Aequorea victoria* (Shimomura *et al*., 1962; Prasher *et al*., 1992). It is a very stable protein that autofluoresces in the presence of UV or blue light illumination and does not require an external substrate. Niedz *et al*. (1995) were the first to show that the wild-type *Aequorea* GFP could be visualized in plant cells as an *in vivo* reporter of plant gene expression. Although wild type GFP was used successfully in plant cell and tissue expression studies, it had some disadvantages, such aberrant splicing in plants and formation of cytotoxic and nonfunctional aggregates. Effective expression in whole plants was achieved upon modification of the GFP-coding sequence (Haseloff *et al*., 1997) that improved fluorescence intensity and thermostability. GFP has a small molecular size, thus facilitating the construction of fusion proteins for subcellular protein localization or protein-protein interactions. GFP visualization is nondestructive and allows the direct imaging of gene products in living tissues in real time at the cellular level. GFP has an autofluorescence capacity and, therefore, does not require addition of cofactors or exogenous substrates to produce light; moreover, it is very stable to heat, extreme pH, and chemical denaturants. Several GFP mutants have been developed through amino acid substitution, resulting in variants with altered excitation and emission spectra, such as variants with shifts to cyan, red, and yellow that are used for colocalization of specific proteins; these are discussed in detail in this special issue by Voß *et al.* (2013). EGFP is one of these GFP variants that is commonly used because of its improved fluorescence intensity (Yang *et al*., 1996).

β-Glucuronidase

β-Glucuronidase (GUS) is a bacterial enzyme encoded by the *E. coli uidA* (*gusA*) gene that occurs in microorganisms, vertebrates, and invertebrates, but not in most higher plants (Jefferson *et al*., 1987). It catalyzes the hydrolysis of a wide variety of β-glucuronides, such as the chromogenic histochemical 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), a colorless compound that is converted by the β-glucuronidase enzyme to an insoluble indigo blue product. It is used for *in situ* histochemical localizations of the β-glucuronidase activity in cells and tissues. The fluorogenic assay, in which β-glucuronidase cleaves the 4-methly-umbelliferyl glucuronide (MUG) substrate into a blue fluorescent compound, is used to quantify promoter strength (Coussens *et al*., 2012). The GUS enzyme is very stable within plants and nontoxic when produced at high levels (Miki and McHugh, 2004), but the assays are destructive.

Luciferase

The luciferase reporter gene (*luc*) originates from the firefly *Photinus pyralis* and encodes the enzyme luciferase (LUC) that catalyzes the ATP-dependent oxidative decarboxylation of luciferin, leading to an excited form of oxyluciferin and to light emission. The flash of light is captured with a luminometer that measures the integrated light output. The total amount of light measured during a given time interval is proportional to the amount of luciferase activity in the sample. Typically, the flash of light decays in seconds; enhanced light intensity and a more sustained light reaction were obtained when coenzyme A was provided to the reaction, thus increasing the sensitivity and reproducibility of the assay. Initially, the *in vivo* LUC activity was detected by spraying plant tissues with a luciferin substrate and squeezing it onto a film for exposure (Ow *et al*., 1986), currently, the faint light from the reporter can be detected with specialized cameras. An advantage of the LUC reporter system is that it permits the nondestructive monitoring of the gene expression patterns, including circadian rhythms, in real time and with great sensitivity (Millar *et al*., 1992; Xu *et al*., 2010). In addition, the luciferase assay is highly sensitive and results can be obtained within minutes. The limitations of the *in vivo* applications were overcome by the development of soluble luciferin forms that allow cell penetrance. Thus, LUC has been used to study regulated reporter gene activities *in vivo* in whole organisms, such as plants, as well as in single cells.

Anthocyanin

Anthocyanins are endogenous pigments responsible for the red, purple, and blue color in flowering plants, form a diverse family of aromatic flavonoid compounds, and play a role in protection against UV, defense response; and attraction of pollinators and seed dispersers. The biosynthesis of anthocyanins is controlled by a conserved triad of transcriptional regulators (an R2R3-MYB protein, a basic helix-loop-helix [bHLH], and a WD40 repeat protein) of which the overexpression results in increased accumulation of anthocyanin pigments that have been exploited to monitor both transient and stable gene expression in plants. Overexpression of one or more types of these regulatory genes led to cell autonomous pigmentation in maize, *Arabidopsis*, and tobacco (Ludwig *et al*., 1990; Lloyd *et al*., 1992; Chu *et al*., 2013). Anthocyanin accumulation is used as a visual marker in cereal transformation and transgenic seeds (Kawahigashi *et al*., 2007; Gao *et al*., 2011). The anthocyanin reporter is nondestructive, requires no exogeneous substrate, and is not toxic; hence, there are no environmental and health concerns related to it.

Promoters for chimeric gene construction

A promoter refers to the region in the genome sequence upstream of a gene transcription start site that controls the gene expression level and the kind of specificity, i.e. constitutive, inducible, tissue-specific, or developmentally regulated. In transgenic research, promoters are used to drive the expression of the selectable marker to select transformed callus and shoots during the transformation procedure, to follow the T-DNA segregation with the gene of interest in subsequent progenies, and to determine the expression level and specificity of the gene of interest in the progeny. Promoters either originate from heterologous species, which avoids gene silencing, or are cisgenic, which is considered similar to plants bred through conventional breeding methods and, therefore, more acceptable to consumers.

Constitutive promoters

Constitutive promoters direct high levels of gene expression in all cell types throughout the entire growth and development period and are used to overproduce proteins of interest to study their function in basic research or to generate superior plants or seeds for agronomical purposes. The *35S CaMV* gene *CaMV35S* (Odell *et al*., 1985) confers a high transgenic expression in most cell types, except in pollen, is independent on environmental conditions, and is well characterized and active in various monocotyledonous and dicotyledonous plants (Benfey and Chua. 1990). The maize *Ubi-1* promoter is derived from the constitutively expressed *Ubi-1* ubiquitin gene (Christensen *et al*., 1992) and is generally used in cereals (Shrawat and Lörz, 2006). Other strong constitutive promoters with a lot of potential in plant biotechnology have been identified in the rice actin genes, *OsAct1* and *OsAct2* (McElroy *et al*., 1990), in the *Brachypodium distachyon* elongation factor and ubiquitin genes, *pBdEF1a* and *pBdUBI10* (Coussens *et al*., 2012; Karimi *et al*., 2013), and in the switchgrass (*Panicum virgatum*) ubiquitin genes, *PvUbi1* and *PvUbi2* (Mann *et al*., 2011).

Organ, tissue, domain or cell type-specific promoters

Organ, tissue, domain or cell type-specific promoters are used when the transgene expression is preferred at a specific site and/or specific time in development to generate specific phenotypes and avoiding adverse effects on plant growth or yield. Several endosperm-specific promoters have been identified and are used to express single or, even, multiple enzymes of biochemical pathways either to dissect the metabolic pathway or to improve the nutritional seed quality (Naqvi *et al*., 2009; Coussens *et al*., 2012). Other promoters have been used for basic or biotechnological research, such as the TA29 tapetum-specific promoter that has been used

successfully for the generation of male sterility in canola (Mariani *et al*., 1990). The *AP1* promoter of the A-type flower gene, *AP1* that is active in floral whorl one and two- has been used to silence the *AP3* gene in whorl two and create a doubled sepaloid flower in *Arabidopsis* and canola (Byzova *et al.*, 2004) in order to avoid reflection of the bright-yellow canopy, capture more sun light and increase yield.

Inducible promoters

Inducible promoters are specifically activated in response to external stimuli. In contrast to constitutive promoters, the fused transgenes can be expressed at a distinct developmental stage for a certain duration or in a specific tissue. Additionally, the promoters are inactive in the absence of inducers and, therefore, have no negative impact on plant development. The promoter activity can be induced by chemical factors, such as tetracycline, ethanol, steroids, copper ions, and herbicides, or by physical factors, such as heat, cold, and light. Promoters that respond to specific chemical compounds, not found naturally in the organism of interest, are of particular interest in genetic engineering because of the manipulation ease. Some of the most commonly used chemically inducible promoters in plants (Padidam, 2003) are briefly described. Tetracyclines are particularly attractive as gene expression inducers, because they are small lipophilic compounds that enter easily into eukaryotic cells by passive diffusion and they have been routinely used in both human and veterinary medicine with negligible side effects. The tetracycline-inducible system consists of three main components: the transcriptional repressor, the tetracycline-responsive operator, and an antibiotic of the tetracycline family. The tetracycline-inducible system has been used successfully to produce valuable pharmaceutical or industrial proteins in plant cell suspension cultures (Bortesi *et al*., 2012). In the steroid-inducible systems, heterelogous proteins are fused to a receptor for glucocorticoid or estrogen and induced by steroids. The glucocorticoid receptor-based steroid-inducible system has significantly advanced the insight into the function of plant transcription factors that control plant developmental pathways (Lloyd *et al*., 1994; Aoyama *et al*., 1995). The ethanol-inducible gene expression system is derived from the filamentous fungus *Aspergillus nidulans* and consists of two elements: the alcohol-regulated transcription factor (ALCR) that binds the *alcA*-derived promoter that regulates the expression of the transgene (Roslan *et al*., 2001). The ethanol-inducible system has been optimized for the production of proteins in plants (Dugdale et al., 2013).

Databases for plant promoter sequences

Functional analysis of genes in transgenic plants often demands selection of promoters with appropriate activity patterns. Promoters commonly used in vectors are very limited and provide only little variation in gene expression patterns. Thus far, with an increasing number of plant genome sequences, it has become necessary to develop a robust computational method for detecting novel plant promoters in transgenic research. To date, a wide variety of programmes for predicting promoters are available, including PlantPAN (Chang *et al*., 2008), GRASSIUS (Yilmaz *et al*., 2008), PlantCARE (Lescot *et al*., 2002) and TransGene Promoters (TGP) database (Smirnova *et al*., 2012). However, promoters identified by prediction programs need to be tested by using reporter genes in plant development and under different stimuli in order to be applicable in transgenic research.

Vectors for higher plant transformation

Binary vectors

A binary vector system (Lee and Gelvin, 2008) consists of two plasmids: the helper plasmid that is constituted of the *Agrobacterium* Ti plasmid without T-DNA, but carries the *vir* genes that are necessary for the T-DNA transfer in the plant host genome and acts *in trans*, and the binary vector derived from the commonly used *E. coli* cloning vectors and carrying the gene of interest, flanked by 25-bp terminal repeats, designated the right and left T-DNA border sequences. The binary cloning vector is a standard molecular tool in the *Agrobacterium*-mediated transformation of higher plants, because it is easy to manipulate *in vitro* by recombinant DNA methods (Bevan, 1984). Cloning vectors can be assembled to facilitate fusion, overexpression, or downregulation of a variety of genes in plant cells. Their basic skeleton includes a gene of interest under a specific promoter in addition to a selectable and/or reporter gene. Superbinary vectors have additionally the *virB*, *virG*, and *virC* virulence genes of the supervirulent pTiBo542 plasmid, requiring yet another intermediate cloning vector and cointegration step in *Agrobacterium,* complicating the cloning (Komori *et al*., 2007). A good alternative strategy to the use of superbinary vectors is the use of the supervirulent *Agrobacterium* strain EHA101 that contains the supervirulent *vir* genes of pTiBo542 in the helper plasmid (Hood *et al*., 1986; Frame *et al*., 2002; Coussens *et al*., 2012).

GATEWAY vectors are a set of versatile and robust T-DNA binary vectors that enable rapid and efficient cloning and transfer of DNA fragments between vector backbones. The utilization of these vectors overcomes the cumbersome conventional cloning procedure, involving DNA restriction and ligation reactions, and the efforts to develop small vectors with unique restriction sites. The

GATEWAY cloning technology takes advantage of the site-specific reversible recombination system of phage λ that enables rapid and efficient cloning and transfer of PCR DNA fragments related to promoters, cDNA, or gDNA between different expression vectors (Hartley *et al*., 2000). The DNA fragment is first captured in a GATEWAY donor vector (pDONR) through a site-specific recombination reaction resulting in a GATEWAY entry clone (pENTR). Subsequently, the DNA fragment can be recombined into many different GATEWAY destination vectors (pDEST) depending on the necessity for overexpression, silencing, and promoter analysis, resulting in an expression clone (pEXPR). Several components are essential in the Gateway cloning procedure: the *att* sites, the *ccd*B gene, and clonase enzymes that recognize the *att* site, thus facilitating the recombination reaction. The GATEWAY *att* sites are phage-derived recombination sites that facilitate directional cloning and maintain orientations and reading frames of the DNA fragments. The *att*B sites that flank the DNA of interest recombine with *att*P sites in a donor vector yielding *att*L sites in a novel entry clone, a reaction catalyzed by the BP clonase enzyme. Similarly, the *att*L sites in the entry vector recombine with the *att*R sites of a destination vector to yield the *att*B sites in a novel expression clone. Directional cloning is enabled by ensuring that only specific sites recombine, for instance, *att*B1 with *att*P1 and not *attP*2 (Hartley *et al*., 2000). The BP clonase enzyme is composed of the phage integrase and the *E. coli* integration host factor, whereas the LR clonase consists of the phage integrase, the *E. coli* integration host factor, and the phage excisionase. GATEWAY vectors can be selected and maintained by the use of *ccd*B gene and antibiotic selection markers. The *ccd*B gene is a negative counterselection marker encoding a protein that interferes with the DNA gyrase, thus inhibiting the *E. coli* growth. *ccdB* is present in the pDONR or pDEST vectors and is replaced by the DNA of interest upon recombination; hence, *E. coli* cells with the correctly recombined plasmids will survive, whereas cells with unreacted vectors or byproduct-containing *ccdB* will fail to grow. Multisite GATEWAY is an extension of the GATEWAY technology, involving additional novel recombination sites with unique specificities to enable the simultaneous cloning of multiple fragments in a single highly efficient and specific *in vitro* LR clonase reaction. The fragments are cloned in an expression vector in a predefined order, orientation, and translation reading frame (Cheo *et al*., 2004).

GATEWAY vectors for plant transformation

GATEWAY destination vectors have been developed to analyze the gene function through *Agrobacterium*-mediated transformation of dicotyledonous or monocotyledonous plants (Karimi *et al*., 2007, 2013; Himmelbach *et al*., 2007). A versatile set of GATEWAY-compatible destination vectors (termed pANIC) has been constructed to be used in monocotyledonous plants for

improvement of transgenic crops either through transgene overexpression or interference RNA (RNAi)-mediated gene suppression (Mann *et al*., 2012). Unique plant GATEWAY RNAi vectors for the functional analysis of the metabolic pathway in root tissues have been described (Muranaka, 2011). Functional elements built as GATEWAY entry clones, such as promoters, terminators, open reading frames, or diverse tags, can be recombined in a single step in the multisite GATEWAY cassettes, thus simplifying design and construction of the recombinant DNA molecules (Karimi *et al*., 2007). GATEWAY MultiSite entry clones are potentially adaptable to any model system and an inventory of the entry clones and destination vectors for the GATEWAY MultiSite cloning has been established (Petersen and Stowers. 2011). A new series of binary GATEWAY cloning vectors (pAUL1-20) has been generated for C-terminal and N-terminal proteins fused in-frame to four different tags: a single hemagglutinin epitope, a streptavidin-tagII, both epitopes combined to a double tag, and a triple tag consisting of the double tag extended by a Protein A tag possessing a 3C protease cleavage site (Lyska *et al*., 2013). GATEWAY-compatible cassettes have been assembled for the expression of multiple genes (Chung *et al*., 2005) in addition to a GATEWAY recycling system for linking multiple expression cassettes (Kimura *et al*., 2013) and a modified multisite hybrid vector for stacking genes in plants (Vemanna *et al*., 2013).

Maize and Arabidopsis transformation platform at the Center for Plant System Biology (PSB), VIB-UGent

At PSB, maize transformation is carried out via the *Agrobacterium tumefaciens*-mediated transformation of immature embryos from the maize B104 inbred line using a super-virulent EHA101 *Agrobacterium* strain. Transformed embryogenic calli are selected using phosphinothricin or hygromycin and stable T0 transgenic shoots are generated through tissue culture regeneration then transferred to the green-house and backcrossed with wildtype B104. On average ten T1 transgenic seed events per construct are produced in a process that takes eight months from the *Agrobacterium* co-cultivation step (Coussens et al., 2012). Users of the platform benefit from the Multisite Gateway vectors optimized for monocots (Karimi et al., 2013), strong constitutive promoters derived from *Brachypodium distachyon* (Coussens et al., 2012) and the recent Golden Gateway collection tools available at the department. Currently fifty constructs per year are transformed into maize and further optimization is in progress to reduce tissue culture timelines, enhance tissue competence for Agrobacterium infection, to increase somatic embryogenesis and plantlet regeneration capacity.

Arabidopsis transformation at PSB is carried out via the *Agrobacterium*-mediated floral dip of Arabidopsis plants at early flowering stages (Clough and Bent, 1998). The dipped plants are allowed to grow to maturity and set seed, the whole process taking 6 weeks from the *Agrobacterium* infection step. The T0 seeds obtained are germinated in tissue culture on high density plates by the user, allowing for selection of transgenic progeny that will produce T1 seeds in fourteen weeks. T3 homozygous seedlings are generated for functional analysis of the transgene activity. No tissue culture or regeneration steps are required and thus the method avoids somaclonal variations and can be carried out by non-specialists. A thousand constructs per year can be transformed in *Arabidopsis* through floral dipping method at PSB.

Perspectives

Higher plant transformation technology has become an adaptable platform for cultivar improvement as well as for studying gene functions in plants. Plant DNA can be altered by introducing into a gene specific nucleotide substitution that change a protein's amino acid sequence, delete genes or chromosomal segments, and insert foreign DNA at precise genomic locations. Such targeted DNA sequence modifications are enabled by sequence-specific nucleases that create double-strand breaks in the genomic loci to be changed. The genomic alteration has recently been achieved through engineered zinc finger nucleases (ZFNs) (Hauschild-Quintern *et al*., 2012; Tzfira *et al*., 2012) and transcription activator-like effector nuclease *(*TALEN)-type transcription factors (Mussolino and Cathomen, 2012). Through an innovative method, designated directed nuclease editor (DNE), selected genes can be incorporated into the plant genome with an enhanced accuracy (http://www.research.bayer.com)

The engineering of a single gene to modify the plant metabolism has largely been promising, but many traits result from many interacting factors that need to be modulated. Such a modulation of complex pathways could be achieved through classical breeding or through the simultaneous engineering of multiple transgenes in nuclear and plastid genomes. It will be interesting to develop versatile molecular toolboxes for the engineering of multiple genes in organelles, such as mitochondria, that hitherto has not been successful. The choice of the preferred technology for multiple transgene engineering will be influenced by many factors, including the T-DNA transfer method, the targeted plant species, the cellular compartmentalization of the pathway of interest, the number of genes to be engineered into the plant, their desired expression levels as well as the available knowledge about metabolite pools, fluxes, and the biochemical regulation of the pathway (Bock, 2013).

Although selectable marker genes are useful tools in the production of transgenic plants by selecting transformed cells from a matrix consisting of mostly untransformed cells, their presence in genetically modified plants and, subsequently, in food, feed, and the environment, are of concern and subject to special governmental regulations in many countries. In addition, they could result in a metabolic burden for the host plants and prevent the reuse of the same selectable markers when a second transformation scheme is needed on the transgenic host. Therefore, innovation in selectable marker removal is necessary to improve existing systems and to develop new technologies. Although the focus of the ZFN and TALEN technologies has been the introduction of local genomic modifications, the ZFN technology has been used for plant selection marker gene deletion. For instance, a preintegrated cassette containing the GUS reporter gene flanked by two ZFN cleavage sites was deleted from a stably transformed plant by crossing it with a second plant expressing a corresponding *ZFN* gene, a method that can also be applied for selectable marker removal (Petolino *et al*., 2010). Furthermore, two identical sets of TALEN-binding sequences can be designed to flank a selectable marker in a transformation vector whereby, after expression of TALEN, double-stranded breaks will be induced at both TALEN-binding sequences and remove the selectable marker (Yau and Stewart, 2013).

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CHAPTER 3

Mutagenesis as a tool for functional analysis of plant genes

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Abstract

Gene functional analysis is a broad subject as various tools and techniques have been developed over time and are still being explored to accurately annotate gene function in an attempt to solve many biological questions. Success in whole genome sequencing of numerous organisms has resulted in many predicted gene sequences but their biological functions are still unknown. In this chapter a brief overview of the genetic techniques used in modulating the Arabidopsis and maize Poly(ADP-ribosyl)ation pathway to study the plant energy homeostasis and stress response are briefly discussed.

3.1 Forward and reverse genetics

Genetics relies on the study of variants, either found in natural populations or induced by mutagenesis. Forward genetics is a scientific approach of determining the genetic basis of a phenotype; it seeks to identify genes involved in a biological pathway through the screening of populations that contain random modifications throughout the genome that can alter gene function. Populations that carry interesting modified alleles are identified by the observation of the phenotypes, and subsequent mapping of the allele within the genome to reveal genes that are associated with the observed biological process (Lawson et al., 2011). In forward genetics, chemicals like ethylmethanesulfonate, γ radiation or movable or heterologous DNA (transposons or T-DNA) are often used to generate mutations in model organisms, subsequent crossing is carried out, mutant individuals are isolated, and then the gene is mapped.

Reverse genetics approach, on the other hand, seeks to find what phenotypes arise as a result of particular genetic sequences. It begins with a gene of interest and seeks to identify the phenotype of mutation of the gene. Success in the whole genome sequencing of many organisms has resulted in many predicted gene sequences but their biological functions are unknown. Thus reverse genetics is a powerful tool for assigning functions to predicted genes. The process of DNA sequence alteration can either be targeted specifically as in the case of gene silencing or homologous recombination or can rely on non-targeted random disruptions (e.g. chemical mutagenesis, radiations, insertional mutagenesis) followed by screening a library of individuals for mutations at a specific location (Tierney and Lamour, 2005).

3.2 Insertional mutagenesis

A direct way to obtain information on the function of a gene is to create a loss of function mutation and study the phenotype of the resulting mutant. One way of disrupting a gene function is by addition of a fragment of DNA into the pre-existing sequence called insertional mutagenesis. Insertion mutants can occur naturally as in the case of transposon or viral mediated DNA integration or can be created through introduction of foreign DNA into a gene sequence. DNA elements that are able to insert at random within chromosomes, such as transposons (Martienssen, 1998) or the T-DNA of Agrobacterium tumefaciens (Van Lijsebettens et al., 1991; Krysan et al., 1999), can be used as mutagens to create loss of function mutations in plants. The foreign DNA not only disrupts the expression of the gene into which it is inserted but also acts as a marker for subsequent identification of the mutation. The mutants are then screened for unusual phenotypes, if any such phenotype is found, then it can be assumed that the insertion has caused the gene relating to the

unusual phenotype to be disrupted and the phenotype identifies the process in which the identified protein functions.

However, insertion mutants do not always result in visible phenotypes due to gene functional redundancy, or they may result in early lethality that obscures late-acting functions when the same gene has multiple functions in development. To overcome this challenge, insertional elements are engineered with reporter cassettes that will report on the expression of the chromosomal gene at the site of insertion. Two types of these reporter cassettes are commonly used: Enhancer trap which contains a reporter fused to a minimal / weak promoter that can respond to nearby endogenous enhancers when the cassette is inserted within or close to a gene and a Gene trap in which the reporter gene is fused to a splice acceptor so that integration within introns leads to read-through transcription and splicing (Martienssen, 1998; Bouchez and Hofte, 1998; Ramachandran and Sundaresan, 2001). GUS gene (Jefferson et al., 1987) is the most frequently used reporter gene because of its accurate detection of the gene product and tolerance of N-terminal translation fusions in its enzyme activity (Jeon et al., 2000). In addition, gene disruptions through insertional mutagenesis nearly always generate recessive loss of function mutations which do not always produce an obvious phenotype due to factors such as functional redundancy. In such cases, increasing the expression level or ectopically expressing a gene can provide dominant gain of function mutations that produce informative mutant phenotypes. This is achieved through activation tagging in which a strong enhancer or promoter is fused to the T-DNA or transposon insert resulting in over-expression of nearby genes through transcriptional activation (Ramachandran and Sundaresan, 2001).

Various populations of mutagenized plants, either with heterologous transposons (mainly maize transposons such as Ac/Ds, En/Spm, or Mu) or the T-DNA of A. tumefaciens, have been produced in several plant species (Arabidopsis, tomato, maize, and rice) (Cooley et al., 1996; Azpiroz-Leehan and Feldmann, 1997; Jeon et al., 2000; Brutnell and Conrad, 2003). The use of T-DNA and transposon insertion mutagenesis for reverse genetics will be discussed further and their application in functional genomics of plants.

3.2.1 T-DNA insertional mutagenesis

The Transfer DNA (T-DNA) of Agrobacterium is a suitable mutagen for the generation of insertional mutant lines because it integrates randomly in the genome and the insertion alleles are stable through multiple generations. Through polymerase chain reactions, it is possible to isolate the individual plants carrying a particular T-DNA mutation of interest. Unlike transposons, T-DNA

insertions will not transpose subsequent to integration within the genome and are therefore chemically and physically stable. In addition, since each transformant yields a stable insertion in the genome there is no need for additional steps to stabilise the insert as in transposon mutagenesis. Insertional mutagenesis using the Agrobacterium mediated T-DNA integration into plant genomes has proven to be very successful and has been used in Arabidospsis, rice and maize (Van Lijsebettens et al., 1994; ` Azpiroz-Leehan and Feldmann, 1997; Krysan et al., 1999; Jeon et al., 2000). Different groups have used this approach to generate population of T-DNA mutagenized Arabidopsis thaliana lines that can be used for reverse genetics including: SAIL (Sessions et al., 2002), SALK lines (Alonso et al., 2003), GABI-Kat (Rosso et al., 2003) and WISCDSLOX (Woody et al., 2007). Modified T-DNA insertions have been used in A. thaliana as gene traps (Babiychuk et al., 1997), promoter traps (Lindsey et al., 1993) and in activation tagging (Weigel et al., 2000). T-DNA insertions have also been used for functional genomics in rice (Jeon et al., 2000).

There are few drawbacks of T-DNA insertion mutagenesis despite the great success in its use. The integration of T-DNA may result in tandem direct or inverted repeats and deletions in one or more border. These occurrences can make the subsequent molecular analysis difficult and affect the success of subsequent strategies such as development of flanking sequence databases. Secondly, complex and multiple insertions are more likely to lead to inaccurate patterns of reporter gene expression when using gene traps or enhancer traps. Finally, the T-DNA approach is very useful for plant species where quick and efficient methods of transformation are available but may not be feasible in plants species where transformation methods are slow or labour intensive (Ramachandran and Sundaresan, 2001).

In Arabidopsis, the introns are small and there is very little intergenic material thus, insertion of a piece of T-DNA of approximately 5 to 25kb in length produces significant disruption of the gene function. Therefore, when a large population of T-DNA transformed lines is available, there is a good chance of finding a plant carrying a T-DNA insert within any gene or interest. Homozygous lethal mutations can be maintained in the population in the form of heterozygous plants. Alternative approaches in the Agrobacterium-mediated transformation techniques such as seed transformation, transformation of intact plants and floral dip method have made the T-DNA insertion a viable method for approaching genome-wide mutagenesis while minimizing the effects of somaclonal variations linked to in vitro culture and regeneration (Krysan et al., 1999). In this work, Arabidopsis T-DNA insertion lines obtained from the SALK mutant collection were used in functional analysis of Nudix hydrolase gene.

3.2.2 Transposon mediated mutagenesis

Transposable elements provide a convenient and flexible means to disrupt many genes generating a large population of insertions. Transposon mediated mutagenesis offers several advantages over T-DNA mutagenesis. First, transposons are single intact elements which allow easy molecular analysis on insertion into a new region. The insertions are less likely to result in faulty patterns of expression if the transposon being used is a gene trap or enhancer trap as there are no direct or inverse repeats during DNA integration. Secondly, since many transposons can be excised from the disrupted gene in the presence of a transposase, phenotypic reversion to wildtype trait is possible. This phenotypic reversion provides ready confirmation that the mutation was caused by the transposon insertion. Thirdly, since several transposons preferentially integrate into genetically linked sites, this property can be used to perform local mutagenesis in particular regions of interest by remobilising the transposons. Finally, transposon mutagenesis is of great advantage in plant species where transformation is inefficient or laborious since new insertions are generated though crossing or selfing rather than through transformation (Ramachandran and Sundaresan, 2001). Maize has a rich collection of native transposon families utilized in large-scale mutagenesis experiments to produce genome-wide genetics resources. The two most popular, *Activator*/*Dissociator* (*Ac/Ds*) and *Mutator* (*Mu*) transposons will be further elaborated.

3.2.2.1 Activator/Dissociator (Ac/Ds) transposons

Ac/Ds were the first transposable elements discovered by Barbara McClintock (McClintock, 1956). The *Ac* element is autonomous whereas the *Ds* element requires an activator element to transpose. These elements share an 11bp terminal inverted repeat sequence and have sequence heterogeneity both in length and content. They have a tendency to preferentially transpose to genetically linked sites (Jones et al., 1990), a feature exploited for directed tagging of a specific gene or performing insertional mutagenesis within a targeted region of the chromosome (Brutnell and Conrad, 2003). Although native to maize, *Ac/Ds* elements have been shown to transpose actively in other species such as tobacco, Arabidopsis, rice, tomato and potatoes where they have been exploited for heterologous gene tagging studies (Nelissen et al., 2003). The primary limit of *Ac* as a tool for forward and reverse genetics in maize lies in its relatively low mutation rates. *Ac* populations are approximately 100-fold less mutagenic than *Mutator* populations (Walbot 2000)

3.2.2.2 Mutator transposons

The Maize *mutator* (*Mu*) transposon is the most active plant DNA transposon discovered to date with approximately 50–100 copies transposing on average in each generation. It was first described by Donald Robertson (1978) thus commonly referred to as Robertson's *Mutator* transposon. The *Mu* transposon family is a two component system consisting of the autonomous *MuDR* element that controls the transposition of itself and that of 12 non-autonomous *Mu* elements (Walbot and Rudenko, 2002). All maize *Mu* elements contain conserved approximately 215 bp terminal inverted repeats (TIRs) and upon insertion generate a 9 bp target site duplication directly flanking the Mu elements. However, each class of these elements contains a unique internal sequence. *Mu* is also one of the most successfully used high-copy number transposon with a high rate of germinal transposition and unlike *Ac*; germinally transmitted excisions of *Mu* are rare (Walbot 2000). *Mu* elements do not exhibit a preference for transposition to a nearby site as in the case of *Ac/Ds* transposons and are therefore suitable for genome-wide mutagenesis screens. *Mu* elements are also reported to have a pronounced preference for insertion at the 5' ends of genes with the strongest preference near the transcription start site. Additionally, regions close to the ends of chromosomes experience more *Mu* insertions than do pericentromeric regions (Liu et al., 2009).

Mu elements accumulate to a high copy number within maize lines harbouring active *Mu* due to their transposition properties. This allows for a relatively small population (~ 40,000 plants) to have a high chance of mutating most genes in the genome. Consequently, several groups have developed *Mu* transposon-tagging populations namely: Trait utility system for corn (TUSC) (Bensen et al., 1995), *Mu*AFLP (Hanley et al., 2000), *RescueMu* (Raizada et al., 2001), Maize Targeted Mutagenesis (MTM) (May et al., 2003), Photosynthetic Mutant Library (Stern et al., 2004), UniformMu (McCarty et al., 2005).

UniformMu population

UniformMu is a large inbred maize population developed specifically for transposon mutagenesis of the maize genome. The population was developed by backcross introgression of an active, autonomous, *MuDR* transposon into colour converted, genetically uniform, W22 maize inbred (McCarty et al., 2005). Colour converted lines were developed through introgression of a mutable *bronze-1 mu-mutable-9* (*bz1-mum9)* anthocyanin biosynthetic gene (Brown and Sundaresan., 1992) into the W22 genetic background. The *bz1-mum9* allele contains a non-autonomous *Mu1* transposon insertion that disrupts the *Bronze 1 (Bz1)* gene. *Bz1* gene encodes a UDP-glucose flavanol glucosyl transferase that catalyzes a key step in biosynthesis of purple anthocyanin pigment in the

seed aleurone. Thus, all UniformMu stock is bronze coloured as opposed to the purple colour of W22 inbred and homozygous for the *bz1-mum9* mutation. *Mu* transposons were stabilized by selecting against somatic transposition based on reversion of the *bz1-mum9* locus in the endosperm. A Mu-active plant shows sectors of purple spots on bronze-colored aleurone, an indicator of the presence of *MuDR* which induces transposition of the *Mu1* element in *bz1-mum9* locus. The spots are due to small, typically single-cell, revertant sectors that produce purple anthocyanin. In the absence of *MuDR*, the *bz1-mum9* allele has a uniformly bronze-colored aleurone (Fig. 1). Analysis of the UniformMu transposon-inactive lines did not show reversion of the *bz1-mum9* locus, and all lines segregated for independent seed mutant phenotypes (Settles et al., 2004; McCarty et al., 2005; Settles et al., 2007).

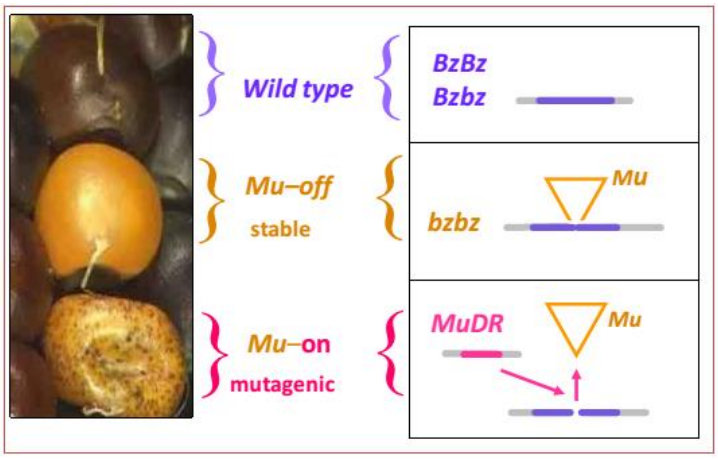

Fig. 1: Color effects on the maize seed aleurone due to the presence or absence of the MuDR in the bronze locus. Figure adopted from UniformMu resource, 2011

The UniformMu population was designed to address specific constraints of high copy transposon populations. Firstly, the heterogenous genetic background that limits detection of tagged mutation and phenotypic analysis of knockouts and secondly, the accumulation of parental mutations. Thus the key features of the UniformMu resource includes: highly mutagenic activity in a homogeneous inbred background, genetic control of Mu activity for suppression of Mu transposition prior to molecular analysis, eliminated parental seed mutations to maximise independence of seed mutations, mapped heritable insertions and a sustainable seed resource (McCarty et al., 2005). UniformMu genetic stocks that are freely distributed by the Maize Cooperation Stock Centre using online tools maintained at MaizeGDB.org. Genetic and molecular analyses of UniformMu insertion lines requires development of genotyping assays that use a gene-specific polymerase chain reaction to follow segregation of transposon insertions in genes of interest. In this work, UniformMu insertions in the Poly(ADP-ribose) Polymerase and Nudix hydrolase genes were screened and ordered for use in functional analysis.

3.3 Genome editing using CRISPR/Cas9

Genome editing is a type of genetic engineering in which DNA is inserted, deleted or replaced at a specific site in the genome of a living organism or cell using engineered nucleases also known as “molecular scissors”. The nucleases create site-specific double stranded breaks (DSB) at desired locations in the genome which stimulates the DNA repair pathways within the cell to make modifications. The repairs occur via two possible pathways, non-homologous end joining (NHEJ) pathway or homologous recombination (HR) pathway (Symington and Gautier, 2011). Double stranded breaks are most frequently repaired by NHEJ pathway which is error prone and can result in the introduction of insertions or deletions resulting in frameshift mutations in the coding sequence of genes. On the other hand, if a double stranded break is introduced in the presence of a homologous donor sequence, then repair may occur via a homology-directed repair pathway. The HR pathway can be exploited by inserting an exogenous sequence that is homologous to the flanking sequences of a DSB which, when used as a template by HR system, would lead to the creation of the desired change within the genomic region of interest.

Creation of site-specific DSB requires precise enzymes which are able to recognize and interact with DNA sequences only at the target site. Normal restriction enzymes, which recognize short sequences that often occur at several sites in the genome cannot be used as they are likely to cut the DNA molecule several times. Targeted genome engineering is performed using enzymes which are able to recognize and interact with DNA sequences that are sufficiently long so as to occur only once, with high probability, in any given genome. A number of commonly used Engineered Nucleases include: Meganucleases (Curtin et al., 2012), Zinc Finger Nucleases (ZFNs, Kim et al., 1996), Transcription Activator Like Effector Nucleases (TALENs, Christian et al., 2010) and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats). In this work CRISPR nuclease was used in editing maize genome and will be discussed further.

Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) editing system is a recently developed tool for the introduction of site-specific double-stranded DNA breaks based on RNA-guided engineered nucleases. The system is adapted from prokaryotic CRISPR/Cas immune system protecting them against invading nucleic acids such as viruses by cleaving the foreign DNA in a sequence specific manner. The most widely used system is the type II CRISPR/Cas9 system from *Streptococcus pyogenes* (Jinek et al., 2012) which incorporates sequences from foreign DNA between CRISPR repeat sequences encoded as arrays within the bacterial host genome. Transcripts resulting from the CRISPR repeat arrays are processed into

CRISPR RNAs (crRNAs), each containing a variable sequence transcribed from the foreign DNA, known as the protospacer sequence, and part of the CRISPR repeat sequence. crRNA hybridizes with another RNA known as the transactivating CRISPR RNA (tracrRNA), and the two RNAs complex with the Cas9 nuclease. When the protospacer is adjacent to short sequences known as protospacer adjacent motifs (PAMs), it guides the Cas9 to cleave complementary target DNA sequences. Protospacer sequences incorporated into the CRISPR locus are not cleaved probably because they are not next to a PAM sequence (for reviews on CRISPR/Cas9 see Doudna and Charpentier, 2014; Hsu et al., 2014; Sander and Joung, 2014).

CRISPR/Cas9 system became an important tool for genome engineering when it was shown that any target DNA sequence of interest could be modified by changing 20 nucleotides in the crRNA and that the targeting specificity of the crRNA could be combined with the structural properties of the tracrRNA in a chimeric guide RNA (gRNA), thus reducing the system from three to two components (Jinek et al., 2012). Fig.2 shows a comparison of the naturally occurring type II and engineered CRISPR/Cas9 systems.

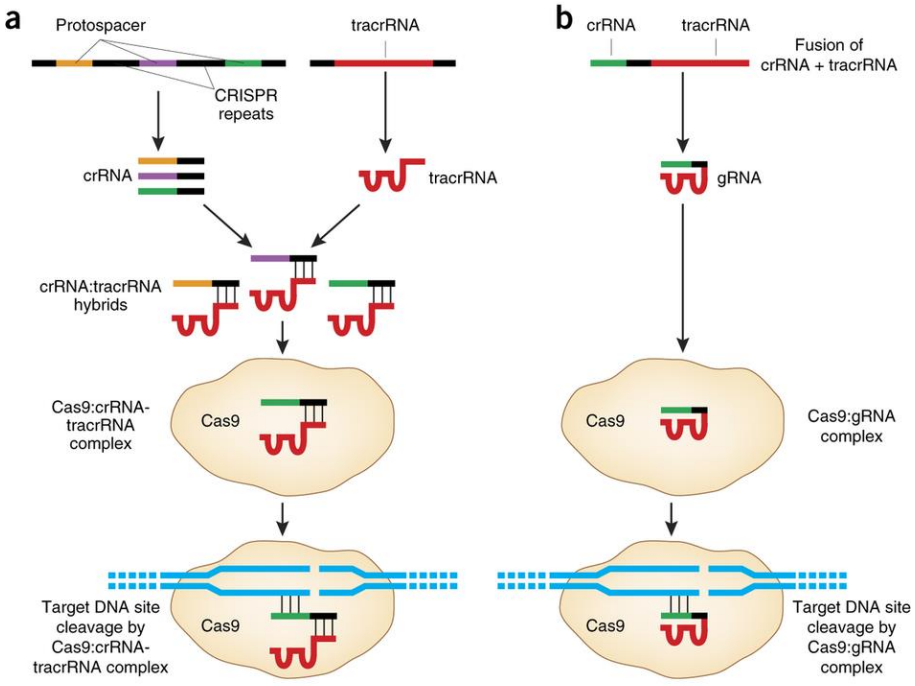

Fig. 2: DNA cleavage using (a) Naturally occurring type II CRISPR/Cas9 system composed of a three component system: crRNA, tracrRNA and Cas9 nuclease in comparison with (b) an engineered CRISPR/Cas9 system with a two component system: gRNA and Cas9 protein (Sander and Joung, 2014).

The two components, Cas9 nuclease and a guide RNA, must be introduced into and/or expressed in cells or an organism to perform genome editing. The 20 nucleotides at the 5' end of the gRNA

directs Cas9 to a specific target DNA site using standard RNA-DNA complementarity base-pairing rules thus the 20 nucleotides of the gRNA always need to correspond to the target DNA sequence. Also importantly, the target sites must lie immediately 5' of the PAM sequence (5' NGG from *S. pyogenes* Cas9 is currently the most widely used in genome engineering. Other PAM sequence variants corresponding to Cas9 from different species are also available). It has also been shown that multiple gRNAs with different sequences can be used to achieve high-efficiency multiplex genome engineering at different loci simultaneously.

The CRISPR/Cas9 system is the latest ground-breaking technology for genome editing and already holds a great promise due to its simplicity, efficiency and versatility. It has been used to precisely and efficiently target, edit, modify, regulate, and mark genomic loci of a wide array of cells and organisms from bacteria to humans and also is widely applied in plants. In this project we used CRISPR/Cas9 system to introduce indels at the maize PARP genes to study drought and oxidative stress tolerance in response to PARP gene mutation.

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CHAPTER 4

Perturbation of the maize PARP gene expression using RNAi hairpin silencing and CRISPR gene editing as a strategy for abiotic and genotoxic stress tolerance

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Contribution: E.N. carried out experiments (CRISPR gene cloning and genotypings, RNAi lines expression analyses, T3 lines generation and DNA damage assay) and wrote the chapter.

Abstract

The role of Poly(ADP-ribose) polymerases in DNA damage repair, chromatin modification, cell cycle, transcription regulation, telomere dynamics and cell death is well characterised in animal science but there are fewer reports in plant science. Significant work has been done in Arabidopsis showing the involvement of Poly(ADP-ribose) polymerases in DNA damage, biotic and abiotic tolerance. In this work we attempt to contribute to the knowledge by studying the function of PARP genes in maize. The ZmPARP gene expression was perturbed using either RNAi hairpin silencing or CRISPR/Cas9 gene editing technology, transgenic lines were characterised and genotoxic stress tolerance evaluated. Analysis of the mild drought stress response of the CRISPR and RNAi maize PARP lines will be shown in chapter 5 together with the mild drought stress data of maize NUDX overexpression lines.

4.1 Introduction

Poly(ADP-ribose) polymerases (PARPs) are a large family of proteins found in a wide variety of organisms from archaebacteria to mammals and plants but are absent in yeast. They display a conserved PARP catalytic domain and catalyze a covalent post-translational protein modification in eukaryotic cells via the polymerization of ADP-ribose units from donor NAD^+ molecules onto nuclear target proteins such as histone and transcription factors, resulting in the attachment of linear or branched poly(ADP-ribose). PARP enzymes are well studied in mammals and reported to be involved in DNA damage repair, chromatin modification, cell cycle, transcription regulation, telomere dynamics and cell death (Ame et al., 2004; Burkle et al., 2005). In plants although not as extensively studied, PARPs are mostly reported in the context of biotic and abiotic stresses (De Block et al., 2005; Vanderauwera et al., 2007; Adams-Phillips et al., 2010; Feng et al., 2015; Song et al.,2015). PARPS are also reported to be involved in DNA repair, cell cycle, genotoxic stress and recently in plant growth by promoting leaf cell number (Lamb et al., 2012; Doucet-Chabeaud et al., 2001; Schulz et al., 2014).

Downregulation of PARP genes through overexpression of an RNAi hairpin construct containing 5'-end of the Arabidopsis AtPARP1 or AtPARP2 genes in *Brassica napus* and Arabidopsis thaliana enhanced the stress tolerance of the plants to a broad range of abiotic stresses such as high light, drought, and heat (De Block et al., 2005). The enhanced stress tolerance upon reduction of PARP activity was partly attributed to maintenance of energy homeostasis due to reduced NAD^+ and ATP consumption and also to alteration in abscisic acid (ABA) levels leading to ABA-mediated stress response likely due to the preserved NAD^+ levels (Vanderauwera et al., 2007).

In a maize translational study, Anami (2010) used the AtPARP2 gene to screen for PARP homologues in maize genomic databases obtaining two maize genes: ZmPARP1 (GRMZM5G831712) and ZmPARP2 (GRMZM2G099231, GRMZM2G124718). Anami (2010) futher used the ZmPARP1 sequence to develop two RNAi hairpin constructs (hpPARP1-555 and hpPARP1-373) targeting the 5' end of the ZmPARP1 gene that were driven by the maize ubiquitin promoter. However, the level of silencing of the endogenous ZmPARP1 gene expression was found to be insufficient probably due to the hairpin construct design or too few number of transgenic events analysed. De Block et al. (2005) showed that Arabidopsis lines carrying an RNAi hairpin construct targeting the AtPARP2 catalytic domain sequence allowed silencing of both AtPARP1 and AtPARP2 which resulted in tolerance to high light stress and was more efficient as compared to the RNAi PARP construct targeting the 5' end of individual genes. This report was supported by a recent study by Song et al. (2015) which shows that PARP2 is the predominant Poly(ADP-Ribose) polymerase in Arabidopsis DNA damage and immune

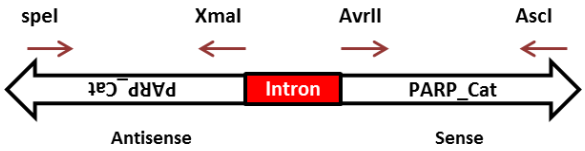
responses. In this study we used the RNAi gene silencing approach and the revolutionary CRISPR gene editing techniques to develop maize lines with perturbed PARP gene expression by targeting the PARP catalytic domain and study their response to DNA damage and drought stresses.

4.2 Results

4.2.1 Cloning, transformation and characterization of the RNAi hpPARP1_Cat silencing construct

A 650bp cDNA sequence fragment from the ZmPARP1 catalytic domain of B73 inbred maize line was amplified with gene specific primers carrying terminal enzyme restriction sites to introduce nucleotide overhangs on the amplified fragment. The PCR fragments were cloned into the StrataClone PCR cloning vector pSC-A-amp/kan and recloned into the pMCG1005 RNAi expression vector. *Avr*II and *Asc*I enzymes were used to put the ZmPARP1_Cat fragment in the forward orientation between the *ALCOHOL DEHYDROGENASE1* (ADHI) intron and rice *Waxy-a* intron, while *Xma*I and *Spe*I enzymes were used to put the ZmPARP1_Cat fragment in the reverse orientation between *OCTOPINE SYNTHASE* 3′ (OCS 3') and the rice *Waxy-a* intron. The hairpin construct was driven by the maize ubiquitin promoter. A *bar* marker gene driven by the cauliflower mosaic virus p35S promoter was used for selection (Fig.1). The expression vector was cloned into EHA101 super virulent Agrobacterium strain and transformed into the B104 maize genotype using the Agrobacterium-mediated transformation system.

(A)



(B)

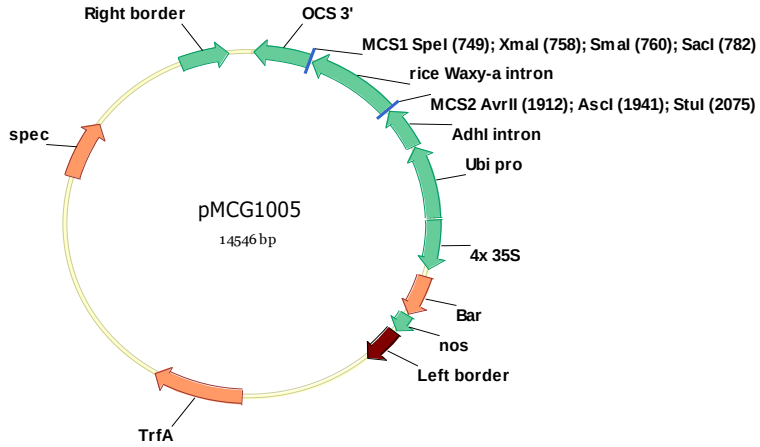


Fig. 1: **A)** Schematic representation of the ZmPARP1 catalytic domain inverted repeat sequence in the RNAi expression vector and the position of enzymes restriction sites indicated (S. Anami, unpublished results). (**B)** pMCG1005 RNAi expression vector used to express the hpZmPARP1_Cat construct. The position of the rice waxy-a intron between the multiple cloning sites (MCS) where the sense and antisense PARP_Cat fragments are cloned, the Ubiquitin promoter (Ubi pro) used to drive the expression of the hpPARP_Cat construct and the Bar marker driven by the p35S promoter are shown.

The cloning was carried out by Sylvester Anami at the Phil Becraft's lab, IOWA and the maize transformation carried out at the IOWA Plant Transformation Facility. 19 T0 events were obtained and backcrossed with pollen from wildtype B104 genotype and the T1 progenies were sent to PSB-VIB-UGent for further characterization and functional analysis.

Bar gene segregation was tested using the ammonium-multiwell assay (De Block et al., 1995; Rasco-Gaunt et al., 1999) in 24 T1 progeny individuals per line in order to determine the number of T-DNA loci. Four of the 19 T1 lines did not show clear bar activity and another four lines had very limited seed stocks thus not used. T1 backcross ratio of 1 positive versus 1 negative indicates the presence of 1 T-DNA locus, of 3 positive versus 1 negative indicates the presence of 2 T-DNA loci. The results for the 11 remaining T1 progenies are summarized in Table 1.

Table 1: Segregation analysis of T1 maize plants expressing the ZmPARP1_cat RNAi hairpin construct. Chi Square test used in predicting the number of T-DNA loci in each line

T1 ID	BAR Activity		H0 hypothesis		No. of T-DNA loci
	Positive	Negative	1:1 χ^2	3:1 χ^2	
ZmPARP1_HP-1	13	11	0,17	5,56	1
ZmPARP1_HP-2	11	13	0,17	10,89	1
ZmPARP1_HP-3	8	16	2,67	22,22	Silencing
ZmPARP1_HP-4	17	7	4,17	0,22	2
ZmPARP1_HP-5	10	14	0,67	14,22	1
ZmPARP1_HP-6	12	11	0,04	6,39	1
ZmPARP1_HP-7	19	5	8,17	0,22	2
ZmPARP1_HP-8	15	9	1,50	2,00	1 or 2
ZmPARP1_HP-9	14	10	0,67	3,56	1
ZmPARP1_HP-10	17	7	4,17	0,22	2
ZmPARP1_HP-11	12	12	0,00	8,00	1

Six T1 lines had a 1:1 segregation ratio of the bar gene activity indicating the presence of one T-DNA locus insertion. Three T1 lines had a 3:1 segregation ratio indicating the presence of 2 T-DNA loci. The T-DNA loci number was confirmed using Chi square statistical testing. Line ZmPARP1_HP-3 had double the number of negative to the positive plants not fitting in the Mendelian segregation ratios, indicating a possibility of a chimeric T0 parent originating from multiple cells or a partial silencing of the hairpin construct.

4.2.2 Expression analysis of RNAi ZmPARP1 lines and generation of T3 homozygous lines

10 T1 hairpin PARP1_Cat lines containing one or two T-DNA loci were analysed for their expression level using the quantitative PCR (QPCR) technique. QPCR primers specific for the 3' end of ZmPARP1 and ZmPARP2 genes downstream of the PARP catalytic domain were designed using NCBI Primer BLAST program (Ye et al., 2012) as shown in Fig. 2.

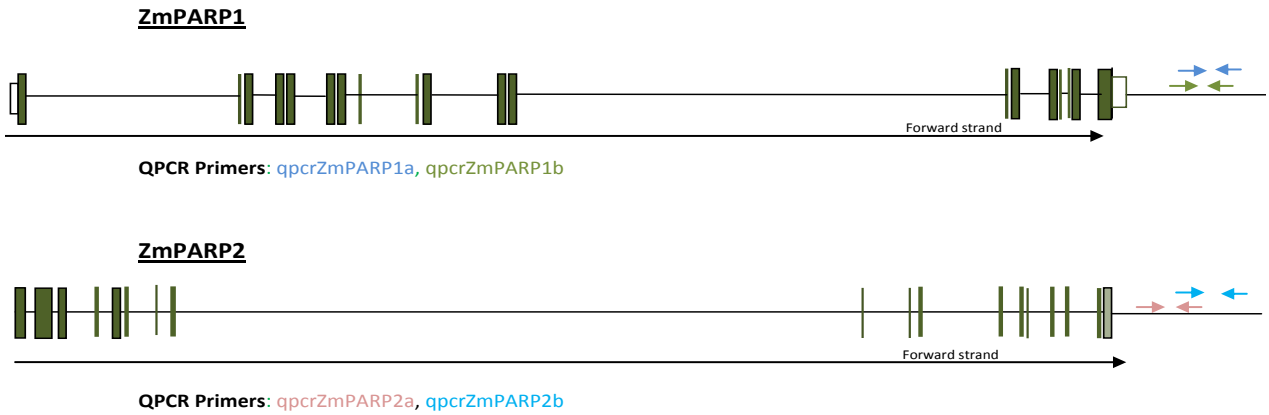


Fig. 2: QPCR primers position shown with arrows on 3' end of ZmPARP1 (GRMZM5G831712) and ZmPARP2 (GRMZM2G099231, GRMZM2G124718) gene models. The green boxes represent the exons, the lines represent the introns and the unshaded boxes represent the 5' or 3' untranslated region/ non-coding exon.

40 T1 plants per line were analysed for their bar gene activity using Pat assay test selecting 15 Pat positive plants containing the T-DNA (5 pools of 3 plants) and 15 Pat negative plants (5 pools of 3 plants) per line for the QPCR expression analysis. 7 out of the 10 T1 hairpin PARP1_Cat lines showed significant downregulation of the ZmPARP1 gene expression relative to the Pat negative plants in their respective populations. The fold change reduction in expression level was determined for each line (Fig. 3). ZmPARP1 gene expression was reduced in a range of 2.8 to 6 fold. For ZmPARP2 gene expression, no significant downregulation was observed in spite of using the PARP catalytic domain sequence, a region which is conserved in the PARP family showing 61% identity between ZmPARP1 and ZmPARP2 catalytic domain. This indicates that almost high identity between the sequence used in designing an RNAi silencing construct and the target sequence is required to obtain suitable RNAi fragments for efficient silencing. The two primer pairs per gene used in the QPCR reaction gave relatively similar expression values thus a single graph is shown for each PARP gene and the fold change values per primer pair indicated in Table 2.

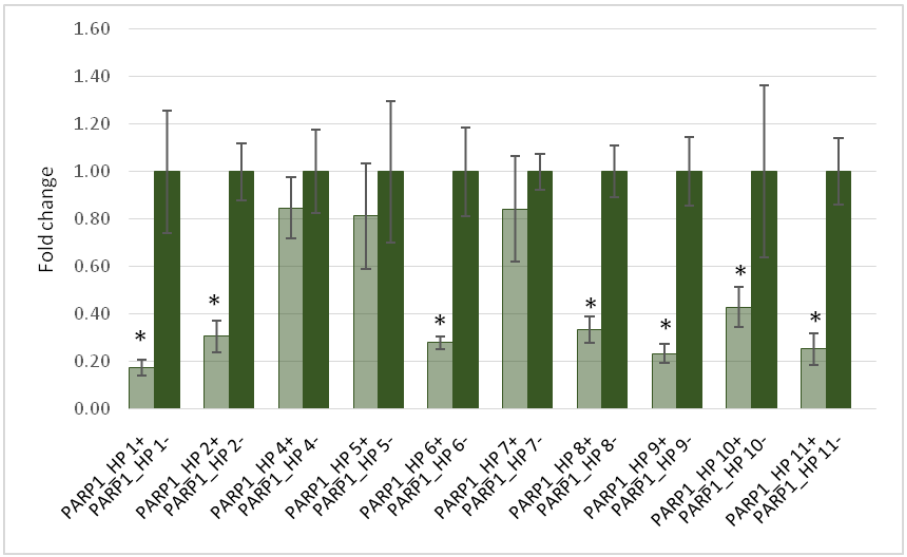


Fig. 3a: Fold change in ZmPARP1 gene expression in T1 ZmPARP1_Cat hairpin silenced lines amplified using ZmPARP1b QPCR primer. Seven lines showed significant downregulation of the ZmPARP gene upon RNAi hp silencing. Error bars indicate standard deviation and asterisks indicate significant difference compared to the respective azygous pat negative line.

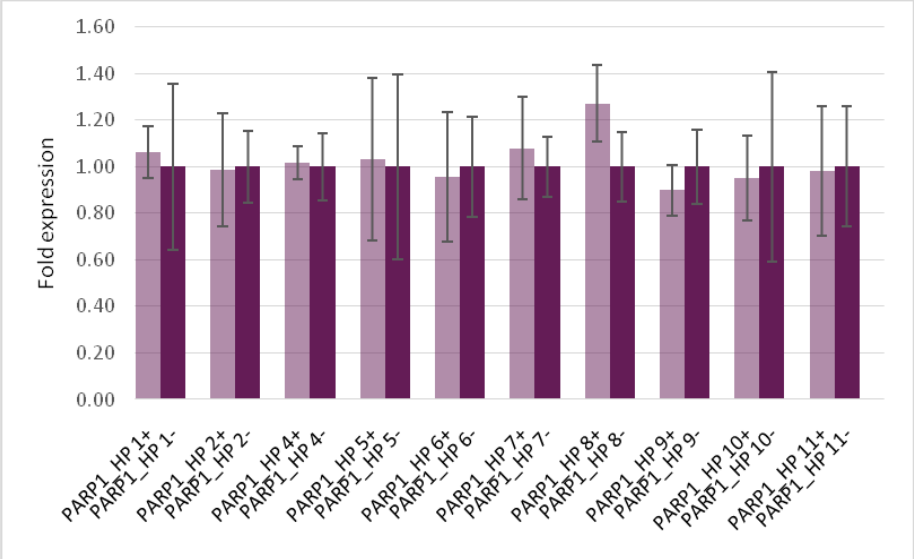


Fig. 3b: Fold change in ZmPARP2 gene expression in T1 ZmPARP1_Cat hairpin silenced lines amplified using ZmPARP2a QPCR primer. None of the lines showed downregulation of the ZmPARP2 gene. Error bars indicate starndard deviation.

Table 2: Fold change in ZmPARP1 and ZmPARP2 gene expression level in T1 ZmPARP1_Cat hairpin silenced lines.

T1 ID	T-DNA	Fold change per QPCR primer pair			
		ZmPARP1a	ZmPARP1b	ZmPARP2a	ZmPARP2b
ZmPARP1_HP-1	1	- 6.0	-5.8	-0.9	-0.9
ZmPARP1_HP-2	1	-3.6	-3.3	-1.0	-1.0
ZmPARP1_HP-4	2	-1.1	-1.2	-1.0	-1.0
ZmPARP1_HP-5	1	-1.3	-1.2	-1.0	-1.0
ZmPARP1_HP-6	1	-4.0	-3.6	-1.0	-1.0
ZmPARP1_HP-7	2	-1.2	-1.2	-0.9	-1.0
ZmPARP1_HP-8	1 or 2	-2.6	-3.0	-0.8	-0.8
ZmPARP1_HP-9	1	-5.0	-4.3	-1.1	-1.1
ZmPARP1_HP-10	2	-2.8	-2.3	-1.1	-1.1
ZmPARP1_HP-11	1	-4.2	-4.0	-1.0	-1.0

T1 lines ZmPARP1_HP-1, ZmPARP1_HP-6, ZmPARP1_HP-9 and ZmPARP1_HP-11 showing significant downregulation of ZmPARP1 gene expression were selected for upscaling to generate T3 homozygous lines. The T1 and subsequent T2 progenies were selfed, Basta leaf painting assay was used for selection of transgenic lines and eventually a homozygous T3 population. The homozygous T3 lines were upscaled for production of sufficient seeds for functional analysis.

4.2.3 Evaluation of DNA damage stress response in T2 RNAi hairpin silenced ZmPARP1 lines

Two T2 RNAi hairpin silenced lines showing downregulation of ZmPARP1 gene were selected for determination of DNA damage stress response upon treatment with hydroxyurea. Lines PARP1_HP-

1-1 and line PARP1_HP-9-1 heterozygous progenies of selfed T1 lines PARP1_HP-1 and PARP1_HP-9 respectively (Table 2) were used in this experiment. Wildtype B104 was used as a control line lacking the RNAi construct and a mutant control line developed using CRISPR editing on a gene that plays a key role in DNA replication (B104 background) was included in the assay for its hypersensitivity to hydroxyurea (Thomas Eekhout and Lieven De Veylder, unpublished results). 24 seeds per line (ZmPARP 1 RNAi lines) and 12 seeds per line (B104 and mutant control lines) per treatment were sown on filter paper rolls (12 seeds per roll), placed in Hoagland's medium and grown in the 24°C maize growth room chamber. 7.5mM hydroxyurea was introduced into Hoagland's medium of the treated plants 5 days after sowing (DAS) and pictures of all roots were taken daily (from 5 to 8 DAS) to determine the effect of hydroxyurea DNA damage perturbation on root growth of the RNAi ZmPARP1 hairpin silenced lines. Bar gene analysis was carried out for the segregating ZmPARP 1 RNAi lines using pat assay and only pat positive lines were used in the analysis. Wt B104 plants grew homogenously both under hydroxyurea treatment and in control conditions. The increase in root length from 5 DAS to 8 DAS was determined daily for each plant and is described as root growth from time point 1 to 3. For example the root growth at time point 1 = (root length at day 6 – root length at day 5). The line graphs on Fig. 4a and 4b show the root growth of pat positive RNAi lines (ZmPARP1_HP 1-1, ZmPARP1_HP 9-1), Wt B104 and a mutant control line without hydroxyurea (a) and with 7.5mM hydroxyurea treatment (b). In addition, Fig. 4c shows the total root length between day 5 and day 8 determined for comparison of the overall performance of the lines using percentage root length reduction calculated as follows: (Total root length without hydroxyurea – Total root length upon hydroxyurea) * 100 / Total root length without hydroxyurea

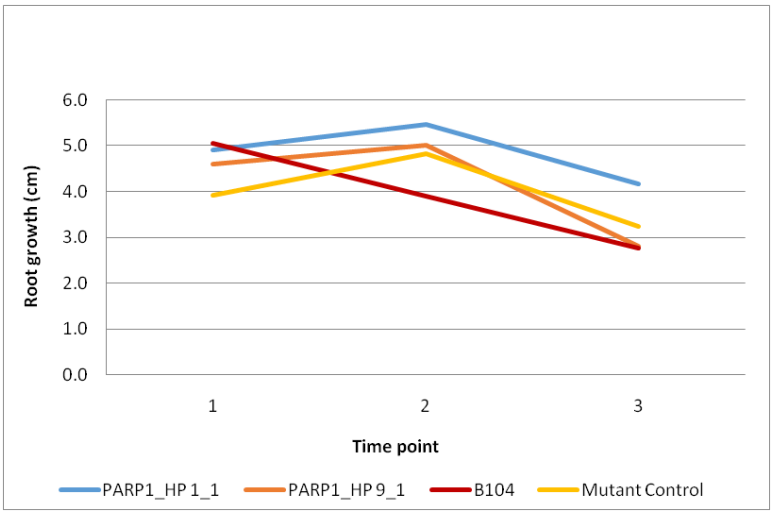


Fig. 4a: Root growth of RNAi ZmPARP1 hairpin silenced lines, mutant control and Wt-B104 without hydroxyurea treatment from time point 1 to 3 (n=12).

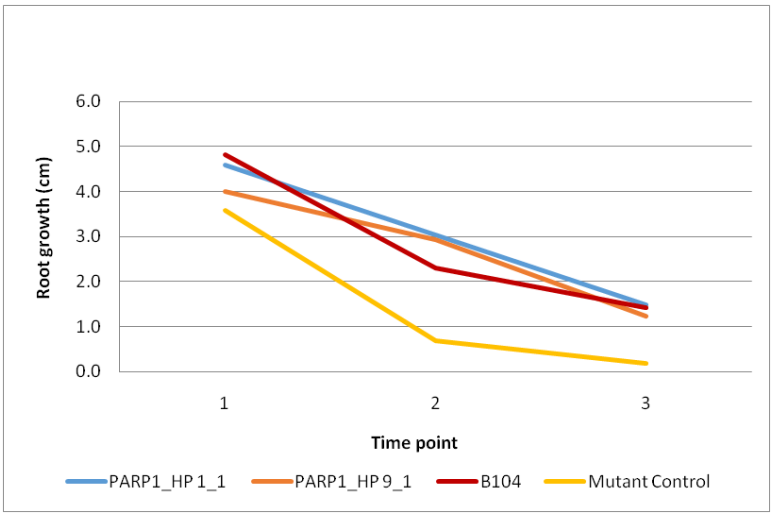


Fig. 4b: Root growth of RNAi ZmPARP1 hairpin silenced lines, mutant control and Wt-B104 with 7.5mM hydroxyurea treatment from time point 1 to 3 (n=12).

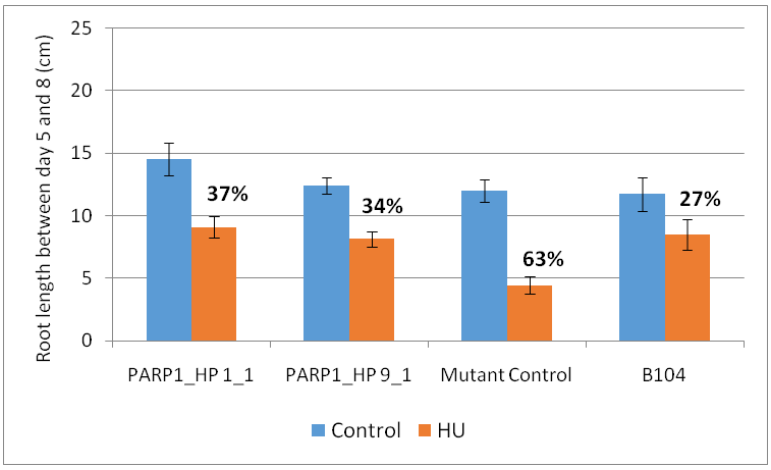


Fig. 4c: Total root length of RNAi ZmPARP1 hairpin silenced lines, mutant control and Wt-B104 without hydroxyurea (control) and with 7.5mM hydroxyurea (HU) treatment beween day 5 and 8. Percentages represent root length reduction under hydroxyurea treatment. Error bars indicate starndard deviation (n=12)

The percentage reduction in root length of lines ZmPARP1_HP 1-1 and ZmPARP1_HP 9-1 was a 10% and 7% higher respectively than Wt B104 upon hydroxyurea treatment indicating a tendency to sensitivity to hydroxyurea-induced DNA damage stress. The mutant control line already known to be hypersensitive to hydroxyurea-induced DNA damage showed an even higher, 36%, reduction in root length in comparison to Wt B104 upon hydroxyurea treatment confirming its hypersensitivity to hydroxyurea. This experiment was exploratory and we could not carry out statistical analysis on the data to provide definite significance values due to a fault in the design of the experiment where only one genotype was used per paper roll instead of all the four genotypes on a single paper roll. In future experiments, several individual plants from all the test genotypes should be grown on one paper roll and also larger sample sizes should be used. Note also that the data presented in the experiment above is obtained from a single experiment. The RNAi ZmPARP1 hairpin silenced lines

were analysed for drought stress tolerance in an automated platform and the results are shown and discussed in Chapter 5 together with ZmNUDX over expression lines.

4.2.4 Cloning and transformation of CRISPR/Cas9 ZmPARP1 & 2 Constructs

Since different reports in plants indicate that downregulation of AtPARP2 was more effective than AtPARP1 (De Block et al., 2005; Song et al., 2015), we found it important to develop new constructs that would specifically target ZmPARP2 gene. The CRISPR/Cas9 gene editing system reported to be fast, simple, versatile and precise in gene editing, was the preferred system for ZmPARPs editing.

To perform gene editing two components must be introduced into and/or expressed in cells or an organism: Cas9 nuclease and a guide RNA (gRNA). 20 nucleotides at the 5' end of the gRNA direct Cas9 to a specific target DNA site using standard RNA-DNA complementarity base-pairing rules thus the 20 nucleotides of the gRNA always need to correspond to the target DNA sequence. The CRISPR/Cas9 gene editing technique is described in chapter 3 section 3.3.

The PARP catalytic domain was the preferred target region due to its central role in PARP protein activity. We designed 3 constructs to target (a) deletion within ZmPARP2 catalytic domain named ZmPARP2_CRISPR construct, (b) deletion within ZmPARP1 catalytic domain named ZmPARP1_CRISPR construct and (c) frameshift mutation in both ZmPARP2 and ZmPARP1 catalytic domains named ZmPARP1&2 construct. Two gRNAs, targeting different regions of the PARP catalytic domain or the catalytic domain of both ZmPARPs, were required for each construct design. The gRNAS were designed using CRISPR P (Lei et al., 2014) an online gRNA designing tool that allows users to search for highly specific Cas9 target site within a DNA sequence of interest. The tool also provides off-target loci prediction for specificity analysis and marks restriction enzyme cutting site of every gRNA for downstream screening of knock-outs. (ZmPARP CRISPR cloning was done in collaboration with Thomas Eekhout and Lieven De Veylder, PSB-VIB-UGent).

Guide RNAs were designed using the B73 reference genome but we checked for any nucleotide polymorphism between B73 and B104 PARP catalytic domain region by comparing the B73 genome against the B104 deep sequencing reads of an in-house database using Integrative Genomics Viewer (Robinson et al., 2011). Fig. 5 shows the position of the gRNAs in the PARP catalytic domain for the respective constructs and the size of deletions expected if Cas9 protein cuts both guide RNAs at the precise cleavage site.

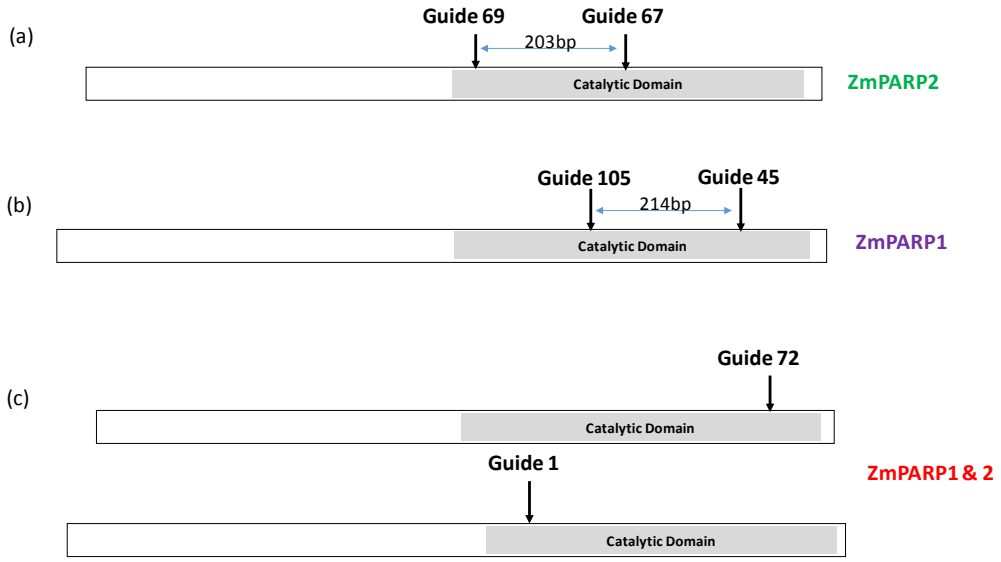


Fig 5: Position of gRNAs within the catalytic domain of (a) ZmPARP2, (b) ZmPARP1 and (c) Both ZmPARP2 & 1

The gRNA pairs were cloned into the pCBC-MT1T2 vector introducing the Bsa1 restriction site and then recloned through golden gate cloning into pBUN411-Sp expression vector (Xing et al., 2014) which contains the Cas9 protein driven by the maize ubiquitin promoter, two gRNAs positions driven by rice ubiquitin promoter (OsU3p) and wheat ubiquitin promoter (TaU3p) respectively and a bar selection marker driven by the p35S promoter as shown in the T-DNA scheme in Fig. 6. The expression vectors were cloned into the EHA101 super virulent Agrobacterium strain (Hood et al., 1986) and transformed into the B104 maize genotype using the Agrobacterium-mediated transformation procedure described by Coussens et al., (2012) by the PSB-VIB maize transformation platform.

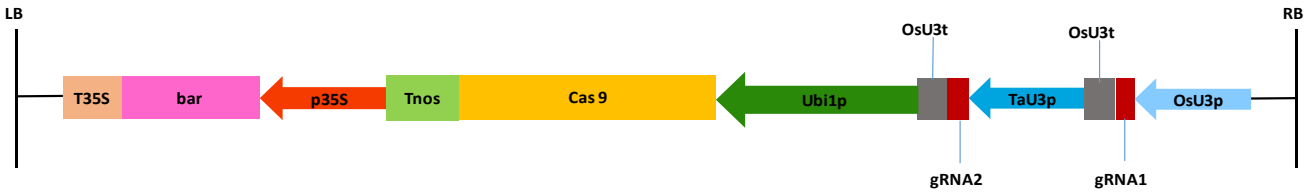


Fig. 6: T-DNA region of pBUN411 vector used to express the ZmPARPs gene editing constructs. Position of two guide RNAs driven by rice ubiquitin promoter (OsU3p) and wheat ubiquitin promoter (TaU3p) is shown in red boxes. The Cas9 protein expression is driven by the Maize ubiquitin promoter and bar selection marker driven by p35s promoter.

4.2.5 Genotyping of CRISPR/Cas9 gene edited ZmPARP lines

20 transgenic T0 shoots carrying the ZmPARP2_CRISPR construct, 11 transgenic T0 shoots carrying the ZmPARP1_CRISPR construct and 9 transgenic T0 shoots carrying the ZmPARP1&2_CRISPR construct were obtained. To determine the type of mutation resulting from the Cas9 activity, we extracted genomic DNA from 2 different leaf samples of each T0 shoot and amplified the ZmPARP

catalytic domain Cas9 target region using gene specific primers binding to sequences flanking the gRNA target sites. The PCR fragments were visualised on a 1% agarose gel, gel purified and sequenced. The sequencing reads were analysed using CLC Main Workbench 6 (Qiagen) and TIDE (Tracking of Indels by Decompositon- Brinkman et al., 2014) softwares. 8 out of the 20 T0 shoots carrying the ZmPARP2_CRISPR construct were found to have homozygous deletions of between 201bp to 233bp in the ZmPARP2 catalytic domain indicating that the Cas9 protein cleaved both alleles of the ZmPARP2 DNA in the precise predicted site of both gRNAs in some but not all shoots (Table 3) The remaining 12 shoots had a wildtype genotype (data not shown). All the 11 T0 shoots carrying the ZmPARP1_CRISPR construct had either a homozygous single nucleotide insert or a homozygous 4bp deletion around the gRNA45 target site but no mutation on the gRNA105 target site indicating a possible failure of the gRNA105 in directing the Cas9 protein to the target site (Table 3). Unexpectedly all the 9 T0 shoots carrying the ZmPARP1&2_CRISPR construct had wildtype genotype also indicating a failure in both gRNA72 and gRNA1 (data not shown). Table 3 shows the mutations obtained through sequence analysis on CLC Main Workbench and TIDE software in the T0 shoots transformed with the ZmPARP2_CRISPR construct and the ZmPARP1_CRISPR construct respectively.

Table 3: Mutations in Pat+ transgenic T0 maize lines carrying the ZmPARP2_CRISPR and ZmPARP1_CRISPR construct respectively. (Hm, homozygous; del, deletion)

T0 Line	**Indel in ZmPARP2**
ZmPARP2_CR 1	233bp Hm del
ZmPARP2_CR 2	Multiple del (1-4bp) & A Insert
ZmPARP2_CR 3	Multiple del (1-7 bp) & A Insert
ZmPARP2_CR 4	208bp Hm del
ZmPARP2_CR 5	203bp Hm del
ZmPARP2_CR 6	233bp Hm del
ZmPARP2_CR 7	201bp Hm del
ZmPARP2_CR 8	201bp Hm del
ZmPARP2_CR 9	201bp Hm del
ZmPARP2_CR 10	205bp Hm del
T0 Line	**Indel in ZmPARP1**
ZmPARP1_CR 1	4bp Hm del
ZmPARP1_CR 2	4bp Hm del
ZmPARP1_CR 3	Hm A Insert
ZmPARP1_CR 4	Hm A Insert
ZmPARP1_CR 5	Ht T/A Insert
ZmPARP1_CR 6	Hm A Insert
ZmPARP1_CR 7	Hm A Insert
ZmPARP1_CR 8	Hm A Insert
ZmPARP1_CR 9	Hm T Insert

ZmPARP1_CR 10	Hm T Insert
ZmPARP1_CR 11	4bp Hm Del

We repeated the genotyping of a leaf sample from a different part of each T0 shoot obtained from the three different constructs to check if the genotype obtained was homogenous in each T0 plants. The genotyping results were identical to the first genotyping showing similar sizes of deletions, insertions or wildtype sequences which indicate no cases of chimerism and the plants were most likely clonal originating from a single transformed cell. The T0 shoots were allowed to grow to maturity and were all selfed to maintain the homozygosity of the CRISPR induced mutation. Pollen from the T0 shoots was also used to backcross wildtype B104 plants to obtain ears with higher seed number and segregated T-DNA.

In this work, T1 genotyping was carried out in progenies of lines carrying ZmPARP2_CRISPR construct arising from selfed T0 shoots: ZmPARP2_CR1, ZmPARP2_CR4, ZmPARP2_CR5 and ZmPARP2_CR8 showing homozygous deletion of sizes 233bp, 208bp, 203bp and 201bp respectively in the ZmPARP2 catalytic domain. 6 individuals arising from each T1 were genotyped and analysed as described above for T0 shoots. Pat assay was carried out to determine the T-DNA segregation in the T1 lines. Table 4 gives a summary of the mutations through sequence analysis on CLC Main Workbench and TIDE software and bar gene segregation in 6 T1 plants per line carrying ZmPARP2_CRISPR construct.

Table 4: Mutations & Bar gene segregation in transgenic T1 maize lines carrying the ZmPARP2_CRISPR construct (Hm, homozygous; del, deletion)

T1 Line	Plant	Indel	Bar gene
ZmPARP2_CR1-1	Plant 1	233bp Hm del	Neg
	Plant 2	233bp Hm del	Pos
	Plant 3	233bp Hm del	Pos
	Plant 4	Multiple Hm del (1-6bp)	Neg
	Plant 5	Hm T and A Insert	Neg
	Plant 6	Hm T and A Insert	Neg
ZmPARP2_CR4-1	Plant 1	208bp Hm del	Pos
	Plant 2	208bp Hm del	Pos
	Plant 3	Multiple Hm del (2 & 11bp)	Pos
	Plant 4	208bp Hm del	Pos
	Plant 5	Hm A del and Hm T insert	Pos
	Plant 6	208bp Hm del	Pos
ZmPARP2_CR5-1	Plant 1	203bp Hm del	Pos
	Plant 2	203bp Hm del	Pos
	Plant 3	203bp Hm del	Pos
	Plant 4	Hm 7bp del & Hm T insert	Pos

	Plant 5	203bp Hm del	Pos
	Plant 6	203bp Hm del	Pos
ZmPARP2_CR8-1	Plant 1	201bp Hm del	Pos
	Plant 2	201bp Hm del	Pos
	Plant 3	201bp Hm del	Neg
	Plant 4	201bp Hm del	Pos
	Plant 5	201bp Hm del	Pos
	Plant 6	201bp Hm del	Pos

Many T1 lines had mutations similar to their T0 parents maintaining the big deletion within the ZmPARP2 catalytic domain. However, a number of the T1 progenies had a different mutation, a single nucleotide insertion, several base pair (2-11bp) deletions or multiple deletions within the target catalytic domain. ZmPARP2_CR8-1 had a uniform 201bp confirming that the Cas9-induced deletion in the T0 plant was indeed homozygous. The other 3 lines (ZmPARP2_CR1-1, ZmPARP2_CR4-1, ZmPARP2_CR 5-1) had segregating phenotypes of Cas9-induced deletions possibly indicating that the deletions observed in T0 plants were not actual homozygous deletion as observed in the CLC main workbench6 analysis software but a wildtype allele was masked. Cas9 activity on the wildtype allele might have resulted in new mutations upon selfing that were not observed in the T0 plants .The T1 plants without T-DNA (negative for the bar gene) and thus with stabilised PARP mutation will be selfed for further testing.

4.2.6 Evaluation of DNA damage stress response in T1 ZmPARP2_CRISPR maize lines

Four selfed T1 ZmPARP2_CRISPR maize lines with big deletions or indels in the ZmPARP2 catalytic domain were evaluated for their DNA damage stress response upon treatment with hydroxyurea. Selfed T1 lines ZmPARP2_CR1-1, ZmPARP2_CR4-1, ZmPARP2_CR 5-1, ZmPARP2_CR8-1 (Table 4) were used in the hydroxyurea induced DNA damage experiment which was set up as described for RNAi hairpin silenced ZmPARP1 lines above. This is a preliminary test using T1 lines with segregating ZmPARP2 mutations which are all expected to result in inhibition of the ZmPARP2 catalytic activity through frameshift mutation in the gene or deletion of a significant part of the PARP catalytic domain sequence. B104 wildtype and a mutant control line hypersensitive to hydroxyurea treatment (described in section 4.2.3) were used as controls. 12 seeds per line per treatment were sown on filter paper rolls, placed in Hoagland's medium and grown in the 24^{o}C maize growth room chamber. 7.5mM hydroxyurea was introduced into Hoagland's medium of the treated plants 5 DAS and pictures of the roots were taken daily (from 5 to 9 DAS). The increase in root length from 5 DAS to 9 DAS was determined daily for each plant and is described as root growth from time point (Tp) 1 to 4. The line graphs on Fig. 7a and b show the root growth of T1 ZmPARP2_CRISPR lines (ZmPARP2_CR1-

1, ZmPARP2_CR4-1, ZmPARP2_CR 5-1, ZmPARP2_CR8-1), Wt B104 and a mutant control line in Hoagland's medium without hydroxyurea (a) and with 7.5mM hydroxyurea treatment (b). The bar graph in Fig. 7c shows the total root length beween day 5 and day 9, percentage root length reduction values are shown for comparison of the overall performance of the lines.

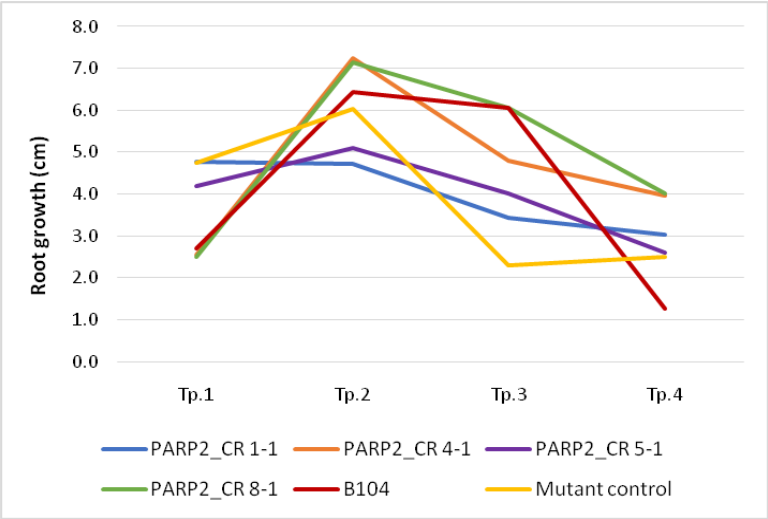


Fig. 7a: Root growth of T1 ZmPARP2_CRISPR lines, mutant control and Wt-B104 without hydroxyurea treatment from time point 1 to 4 (n=12).

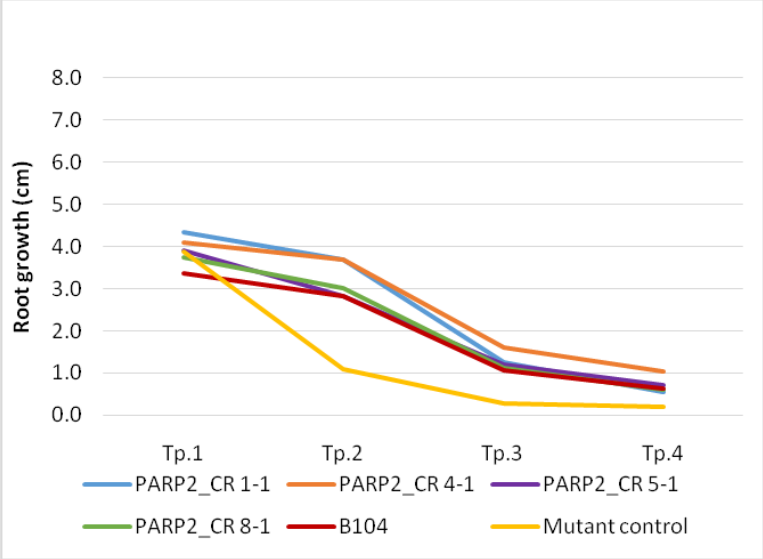


Fig. 7b: Root growth of T1 ZmPARP2_CRISPR lines, mutant control and Wt-B104 with hydroxyurea treatment from time point 1 to 4 (n=12).

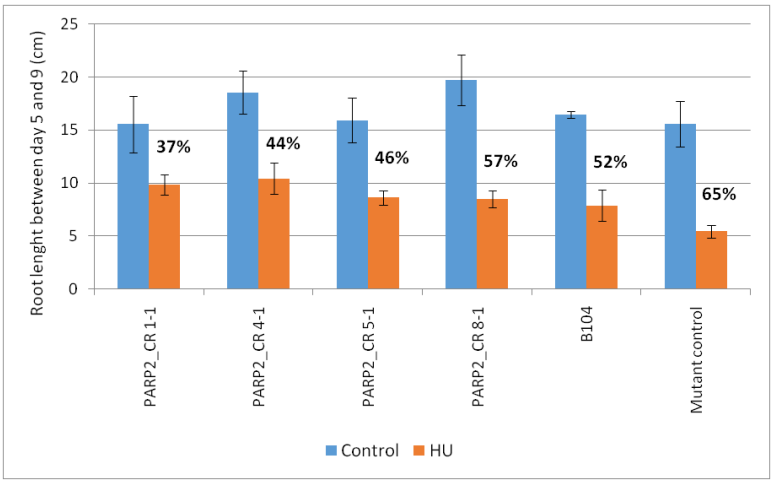


Fig. 7c: Total root length of T1 ZmPARP2_CRISPR lines, mutant control and Wt-B104 without hydroxyurea (control) and with 7.5mM hydroxyurea (HU) treatment beween day 5 and 9. Percentages represent root length reduction under hydroxyurea treatment. Error bars indicate starndard deviation (n=12)

Upon treatment with hydroxyurea, the percentage reduction in root length of lines ZmPARP2_CR1-1, ZmPARP2_CR4-1 and ZmPARP2_CR 5-1 was 15%, 8% and 6% lower than Wt B104 respectively indicating a tendency to tolerance to the hydroxyurea-induced DNA damage while line ZmPARP2_CR8-1 showed 5% more reduction in root length in comparison to Wt B104 upon hydroxyurea treatment indicating a tendency to sensitivity to hydroxyurea-induced DNA damage. The mutant control line known to be hypersensitive to hydroxyurea-induced DNA damage showed a 13% reduction in root length in comparison to Wt B104 upon hydroxyurea treatment. A repeat of this experiment following a similar set up, the same T1 ZmPARP2_CRISPR lines and mutant control line but a different Wt B104 seed stock with more homogeneous germination is shown in Figure 8a, 8b and 8c.

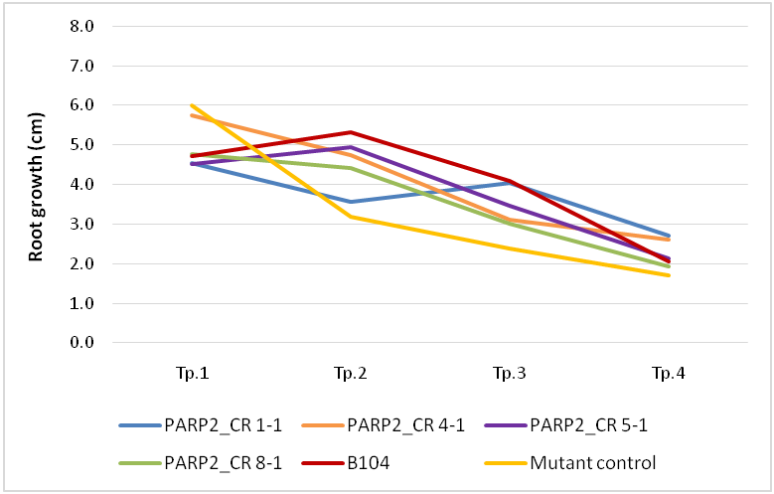


Fig. 8a: Root growth of T1 ZmPARP2_CRISPR lines, mutant control and Wt-B104 without hydroxyurea treatment from time point 1 to 4 (n=12) (Expt.2).

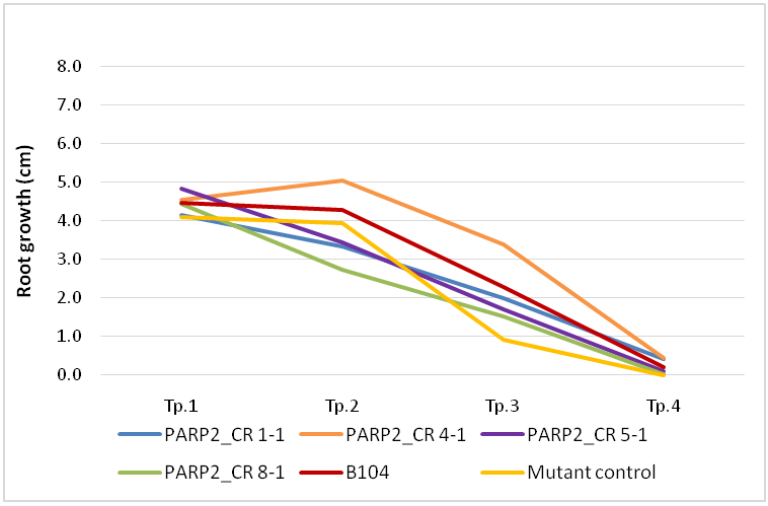


Fig. 8b: Root growth of T1 ZmPARP2_CRISPR lines, mutant control and Wt-B104 with hydroxyurea treatment from time point 1 to 4 (n=12) (Expt. 2).

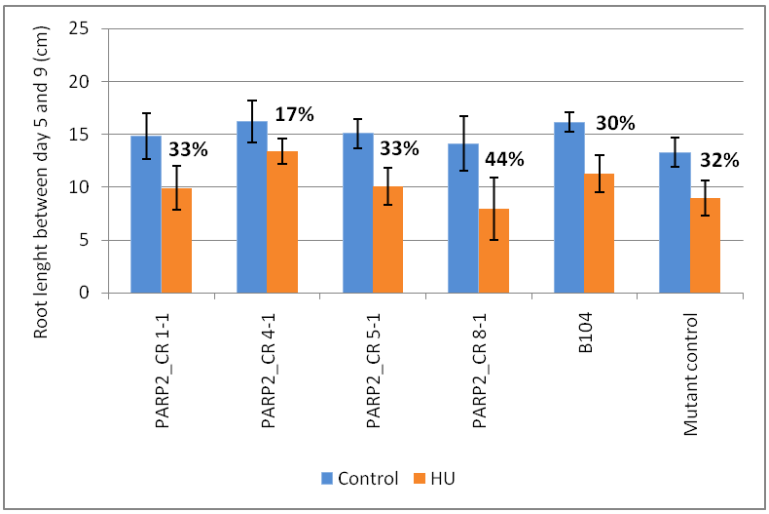


Fig. 8c: Total root length of T1 ZmPARP2_CRISPR lines, mutant control and Wt-B104 without hydroxyurea (control) and with 7.5mM hydroxyurea (HU) treatment beween day 5 and 9 (Expt 2). Percentages represent root length reduction under hydroxyurea treatment. Error bars indicate starndard deviation (n=12)

The percentage root length reduction in line ZmPARP2_CR1-1, ZmPARP2_CR 5-1 and ZmPARP2_CR8-1 was 3%, 3% and 14% higher respectively than Wt B104 upon hydroxyurea treatment indicating a tendency to sensitivity to hydroxyurea-induced DNA damage while line ZmPARP2_CR4-1 had a 13% lower root growth reduction in comparison to Wt B104 upon hydroxyurea treatment indicating a tendency to tolerance. The mutant control line showed a 2% reduction in root length in comparison to Wt B104 upon hydroxyurea treatment. Similar to the hydroxyurea-induced DNA damage experiment in section 4.2.3, the data presented in this section is obtained from a single experiment. In addition, we could not carry out any statistical analysis on the data obtained due to a fault in the design of the experiment. Each genotype was grown on a separate paper roll instead of a single paper roll resulting on confounding genotype and treatment effects. To resolve the genotype confounding effect, each paper should contain all 4 genotypes, thus 4 paper rolls each with 3

seedlings of each genotype. The effect of treatment is also confounded with the paper, however, to this, there is no solution thus it would be important to randomize as much as possible (Véronique Storme, statistician, PSB-VIB-UGent). More DNA damage assays will be carried out in selfed T2 lines without T-DNA or back-crossed to B104 and selfed in T1 carrying more uniform and stable ZmPARP2 mutations. Lines ZmPARP2_CR1-1, ZmPARP2_CR 4-1 and ZmPARP2_CR8-1 were selected for further analysis under water deficit stress in an automated platform (Chapter 5).

4.3 Discussion

We have been able to disrupt the ZmPARP1 and ZmPARP2 genes through the use of RNAi hairpin silencing and CRISPR gene editing technologies respectively in B104 maize. ZmPARP1 gene expression was downregulated in a range of 2.8 to 6 fold by overexpressing an RNAi hairpin silencing construct designed using a 650bp inverted repeat ZmPARP1 catalytic domain sequence separated by the rice waxy intron. The construct was expected to disrupt both ZmPARP genes since the catalytic domain used is the most conserved region of the ZmPARPs showing 61% identity, however, only ZmPARP1 was downregulated. This outcome can possibly be attributed to the lack of at least 21-26bp sequence with 100% identity in the maize PARPs necessary for the formation of small interfering RNAs (siRNAs). In Arabidopsis, silencing of both AtPARP1 and AtPARP2 was achieved through the use of a hairpin construct that contains AtPARP2 catalytic domain sequence. The success can be attributed to a 24bp block in AtPARP1 and AtPARP2 catalytic domain showing 100% sequence identity (De Block et al., 2005). We later adopted the CRISPR/Cas9 gene editing system to develop new constructs for ZmPARPs gene disruption and successfully obtained 8 out of 20 T0 shoots with a 201-233bp deletion in the ZmPARP2 catalytic domain and 11 T0 shoots with small insertion or deletions at one guide RNA target site (gRNA45) in ZmPARP1. Most of the mutations in both ZmPARP genes were identified as homozygous (Table 3) upon sequence analysis using CLC Main Workbench and TIDE software. However, sequence analysis of selfed T1 progenies carrying the ZmPARP2_CRISPR construct showed a segregation of the Cas9-induced deletion phenotypes in 3 out of 4 populations indicating that their T0 parents were not homozygous but a wildtype allele was masked. This phenomenon has been observed in T1 CRISPR edited maize lines by different groups at PSB (personal communications) thus one has to use T2 progenies of selfed T1 lines showing homozygous Cas9-induced mutation to be certain of the zygosity. No mutations were obtained in regions targeted by gRNA105 of ZmPARP1_CRISPR construct, gRNA72 and gRNA1 of ZmPARP1&2_CRISPR construct indicating possible failure of the gRNAs in directing the Cas9 protein to the target sites. Through the many studies undertaken using the CRISPR/Cas9 gene editing system, a few factors that affect its effectiveness have been identified: Cas9 activity, delivery methods, target site selection, gRNA design, off-target effects and the incidence of homology

directed repair (for review see Peng et al., 2015). In our case, the pBUN411-Sp expression vector carrying the Cas9 protein and Agrobacterium-mediated gene delivery system used was similar across all constructs thus eliminating the first two factors. Individual gRNAs are also reported to vary greatly in their efficacy to guide Cas9 for genome editing. Design of an ideal gRNA entails: determination of a target gene locus, finding suitable sequences within the gene for CAS9 targeting, checking for potential off-target binding and selecting the gRNA that lies in the preferred binding region. In addition, several criteria of nucleotides type, position, or percentage in a guideRNA are given. Fortunately, these criteria are now integrated in the growing number of computational tools that facilitate the design of highly efficient and specific gRNAs. In our study we used CRISPR P tool, a web application for single gRNA design in more than 20 plant species (Lei et al., 2014). It is however recommended to design and screen the activity of a pool of guide RNAs for better gene editing efficiency. In maize, a quick confirmation of the efficiency of guide RNAs can be accomplished by cloning the guide RNAs and CAS9 DNA in a suitable expression vector, transforming the construct into maize protoplast cells and screening for CAS9 mutations in the transformed cells.

In both plants and animal, studies have shown that PARP protein is a key player in DNA damage response due to its active role in maintenance of genome integrity. Juarez-Salinas et al. (1979) reported that in the absence of DNA single and double strand breaks, poly(ADP-ribosyl)ation seems to be a very rare event in living cells, but it can increase over 100-fold upon DNA damage. In Arabidopsis, Doucet-Chabeaud et al. (2001) reported that ionising radiation-induced DNA damage triggers rapid and massive accumulation AtPARP1 and AtPARP2 transcripts. Also a recent study by Song et al. (2015) showed that more predominantly, mutant *parp2* plants and to a lesser extent mutant *parp1* are sensitive to DNA damage induced by bleomycin, mitomycin C or gamma radiations. Having developed maize lines with reduced ZmPARP1 and ZmPARP2 expression, we were curious to find out if similar DNA damage responses can be observed in maize. We used the hydroxyurea induced DNA damage assay that is already optimised at PSB-VIB-UGent (Lieven De Veylder's lab) and germinated the maize in a paper roll hydroponics system established at PSB-VIB-UGent (Jansen et al., 2013). Since root tips have actively dividing cells, root growth was a suitable parameter to the plant's response to hydroxyurea DNA damage. A T-DNA insertion mutant control line showing root growth inhibition upon hydroxyurea induced DNA damage stress was a suitable control for the assay. The RNAi ZmPARP1 hairpin silenced lines ZmPARP1_HP 1-1 and ZmPARP1_HP 9-1 showed a 10% and 7% higher percentage reduction in root length thus more root growth inhibition upon hydroxyurea induced DNA damage stress compared to Wt B104 indicating a tendency to sensitivity to hydroxyurea-induced DNA damage stress. Evaluation of the DNA damage response in T1 ZmPARP2_CRISPR maize lines resulted in mixed phenotypes one line, ZmPARP2_CR4-

1, showing tendency to tolerance and the other lines, ZmPARP2_CR1-1, ZmPARP2_CR 5-1 and ZmPARP2_CR8-1 , showing tendency to tolerance or sensitivity to hydroxyurea induced DNA damage depending on the experimental repeat. The data from the two experiments are difficult to give a clear interpretation and will thus be repeated using T2 lines having a stable and uniform ZmPARP2 mutation.

Hydroxyurea, used to induce DNA damage in this work, inhibits the activity of ribonucleotide reductase enzyme that catalyses the reduction of ribonucleotides to deoxyribonucleotides, consequently limiting dNTPs availability for the DNA polymerase and arresting the replication folk (Koç et al., 2004) resulting in site specific DNA damage and single stranded DNA break. Bleomycin on the other hand is an antibiotic compound whose mechanism of DNA strand scission is unresolved but has been shown to easily penetrate into the cells and induce double stranded breaks in DNA (Povirk, 1996). Amor et al. (1998) showed the role of PARP in regulating two opposing processes, DNA repair and programmed cell death depending on the severity of the DNA damage where minor DNA damage caused by mild oxidative stress would be repaired, while higher levels of DNA damage, resulting from severe environmental stresses or following attack by avirulent pathogens, could super-activate the PARP enzyme, resulting in NAD depletion and activation of programmed cell death. Additionally, Doucet-Chabeaud et al. (2001) noted that the quality of DNA damage is important for specific PARP gene induction in Arabidopsis. Since PARP is known to bind to both single and double stranded breaks (Puchta et al., 1995) we propose exploring the use of Bleomycin as a DNA damaging agent in further determination of DNA damage stress response of maize lines with reduced ZmPARP1 or ZmPARP2 gene expression levels.

4.4 Materials and Methods

4.4.1 Plant material and growth conditions

B104 maize genotype used in this study was grown either in growth room or green-house conditions. The maize growth room conditions include: 24^{o}C temperature, 55% relative humidity, 230µE/m-2 sec-1 light intensity and 16hrs of lighting. The maize green-house conditions include: 22-26^{o}C temperature, 45% relative humidity, 300µE/m-2 sec-1 light intensity and 16hrs of lighting. Maize seeds were sown on trays containing jiffy soil (sphagnum peat moss) placed in the maize growth room where the seedlings were grown for 3 to four weeks then transferred to bigger soil pots (Saniflor beroepspotgrond met osmocote) and placed in the green-house where they grew to maturity.

4.4.2 CRISPR/Cas9 gene cloning

Guide RNA pairs specific for the respective ZmPARP1 or 2 catalytic domains were designed using the CRISPR P tool (Table 5, Lei et al., 2014), cloned into the pCBC-MT1T2 vector (Xing et al., 2014) using forward and reverse PCR primers that incorporate the two guide RNAs and a BSA1 restriction site (Table 6) in a PCR reaction using iProof™ High Fidelity PCR Kit (Bio-Rad) following the manufacturer's protocol. The resulting PCR product was run on a 1% agarose gel, purified and recloned through golden gate cloning into pBUN411-Sp expression vector (Xing et al., 2014) and expressed in competent Dh5α *E. coli* cells. Colony PCR was carried out using Taq DNA polymerase Kit (Qiagen) following manufacture's protocol and the plasmids sequenced before transformation into EHA101 super virulent Agrobacterium strain (Hood et al., 1986) and into B104 maize genotype using the Agrobacterium-mediated transformation procedure described by Coussens et al., (2012) by the PSB-VIB-UGent maize transformation platform.

4.4.3 Maize transformation, bar gene selection and assays

Immature embryos of the maize inbred line, B104, were transformed with the hpPARP construct at the IOWA transformation facility according to Frame et al. (2006). Immature embryos of the maize inbred line, B104, were transformed with the CRISPR/CAS9 PARP constructs at the PSB-VIB maize transformation platform according to the procedure described by Coussens et al. (2012), in which 2,4-D was replaced by dicamba (3.32mg/l). Transgenic plant materials were selected using a bar marker gene whose activity was identified using either of three assays: Pat assay, Ammonium-multiwell assay or Basta leaf painting assay. Pat assay test was carried using AgraStrip LL Strip test kit following manufacturer's instructions, Ammonium-multiwell assay was carried out according to De Block et al. (1995) and Rasco-Gaunt et al. (1999) and Basta leaf painting was conducted as described in Rasco-Gaunt et al. (1999).

4.4.4 RNA preparation, cDNA synthesis and QPCR expression analysis

1cm leaf 4 meristematic tissue from approximately 12 days old maize seedlings were harvested for QPCR expression analysis. 5 pools each of 3 pat positive plants and 5 pools each of 3 pat negative plants per line were made. RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) and cDNA prepared using SensiFAST cDNA Synthesis Kit (Bioline) following the manufacture's protocols. QPCR experiments were performed in a LightCycler480 Real -Time SYBR Green PCR System (Roche) and all reactions were performed in three technical replicates. Expression levels were normalised to reference genes 18SrRNA and EF1α (Genebank accession X00794.1 and NM_001112117.1 respectively).

4.4.5 DNA Isolation and PCR genotyping

DNA was isolated from maize leaf tissue using the Genomic DNA purification kit (Promega) and PCR carried out using the iProof High-Fidelity PCR kit (Bio-Rad) following the manufacture's protocol. The PCR products were run on a 1% agarose, gel purified and sequenced. The sequencing reads were analysed using CLC Main Workbench 6 (Qiagen) and TIDE (Tracking of Indels by Decompositon-Brinkman et al., 2014) softwares.

4.4.6 Hydroxyurea DNA damage paper roll assay

DNA damage is induced through the use of hydroxyurea, a chemical that inhibits the activity of ribonucleotide reductase enzyme that catalyses the reduction of ribonucleotides to deoxyribonucleotides, consequently limiting dNTPs availability for the DNA polymerase. The assay was set up in a paper roll hydroponics system using Hoagland's medium solution (Jansen et al., 2013). Maize seeds were germinated on a paper roll in which 12 seeds were sandwiched between two filter papers that were covered with paper towels then rolled into a cylindrical shape. The paper roll was placed in a 500 ml jar covered with foil paper and 150ml Hoagland's solution added to the jar. Paper roll sets were prepared per maize genotype and per treatment. The maize seeds were pre-germinated for 4 days at the 24^{o}C maize growth room and on the 5th day 7.5mM hydroxyurea was added to the treatment jars. The medium was refreshed every two days and the plants allowed to grow till 9 days after sowing (DAS) .The paper rolls were unwrapped and pictures of the root growth taken daily from the 5th to the 9th DAS. Image analysis for root length measurement was carried out using Image J software (https://imagej.nih.gov/ij). Fig. 9 shows a picture of maize seedlings growing on an unwrapped paper roll.

Fig.9: B104 maize seedling grown in paper rolls at 5 DAS, under Hydroxurea treatment

4.4.7 List of guide RNAs

Table 5: Selected guide RNAs for ZmPARP1 and ZmPARP2 gene editing using CRISPR P (Lei et al., 2014)

Gene/ construct	gRNA	Sequence	Location	Off targets			
				No.	Intron	UTR	Exon
ZmPARP2	Guide-69	AACACACTCTGGTTATACGG**TGG**	Exon 6	104	-	-	2
	Guide-67	TCTCGGTTGAGCAACTGGGC**TGG**	Exon 7	42	-	1	1
ZmPARP1	Guide-45	GAGCGCACAATACTGTTATG**TGG**	Exon 18	50	-	-	1
	Guide-105	TTCAAGGTTGACGAATTTTG**TGG**	Exon 17	88	-	-	1
ZmPARP2-1	Guide-72	GATGCTAATAACCTGCCCAA**AGG**	Exon 9	50	1	2	2
	Guide-1	TCACTGTGACATCACCCCGC**TGG**	Exon 15	12	-	2	1

4.4.8 List of primers

Table 6: Primer sequences used in the study

Primer	Sequence (5'->3')	Remark
18SrRNA_Q1	ACCTTACCAGCCCTTGACATATG	RNAi hairpin PARP lines QPCR primers
18SrRNA_Q2	GACTTGACCAAACATCTCACGAC	
EF1A_Q1	AGTCCGTTGAGATGCACCATG	
EF1A_Q2	CACATACCCACGCTTCAGATCC	
qPCRZmPARP1aF	AGGCGATGCCTCTTTTGGTA	
qPCRZmPARP1aR	GATTGGTGCTATTCGCGCTG	
qPCRZmPARP1bF	GAAGCAGAGTTAGGCGATGC	
qPCRZmPARP1bR	CTGATGCCCAAGCAACACTT	
qPCRZmPARP2aF	ACGTAGACCAGATAAGAATGCGG	
qPCRZmPARP2aR	TCTCAGCAACAGTTTCAGCTC	
qPCRZmPARP2bF	AGAGCTGAAACTGTTGCTGAG	
qPCRZmPARP2bR	GGGGCTAGAAGCCTGAAACC	
PRPF-AvrII	AGGCCTAGGTGAAACTTCACTGTGACATCACCC	RNAi hairpin PARP lines cloning primers
PRPR-AscI	TCAGGCGCGCCGTGATGGAAACGCACCTTCAGCAA	
PRPF-Xma1	TGCCCGGGTGAAACTTCACTGTGACATCACCC	
PRPR_SpeI	AGGACTAGTGATGGAAACGCACCTTCAGCAA	
ZmPARP2_69-67aF	GATCCGTTGTATGCTCGATA	CRISPR PARP lines Genotyping primers
ZmPARP2_69-67aR	TAACCAGTAACAGGTGCTTC	
ZmPARP1_105-45aF	CAAGATGCTATTATGGCACG	
ZmPARP1_105-45aR	TTCACAAACTCTGACTCCAG	
ZmPARP2_72aF	GAAGCACCTGTTACTGGTTA	
ZmPARP2_72aR	CATGTTAGGTGCTGTTTGAC	
ZmPARP1_1aF	CGGGATGAGGATGATTTGAT	
ZmPARP1_1aR	TCCATCTCGATCAAGTGAAAA	
OsU3p-FW	GACAGGCGTCTTCTACTGGTGCTAC	CRISPR PARP lines cloning primers
TaU3p-REV	CTCACAAATTATCAGCACGCTAGTC	
TaU3p-FW	TTGACTAGCGTGCTGATAATTTGTG	
MT1-BSF_G69	ATATATGGTCTCTGGCGAACACACTCTGGTTATACGGGTT	
MT1-F0_G69	TGAACACACTCTGGTTATACGGGTTTTAGAGCTAGAAATAGC	
MT2-R0_G67	AACGCCCAGTTGCTCAACCGAGACGCTTCTTGGTGCC	

MT2-BSR_G67	ATTATTGGTCTCTAAACGCCCAGTTGCTCAACCGAGAC	
MT1-BSF_G105	ATATATGGTCTCTGGCGTTCAAGGTTGACGAATTTTGGTT	
MT1-F0_G105	TGTTCAAGGTTGACGAATTTTGGTTTTAGAGCTAGAAATAGC	
MT2-R0_G45	AACCATAACAGTATTGTGCGCTCGCTTCTTGGTGCC	
MT2-BSR_G45	ATTATTGGTCTCTAAACCATAACAGTATTGTGCGCTC	
MT1-BSF_G72	ATATATGGTCTCTGGCGATGCTAATAACCTGCCCAAGTT	
MT1-F0_G72	TGATGCTAATAACCTGCCCAAGTTTTAGAGCTAGAAATAGC	
MT2-R0_G1	AACGCGGGGTGATGTCACAGTGACGCTTCTTGGTGCC	
MT2-BSR_G1	ATTATTGGTCTCTAAACGCGGGGTGATGTCACAGTGAC	

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CHAPTER 5

Functional analysis of altered NUDX gene expression in maize for water deficit and oxidative stress tolerance

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Contribution: E.N. carried out experiments (OE NUDX lines gene cloning & expression analysis, Mu transposon lines genotyping & expression analysis, paraquat oxidative stress assay, water deficit stress analysis on endogenous NUDX and on transgenic lines shrimpy experiment 1 and partly shrimpy experiment 2) and wrote the chapter.

Abstract

Nudix hydrolases catalyze the hydrolysis of intact and damaged oxidised nucleoside diphosphates and triphosphates, nucleotide sugars, coenzymes, dinucleoside polyphosphates, and RNA caps in various organisms such as bacteria, yeast, algae, nematodes, vertebrates, and plants. Regulation of ADP-ribose and/or NADH levels through the hydrolysis activity of ADP-ribose/NADH pyrophosphohydrolase-specific NUDX genes play a vital role in biotic and abiotic stress response in Arabidopsis. In this work, we altered the maize NUDX gene expression using overexpression constructs and screened for corresponding Mutator transposon knock out lines to study the role of Maize NUDX genes in water deficit and oxidative stresses.

5.1 Introduction

NUDX is an acronym for the Nudix hydrolase family, a name given because all the enzymes characterized from that family hydrolyse a Nucleoside Diphosphate substrate linked to a moiety "X". Nudix hydrolases catalyse a number of compounds with varying degree of substrate specificity including: dinucleoside polyphosphates, ADP-ribose, NADH, nucleotide sugars, or ribo- and deoxyribonucleoside triphosphates, coenzymeA, mRNA cap and FAD (chapter 1, table 2). Accumulation of these metabolites may be toxic to the cell thus Nudix hydrolases were postulated to play a role in modulating their intracellular levels (Bessman et al., 1996). The Nudix hydrolase family is widely distributed in viruses, archaea, bacteria and eukaryotes and characterized by a conserved Nudix motif (Bessman et al., 1996). Studies of the Nudix hydrolase family have been carried out in *E. coli, S. cerevisiae*, mouse and humans confirming their housecleaning roles of eliminating toxic metabolites such as oxidised nucleotides and controlling the level of pathway metabolic intermediates and signalling compounds (Bessman et al., 1996; McLennan et al., 2006). In plants, Nudix hydrolase activity has been reported in Arabidopsis, rice, tomato, poplar and barley among others with varying number of NUDX genes up to over 50 (see Chapter 1 Table 1). Arabidopsis thaliana contains 28 Nudix hydrolase genes and their proteins are be classified into different subfamilies based on their preferred substrate (chapter 1, table 2). Regulation of ADP-ribose and/or NADH levels through the hydrolysis activity of ADP-ribose/NADH pyrophosphohydrolase specific NUDX genes, AtNUDX2, 6 and 7, has been reported to play a vital role in biotic and abiotic stress response (Bartsch et al., 2006; Ge et al., 2007; Ogawa et al., 2009; Ishikawa et al., 2009 & 2010, Adams-Phillips et al., 2010). Overexpression (OE) of AtNUDX2 in Arabidopsis thaliana enhanced tolerance to paraquat-induced oxidative stress resulting from maintenance of NAD^+ and ATP levels by nucleotide recycling from free ADP-ribose molecules under stress conditions (Ogawa et al., 2009). Similary, Arabidopsis transgenic plants with high expression levels of AtNUDX7 showed enhanced tolerance to paraquat-induced oxidative stress while T-DNA mediated loss-of function AtNUDX7 Knock out (KO) plants showed decreased tolerance to paraquat-induced oxidative stress. The depletion of NAD^+ and ATP resulting from the activation of the Poly(ADP-ribosyl)ation reaction under oxidative stress was completely suppressed in the OE-AtNUDX7 plants while accumulation of NAD^+ and ATP was observed in the KO-AtNUDX7 plants (Ishikawa et al., 2009). Additionally, Genevestigator based metadata analysis indicate that gene expression of AtNUDX6 and 7 are upregulated under pathogenic attacks, chemical and hormonal treatments, drought and genotoxic stresses (Chapter 1, Fig. 8).

We identified three maize homologs of Arabidopsis AtNUDX7 protein (AEE83169) through BLAST searches in maize protein databases: ZmNUDX2 (GRMZM2G101693), ACN26985 (GRMZM2G162605)

& ZmNUDX8 (GRMZM2G175816) with 49%, 49% and 47% amino acid sequence identity to AtNUDX7 respectively. The ZmNUDX genes are highly similar with main differences seen in the 5' end region (Fig. 1). In addition, ACN26985 is highly homologous to ZmNUDX2 (94%), the main difference being the additional 80 amino acids at the 5' end thus we only used ZmNUDX2 for overexpression analysis.

Fig. 1: Schematic representation of a multiple sequence alignment of NUDX amino acid sequences highlighting key differences and similarities in AtNUDX7 and its 3 maize homologues (ZmNUDX2, ACN26985 and ZmNUDX8). The similar red colour in ZmNUDX2 and Zm-ACN26985 indicates their high sequence similarity and the black colour of the Nudix box indicates a highly conserved region.

AtNUDX7 and its two maize homologs ZmNUDX2 and ZmNUDX8 were overexpressed in B104 maize and knock out Mutator transposon insertion in ZmNUDX gene identified to investigate the role of NUDX in modulating water deficit and oxidative stress tolerance in maize. In this work the term T0 refers to the primary transformant (initial transformed line) and T1, T2, T3… refers to the subsequent sexual generations from the primary transformant.

5.2 Results

5.2.1 Cloning, transformation and characterization of AtNUDX / ZmNUDX overexpression lines

AtNUDX7 (AT4G12720) gene and its two maize homologs ZmNUDX2 (GRMZM2G101693) and ZmNUDX8 (GRMZM2G175816) were cloned into maize using the Gateway cloning system (Invitrogen). Two sets of constructs were developed driven either by the *Brachypodium distachyon* promoter, pBdEF1α, (Coussens et al., 2012) or by the maize ubiquitin promoter, pZmUBIL, (Christensen et al., 1992). The coding region of AtNUDX7, ZmNUDX2 and ZmNUDX8 was amplified from Arabidopsis cDNA or maize gDNA / cDNA using gene specific forward primers precisely starting at their respective 5' start and reverse primers ending at their 3'stop sequences and each PCR fragment cloned into a pDONR221 donor vector. The inserts in the pDONR221 entry clones were then recloned into pBbm42GW7 monocot multisite gateway vectors (Karimi et al., 2013) including either the pEN-L4-BdEF1a-R1 or the pENTRY-L4-UBIL-R1 entry clones that provide the promoter (https://gateway.psb.ugent.be). The expression vectors developed were cloned into the EHA101

super virulent Agrobacterium strain (Hood et al., 1986) and transformed into the B104 maize genotype using the Agrobacterium-mediated transformation procedure described by Coussens et al. (2012) by the PSB-VIB maize transformation platform. Fig. 2 shows a general scheme of the T-DNA constructs developed for overexpression of AtNUDX7, ZmNUDX2 and ZmNUDX8 genes using either pBdEF1α or pZmUBIL promoters.

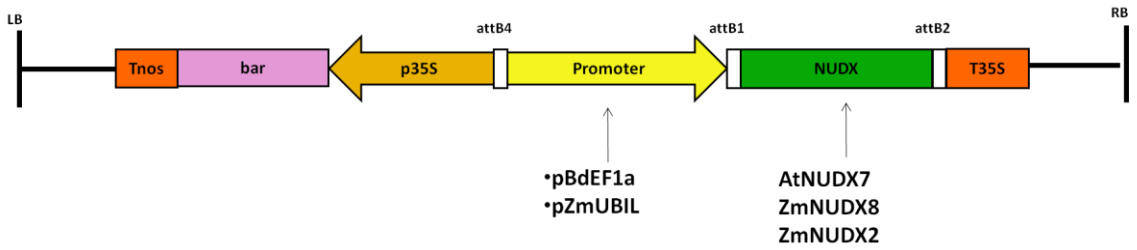

Fig. 2: Schematic representation of the T-DNA constructs for overexpression of NUDX genes (AtNUDX7, ZmNUDX8, ZmNUDX2) using either pBdEF1a or pZmUBIL promoters and a bar gene selection marker

The pBdEF1α-ZmNUDX constructs were designed using maize genomic DNA sequences, pZmUBIL-ZmNUDX constructs designed using the maize cDNA sequences and all AtNUDX7 constructs designed using Arabidopsis cDNA sequence. Maize genomic DNA was used for cloning due to initial unsuccessful attempts in using maize cDNA for cloning. We noted that one has to use a high concentration of maize cDNA (above 500ng/ul) in the PCR amplification reaction to obtain high number of amplicons visible on the agarose gel. Thus the subsequent pZmUBIL-ZmNUDX constructs were developed using maize cDNA. Attempts to clone the third maize gene GRMZM2G162605 (ACN26985) were unsuccessful, possibly due to its larger gDNA size, 3.4 Kbp as opposed to ZmNUDX2 and ZmNUDX8 with 2.5Kbp and 2.1Kbp gDNA sizes respectively. The transgenic T0 shoots obtained were backcrossed with wildtype B104 maize plants generating T1 lines with heterozygous T-DNA insertion. The T1 lines were analyzed for bar gene segregation using PAT assay or ammonium-multiwell assay. In addition, T-DNA intactness was determined in a representative number of lines per construct through PCR analysis using primers binding to the promoter and terminator region flanking the NUDX gene of interest in the respective constructs. Table 1 shows a summary of the segregation data obtained.

Table 1: Segregation data of transgenic T1 maize lines carrying different NUDX OE constructs

Construct	DNA type	Total T1 lines obtained	No. of lines with 1 T-DNA	Lines checked for T-DNA intactness	No. of lines used in QPCR
pBdEF1α-AtNUDX7	cDNA	8	6	3	6
pZmUBIL-AtNUDX7	cDNA	6	5	3	5
pBdEF1α-ZmNUDX2	gDNA	23	10	7	6
pZmUBIL-ZmNUDX2	cDNA	2	2	2	2
pBdEF1α-ZmNUDX8	gDNA	15	6	6	5

Several lines per construct had 1 T-DNA locus insertion (Table 1). However, 4 out of 15 lines carrying pBdEF1α-ZmNUDX8 construct and 3 out of 23 lines carrying the pBdEF1α-ZmNUDX2 resulted in a silencing phenotype where all the 40 individual plants tested were pat negative probably due to use of the strong *Brachypodium distachyon* promoter. A few lines per construct contained 2 T-DNA loci or a chimera phenotype showing non-Mendelian segregation of the bar gene with more pat negative plants than pat positive plants (data not shown). We did not obtain any T0 shoots with the pUBIL-ZmNUDX8 construct however this can not be considered as a phenotype of ZmNUDX8 gene since it was possible to amplyfiy the same gene using pBdEF1α promoter. The lines checked for presence of transgene using forward primers binding either to the pBdEF1α or pZmUBIL promoter and reverse primer binding to the T35S terminator confirmed that the complete gene of interest was inserted because of the positive bands of the correct sizes. A number of lines with 1 or 2 T-DNA locus insertions per construct were selected for QPCR expression analysis.

5.2.2 Expression analysis of overexpression NUDX maize lines and generation of T3 homozygous lines

40 T1 plants per line were analysed for bar gene activity using the Pat assay test in order to select 15 Pat positive plants containing the T-DNA (5 pools of 3 plants) and 15 Pat negative plants (5 pools of 3 plants) per line for the QPCR expression analysis. In maize lines carrying the pBdEF1α-ZmNUDX8 construct, T2 plants, obtained from selfing of T1 parents, were used for QPCR analysis following the same set up. Primers specific for the 3' end of each NUDX gene were designed using NCBI Primer BLAST program (Ye et al., 2012). In Fig. 3a, 4a and 5a the primer position for each gene is shown in a model on top of its respective gene expression graph (the green boxes represent the exons, the lines represent the introns and the unshaded boxes represent the untranslated 5' or 3' ends / non coding-exon). Fig. 3b shows the relative expression level of AtNUDX7 gene and Fig. 4b & 5b show the fold expression of ZmNUDX2 and ZmNUDX8 genes. Each bar represents a mean value scoring of 15 plants.

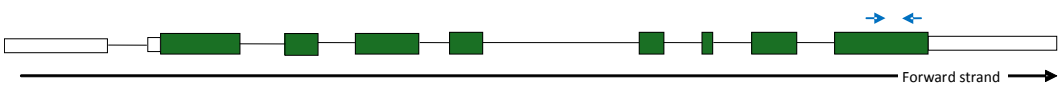

Fig. 3a: Position of the QPCRAtNUDX7b primer pair shown with arrows at the 3' end of the AtNUDX7 gene model. The green boxes represent the exons, the lines represent the introns and the unshaded boxes represent the 5' or 3' untranslated region / non-coding exon

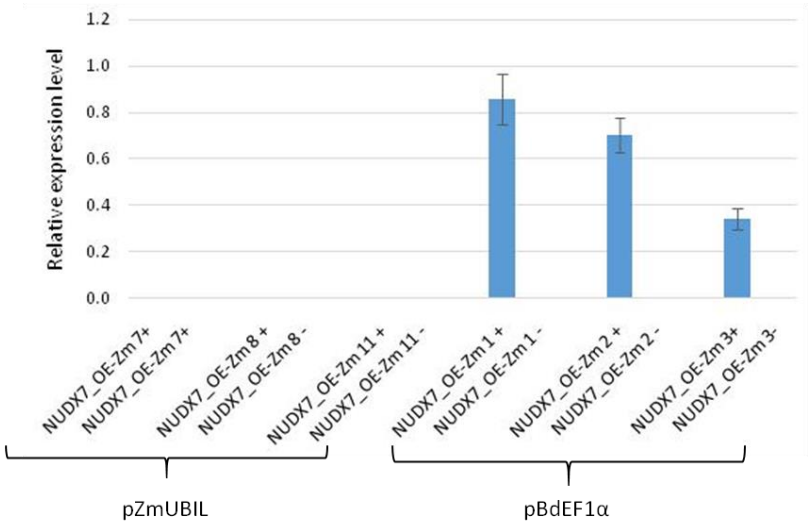

Fig. 3b: Relative expression levels of AtNUDX7 gene in 6 T1 transgenic maize lines carrying the pZmUBIL-AtNUDX7 or pBdEF1α-AtNUDX7 construct amplified using QPCRAtNUDX7b primer. Error bars indicate standard deviation (n=15).

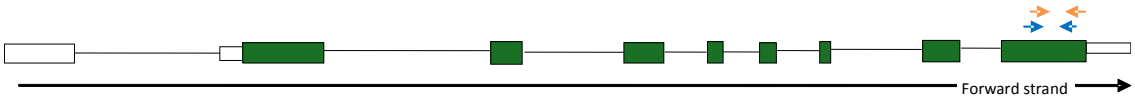

Fig. 4a: Position of the qPCRZmNUDX2b (blue) and qPCRZmNUDX2c (orange) primer pairs shown with arrows at the 3' end of the ZmNUDX2 gene model. The green boxes represent the exons, the lines represent the introns and the unshaded boxes represent the 5' or 3' untranslated region / non-coding exon

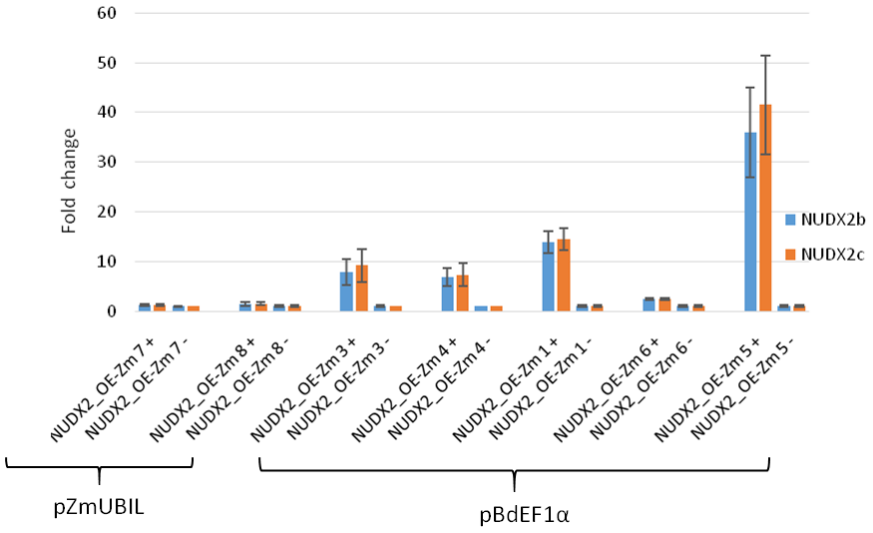

Fig. 4b: Fold change in ZmNUDX2 gene expression in 7 T1 transgenic maize lines carrying the pZmUBIL-ZmNUDX2 or pBdEF1α-ZmNUDX2 construct amplified using QPCRZmNUDX2b and QPCRZmNUDX2c primers. Error bars indicate standard deviation (n=15).

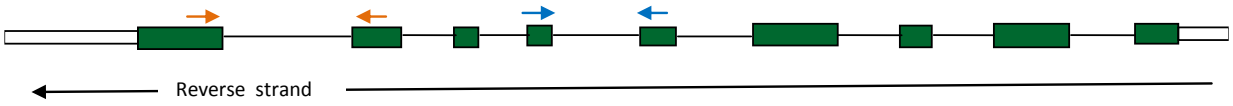

Fig. 5a: Position of the qPCRZmNUDX8e (blue) and qPCRZmNUDX8f (orange) primer pairs shown with arrows in the ZmNUDX8 gene model. The green boxes represent the exons, the lines represent the introns and the unshaded boxes represent the 5' or 3' untranslated region.

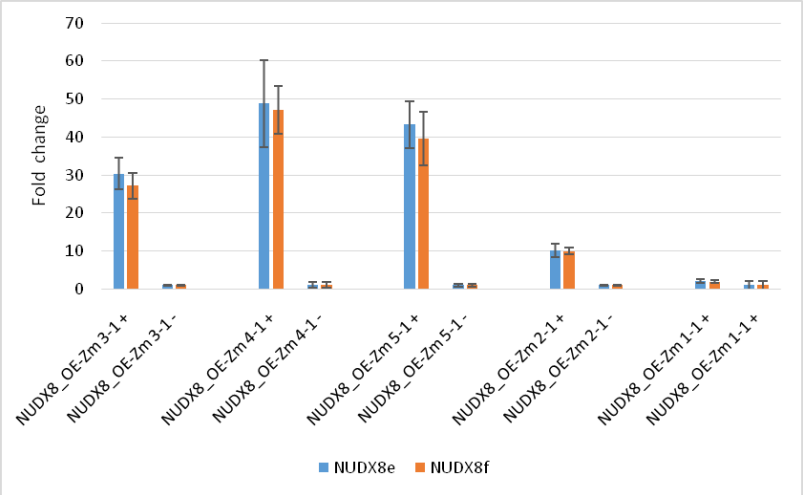

Fig. 5b: Fold change in ZmNUDX8 gene expression in 5 T2 transgenic maize lines carrying the pBdEF1α-ZmNUDX8 construct amplified using QPCRZmNUDX8e and QPCRZmNUDX8f primers. Error bars indicate standard deviation (n=15).

Table 2: Summary of the Pat+ T1 or T2 transgenic maize lines carrying the NUDX OE constructs used in QPCR analysis. Fold change in expression of ZmNUDX2/8 genes is shown. Highlighted lines were selected for generation of T3 seeds for functional analysis. Functional assays carried out in the different lines were water deficit (W.D) and Oxidative stress (O.S).

OE construct	ID	Generation	Parents	T-DNA Loci	Fold change	Functional assay
pBdEF1α-AtNUDX7	NUDX7_OE-Zm 1	T1	B104 X 193-83-B	1		W.D & O.S
	NUDX7_OE-Zm 2	T1	B104x193-83-Bb	1		W.D
	NUDX7_OE-Zm 3	T1	B104 X 193-83-C	1		W.D & O.S
	NUDX7_OE-Zm 4	T1	B104 X 195-83-Da	1		
	NUDX7_OE-Zm 5	T1	B104 X 195-83-Db	1		
	NUDX7_OE-Zm 6	T1	B104 X 195-83-Eb	1		
pZmUBIL-AtNUDX7	NUDX7_OE-Zm 7	T1	B104x281-114-Aa	1 or 2		
	NUDX7_OE-Zm 8	T1	B104x281-114-Ab	1		
	NUDX7_OE-Zm 9	T1	B104x281-114-Ac	1		
	NUDX7_OE-Zm 10	T1	B104x281-114-Ba	1		
	NUDX7_OE-Zm 11	T1	B104x281-114-Bb	1		
pBdEF1α-ZmNUDX2	NUDX2_OE-Zm 1	T1	B104 X 205-82-C	1	16,1	W.D & O.S
	NUDX2_OE-Zm 2	T1	B104 X 205-82-E	Chimera	1,3	
	NUDX2_OE-Zm 3	T1	B104x240-82-Cc	1	8,5	
	NUDX2_OE-Zm 4	T1	B104x240-82-Ge	2	7,1	

	NUDX2_OE-Zm 5	T1	B104x241-82-Bc	1	38,7	W.D
	NUDX2_OE-Zm 6	T1	B104x241-82-D	1	2,4	
pZmUBIL-ZmNUDX2	NUDX2_OE-Zm 7	T1	B104x268-107-Aa	1	1,2	
	NUDX2_OE-Zm 8	T1	B104x268-107-Ab	1	1,5	
pBdEF1α-ZmNUDX8	NUDX8_OE-Zm 1-1	T2	T1 (2013-232-03)	1 or 2	2	
	NUDX8_OE-Zm 2-1	T2	T1 (2013-232-07)	1	10,1	W.D
	NUDX8_OE-Zm 3-1	T2	T1 (2013-232-09)	1	28,8	
	NUDX8_OE-Zm 4-1	T2	T1 (2013-232-12)	1	48	W.D
	NUDX8_OE-Zm 5-1	T2	T1 (2013-234-02)	1	41,5	

Comparison of the expression levels of AtNUDX7 gene in pBdEF1a-AtNUDX7 and pZmUBIL-AtNUDX7 lines can be observed in Fig. 3b, since the gene does not exist endogenously, we could not calculate the fold expression for these lines. Lines carrying the pZmUBIL promoter in their constructs had generally much lower expression levels than lines carrying the pBdEF1α and thus were not used further in the functional analysis. The highlighted lines showing different overexpression levels of their respective NUDX genes were selected for upscaling to generate T3 homozygous lines. The T1 and subsequent T2 progenies were selfed, Basta leaf painting assay was used for selection of transgenic lines and eventually a homozygous T3 population. The homozygous T3 lines were upscaled for production of sufficient seeds for functional analysis.

5.2.3 Screening, genotyping and expression analysis of Mutator transposon insertion in ZmNUDX genes

We screened for Mutator (Mu) transposon insertions in ZmNUDX genes to obtain lines with a disrupted ZmNUDX activity for functional study and for use as control lines in functional analysis of OE NUDX lines. Two mutator transposon insertions were identified in the ZmNUDX8 gene: Mu1055846 (Stock UFMu-07274) and Mu1047296 (stock UFMu-06482) in exon 1 and exon 8 respectively, but no Mu insertion was found in the ZmNUDX2 gene. The Mu transposon insertions from the UniformMu collection (McCarty et al., 2005) were identified in the Maize Genomic Database (MaizeGDB) genome browser and ordered from the maize genetics cooperation stock center, 15 sib-pollinated F3 kernels per stock (http://www.maizegdb.org/uniformmu) both carrying a heterozygous Mu insertion were received. To confirm the presence of the Mu insert, PCR genotyping was carried out in 8 plants per line using gene-specific primers binding to regions flanking the Mu transposon insertion site (500bp upstream and downstream of the insertion site) in combination with transposon-specific primers (TIR6 / TIR8). Heterozygous insertion of the Mu transposons Mu1055846 and Mu1047296 were confirmed in exon 1 and exon 8 respectively of the ZmNUDX8 gene in the W22 maize inbred line background. F3 lines containing the Mu transposon inserts were selfed to generate F4 lines with homozygous Mu insertion. PCR genotyping of F4 plants

was carried out in a procedure identical to the F3 lines genotyping. We obtained 8 plants with homozygous Mu1055846 transposon insertion in ZmNUDX8 exon 1, PCR genotyping of 6 of these lines using gene-specific primers and also gene-specific primers in combination with transposon-specific primer is shown in Table 3 and Fig. 6. Interestingly, no homozygous insertion was obtained from the progenies of selfed F3 lines carrying the Mu1047296 transposon in heterozygous condition, suggesting a lethal phenotype in homozygous status. Four F4 lines carrying the homozygous Mu1055846 insert (UFMu-07274 stock) in ZmNUDX8 were analysed for the ZmNUDX8 gene expression levels using QPCR (Mu-KD1, Mu-KD2, Mu-KD-3 and Mu-KD4). An additional line showing wildtype segregation of the Mu1055846 insert (named Mu-Neg) and a wildtype W22 line were included in the QPCR analysis as controls. Fig. 7a shows the Mu transposon insertion in exon 1, QPCR primer positions in ZmNUDX8 and Fig. 7b, the gene expression level for each line where each bar represents a mean value scoring of 12 plants.

Table 3: PCR genotyping of F4 ZmNUDX8 lines carrying the Mu1055846 insert (UFMu-07274 stock)

Line ID	Genotype	DNA samples	PCR prod. Gene specific primers	PCR prod. Gene specific + TIR 6
Mu-KD1	Homozygous	1-4	-	+
Mu-KD2	Homozygous	5-8	-	+
Mu-KD3	Homozygous	9-12	-	+
Mu-KD4	Homozygous	13-16	-	+
Mu-KD5	Homozygous	17-20	-	+
Mu-KD6	Homozygous	21-24	-	+
Mu-Neg	Negative (Wt)	25-27	+	-
Wt-W22	Wt	29-31	+	-

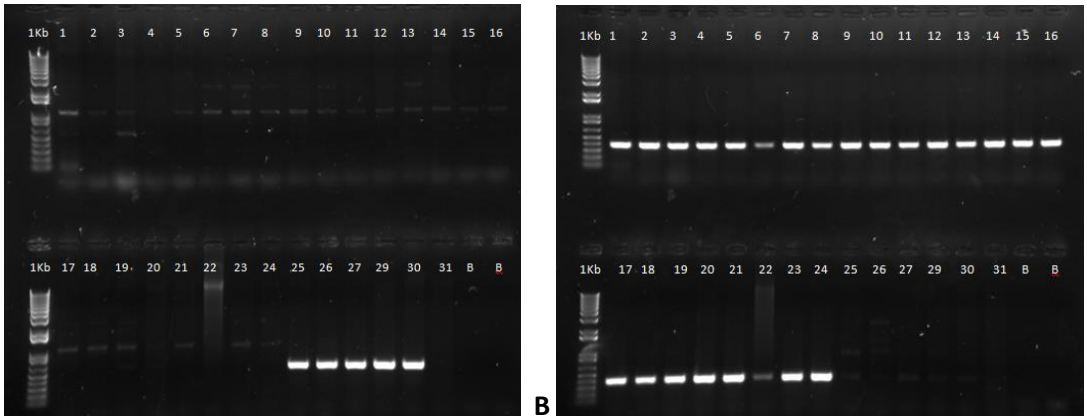

Fig. 6 Agarose gel images displaying the PCR products obtained using gene specific primers (A) or gene specific + transposon specific, TIR6 primer (B) on genotyping Mu1055846 insert in ZmNUDX8 F4 Mu lines

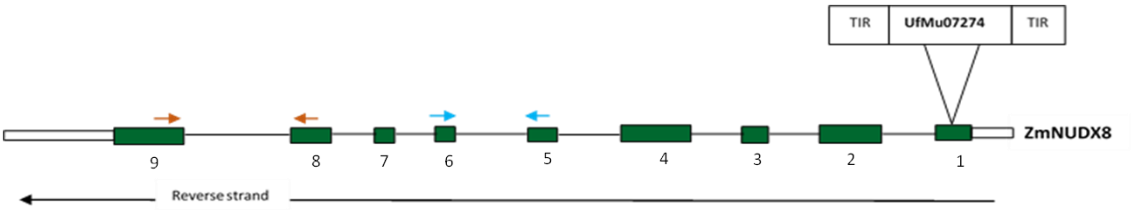

Fig. 7a: Mu transposon insertion in exon 1 of the ZmNUDX8 gene model and positions of QPCR primers pairs QPCRZmNUDX8e (blue) and QPCRZmNUDX8f (orange) shown with arrows. The green boxes represent the exons, the lines represent the introns and the unshaded boxes represent the 5' or 3' untranslated region.

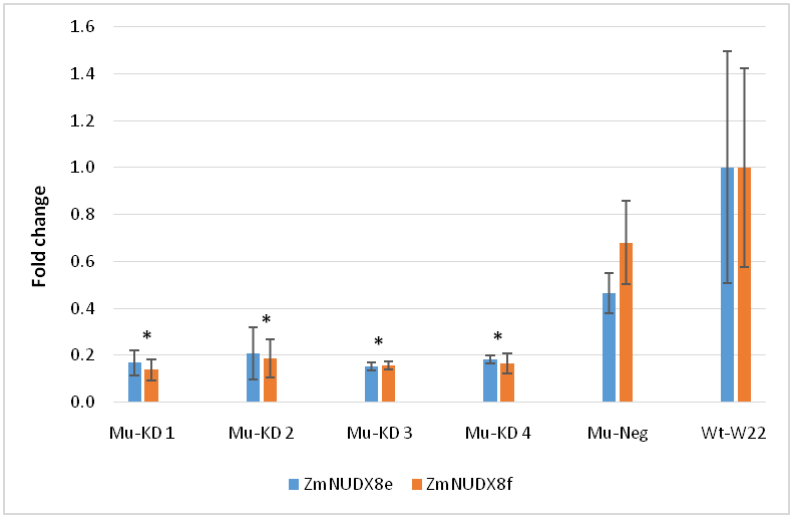

Fig. 7b: Fold change in ZmNUDX8 gene expression in homozygous Mu transposon insertion lines (Mu-KD1 to 4), Wt segregating Mu line (Mu-Neg) and Wt-W22 line using primers QPCRZmNUDX8e and QPCRZmNUDX8f. Error bars indicate standard deviation (n=12) and significance difference in comparison to Wt-W22 (student's t-test) is indicated in asterisk ($p<0.05$)

The four F4 lines carrying the Mu1055846 insert (Mu-KD1, Mu-KD2, Mu-KD-3 and Mu-KD4) showed a range of 5 to 6 fold down regulation of the ZmNUDX8 gene expression levels in comparison to the Wt-W22 and will be used further in functional analysis assays. The negative segregating line, Mu-Neg showed a wild type expression level of ZmNUDX8 comparable to Wt-W22.

5.2.4 Evaluation of the paraquat-induced oxidative stress response of T1 OE NUDX maize lines

Paraquat (Methyl Viologen) is a phytotoxic chemical agent that induces oxidative stress in plants principally targeting the chloroplast membrane under normal light. The chemical interferes with photosynthesis by acting as an alternative electron acceptor, transferring electron from Fe-S proteins of photosystem I to molecular oxygen producing reactive oxygen species. Leaf explants placed in a solution containing paraquat leak ions into the solution. By measuring the conductivity of the floating solution it is possible to detect the level of ion leakage and membrane damage as a result of paraquat activity. Plants overexpressing the NUDX gene are expected to be more tolerant to the oxidative stress (Ishikawa et al., 2009) and therefore to have reduced electrolyte leakage compared to the azygous plants. The assay was first optimized for use in B104 maize by determining

suitable paraquat concentrations where the explants leaked ions into the floating solution but their cellular activity maintained. 1 cm^2 leaf disks from leaf 7 division zone were used in the assay; 7 leaf explants per concentration were used. The explants were incubated in the dark for 20hrs, exposed to low light (30µMol m^{-2} s^{-1}) for 2 hours then incubated for another 20hrs in the dark at 28°C. Electrolyte leakage was measured using a conductivity meter in Micro Siemens per centimeter (µS.cm -1). The graph in Fig 8 shows a steady increase in ion leakage upon increase in paraquat concentration in wildtype B104. Paraquat (PQ) concentrations 0 µM, 0.05µM, 0.07µM and 0.1µm were selected for further use.

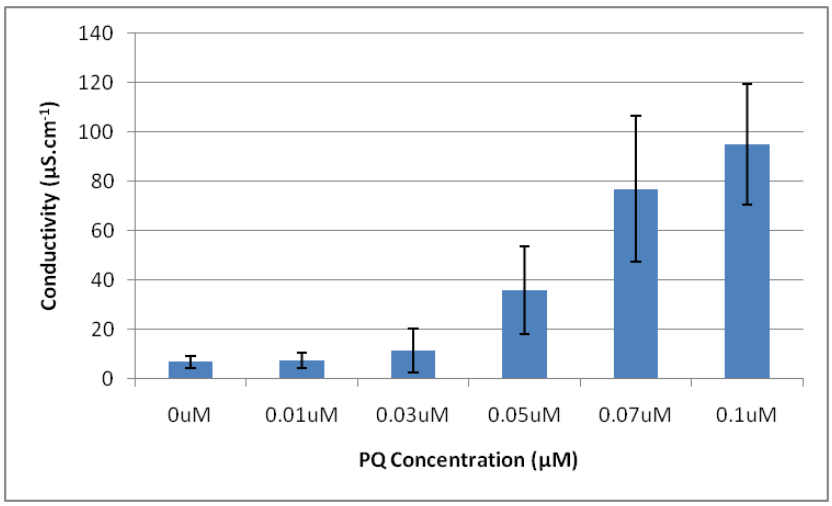

Fig 8: Optimisation of the paraquat concentration in WT B104 maize plants. Steady increase in ion leakage from Wt B104 leaf disks, of maize leaf 7 division zone, upon increase in paraquat concentration. Error bars indicate standard deviation (n=7)

Transgenic T1 lines NUDX7_OE-Zm1 and NUDX7_OE-Zm3 expressing the pBdEF1α-AtNUDX7 construct and T1 line NUDX2_OE-Zm1 expressing pBdEF1α-ZmNUDX2 (Table 2) were used for evaluation of oxidative stress response under paraquat treatment. 1cm^2 leaf disk from leaf 5 division zone were placed in 0uM, 0.05µM, 0.07µM and 0.1µm paraquat. The graphs in Fig. 9 show a comparison of the electrolyte leakage in 20 pat positive plants (transgenic) and 20 pat negative plants (azygous) per line upon paraquat treatment.

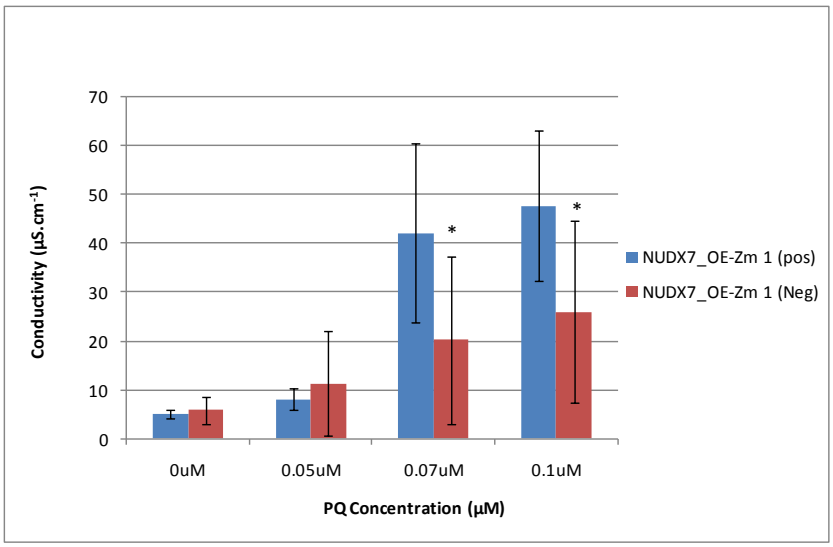

Fig. 9a: Ion leakage in NUDX7_OE-Zm1 transgenic (pos) maize line carrying the pBdEF1α-AtNUDX7 construct and its non-transgenic (Neg) segregating line in different concentrations of paraquat. Error bars indicate standard deviation (n=20) and significance difference in comparison to Wt-W22 (student's t-test) is indicated in asterisk ($p<0.05$)

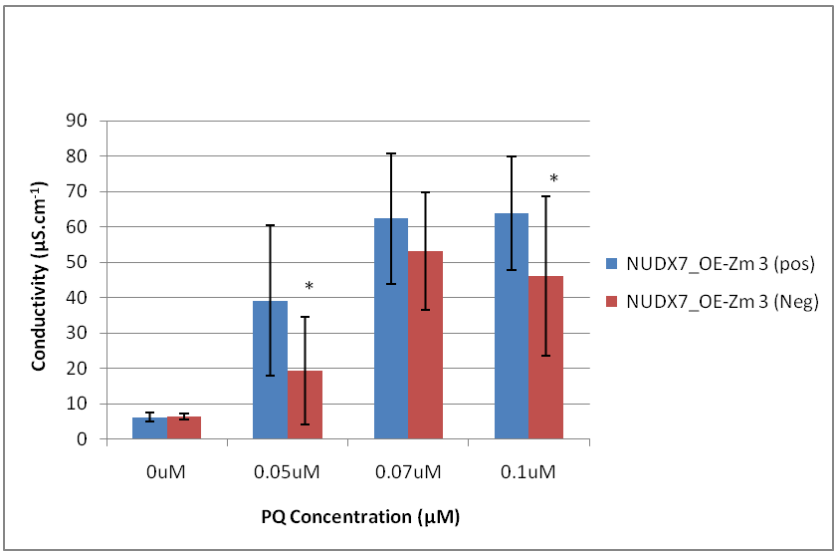

Fig. 9b: Ion leakage in NUDX7_OE-Zm3 transgenic (pos) maize line carrying the pBdEF1α-AtNUDX7 construct and its non-transgenic (Neg) segregating line in different concentrations of paraquat. Error bars indicate standard deviation (n=20) and significance difference in comparison to Wt-W22 (student's t-test) is indicated in asterisk ($p<0.05$)

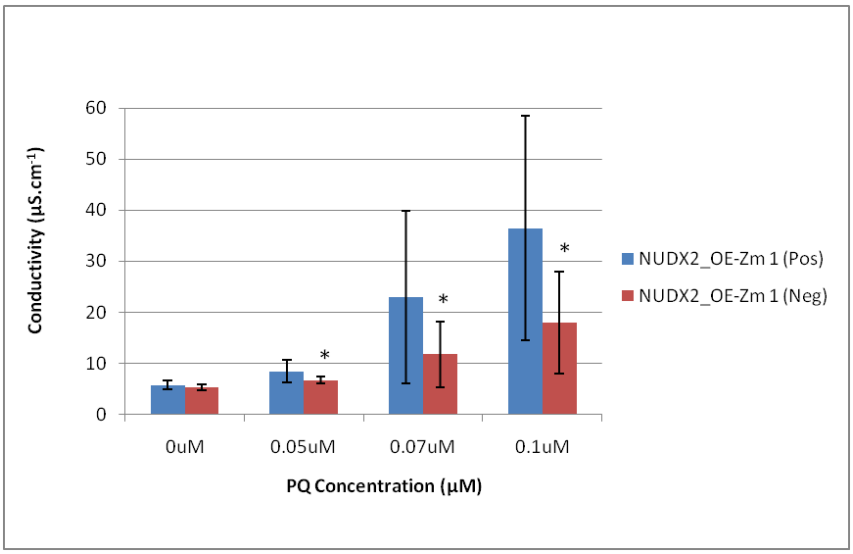

Fig. 9c: Ion leakage in NUDX2_OE-Zm1 transgenic (pos) maize line carrying the pBdEF1α-ZmNUDX2 construct and its non-transgenic (Neg) segregating line in different concentrations of paraquat. Error bars indicate standard deviation (n=20) and significance difference in comparison to Wt-W22 (student's t-test) is indicated in asterisk ($p<0.05$)

In the three lines tested, NUDX7_OE-Zm1 and NUDX7_OE-Zm3 overexpressing the pBdEF1α-AtNUDX7 construct and NUDX2_OE-Zm1 overexpressing pBdEF1α-ZmNUDX2, we observed high standard deviation of the conductivity at higher paraquat concentrations. This is possibly due to the use of tissue from a division zone of the leaf with high cell division rate thus fluctuating ion concentration, tissue from mature zone of the leaf should be considered in a future experiment. The transgenic (pat positive) plants in all lines showed significantly higher ion leakage than azygous (pat negative) samples indicating sensitivity to paraquat induced oxidative stress.

5.2.5 Analysis of endogenous WT B104 ZmNUDX gene expression under water deficit stress

A water deficit experiment was carried out to determine the inducibility of ZmNUDX genes under different water deficit conditions. B104 WT maize plants were grown in an automated weighing, watering and imaging platform (SHRIMPY, PSB-VIB-UGent) under three conditions: well-watered, mild water deficit and severe water deficit. 75 wildtype B104 plants were grown in 500g of soil pot, 25 plants per treatment, in a growth chamber at 25.5^{o}C and 16hrs of light. The well-watered plants were watered daily while the mild water deficit plants were allowed to dry for 5-7 days until the pot weighed 419g and maintained at this weight by watering and the severe water deficit plants were not watered at all. After growing for 10 days in the SHRIMPY platform, 1 cm tissue was harvested from leaf 3 division, elongation and mature zones (21 plants per condition, 7 pools of 3 plants) for QPCR analysis. In Fig. 10a, a schematic representation of a maize leaf with the different zones marked and in Fig. 10b, c & d the expression levels of three maize NUDX genes ZmNUDX2, ZmNUDX8

and Zm-ACN in division, elongation and mature zones under the 3 different water deficit conditions is shown. Each bar represents a mean value scoring of 21 plants.

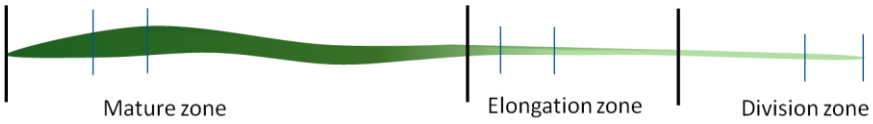

Fig. 10a: Scheme of a maize leaf showing the mature, elongation and division zones (black lines) and 1 cm region of harvest (blue lines)

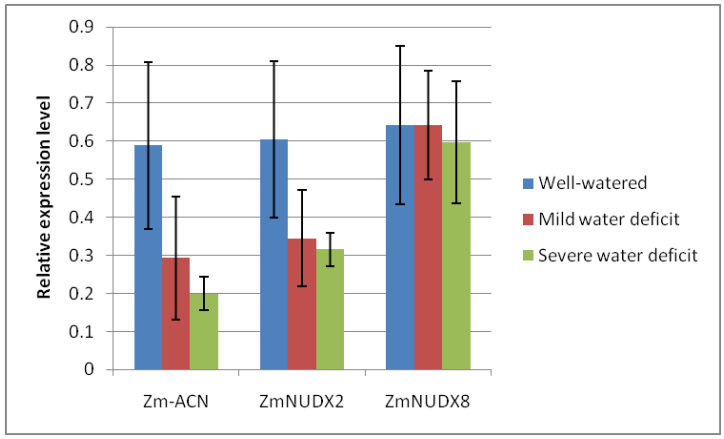

Fig. 10b: Expression of ZmNUDX genes in leaf 3 division zone of WT B104 plants under well-watered, mild and severe water deficit conditions. Error bars indicate standard deviation (n=21)

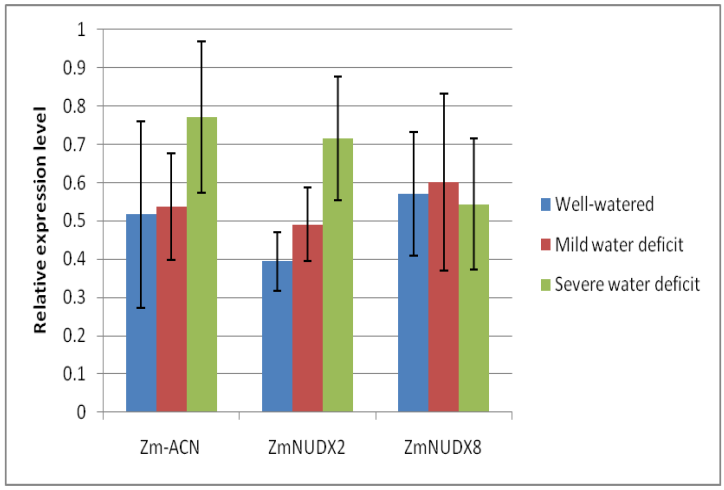

Fig. 10c: Expression of ZmNUDX genes in leaf 3 elongation zone of WT B104 plants under well-watered, mild and severe water deficit conditions. Error bars indicate standard deviation (n=21)

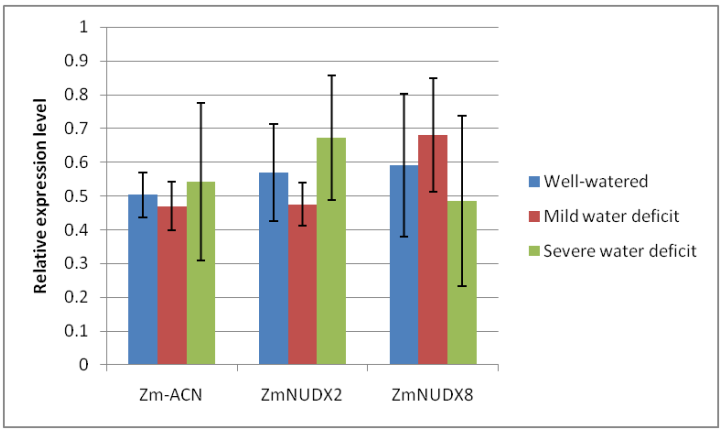

Fig. 10d: Expression of ZmNUDX genes in leaf 3 mature zone of WT B104 plants under well-watered, mild and severe water deficit conditions. Error bars indicate standard deviation (n=21)

The gene expression graphs indicate that endogenous ZmNUDX genes were not induced under mild or severe water deficit conditions. Genevestigator based metadata analysis of ZmNUDX8 and ACN26985 NUDX transcripts (Zimmermann et al., 2004) also showed that ZmNUDX genes are not induced under moderate or severe drought perturbations (Fig. 11).

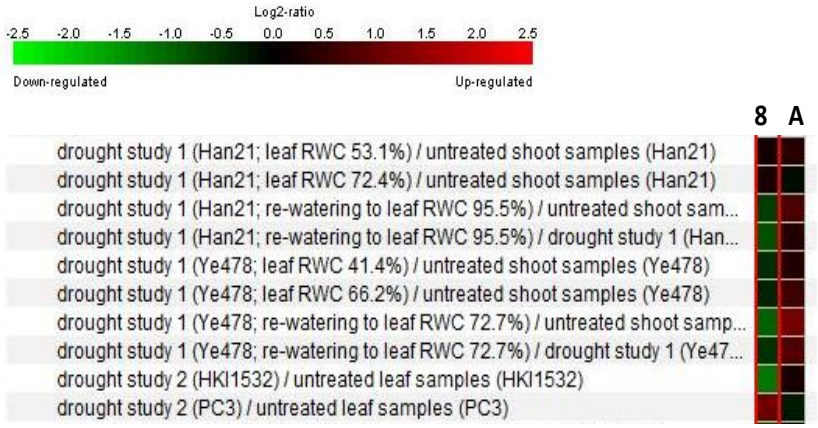

Fig. 11: Heat map showing gene expression levels of ZmNUDX8 (8) and ACN26985 (A) under drought stress perturbations using Genevestigator based metadata analysis software (Zimmermann et al., 2004). Expression level ranges from -2.5 (light green) to +2.5 (Red) compared to the expression in untreated controls

5.2.6 Evaluation of T3 OE NUDX, RNAi PARP1 & ZmNUDX8 Mu KO maize lines under water deficit in an automated platform (SHRIMPY experiment 1)

A water deficit experiment was set up in SHRIMPY, an automated, high-throughput phenotyping platform for maize seedlings at PSB-VIB-UGENT, for which two conditions were selected, a well-watered control and a soil water deficit treatment. The irrigation of plants in SHRIMPY is based on the daily measurement of the gravimetric soil water content and its adjustment to preset values according to the requirements of the treatment. A soil water content of 2.40 and 1.00 g H2O * g dry soil-1 was chosen for the well-watered control and the soil water deficit treatment respectively.

These values correspond to a soil water potential of -0.01 MPa and -6 MPa, respectively. This experiment was carried out in collaboration with Kirin De Muynck and Hilde Nelissen of the Yield group, PSB-VIB. 10 genotypes including T3 homozygous transgenic lines (NUDX OE and RNAi PARP1) and F4 homozygous Mu transposon knockout (KO) lines (Table 4) were used in the experiment. Seeds of all genotypes were soaked in water overnight to enhance germination. 7 seedlings per genotype were grown in the well-watered treatment and 8 seedlings per genotype in the water deficit treatment. A fixed randomisation pattern was used taking into consideration wall effect (plants closer to the wall were noted to grow faster) and temperature gradient in the growth room. The plants were also watered at the same time daily to avoid biases. The plants were allowed to grow for approximately one month and each plant was harvested two days after attaining V-Stage 5 (leaf 5 with a visible collar). Leaf 4 was selected for measurement of the yield parameters because it is the first leaf growing independent of the kernel. Plants with more than 5 days delay in apprearance of leaf 4 were not used further in the analysis apart from the Mutator transposon lines (W22 background) in which leaf 4 appeared about 10 days later. Several parameters of leaf 4 were measured: daily leaf length, final blade weight, final blade + sheath weight, final blade width and total leaf area. In addition the total biomass (wet weight) and total dry weight of each plant was determined. The leaf 4 length was measured daily from the base of the plant to the leaf tip and from appearance until the leaf 4 stops growing, and was used in determining the Leaf Elongation Ratio (LER). LER = (L2-L1) / (Tp2-Tp1) where L1 and L2 are the lengths of leaf 4 measured at time point 1 (Tp1) and 2 (Tp2). In Fig. 12, the LER per genotype and wildtype B104 line graphs under well-watered control and water deficit treatment is shown from time points 1-14.

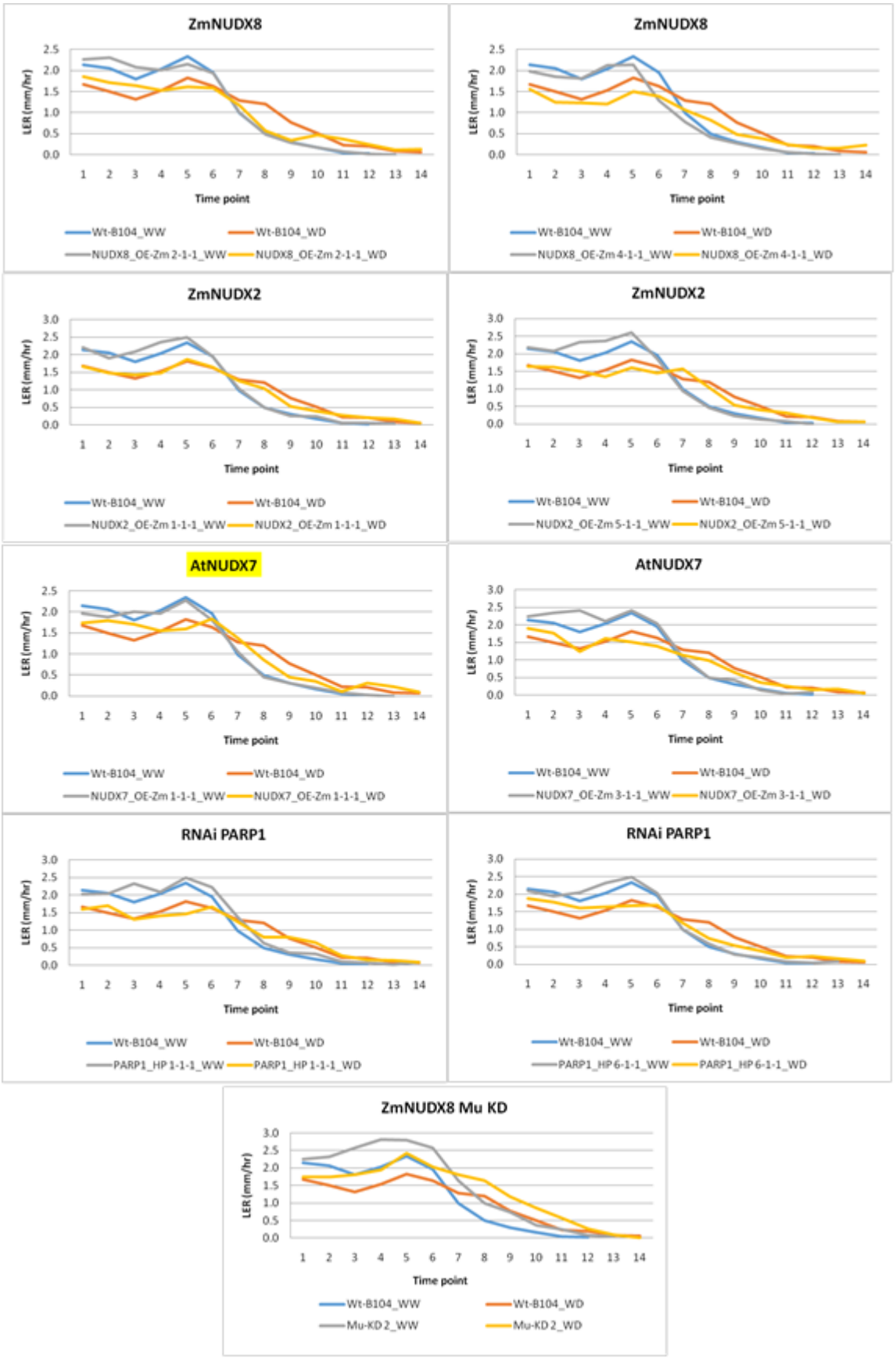

Fig. 12: Leaf 4 elongation rates from timepoint 1 to 14 of OE NUDX, RNAi PARP1 and ZmNUDX8 Mu KD lines in comparison with Wt B104 under well-watered (WW) and water deficit treatment (WD). (n=7 for WW and n=8 for WD)

An increased or fluctuation of the LER from time point 1 to 5 was observed in both stressed and well-watered control lines with a peak at time point 5 then a gradual decrease of the LER to the last time point 14. Generally, the well-watered control plants had a higher LER than the water deficit plants. To compare the growth performance between the genotypes, percentage leaf 4 growth rate in water deficit condition was determined for each genotype as follows: (Average of Tp1 to Tp5 LER in water deficit)*100 / (Average of Tp1 to Tp5 LER in well-watered).

Table 4: Genotypes in water deficit experiment 1 and their percentage leaf 4 growth in water deficit condition

Construct / Mutation	Lines	Fold change	% L4 Growth in W. Deficit
pBdEF1α-ZmNUDX8	NUDX8_OE-Zm 2-1-1	10.1	77.3
	NUDX8_OE-Zm 4-1-1	48	68.0
pBdEF1α-ZmNUDX2	NUDX2_OE-Zm 1-1-1	16.1	71.7
	NUDX2_OE-Zm 5-1-1	38.7	65.1
pBdEF1α- AtNUDX7	NUDX7_OE-Zm 1-1-1	See Fig. 3b	83.2
	NUDX7_OE-Zm 3-1-1	See Fig. 3b	69.8
RNAi PARP1	PARP1_HP 1-1-1	-5.9	68.2
	PARP1_HP 6-1-1	-3.8	78.8
ZmNUDX8 Mu KD	Mu-KD 2	-5.1	75.7
Wildtype	Wt-B104		75.0

Leaf 4 growth rate in wildtype B104 was reduced by 25% upon water deficit treatment. Moderate OE line NUDX8_OE-Zm 2-1-1 and moderate downregulated line PARP1_HP 6-1-1 had a 2.3% and 3.8% higher leaf 4 growth rate upon water deficit treatment than WT B104. A high OE line NUDX7_OE-Zm 1-1-1 carrying an OE construct pBdEF1α-AtNUDX7 had an interestingly higher leaf 4 growth rate of 8.2% upon water deficit treatment than Wt B104 indicating a trend of tolerance to water deficit stress. This line will be included in a repeat of the water deficit experiment to confirm the results.

Some end point parameters (Fig. 13) were measured to further determine the response of the 10 genotypes to water deficit treatment including leaf 4 (blade weight, blade + sheath weight, blade width, length and total leaf area), total plant biomass (wet weight) and total plant dry weight.

Fig. 13: End point parameters measured to compare OE NUDX, RNAi PARP1, ZmNUDX8 Mu KD line and Wt B104 under well-watered (WW) and water deficit (WD) treatment. Percentages represent reductions upon water deficit treatment. The yellow arrow points to a high OE line NUDX7_OE-Zm 1-1-1 where a notable trend of lower percentage reduction compared to Wt-B104 was observed in several parameters upon water deficit treatment. Error bars indicate standard deviation, (n=7 for WW and n=8 for WD)

Upon water deficit treatment, the high OE AtNUDX7 line NUDX7_OE-Zm 1-1-1 had again notable phenotypes showing consistently a trend of better performance (lower reduction ratios) upon water deficit stress than Wt B104 in a number of leaf 4 parameters tested (blade weight, blade + sheath weight, blade width and total leaf area) and also in the total plant dry weight (Fig. 13). Furthermore, the leaf 4 blade width of line NUDX7_OE-Zm 1-1-1 was 11% higher than Wt B104 upon water deficit treatment, though not statistically significant, but indicating a trend towards water deficit stress tolerance (Fig. 13). RNAi PARP1 line, PARP1_HP 1-1-1, had an 8% lower leaf 4 blade weight, 7% lower blade + sheath weight, 11% lower total leaf 4 area and 10% lower leaf 4 length than wildtype B104 upon water deficit stress indicating a trend to sensitivity to water deficit stress (Fig. 13). The ZmNUDX8 Mutator transposon insertion line (Mu-KD2) had generally bigger plants thus the higher values than all other lines in the all parameters measured both under well-watered control and water deficit stress conditions. However, a comparison between its well watered and water deficit treated plants showed no significant difference in the leaf 4 blade width, leaf 4 area and total plant dry weight indicating that the Mu transposon lines are possibly tolerant to water deficit stress. Since the Mutator transposon lines are developed in a W22 background, we could not compare its growth reduction to that of wildtype B104. In a repeat experiment, a wildtype W22 line will be included.

5.2.7 Evaluation of T3 OE AtNUDX7, CRISPR PARP2 & ZmNUDX8 Mu KO maize lines under water deficit in an automated platform (SHRIMPY experiment 2)

A water deficit experiment on SHRIMPY was carried out in the identical set up described above. The T3 high OE line NUDX7_OE-Zm 1-1-1 carrying the pBdEF1α-AtNUDX7 construct was repeated and another AtNUDX7 T3 high OE line, NUDX7_OE-Zm 2-1-1 added (Fig 3b). Also, in addition to the F4 homozygous Mu-KD2, a ZmNUDX8 Mu KD line in W22 genotype background from the previous water deficit experiment, a wildtype W22 line was included as a control. We also tested three T1 lines carrying ZmPARP2_CRISPR construct (ZmPARP2_CR 1-1, ZmPARP2_CR 4-1, and ZmPARP2_CR 8-1- see chapter 4, Table 5 for CRISPR line details) and wildtype B104 control line. In Fig. 14, the LER per genotype and its respective wildtype control line graph under well-watered control and water deficit treatment is shown from Time points (Tp) 1 to 13. In addition, Table 5 shows the percentage leaf 4 growth rate in water deficit condition.

Fig. 14: Leaf 4 elongation rates from timepoint 1 to 13 of OE NUDX7, ZmPARP2-CRISPR and ZmNUDX8 Mu KD lines in comparison with Wt B104 or UFMu-Wt controls under well-watered (WW) and water deficit treatment (WD). (n=7 for WW and n=8 for WD)

Table 5: Genotypes in water deficit experiment 2 and their percentage leaf 4 growth in water deficit condition

Construct / Mutation	Lines	% Growth in W. Deficit
WT B104	Wt-B104	70.3
pBdEF1α- AtNUDX7	NUDX7_OE-Zm 1-1-1	86.8
	NUDX7_OE-Zm 2-1-1	74.3
ZmPARP2_CRISPR	PARP2_CR 1-1	72.5
	PARP2_CR 4-1	64.7
	PARP2_CR 8-1	74.5
ZmNUDX8 Mu KD	Mu-KD 2	61.6
WT W22	Wt-W22	75.2

Similar to the first water deficit experiment, an increased or fluctuation of the LER from time point 1 to 5 was observed in both stressed and well-watered control lines with a peak at time point 5 then a gradual decrease of the LER to the last time point. The percentage leaf 4 grow rate in Table 5 above give an indication of the leaf 4 growth performance of these lines. Leaf 4 growth rate in Wt B104 was reduced by 30% upon water deficit treatment. Similar to the first water deficit experiment, high OE AtNUDX7 line NUDX7_OE-Zm 1-1-1 had again a notable phenotype with a 16.5% higher leaf 4 growth rate upon water deficit treatment in comparison to Wt B104 indicating a trend of tolerance. High OE AtNUDX7 line NUDX7_OE-Zm 2-1-1 and ZmPARP2_CRISPR lines PARP2_CR 1-1 and PARP2_CR 8-1 had leaf 4 growth rates of 4%, 2.2% and 4.2% higher than Wt B104 respectively upon water deficit treatment. The Wt-W22 control line had poor growth of plants and thus it was difficult to compare it with the ZmNUDX8 Mu-KD2 line. End point parameters measured to further determine the response of the 8 genotypes to water deficit treatment include leaf 4 (blade weight, blade + sheath weight, blade width and total leaf area), total plant biomass (wet weight) and total plant dry weight (Fig. 15).

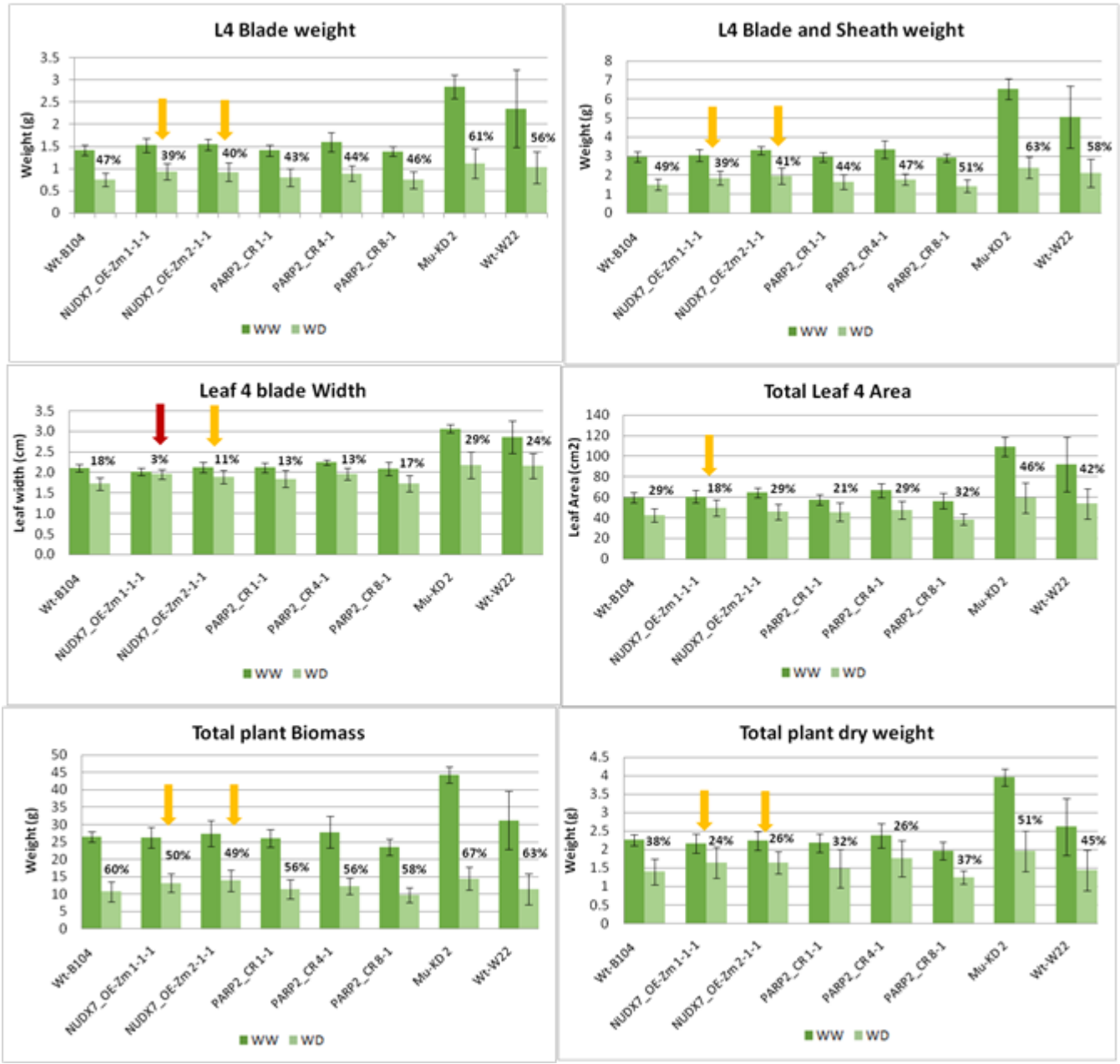

Fig. 15: End point parameters measured to compare OE NUDX7 and ZmPARP2-CRISPR, Wt B104, ZmNUDX8 Mu KO lines and UFmu-Wt under well watered (WW) and water deficit (WD) treatment. Percentages represent reductions upon water deficit treatment. The yellow arrow points to high overexpression lines, NUDX7_OE-Zm 1-1-1and NUDX7_OE-Zm 2-1-1, where a notable trend of lower percentage reduction compared to Wt-B104 was seen in several parameters upon water deficit treatment. The red arrow points to NUDX7_OE-Zm 1-1-1 with a 15% significantly higher leaf 4 blade width than Wt B104 upon water deficit stress (P = 0.0158, two-way analysis of variance with custom hypothesis Wald tests (corrected for multiple testing Sidak step-down)). Error bars indicate standard deviation, (n=7 for WW and n=8 for WD).

The two high OE AtNUDX7 lines, NUDX7_OE-Zm 1-1-1and NUDX7_OE-Zm 2-1-1, had interesting phenotype showing a consistent trend of better performance (lower reduction ratios) than Wt-B104 in most of the parameters tested upon water deficit stress apart from the total leaf 4 area of line NUDX7_OE-Zm 2-1-1 which was similar to that of the B104 control (Fig. 15). The leaf 4 blade width of

line NUDX7_OE-Zm 1-1-1 marked with a red arrow in Fig. 13 was 15% significantly higher than Wt B104 upon water deficit stress (P = 0.0158, two-way analysis of variance with custom hypothesis Wald tests (corrected for multiple testing using Sidak step-down)) indicating tolerance to water deficit stress. The total biomass of high OE lines NUDX7_OE-Zm 1-1-1 and NUDX7_OE-Zm 2-1-1 was 10% and 11% higher respectively and the total plant dry weight was 14% and 12% higher respectively than Wt B104 upon water deficit treatment though the differences are not statistically significant but indicating a trend towards water deficit stress tolerance. Notably also the ZmPARP2_CRISPR lines (ZmPARP2_CR1-1, ZmPARP2_CR4-1, and ZmPARP2_CR8-1) had a slightly better performance (lower reduction ratios) than Wt B104 in most of the parameters tested indicating slight tolerance though not statistically significant (Fig. 15). To determine the power of this experiement, we used leaf 4 blade width as an example. Based on the sample size, effect size and standard deviation, a statistical power of 0.46 was obtained indicating that the probability of obtaining a genotype showing statistically significant leaf 4 blade width in this experiment is 46%. To increase the power of the experiment, more samples than 7 or 8 plants per genotype should be considered in the future experiments.

5.3 Discussion

We have developed single locus T-DNA insertion maize lines showing high overexpression of AtNUDX7, ZmNUDX2 and ZmNUDX8 genes driven by a strong *Brachypodium distachyon* pBdEF1α promoter and also OE AtNUDX7 and ZmNUDX2 lines driven by maize Ubiquitin promoter. We also identified a homozygous Mutator transposon insertion in exon 1 of ZmNUDX8 gene showing 5 to 6 fold down regulation of the ZmNUDX8 gene activity. 4 out of 15 T1 lines carrying pBdEF1α-ZmNUDX8 construct and 3 out of 23 T1 lines carrying the pBdEF1α-ZmNUDX2 resulted in a silencing phenotype where all the 40 individual plants tested were pat negative. The T0 parents of these plants were Pat positive and PCR genotyping of some the Pat negative T1 lines showed a segregation of the transgene. This indicates that the silencing is post transcriptional and may be as a result of the use of the strong *Brachypodium distachyon* pBdEF1α promoter (Coussens et al., 2012) that may have produced abnormally high transcript levels that triggered a feedback mechanism leading to RNA degredation, a phenomenon earlier described by Elmayan and vaucheret, (1996) where a strongly expressed bacterial *UidA* gene cloned between the *35S* promoter with a double enhancer resulted in post transcription silencing in tobacco plants. Interestingly however, OE lines generated using the pZmUBIL promoter had persistently much lower fold expression levels in comparison to pBdEF1α lines (Fig. 3b, 4b and Table 2). We thus used the Pat positive pBdEF1α overexpression lines in the functional assays which did not result in further gene silencing events in T2 or T3 generations.

Two pBdEF1α-AtNUDX7 and one pBdEF1α-ZmNUDX2 maize lines evaluated for their oxidative stress response using paraquat treatment showed higher sensitivity in comparison to their respective azygous controls. This is in contrast to previous reports which indicate that modulation of AtNUDX2 and AtNUDX7 conferred enhanced oxidative stress tolerance in Arabidopsis as assessed by plant physical phenotype, survival rates, chlorophyll content and root growth (Ogawa et al., 2009; Ishikawa et al., 2009). Indeed several factors may have contributed to this contrasting results, it is possible that the NUDX genes do not confer oxidative stress tolerance to maize plants or testing more transgenic lines per construct would result in a line showing tolerance to oxidative stress. In addition, the type of assay used to measure oxidative stress tolerance was different, we used leaf disk assay and measured the ion leakage in the floating solution, an assay confirmed for oxidative stress tolerance determination in transgenic maize (Van Breusegem et al., 1999). The concentrations of paraquat used are also quite different, we used a range of 0.03µM – 0.1µM while in the former report in Arabidopsis 0.3 µM and 3µM were used raising the question of dose-response effects (Claeys et al., 2014). The amount of light used during paraquat treatment also varied, we used 30µMol m^{-2} s^{-1} while in the reports 100µMol m^{-2} s^{-1} and 1600µMol m^{-2} s^{-1} was used. We propose testing more transgenic lines using the leaf disk paraquat assay and development of new assays to counter check results in oxidative stress assays in maize.

We report that overexpression of the Arabidopsis AtNUDX7 gene in maize using *Brachypodium distachyon* pBdEF1α promoter in a high overexpression line NUDX7_OE-Zm 1-1-1 resulted in significant tolerance to water deficit stress. The notable phenotypes of OE AtNUDX7 lines include: Lines NUDX7_OE-Zm 1-1-1 with a 15% significantly higher leaf 4 blade width than Wt B104 upon water deficit stress (P = 0.0158, two-way analysis of variance with custom hypothesis Wald tests (corrected for multiple testing using Sidak step-down)), High OE lines lines NUDX7_OE-Zm 1-1-1 and NUDX7_OE-Zm 2-1-1 with 10% and 11% higher total plant biomass respectively and 14% and 12% higher total plant dry weight respectively than Wt B104 upon water deficit treatment. Also line NUDX7_OE-Zm 1-1-1 showed an 8.2% higher leaf 4 growth rate than Wt B104 in the first experiment and 16.5% higher leaf 4 growth rate than Wt B104 upon water deficit treatment. These findings indicate that the ADP-ribose specific NUDX genes play a role in maize plant response to water deficit stress. ADP-ribose specific Nudix hydrolases are involved in nucleotide recycling in the PAR pathway by hydrolysing free ADP-Ribose molecules into ribose-5-phosphate and AMP, a ready precursor for ATP synthesis (Rossi et al., 2002; Ogawa et al., 2005; Ishikawa et al., 2009). Upon drought stress, there is an increased production of ROS (Cruz de Carvalho, 2008) which induce the PARP protein activity (Vira´g and Szabo´, 2002) and thus the PAR pathway. Activation of the PAR pathway will resultantly activate the nucleotide recycling action of ADP-ribose specific Nudix hydrolases and re-

establishing the energy levels by supplying an ATP source. We thus propose that overexpression of AtNUDX7 in maize plants enhanced the water deficit stress tolerance through enhancing the recycling step of ADP-ribose in the PAR energy homeostasis pathway. The ZmPARP2_CRISPR lines showed a trend of higher leaf 4 growth rates and also other leaf 4 parameters and biomass measurements relative to Wt B104 upon water deficit treatment. The marginal responses of the ZmPARP2-CRISPR lines could be due to the use of T1 lines with a segregation of the Cas9-induced deletion phenotypes (Chapter 4, Table 5). A repeat of this experiment with T2 lines without T-DNA or back-crossed to B104 and selfed in T1 carrying more uniform and stable ZmPARP2 mutations is proposed.

5.4 Materials and methods

5.4.1 Plant material and growth conditions

The Uniform Mu Mutator lines UFMu-07274 and UFMu-06482 (http://www.maizegdb.org/uniformmu) and B104 maize genotype (Hallauer et al., 1997) used in this study were grown either in growth room or green-house conditions. The maize growth room conditions include: 24^{o}C temperature, 55% relative humidity, 230µE m^{-2} s^{-1} light intensity and 16hrs of lighting. The maize green-house conditions include: 22-26^{o}C temperature, 45% relative humidity, 300µE m^{-2} s^{-1} light intensity and 16hrs of lighting. Maize seeds were sown on trays containing jiffy soil (sphagnum peat moss) placed in the maize growth room where the seedlings were grown for 3 to four weeks then transferred to bigger soil pots (Saniflor beroepspotgrond with osmocote) and placed in the green-house where they grew to maturity.

5.4.2 NUDX OE constructs cloning

cDNA/ gDNA sequence coding for AtNUDX7, ZmNUDX2 and ZmNUDX8 was cloned into pDONR221 donor vector and then recloned into pBbm42GW7 monocot multisite gateway vector (Karimi et al., 2013) including either the pEN-L4-BdEF1a-R1 or the pENTRY-L4-UBIL-R1 promoter entry clones following the Gateway Technology Manual (Invitrogen). The expression vectors developed were cloned into the EHA101 super virulent Agrobacterium strain (Hood et al., 1986) and transformed into the B104 maize genotype using the Agrobacterium-mediated transformation.

5.4.3 Maize transformation, bar gene selection and assays

Immature embryos of the maize inbred line, B104, were transformed with the NUDX OE constructs at the PSB-VIB maize transformation platform according to the procedure described by Coussens et al. (2012), in which 2,4-D was replaced by dicamba (3.32mg/l). Transgenic plant materials were selected using a bar marker gene whose activity was identified using either of three assays: Pat

assay, Ammonium-multiwell assay or Basta leaf painting assay. Pat assay test was carried using AgraStrip LL Strip test kit following manufacturer's instructions, Ammonium-multiwell assay was carried out according to De Block et al. (1995) and Rasco-Gaunt et al. (1999) and Basta leaf painting was conducted as described in Rasco-Gaunt et al. (1999).

5.4.4 RNA preparation, cDNA synthesis and QPCR expression analysis

1cm leaf tissue from approximately 10-12 days old maize seedlings were harvested for QPCR expression analysis and samples pooled as described in the results for each QPCR analysis. RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) and cDNA prepared using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacture's protocols. QPCR experiments were performed in a LightCycler480 Real -Time SYBR Green PCR System (Roche) and all reactions were performed in three technical replicates. Expression levels were normalised to reference genes 18SrRNA and EF1α (Genebank accession X00794.1 and NM_001112117.1 respectively).

5.4.5 PCR genotyping

DNA was isolated from maize leaf tissue using the Genomic DNA purification kit (Promega) and PCR carried out using the Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes) or Taq DNA Polymerase (Invitrogen) following the manufacture's protocol. Gene-specific primers alone or in combination with transposon-specific primers (TIR 6/ TIR 8) were used to amplify the Mu insert. The PCR products were run on a 1% agarose. PCR product obtained using gene-specific primers alone indicate the presence of a wildtype allele while PCR products obtained using transposon-specific + gene-specific primer indicates the presence of an insert.

5.4.6 Paraquat induced ion leakage leaf disk assay

$1cm^2$ fresh leaf disks from division zone of leaf 5 or 7 were placed in 3ml of paraquat solution (concentrations 0µM, 0.03µM, 0.05µM, 0.07µM and 0.1µM) in 12 multiwell plates, all solutions prepared using Milli Q water. 20 pat positive leaf disks and 20 pat negative leaf disks per line per concentration were incubated in the dark for 20hrs, exposed to low light for 2 hours at 30µMol m^{-2} s^{-1} then incubated for another 20hrs in the dark at 28^{o}C as described by Van Breusegem et al. (1999). Ion leakage was measured as an increase in conductance of the floating solution using a K610 Conductivity meter (Consort Turnhout, Belgium) in Micro Siemens per centimeter (µS.cm^{-1}).

5.4.7 Water deficit assay in an automated platform

In the first experiment Wt-B104 plants were grown on SHRIMPY under different water deficit conditions and gene expression levels of endogenous NUDX genes determined. The experimental designed is described in a scheme in Fig. 16.

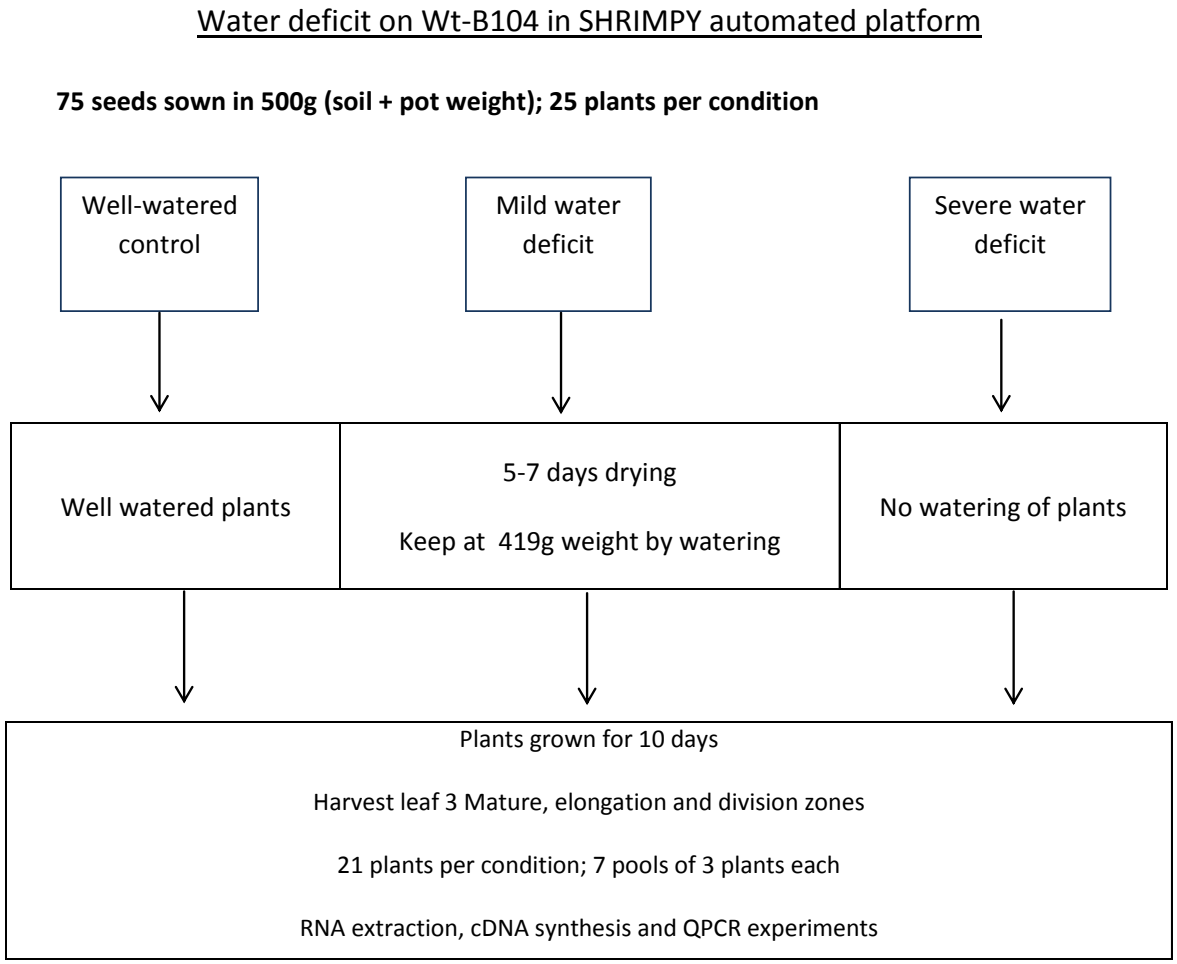

Fig. 16: Experimental set-up to determine induction of endogenous NUDX genes under water deficit conditions

Phenotyping of the OE NUDX, RNAi PARP1, CRISPR-PARP2 and Mutator insertion lines was carried out under water deficit conditions in SHRIMPY automated platform for which two conditions were selected, a well-watered control and a soil water deficit treatment. A soil water content of 2.40 and 1.00 g H2O * g dry soil-1 was chosen for the well-watered control and the soil water deficit treatment, respectively. These values correspond to a soil water potential of -0.01 MPa and -6 MPa, respectively. Seeds of all genotypes were soaked in water overnight to enhance germination. 7 seedings per genotype were grown in the well-watered treatment and 8 seedlings per genotype in the water deficit treatment. A fixed randomisation pattern was used taking into consideration wall effect (plants closer to the wall were noted to grow faster) and temperature gradient in the growth

room. The plants were also watered at the same time daily to avoid biases. The plants were allowed to grow for approximately one month and each plant was harvested two days after attaining V-Stage 5 (leaf 5 with a visible collar). Plants with more than 5 days delay in apprearance of leaf 4 were not used further in the analysis apart from the Mutator transposon lines (W22 background) in which leaf 4 appeared about 10 days later. Several parameters of leaf 4 were measured: daily leaf length, final blade weight, final blade + sheath weight, final blade width and total leaf area. In addition the total biomass (wet weight) and total dry weight of each plant was determined. The leaf 4 length was measured daily from the base of the plant to the leaf tip and from appearance until the leaf 4 stops growing, and was used in determining the Leaf Elongation Ratio (LER).

5.4.8 Data analysis on water deficit experiment

Statistical data analysis was carried out for all the end point growth parameters measured in the water deficit experiments on SHRIMPY automated platform with an interest of determining the different effect upon water deficit of each transgenic line compared to the control line. A two-way analysis of variance was conducted for each end point parameter (dependent variables). The model included the factors genotype and treatment and the interaction term. When the interaction term was significant at the 5% significant level, Wald tests were performed to estimate the significance of the difference in effect upon water deficit of each genotype versus the control genotype. P-values were adjusted for multiple testing using Sidak step-down as implemented in SAS. The analysis was conducted with the glm procedure from SAS (Version 9.4 of the SAS System for windows 7 64bit Copyright 2002-2012 SAS Institute Inc. Cary, NC, USA, www.sas.com). Correction for multiple testing of the interaction effect was done with the multtest procedure. The statistical analysis was carried out by Véronique Storme, statistician, PSB-VIB-UGent.

5.4.9 List of primers

Table 6: Primer sequences used in the study

Primer	Sequence (5'->3')	Experiment
NUDX2F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACCATGTCAAGTTCCATAATTTCAACAGT	OE NUDX gene cloning + Att sites
NUDX2R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACCCTGAGGCCCTGTCCAG	
NUDX8F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACCATGGAGAGCGGCTTGCTTGACACCG	
NUDX8R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGGCGGCGCTGCAGTTCAC	
AtNUDX7F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACCATGGGTACTAGAGCTCAGCAG	
AtNUDX7R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGAGAGAAGCAGAGGCTTG	
18SrRNA_Q1	ACCTTACCAGCCCTTGACATATG	Housekeeping genes
18SrRNA_Q2	GACTTGACCAAACATCTCACGAC	
EF1A_Q1	AGTCCGTTGAGATGCACCATG	
EF1A_Q2	CACATACCCACGCTTCAGATCC	
qpcrAtNUDX7bF	CTTGGGATTCGCCATTGTG	NUDX gene expression analysis
qpcrAtNUDX7bR	CATGATCCGCATTGCAGTAGAT	
qpcrZmNUDX2bF	ATGCAGGCTTTTCGCCAATC	
qpcrZmNUDX2bR	CAGATCCCTCCGGTTCATGT	
qpcrZmNUDX2cF	GCAGGCTTTTCGCCAATCTC	
qpcrZmNUDX2cR	GTCCAGATCCCTCCGGTTCA	
qPCRNUDX8eF	GTGGATCTAGCCGAGTTCGT	
qPCRNUDX8eR	GGGAGCATATTCGGTTCATC	
qpcrZmNUDX8fF	CTGAGGCCTGTGTCGAGTG	
qpcrZmNUDX8fR	GATGAAGGGCTGCTTCACGA	
pBdEF1α2	GATGCTGTCTGTGTACTG	OE NUDX lines genotyping
pZmUbi-L1	TGGTACTGTTTCTTTTGTCG	
T35S1	ACCCTAATTCCCTTATCTGG	
UFMu-07274_F3	ACGCATACGGCGTGTCCGGCCC	Mu transposon insertion genotyping
UFMu-07274_R3	CGCCGCCCGTCTCCGACCAGT	
TIR 6	AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC	
Bc11R692	GTCTTGGCCGTGTACTGGAT	
TIR8.1	CGCCTCCATTTCGTCGAATCCCCTS	
TIR8.2	CGCCTCCATTTCGTCGAATCCSCTT	
TIR8.3	SGCCTCCATTTCGTCGAATCCCKT	
TIR8.4	CGCCTCCATTTCGTCGAATCACCTC	

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CHAPTER 6

Functional analysis of Maize and Arabidopsis NUDX genes in Arabidopsis for seed yield, oxidative stress and mild drought stress responses using overexpression and KO lines

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Contribution: E. N. carried out experiments (Gene cloning and expression analysis of OE NUDX constructs in Col-0 and *nudx7* mutant backgrounds, screening and identification of *nudx7* mutant line, oxidative stress assay on OE / KO NUDX lines, partly participated in seed yield and yield related parameter analysis and also partly in mild drought stress experiment) and wrote the chapter

Abstract

Arabidopsis NUDX7 gene has been extensively studied and identified as a multiple stress induced gene with an impact on both biotic and abiotic stress responses. The gene has also been proposed as the predominant ADP-ribose pyrophosphatase in Arabidopsis cells. In this study, new functional analysis approaches for testing oxidative and drought stress tolerance in Arabidopsis lines with altered NUDX gene expression is reported. Three AtNUDX7 overexpression Arabidopsis lines showed significant tolerance to paraquat induced oxidative stress. In addition an AtNUDX7 and two ZmNUDX Arabidopsis overexpression lines showed a tendency to mild drought stress tolerance. We also make a first reporting of altered seed yield and yield related parameters in lines with modulated AtNUDX7 gene expression. Several lines overexpressing AtNUDX7 in Col-0 background showed a significant increase in total seed weight, seed number seed size and mass in addition to early flowering time and reduced rosette leaf number.

6.1 Introduction

Nudix hydrolases (NUDX) from Arabidopsis thaliana are so far the most substantially characterized in higher plants. AtNUDX7 shows preferential activity for ADP-ribose and NADH when expressed in E. coli cells (Ge et al., 2007) and has been proposed as the predominant NADH and ADP-Ribose pyrophosphatase in Arabidopsis cells (Ishikawa et al., 2009). AtNUDX7 gene has been extensively studied and identified as a multiple stress induced gene with impact on both biotic and abiotic stress responses (Bartsch et al., 2006; Jambunathan and Mahalingam, 2006; Ge et al., 2007; Adams-Phillips et al., 2008; Ishikawa et al., 2009; Jambunathan et al., 2010). The gene also plays a role in seed germination as *Atnudx7* mutants accumulated high levels of ABA resulting in reduced seed germination phenotype (Zeng et al., 2014).

Crop yield is a complex trait determined by a combination of genetic, epigenetic, and environmental factors that interact in unpredictable ways resulting in non-linear relationships between genotypes and phenotypes. There have been joined efforts from various disciplines including plant breeding, genetics, molecular biology, plant physiology and agronomy to develop improved plant varieties with better adaptability and productivity under various environmental conditions. The maximum yield a crop variety can reach under optimal growth and harvest conditions, termed “potential yield”, is determined by genetic and epigenetic constitution of the crop thus a crop’s potential yield can be improved by genetic manipulations. Despite the known complexity of crop yield genetics, growing evidence shows that yield can be increased by genetic modification of single genes. Van Camp (2005) describes positive results that have been obtained by using single gene modification to target different yield constituents such as photosynthesis, starch biosynthesis, plant architecture and transcriptional networks controlling plant development thus indicating that there is ample room for further yield improvement by genetic means. Another approach to improving crop yield is genetic engineering to produce higher seed yield. Seed size and number are described as the two main components contributing to seed yield. In this study, we analyzed seed yield parameters: seed size, seed number, total seed weight and mass per seed, and other yield-associated parameters (inflorescence height, rosette number and flowering time) according to Van Daele *et al*. (2012) in Arabidopsis lines, in which the activity of the AtNUDX7 gene has been overexpressed or knocked down.

Reactive oxygen species (ROS) are formed by the inevitable leakage of electrons from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a by-product of various metabolic pathways localized in different cellular compartments even under optimal

conditions, which reduce molecular oxygen. During biotic and abiotic stress conditions, the levels of ROS increase potentially resulting in oxidations of DNA, proteins and lipids. Modulation of AtNUDX2 and AtNUDX7 has been reported to confer enhanced oxidative stress tolerance in Arabidopsis plants (Ogawa et al., 2009; Ishikawa et al., 2009). We explored these studies further by determining the rosette area under paraquat-induced oxidative stress of Arabidopsis plants whose AtNUDX7 gene activity has been overexpressed or knocked down.

Modulation of the poly(ADP-ribosyl)ation (PAR) pathway via the down-regulation of the PARP gene in Arabidopsis and *Brassica napus* by RNAi gene silencing resulted in plants with reduced NAD^+ depletion and ATP consumption and were tolerant to a broad range of abiotic stresses such as high light, drought, and heat (De Block et al., 2005). In this study the ADP-ribose specific NUDX protein, a downstream PAR pathway protein, was modulated through overexpression of Arabidopsis and maize NUDX genes in Arabidopsis and the plants were evaluated for mild drought stress response in an automated platform.

6.2 Results

6.2.1 Generation of overexpression NUDX transgenic lines and identification of a nudx7 mutant line

6.2.1.1 OE of AtNUDX and ZmNUDX genes in the Arabidopsis Col-0 background

The complete coding sequence of the AtNUDX7 (AT4G12720) gene and its two maize homologs ZmNUDX2 (GRMZM2G101693) and ZmNUDX8 (GRMZM2G175816) were transformed into Arabidopsis thaliana Col-0 ecotype using the Gateway cloning system (Invitrogen). The coding region of each gene was amplified from Arabidopsis or maize cDNA using gene specific primers and each PCR fragment cloned into a pDONR221 donor vector. The inserts in the pDONR221 entry clones were then recloned into the pK2GW7 Gateway expression vector driven by the cauliflower mosaic virus p35S promoter (Karimi et al., 2007), expressed in pMP90 Agrobacterium cells and further transformed into Arabidopsis using the floral dip system (Clough and Bent, 1998) at PSB-VIB. Fig. 1 shows a general scheme of the T-DNA constructs developed for overexpression of AtNUDX7, ZmNUDX2 and ZmNUDX8 genes in the Col-0 background.

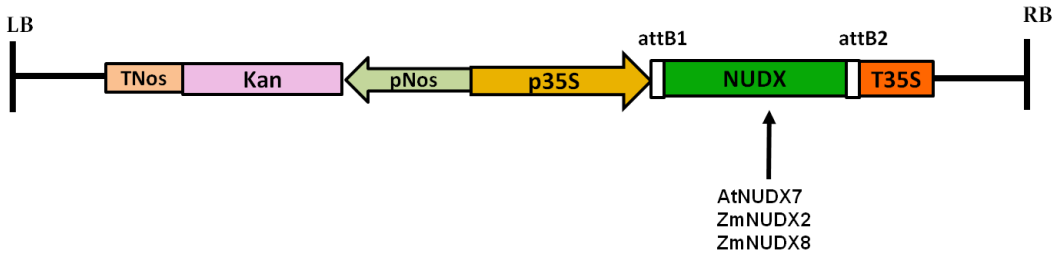


Fig. 1: General scheme of the T-DNA construct for overexpression of NUDX genes using the p35S promoter in the Arabidopsis Col-0 background.

T1 seeds, obtained from the floral dipped T0 plants, were screened for the presence of the T-DNA through high density plating on selective medium containing kanamycin, resistant seedlings were transferred to soil for the generation of T2 seeds. Subsequent segregation analysis through selection on MS medium with kanamycin was performed to generate T3 homozygous lines with 1 T-DNA locus.

6.2.1.2 Identification of a KO nudx7 mutant line

We screened for lines with a T-DNA insertion in the AtNUDX7 gene in the Col-0 background (www.arabidopsis.org) and three seed stocks were obtained SALK-046441, SALK-046825 and SALK-088538C with T-DNA insertion in exon1, exon 1 and the 5' untranslated region, respectively from NASC (Nottingham Seed Stock Center). Upon PCR genotyping and screening for a homozygous T-DNA insertion line on MS medium with Kanamycin, we obtained a T3 line, SALK-046441_1, with a single locus homozygous T-DNA insertion in exon 1. Expression analysis of the KO-nudx7 mutant line is described in section 6.2.2. The line was preferentially selected for use as a control line in functional analyses since a T-DNA insert in exon 1 is more likely to disrupt the gene activity than one in the untranslated region. The line was also used as a background for OE of ZmNUDX genes in Arabidopsis.

6.2.1.3 OE of ZmNUDX in the Arabidopsis nudx7 SALK mutant background

The OE constructs containing the ZmNUDX2 or the ZmNUDX8 genes and driven by the pBdEF1α or pZmUBIL promoters in the pBbm42GW7 gateway expression vector (Karimi et al., 2013), developed for maize transformation (Chapter 5), were expressed in EHA101 Agrobacterium cells and further transformed into the KO-nudx7 Arabidopsis mutant line (SALK-046441_1) using floral dip transformation. Transgenic seedlings were selected through high density plating on selective medium and subsequent segregation analysis through selection on phosphinothricin was performed to generate T3 homozygous lines with 1 T-DNA locus. Fig 2 is a general scheme of the T-DNA constructs developed for overexpression of ZmNUDX2 and ZmNUDX8 genes using pBdEF1α or pZmUBIL in the nudx7 mutant background.

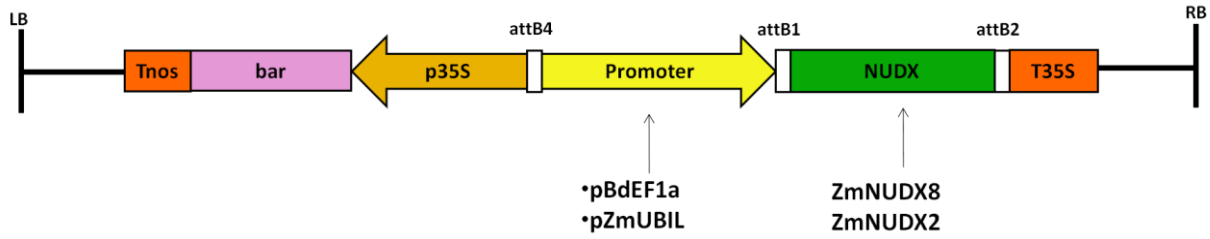


Fig. 2: General scheme of the T-DNA construct for overexpression of the maize NUDX genes using pBdEF1α or pZmUBIL in the nudx7 mutant background

6.2.1.4 pBdEF1α and pZmUBIL monocot promoters are active in Arabidopsis

The pBdEF1α and pZmUBIL promoters were used to drive the expression of the GUS gene containing a PIV2 intron in Arabidopsis. We transformed pBdEF1α-GUS and pZmUBIL-GUS constructs expressed in pBbm42GW7 and pXBb7-SI-UBIL Gateway vectors respectively into EHA101 Agrobacterium cells and further into Arabidopsis Col-0 using floral dip transformation. T1 seed were germinated on high density plates with phosphinothricin selection and resistant seedlings transferred to soil. Leaves from 32 days old T1 seedlings were tested for GUS activity as shown in Fig. 3.

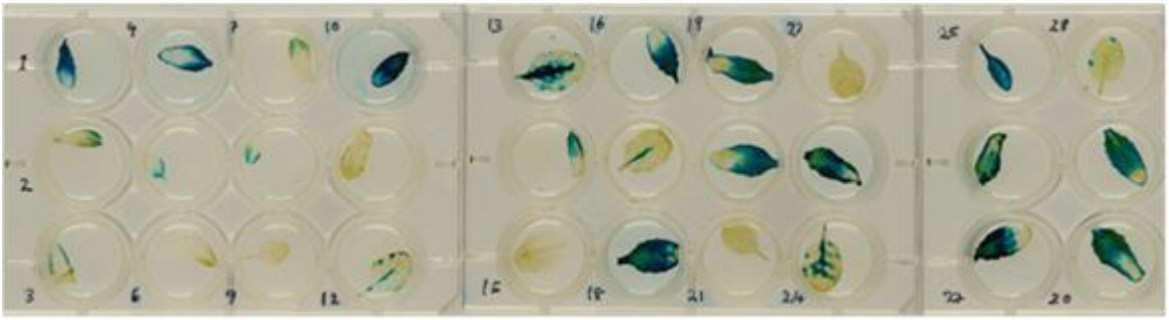


Fig. 3: GUS activity in Arabidopsis leaves of T1 seedlings transformed with pBdEF1α-GUS (1-15) and pZmUBIL-GUS (16-30).

The GUS activity in leaves of Arabidopsis seedling from both constructs indicates that pBdEF1α and pZmUBIL monocot promoters are able to drive the expression of the GUS gene in dicots. This experiment was carried out to confirm that the pBdEF1α and pZmUBIL promoters can be used to drive the expression of the ZmNUDX2 and ZmNUDX8 maize genes in Arabidopsis.

6.2.2 Expression analysis of NUDX overexpression and Knock-out lines

2 week-old seedlings of T3 lines with a single locus homozygous T-DNA insertion, growing on MS medium were used in the QPCR expression analysis. 4 pools of 5 seedlings per line for the p35S-AtNUDX7 line, nudx7 mutant line (SALK 046441_1) and Wt Col-0 control were made. For the p35S-ZmNUDX2 and ZmNUDX8 lines and their Wt Col-0 control, 3 pools of 5 seedlings were made. RNA was isolated from each pool using the RNeasy Plant Mini Kit (Qiagen) and cDNA prepared using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) or SensiFAST cDNA Synthesis Kit

(Bioline). QPCR experiments were performed in a LightCycler480 Real -Time SYBR Green PCR System (Roche). Fig 4a, 5a and 6a show the position of gene specific primers used in the QPCR amplification. Fig. 4b, 5b and 6b show the relative expression levels of the NUDX genes in OE or KO Arabidopsis lines. Fig. 4c shows a different QPCR expression analysis using freshly prepared RNA (3 pools of 5 seedlings per line) and similar set up as described above to confirm the knock out of AtNUDX7 gene in the nudx7 mutant line (SALK 046441_1) using gene specific primer pair shown in Fig 4a.

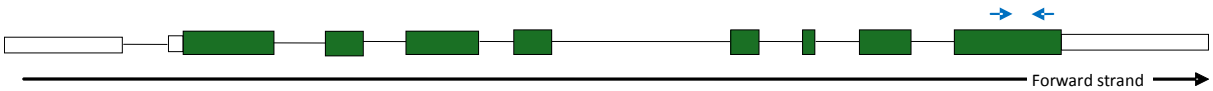


Fig. 4a: Position of the QPCRAtNUDX7b primer pair shown with arrows at the 3' end of the AtNUDX7 gene model. The green boxes represent the exons, the lines represent the introns and the unshaded boxes represent the 5' or 3' untranslated region / non-coding exon

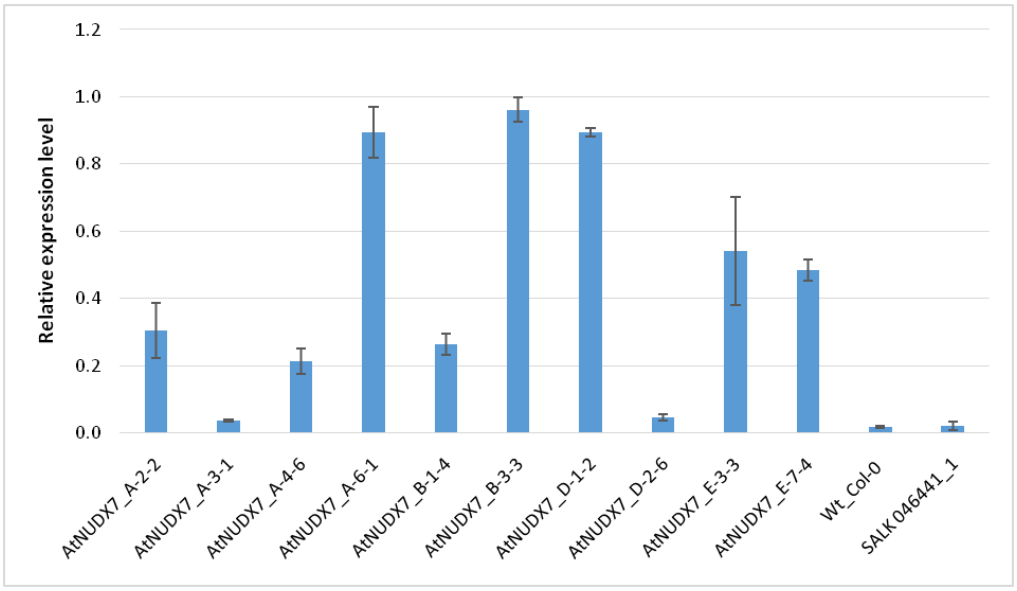


Fig. 4b: Relative expression levels of the AtNUDX7 gene in OE p35S-AtNUDX7 in Col-0 lines, Wt Col-0 and KO-nudx7 (SALK-046441_1) lines amplified using QPCRAtNUDX7b primer. Error bars indicate standard deviation (n=20)

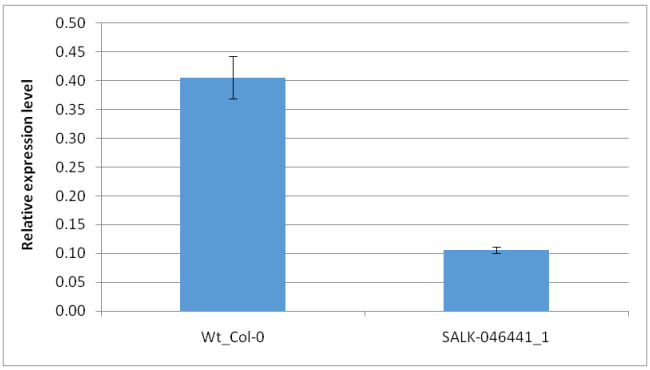


Fig. 4c: Relative expression levels of the AtNUDX7 gene in Wt Col-0 and nudx7 mutant line (SALK-046441_1) amplified using QPCRAtNUDX7b primer. Error bars indicate standard deviation (n=15)

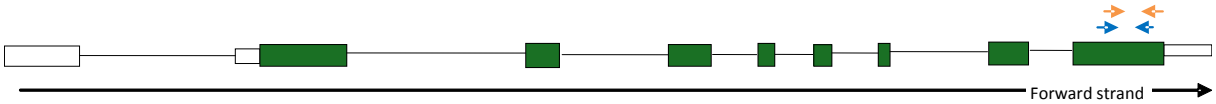


Fig. 5a: Position of the qPCRZmNUDX2b (blue) and qPCRZmNUDX2c (orange) primer pairs shown with arrows at the 3' end of the ZmNUDX2 gene model. The green boxes represent the exons, the lines represent the introns and the unshaded boxes represent the 5' or 3' untranslated region / non-coding exon

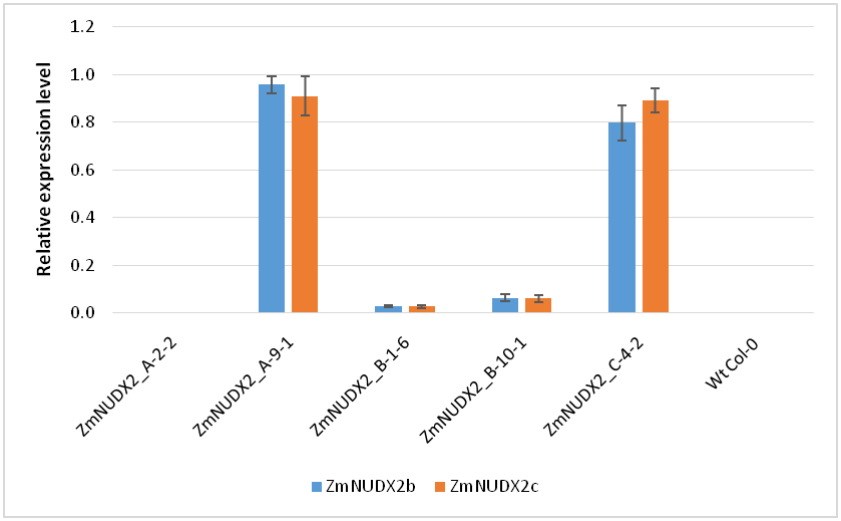


Fig. 5b: Relative expression levels of the ZmNUDX2 gene in OE p35S-ZmNUDX2 in Col-0 and Wt_Col-0 lines amplified using qPCRZmNUDX2b and qPCRZmNUDX2c primers. Error bars indicate standard deviation (n=15)

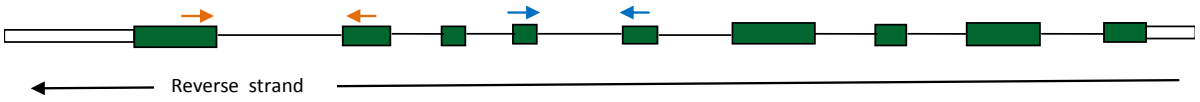


Fig. 6a: Position of the qPCRZmNUDX8e (blue) and qPCRZmNUDX8f (orange) primer pairs shown with arrows in the ZmNUDX8 gene model. The green boxes represent the exons, the lines represent the introns and the unshaded boxes represent the 5' or 3' untranslated region

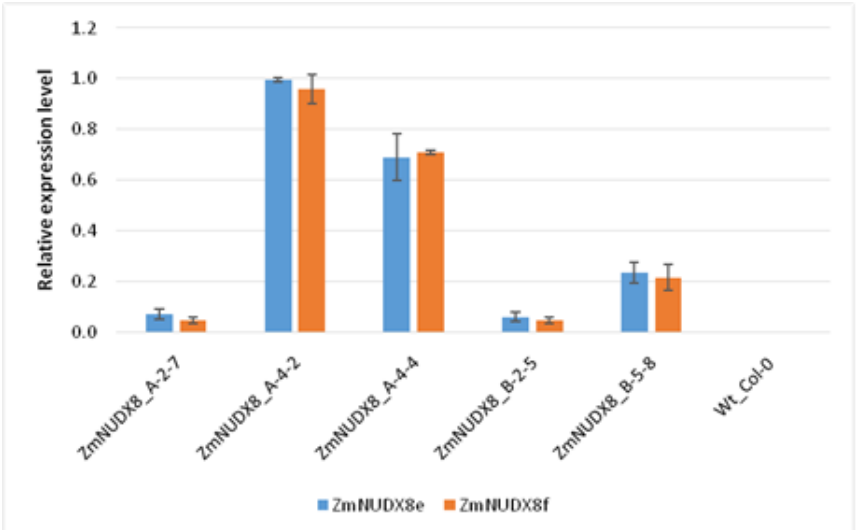


Fig. 6b: Relative expression levels of the ZmNUDX8 gene in OE p35S-ZmNUDX8 in Col-0 and Wt_Col-0 lines amplified using QPCRZmNUDX8e and QPCRZmNUDX8f primers. Error bars indicate standard deviation (n=15).

Varying levels of overexpression of NUDX genes was observed in the p35S-AtNUDX7 in Col-0 and the p35S-ZmNUDX2 and ZmNUDX8 lines in Col-0. The p35S-AtNUDX7 line in Col-0, showing high, medium and low level of AtNUDX7 gene expression were used in paraquat-induced oxidative stress assay, a water deficit experiment and evaluated for seed yield and yield contributing parameters. The p35S-ZmNUDX2 and p35S-ZmNUDX8 lines in Col-0 were only used in the water deficit experiment in this work and will be considered for oxidative stress experiments in the future. T3 lines carrying the pBdEF1α-ZmNUDX2, pBdEF1α-ZmNUDX8, pZmUBIL-ZmNUDX2 or pZmUBIL-ZmNUDX8 in the KO-nudx7 Arabidopsis mutant background were recently generated and are currently being screened for homozygous lines carrying 1 T-DNA insertion. These lines will be suitable for studying the ZmNUDX gene activity in Arabidopsis as they should complement the knocked down AtNUDX7 gene activity.

6.2.3 Evaluation of rosette area of OE and KO AtNUDX7 lines under paraquat-induced oxidative stress

T3 lines carrying the p35S-AtNUDX7 construct in Col-0 background and the KO nudx7 SALK-046441_1 mutant line were analysed for their response to paraquat (PQ) induced oxidative stress. The T3 seeds were sterilised and sown on 0.5 MS medium containing 25nM PQ. 8 seeds per genotype and 4 genotypes per plate (3 mutant lines and 1 wildtype) were sown and a duplicate of each plate made. Thus a total of 16 plants per genotype were tested. A control experiment was made in the identical set up but paraquat was not added to the medium. The plates were placed at 4°C in the dark for two days for vernalisation and then in the Arabidopsis tissue culture room at 21°C with 16 hours of light/ 8 hours of dark and 60µmol m^{-2} s^{-1} . The first day in the tissue culture room is considered as the first day after stratification (DAS). Pictures of the plates are taken daily from 7 DAS to 17 DAS using the PRAC (Plant Rosette Area Calculator) set up in Frank Van Breusegem's lab, PSB-VIB-UGent. The rosette area of each plant is determined daily using Image J software (https://imagej.nih.gov/ij) and a rosette area calculator macro designed by Davy Opdenacker, PSB-VIB-UGent. The average rosette area per day for each genotype and per PQ concentration is shown in the line graphs in Fig. 7a, 7b, 7c and 7d. Fig. 7e shows pictures of the Arabidopsis seedlings grown in MS medium with 0nM or 25nM PQ treatment at the last time point, 17 DAS. The overexpression p35S-AtNUDX7 lines used in the assay are described in Table 1.

Table 1: T3 OE p35S-AtNUDX7 lines in Col-0 used in the paraquat-induced oxidative stress assay and seed yield parameter analysis

Line	Expression level
p35S- AtNUDX7_B-3-3	High OE
p35S-AtNUDX7_D-1-2	High OE
p35S-AtNUDX7 _E-7-4	Moderate OE

p35S-AtNUDX7_E-3-3	Moderate OE
p35S-AtNUDX7_A-2-2	Weak OE
Salk 046441_1 (KO nudx7)	Low
Col-0 (Wt)	Control

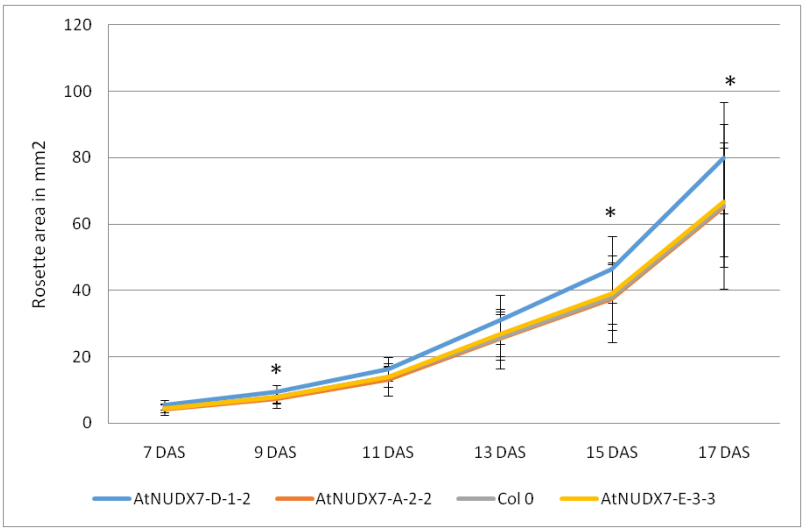


Fig. 7a: Rosette area of three p35S-AtNUDX7 lines in Col-0 and Wt Col-0 at 0nM PQ treatment from 7-17 DAS. Error bars represent +/- StDev (n=16), significant differences compared to Wt Col-0 (Student's t-test) are indicated in sign * = D-1-2 significance indicated with single sign, $p < 0.05$

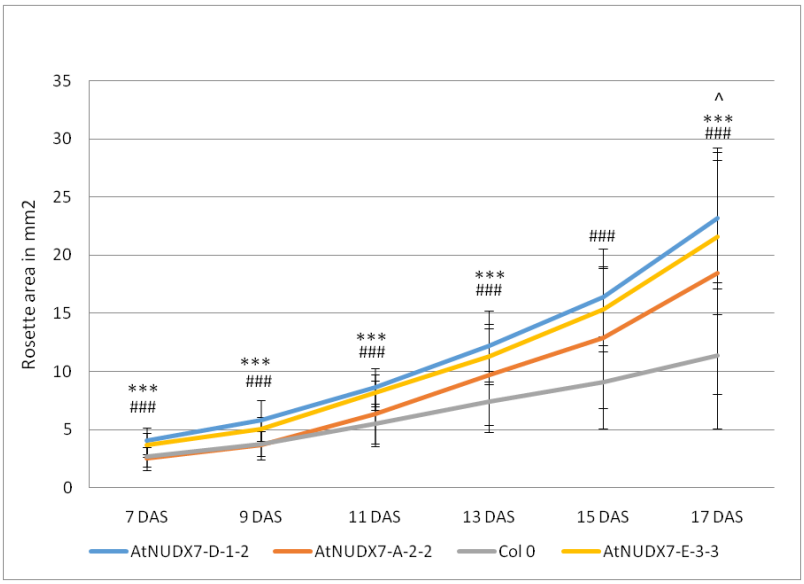


Fig. 7b: Rosette area of three p35S-AtNUDX7 lines in Col-0 and Wt Col-0 at 25nM PQ treatment from 7-17 DAS. Error bars represent +/- StDev (n=16), significant differences compared to Wt Col-0 (Student's t-test) are indicated in signs * = D-1-2, ^ = A-2-2 and # = E-3-3 significance indicated with single sign ($p<0.05$) double ($p<0.01$) and triple ($p<0.001$).

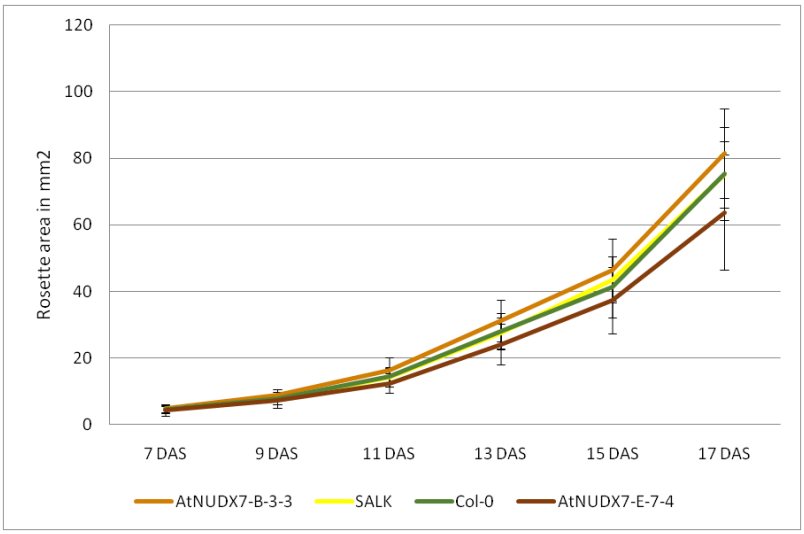


Fig. 7c: Rosette area of two p35S-AtNUDX7 lines in Col-0, KO nudx7 Salk-046441_1 and Wt Col-0 at 0nM PQ treatment from 7-17 DAS. Error bars represent +/- StDev (n=16). No significant differences from Col-0 obtained for all genotypes at all time points.

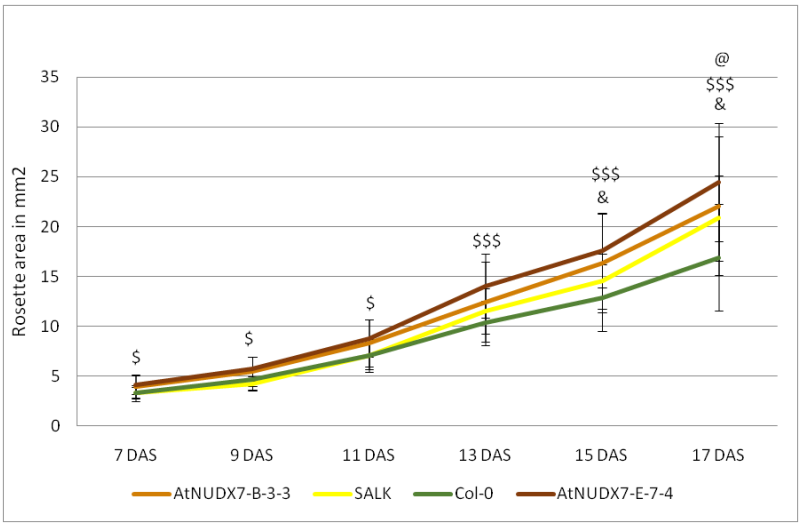
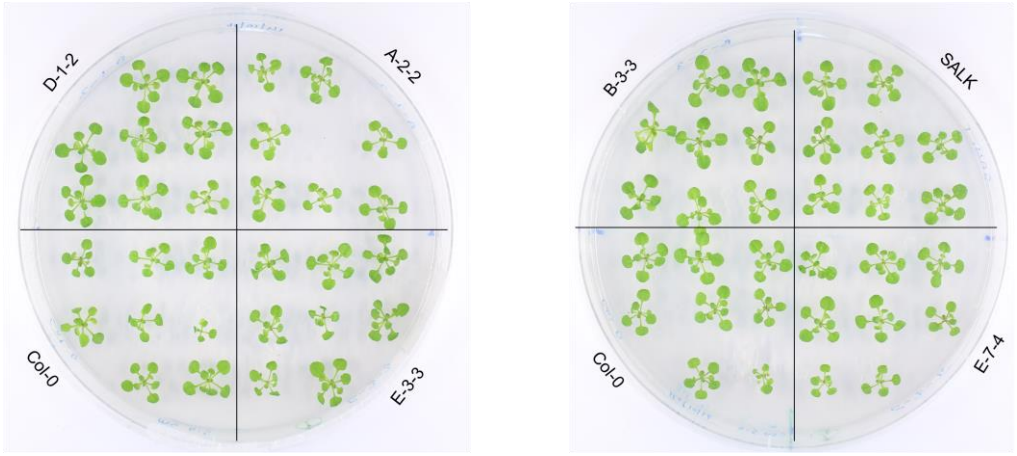


Fig. 7d: Rosette area of two p35S-AtNUDX7 lines in Col-0, KO nudx7 Salk-046441_1 and Wt Col-0 at 25nM PQ treatment from 7-17 DAS. Error bars represent +/- StDev (n=16), significant differences compared to Wt Col-0 (Student's t-test) are indicated in signs & = B-3-3, @ = Salk-046441_1, $ E-7-4 significance indicated with single sign ($p<0.05$) double ($p<0.01$) and triple ($p<0.001$).

0nM Paraquat



25nM Paraquat

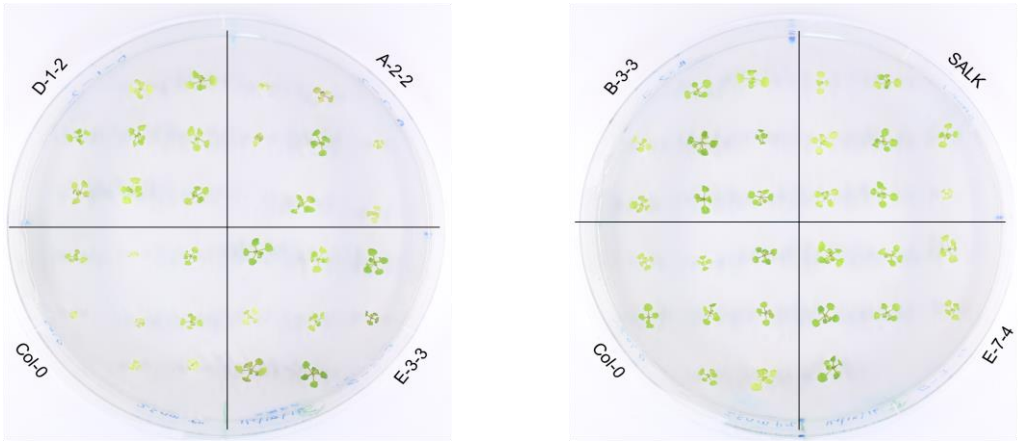


Fig. 7e: Arabidopsis seedlings of OE p35S-AtNUDX7 lines in Col-0, KO nudx7 (SALK-046441_1) and Wt Col-0 lines grown in MS medium containing 0nM or 25nM PQ treatment at 17 DAS

The moderate OE lines p35S-AtNUDX7_E-7-4 and p35S-AtNUDX7_E-3-3 had significantly higher rosette area than Wt Col-0 at 25nm paraquat treatment at all time points. Although the high OE line p35S-AtNUDX7_D-1-2 showed a slightly higher rosette area than Col-0 line without paraquat induction, upon treatment with 25nM paraquat the significant difference in rosette area is much higher than Col-0 line. The significantly higher rosette area of the three OE lines than Wt Col-0 is an indication of tolerance to paraquat-induced oxidative stress upon overexpression of the AtNUDX7 gene. The rosette growth of the KO nudx7 Salk 046441_1 was notably similar to Col-0 Wt line with and without paraquat treatment and only showed a slightly higher growth difference at 17 DAS. This possibly indicates that the KO of the AtNUDX7 gene may not have been sufficient to reduce its enzymatic activity because other endogenous AtNUDX genes may have complemented its function.

Noteworthy: The data shown in the paraquat-induced oxidative stress assay was obtained from a single experiement. In addition, the statistical analysis on this assay using Student's t-test assumed

independence of the time points. However, there is a correlation between observations done on the same plants at the different timepoints (longitudinal data) thus the data are no longer independent and cannot be sufficiently analysed with a Student's t-test or an analysis of variance. The data is clustered since the genotypes were grown on 2 separate plates. There is also a need to consider correlations between observations on plants growing on the same plate. This is typically analysed with a mixed model (Verbeke and Molenberghs, 2009). The fixed effect part of the model will contain the main effects of genotype, treatment, time and all possible interaction terms. A random effect for plate is added to the model. Several structures for the variance-covariance matrix of the residuals can be tested based on a saturated mean model (i.e. considering all independent variables as categorical variables and including all interaction effects): unstructured, (heterogenous) compound symmetry, (heterogenous) autoregressive, and (heterogenous) banded toeplitz. The best structure can be chosen based on AIC values. The Kenward-Roger approximation for computing the denominator degrees of freedom for the tests of fixed effects can be applied. Finally, user-defined contrasts can be estimated with Wald tests. Appropiate adjustment of p-values can be done with the MaxT method as implemented in SAS and residual diagnostics carefully examined (Westfall et al., 2011). All longitudinal analyses can be performed with the mixed and plm procedure of SAS (Version 9.4 of the SAS System for windows 7 64bit Copyright © 2002-2012 SAS Institute Inc. Cary, NC, USA (www.sas.com). (Future experimental set-up obtained on personal communication with Véronique Storme, statistician, PSB-VIB-UGent).

6.2.4 Evaluation of seed yield and yield contributing parameters in OE and KO AtNUDX7 lines

The seed yield parameters: total seed weight, seed number, seed size and mass per seed and other yield-associated parameters (flowering time, rosette leaves number and inflorescence height) were analysed according to Van Daele *et al*., 2012. T3 OE p35S-AtNUDX7 lines in Col-0, KO nudx7 Salk 046441_1 line and a Wt-Col 0 described in Table 1 were used in the experiment (except p35S: AtNUDX7_E-3-3 in Col-0). Plants used for total seed weight and seed size determination were grown in the green-house for a period of 3.5 months. The seeds were harvested from each plant, cleaned and the total seed weight per plant determined by weighing all the seeds harvested from each plant (25 plants per genotype) and determing the mean seed weight per plant. For seed size determination, seed area was measured by analysis of images of 200-400 seeds per plant (10 plants per genotype) using Image J software and a macro developed by Hannes Van Haeren, PSB-VIB-UGent. The mass of the 200-400 seeds per plant (10 plants per genotype) was obtained by weighing the seeds on a scale. The mass per seed parameter was then calculated by dividing this mass to the respective number of seeds that was counted through image analysis on Image J software.

Plants used for determination of inflorescent height and number of seeds per 10 silliques and were grown in the gree-house in separate pots harvested at approximately 2 months old when the plants reached maturity and no more increase in length was observed. Inflorescent height of 16 plants per genotype was determined by recording the length of a stretched primary inflorescent. The number of seeds per 10 silliques was determined by counting seeds from 10 yellow or brown unopened siliques from the middle of the inflorescence of each plant, 16 plants per genotype. The seed number parameter is described as average number of seeds per 10 siliques (Fig. 8). Similar to seed size, seed number counting was done through image analysis on Image J software (https://imagej.nih.gov/ij). Plants used in the determination of flowering time and rosette leaves number were grown in Arabidopsis growth room conditions. Flowering time was calculated as the difference between the first day of appearance of the flower bud and the day after sowing (DAS) for each of the 25 plants per genotype. Rosette leaf number (excluding cotyledons) was counted at the first day of flower bud appearance for each of the 25 plants per genotype. The graphs in Fig. 8 give an overview of the seed yield and yield related parameters determined of the OE p35S-AtNUDX7 lines in Col-0, KO nudx7 and Wt Col-0 control line.

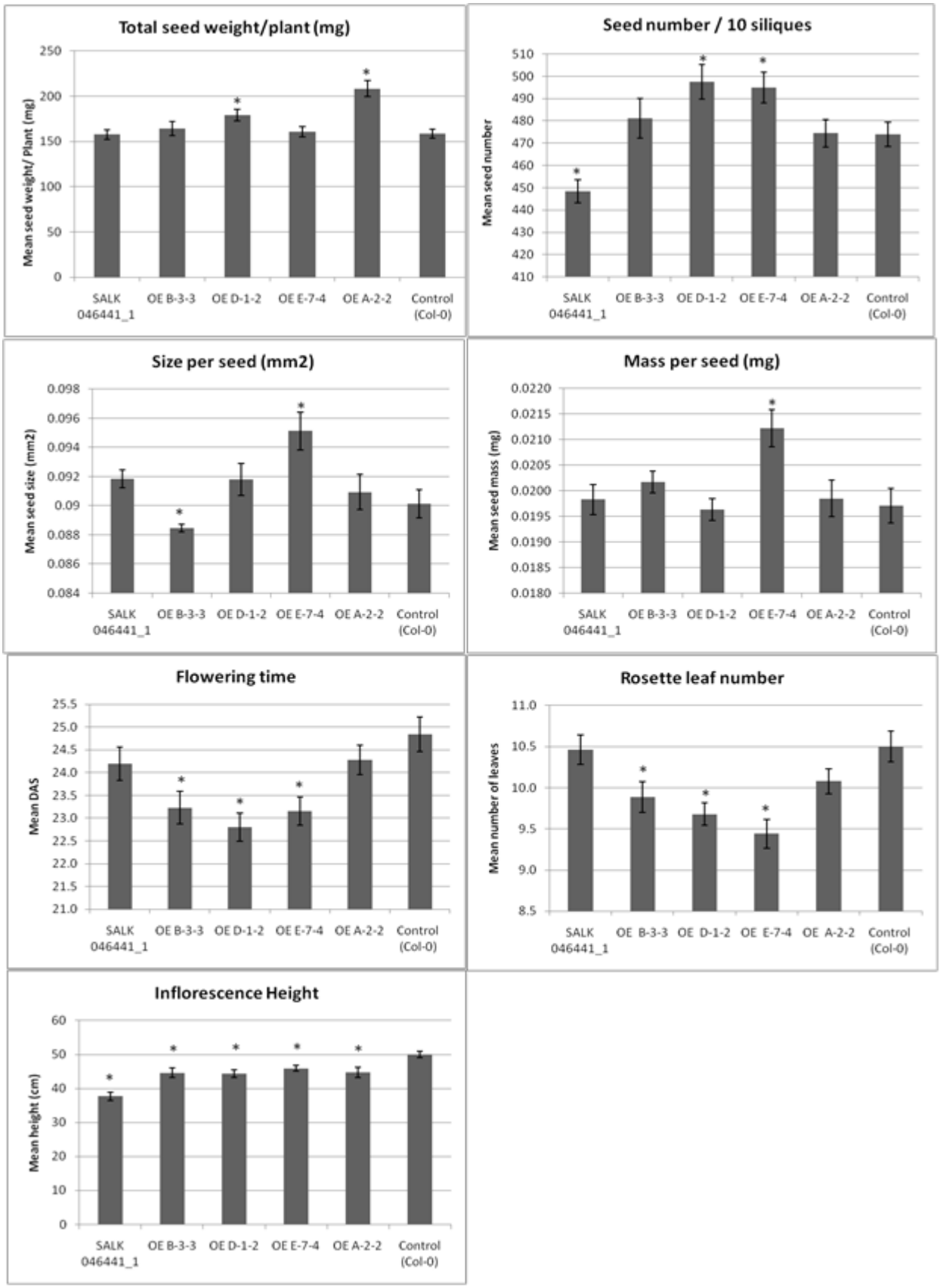


Fig. 8: Seed yield and yield contributing parameters of p35S-AtNUDX7 lines in Col-0 OE, KO nudx7 SALK-046441_1 and Wt Col-0 control lines. Error bars indicate standard error of the mean. Significant difference compared to Wt Col-0 (Student's t-test) is indicated in asterisks ($p<0.05$).

A heat map of the seed yield and yield contributing parameters was constructed to better visualise the differences in the performance of the overexpression and KO nudx7 lines in comparison to Wt Col-0 (Fig. 9).

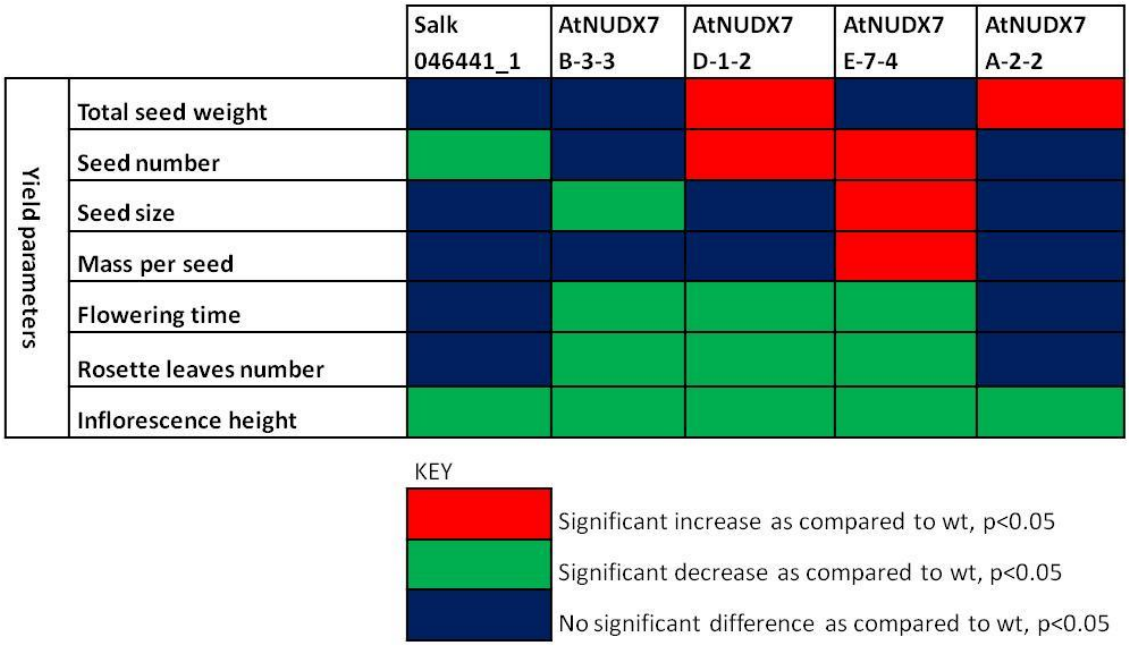


Fig. 9: Heat map of seed yield and yield-associated parameters in five Arabidopsis NUDX genotypes OE p35S-AtNUDX7 lines in Col-0 and KO nudx7 Salk 046441_1 line. Significant differences determined using Student's t-test.

High OE line p35S-AtNUDX7_D-1-2 and low OE line p35S-AtNUDX7_A-2-2 had a significantly higher total seed weight per plant in comparison to Wt Col-0. The increase in total seed weight of line p35S-AtNUDX7_D-1-2 can be linked to its significant increase in seed number. Line p35S-AtNUDX7_E-7-4 had remarkable consistence in the significant increase in three seed yield parameters: seed number, seed size and mass per seed in comparison to Wt Col-0 and a reduction in flowering time, rosette leaf number and inflorescent height. Early flowering time is often correlated with a reduced leaf number, which was confirmed in this experiment where high and moderate OE lines p35S-AtNUDX7_B-3-3, p35S-AtNUDX7_D-1-2 and p35S-AtNUDX7_E-7-4 flowered earlier and had fewer rosette leaves than Wt Col-0. All the genotypes analysed showed a significantly smaller inflorescent height compared to the Wt Col-0.

6.2.5 Evaluation of OE AtNUDX7, ZmNUDX in Col-0, KO nudx7 and RNAi hpPARP2 lines under mild drought stress in an automated platform (WIWAM)

To study the response of the transgenic OE or KO lines to water deficit conditions, a mild drought stress experiment was set up on an automated weighing, imaging and watering (WIWAM) high-

throughput phenotyping platform at PSB-VIB-UGent according to established protocols (Skirycz et al., 2011; Clauw et al., 2015). The mild drought stress experiment was selected because it has been proposed to be a better test for superior growth performance thus, biomass yield gain, during water deficit conditions as opposed to severe drought stress treatment that activates plant survival mechanisms including redistribution and saving of resources leading to growth reduction to ensure reproduction even when the stress becomes extreme at the expense of yield (Skirycz et al., 2011). Two conditions were selected for the experiment, well-watered control for which the soil water content was maintained at a constant value of 2.19 g H_2O/g dry soil during the entire experiment and mild drought stress for which the plants were grown for 9 days in well-watered conditions, then the daily target soil water content was lowered and maintained at 1.19 g H_2O/g dry soil until the end of the experiment (21 DAS). 16 plants per genotype and 12 genotypes in total were used in the experiment as described in Table 2. Three Wt Col-0 lines were used corresponding with the age of three line sets: OE AtNUDX7 + Salk lines, OE ZmNUDX lines and hpPARP2 RNAi line. The final shoot area and stress sensitivity slopes were determined from the final shoot area are shown in Fig. 10-12. This experiment was carried out in collaboration with Stijn Dhondt of the Yield group, PSB-VIB-UGent.

Table 2: Lines used in the mild drought stress experiment

Genotype	**Expression level**
AtNUDX7-OE B-3-3	High
AtNUDX7-OE D-1-2	High
AtNUDX7-OE E-7-4	Medium
AtNUDX7-OE E-3-3	Medium
AtNUDX7-OE A-2-2	Low
Salk 046441_1	Knock-out
Col-0	Wt (AtNUDX7 lines control)
ZmNUDX2-OE A-9-1-2	High
ZmNUDX8-OE A-4-2-2	High
Col-0	Wt (ZmNUDX lines control)
hpPARP2 427-26-43	Knock-down
Col-O	Wt (hpPARP2 line control)

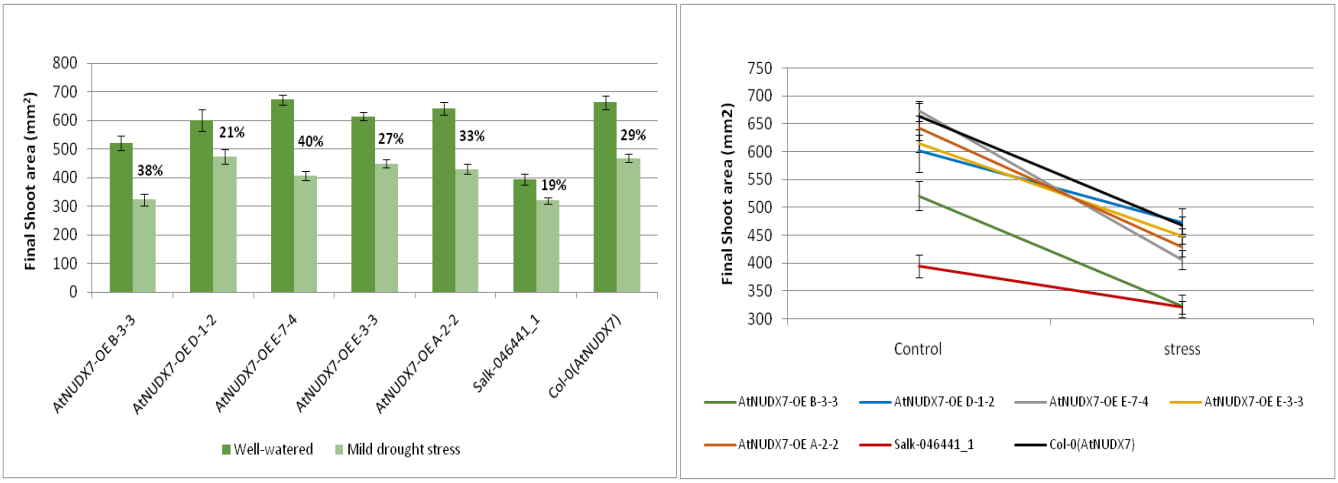


Fig. 10: Final shoot area and stress sensitivity slopes of p35S-AtNUDX7 lines in Col-0, K0 nudx7 and Wt Col-0 under well-watered and mild drought stress treatments. Percentages represent reductions under mild drought relative to the control. Error bars indicate standard error of the mean (n=16).

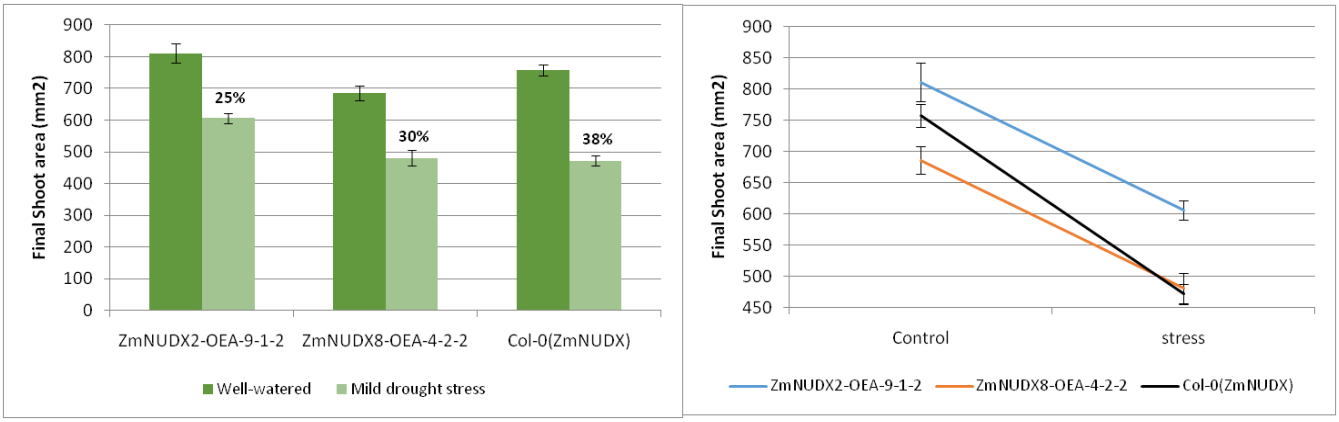


Fig. 11: Final shoot area and stress sensitivity slopes of p35S-ZmNUDX2 & p35S-ZmNUDX8 lines in Col-0 and Wt Col-0 under well-watered and mild drought stress treatments. Percentages represent reductions under mild drought relative to the control. Error bars indicate standard error of the mean (n=16).

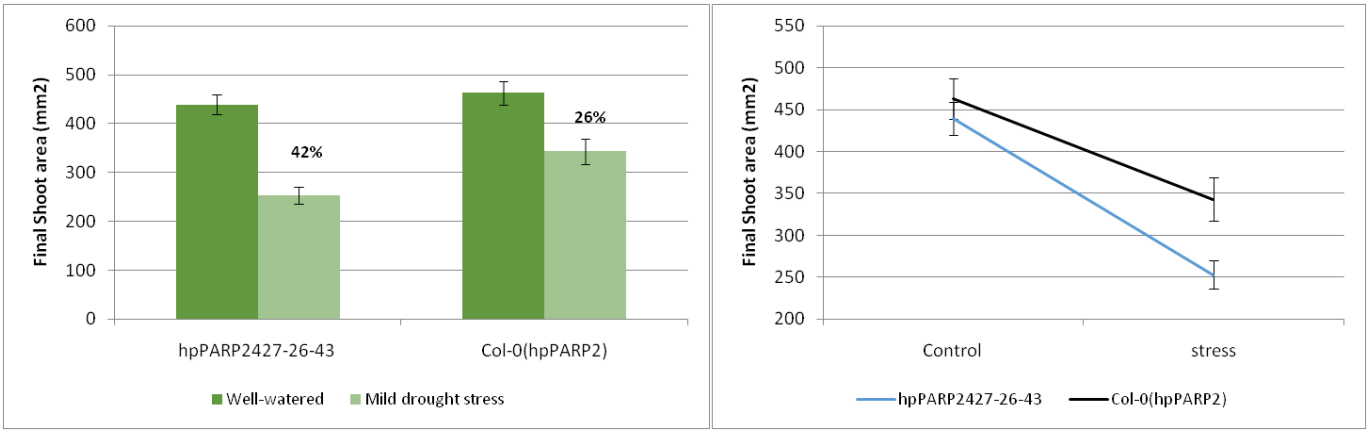


Fig. 12: Final shoot area and stress sensitivity of RNAi hpPARP2 line and Wt Col-0 under well-watered and mild drought stress treatments. Percentages represent reductions under mild drought relative to the control. Error bars indicate standard error of the mean (n=16).

Shoot growth has been described as a sensitive, relevant, and easily measured phenotype for assessing stress tolerance over a wide range of stress levels (Claeys et al., 2014). In this experiment the shoot area (rosette area) was used as an indicator of mild drought stress response. Stress sensitivity slopes were made using the final shoot areas measurement of each line under well-watered (control) and mild drought (stress) treatments to visualise the performance differences of the lines. Lines with a steeper slope are more sensitive to stress while lines with a gentler slope are more stress tolerant. The final shoot area of plants under mild drought stress was observed to be generally lower than under well-watered condition with relatively 20-40% growth reduction. Among the Arabidopsis NUDX7 modulated lines, high OE AtNUDX7_D-1-2 in Col-0, moderate OE line AtNUDX7_E-3-3 and KO nudx7 Salk-046441_1 line had 8%, 2% and 10% increased shoot area respectively in comparison to the wildtype Col-0 under mild drought stress treatment though the differences are not statistically significant but indicating a trend towards tolerance to mild drought stress (Fig. 10). However the Salk-046441_1 mutant line had a 10% significantly larger shoot area in comparison to the wildtype Col-0 under mild drought stress treatment ($P = 0.0244$, two-way analysis of variance with custom hypothesis Wald tests (corrected for multiple testing using Sidak step-down)) indicating tolerance to mild drought stress. However Salk-046441_1 mutant had generally smaller plants and consequently lower shoot areas which may cause a false stress tolerance impression. The two maize NUDX OE lines, ZmNUDX2_A-9-1-2 and ZmNUDX8_A-4-2-2 lines in Col-0 had 13% and 8% larger shoot area respectively relative to their wildtype Col-0 under mild-drought stress though the differences are not statistically significant but indicating a trend towards tolerance to mild drought stress (Fig. 11). The AtPARP2 RNAi line had 16% reduced shoot area relative to wildtype Col-0 under mild drought stress though the difference is not statistically significant but indicating a trend towards sensitivity to mild drought stress (Fig. 12). Note that the data presented here is from a single experiment.

6.3 Discussion

In this study we overexpressed the AtNUDX7, ZmNUDX2 and ZmNUDX8 genes in Arabidopsis thaliana Col-0 ecotype using p35S promoter through Gateway cloning system and Agrobacterium floral dip transformation method and obtained varying levels of the NUDX gene expressions which were selected for functional analysis. We identified a KO nudx7 mutant line with a single locus homozygous T-DNA insertion in Exon 1, SALK-046441_1, which was used as a control in the functional assays. In addition we developed new constructs for OE of ZmNUDX2 and ZmNUDX8 using the maize ubiquitin promoter, pZmUBIL, (Christensen et al., 1992) and a strong constitutive Brachypodium distachyon promoter, pBdEF1α, (Coussens et al., 2012) in KO-nudx7 Arabidopsis mutant line (SALK-046441_1). These lines will be suitable for studying the ZmNUDX gene activity in

Arabidopsis as they complement the knocked down AtNUDX7 gene activity. We confirmed through GUS gene expression that the heterologous monocot promoters, pZmUBIL and pBdEF1α, are active in Arabidopsis and propose that they can be used in other dicots as well. Koyama et al. (2005) showed that a promoter of Arabidopsis phosphate transporter gene drives root-specific expression of a transgene in rice, indicating that dicots promoters can also function in monocots.

We observed that moderate OE lines p35S-AtNUDX7_E-7-4 and p35S-AtNUDX7_E-3-3 and high OE line p35S-AtNUDX7_D-1-2 in Col-0 showed a significantly higher rosette area than Wt Col-0 upon treatment with 25nm paraquat indicating that overexpression of the AtNUDX7 gene confers significant tolerance to paraquat-induced oxidative stress. These results support previous studies in which modulation of AtNUDX7 or AtNUDX2 genes conferred enhanced tolerance to oxidative stress in Arabidopsis. Indeed, Arabidopsis plants having high expression levels of AtNUDX7 showed enhanced tolerance to 3µM paraquat-induced oxidative stress as assessed by the survival rates and chlorophyll content (Ishikawa et al., 2009). Similarly, overexpression of AtNUDX2 gene in Arabidopsis resulted in plants with suppressed root growth inhibition upon treatment with 0.3µM and 3µM paraquat in comparison to control plants that were severely inhibited. The OE AtNUDX2 plants also showed a higher survival rate and higher chlorophyll content compared to the control lines (Ogawa et al., 2009). To the best of our knowledge, our study is the first reporting of paraquat-induced stress tolerance in OE AtNUDX7 lines as assessed by the rosette area parameter. Notably the rosette growth of the KO nudx7 Salk-046441_1 was similar to Col-0 Wt line with and without paraquat treatment possibly indicating that the KO of the AtNUDX7 gene may not have been sufficient to reduce its enzymatic activity because other endogenous AtNUDX genes might have complemented its function.

In spite of its known complexity, modulation of single genes has been shown to play a role in crop yield increase for instance overexpression of DWARF4, a gene in the brassinosteroid biosynthetic pathway, resulted in increased vegetative growth and seed yield in Arabidopsis (Choe et al., 2001). In addition, modulating the expression of the maize PLASTOCHRON1 (ZmPLA1) gene, encoding a cytochrome P450, resulted in increased organ growth, seedling vigour, stover biomass and seed yield by extending cell division duration (Sun et al., 2017). We thus attempted to determine the yield performance of plants with a modulation of the AtNUDX7 gene expression, a gene involved in the poly(ADP-ribosyl)ation energy metabolic pathway. Interesting seed yield and yield-associated phenotypes were observed. A high OE line p35S-AtNUDX7_D-1-2 and low OE line p35S-AtNUDX7_A-2-2 had a significantly higher total seed weight per plant than the wildtype control. The increase in total seed weight of line p35S-AtNUDX7_D-1-2 could directly be attributed to the increased seed

number. A moderate AtNUDX7 OE line, p35S-AtNUDX7_E-7-4 in Col-0 had remarkable consistence in the significant increase in three seed yield parameters analyzed: seed number, seed size and mass per seed and a reduction in flowering time, rosette leaf number and inflorescent height in comparison to Wt Col-0. Based on these results, we hypothesize that increased total seed weight for the p35S-AtNUDX7_E-7-4 line was most likely not due to branching number or siliques number increase, though these parameters were not analyzed in this study. Reduced leaf number in the 4 overexpression genotypes coincided with earlier flowering confirming the correlation of early flowering time and reduced leaf number (Alonso-Blanco et al., 1998). The early flowering going together with increased yield parameters is a remarkable combination of beneficial traits as observed in line p35S-AtNUDX7_D-1-2, p35S-AtNUDX7_E-7-4 that had either increased total seed weight, seed number, seed size or mass per seed and flowered early. This is the first report showing the involvement of the AtNUDX7 in seed yield and yield-contributing parameters in Arabidopsis.

We also report that overexpression of Arabidopsis and maize NUDX genes in Arabidopsis Col-0 using the p35S promoter resulted in three lines showing a tendency to mild drought stress tolerance: p35S-AtNUDX7_D-1-2, p35S-ZmNUDX2_A-9-1-2 and p35S- ZmNUDX8_A-4-2-2 with 8%, 13% and 8% higher shoot area respectively relative to Wt Col-0 upon mild drought stress. These findings indicate that the ADP-ribose specific NUDX genes may play a role in Arabidopsis plant response to mild drought stress. ADP-ribose specific Nudix hydrolases are involved in nucleotide recycling in the PAR pathway by hydrolysing free ADP-Ribose molecules into ribose-5-phosphate and AMP, a ready precursor for ATP synthesis (Rossi et al., 2002; Ogawa et al., 2005; Ishikawa et al., 2009). Upon drought stress, there is an increased production of ROS (Cruz de Carvalho, 2008) which induce the PARP protein activity (Vira´g and Szabo´, 2002) and thus the PAR pathway. Activation of the PAR pathway will resultantly activate the nucleotide recycling action of ADP-ribose specific Nudix hydrolases and re-establishing the energy levels by supplying an ATP source. We thus propose that overexpression of AtNUDX7, ZmNUDX2 and ZmNUDX8 genes in Arabidopsis plants may enhance mild drought stress tolerance through enhancing the recycling step of ADP-ribose in the PAR energy homeostasis pathway. The KO nudx7 Salk 046441_1 had a significantly lower shoot area both under mild drought stress and under well-watered condition than Wt Col-0 indicating that the AtNUDX7 may also play a direct or indirect role in Arabidopsis growth or growth regulation. Smaller size of mutant nudx7 plants grown under normal growth condition has previously been reported (Jambunathan and Mahalingam, 2006). This study is the first reporting of mild drought stress testing in Arabidopsis lines with an alteration of NUDX7 gene expression or expressing maize NUDX genes.

Several studies have shown a correlation of drought stress and oxidative stresses due to increased ROS accumulation (Moran et al., 1994; Sgherri et al., 1995; Loggini et al., 1999 and Boo et al., 1999). In our study, we observed that p35S-AtNUDX7_D-1-2, p35S-AtNUDX7_E-3-3 lines in Col-0 showed tolerance to paraquat induced oxidative stress and a tendency to mild drought stress tolerance. Additionally p35S-AtNUDX7_D-1-2 and p35S-AtNUDX7_E-7-4 lines in Col-0 had both oxidative stress tolerance and improved seed yield phenotypes. The p35S-AtNUDX7_D-1-2 line in Col-0 remarkably showed tolerance to oxidative stress, a tendency to mild drought stress tolerance and increased total seed number and seed weight. These lines showing multiple phenotypic traits are interesting for repeat experiments in further investigation.

6.4 Materials and methods

6.4.1 Plant growth conditions

Arabidopsis genotypes used in this study were grown either in tissue culture room, growth room or green-house conditions. Tissue culture room conditions are 21°C temperature, 16 hours of light/ 8 hours darkness and 80µmol m^{-2} s^{-1} light intensity. Arabidopsis growth room conditions include: 22^0C temperature, 55% relative humidity, 100µmolm^{-2} s^{-1} light intensity and 16 hours of light. Arabidopsis green-house conditions: 21^0C temperature, 55% -60% relative humidity and 16 hours light. In vitro plants in tissue culture room were grown in full strength or half strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) sucrose while plants in growth room and green-house condition were grown on jiffy soil (www.jiffygroup.com/jiffy).

6.4.2 Transgenic lines generation and selection and expression analysis

AtNUDX7 (AT4G12720), ZmNUDX2 (GRMZM2G101693) and ZmNUDX8 (GRMZM2G175816) cDNA/ gDNA were transformed into either Arabidopsis thaliana Col-0 ecotype or SALK-046441_1 using the Gateway cloning system (Invitrogen) and Agrobacterium floral dip transformation system as described for each construct in the results section. Selection of transgenic plants and T3 homozygous lines was carried out on kanamycin or phosphinothricin selective medium. Histochemical GUS activity analysis on pBdEF1α-GUS and pZmUBIL-GUS T2 lines was carried out as described by Jefferson et al (1987) and De Block and Van Lijsebettens (1998). For QPCR expression analysis, RNA was isolated from two week-old Arabidopsis seedlings using the RNeasy Plant Mini Kit (Qiagen) and cDNA prepared using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) or SensiFAST cDNA Synthesis Kit (Bioline) following manufacture's instruction. QPCR experiments were performed in a LightCycler480 Real -Time SYBR Green PCR System (Roche). All reactions were

performed in three technical replicates and expression levels were normalised to reference genes *SAND* (AT2G28390), *PP2A* (AT1G13320) and *YLS8* (AT5G08290).

6.4.3 Rosette area determination in paraquat-induced oxidative stressed plants

Seeds were sterilised using 70% Ethanol and 5% bleach and sown on 0.5 MS medium supplemented with 25nM PQ. 8 seeds per genotype and 4 genotypes per plate (3 mutant lines and 1 wildtype) were sown and a duplicate of each plate made. A control experiment was made in the identical set up but paraquat was not added to the MS medium. The plates were placed at 4°C in the dark for two days for vernalisation and then in the Arabidopsis tissue culture room at 21°C with 16 hours of light/ 8 hours of dark and 60µmol m^{-2} s^{-1} light intensity. Pictures of the plates are taken daily from 7 DAS to 17 DAS using PRAC (Plant Rosette Area Calculator) in Frank Van Breusegem's lab, PSB-VIB. Rosette area was determined daily using Image J software (https://imagej.nih.gov/ij) and a rosette area calculator macro designed by Davy Opdenacker, PSB-VIB.

6.4.4 Determination of seed yield and yield related parameters

All the Arabidopsis genotypes used in determination of seed yield and yield related parameters were grown in Arabidopsis green-house conditions apart from plants used in determining flowering time and rosette leaves number that were grown in Arabidopsis growth room conditions. The seed yield parameters: total seed weight, seed number, seed size and mass per seed and other yield-associated parameters (flowering time, rosette leaves number and inflorescence height) were analysed as described in results section according to Van Daele *et al*., 2012.

6.4.5 Mild drought stress on WIWAM

The experiment was set up on an automated weighing, imaging and watering (WIWAM) high-throughput phenotyping platform at PSB-VIB according to established protocols (Skirycz et al., 2011; Clauw et al., 2015). WIWAM is placed in an Arabidopsis growth room with 21°C Temp, 55% relative humidity, 16 hours of day and 8 hours of night and 100 µmol m^{-2} s^{-1} light intensity. Seeds were stratified two days before sowing on 80-90 grams soil pots which were placed in randomised positions on WIWAM. Soil water content for the well-watered control plants was set at a constant value of 2.19 g H_2O/ g dry soil during the entire experiment. For the mild drought stress treatment, plants were grown for 9 days in well-watered conditions and then the daily target soil water content was lowered and maintained at 1.19 g H2O/g dry soil until the end of the experiment (21 DAS). The final shoot area and stress sensitivity slopes were determined from images of the rosette of each plant taken.

6.4.6 Data analysis on mild drought stress experiment

Statistical data analysis was carried out for the final shoot areas measured in the mild drought stress experiment on WIWAM automated platform with an interest of determining the different effect upon mild drought stress of each transgenic line compared to the respective control line. A two-way analysis of variance was conducted for the shoot area variable. The model included the factors genotype and treatment and the interaction term. When the interaction term was significant at the 5% significant level, Wald tests were performed to estimate the significance of the difference in effect upon water deficit of each genotype versus the control genotype. P-values were adjusted for multiple testing using Sidak step-down as implemented in SAS. The analysis was conducted with the glm procedure from SAS (Version 9.4 of the SAS System for windows 7 64bit Copyright 2002-2012 SAS Institute Inc. Cary, NC, USA, www.sas.com). Correction for multiple testing of the interaction effect was done with the multtest procedure. The statistical analysis was carried out by Véronique Storme, statistician, PSB-VIB-UGent.

6.4.7 List of primers

Table 3: Primer sequences used in the study

Primer	Sequence (5'->3')	Experiment
NUDX2F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACCATGT CAAGTTCCATAATTTCAACAGT	OE NUDX gene cloning + Att sites
NUDX2R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACCCTGAGGCCCTGTCCAG	
NUDX8F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACCATG GAGAGCGGCTTGCTTGACACCG	
NUDX8R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGGCGGCGCTGCAGTTCAC	
AtNUDX7F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACCATG GGTACTAGAGCTCAGCAG	
AtNUDX7R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGAGAGAAGCAGAGGCTTG	
qpcrAtNUDX7bF	CTTGGGATTCGCCATTGTG	NUDX gene expression analysis
qpcrAtNUDX7bR	CATGATCCGCATTGCAGTAGAT	
qpcrZmNUDX2bF	ATGCAGGCTTTTCGCCAATC	
qpcrZmNUDX2bR	CAGATCCCTCCGGTTCATGT	
qpcrZmNUDX2cF	GCAGGCTTTTCGCCAATCTC	
qpcrZmNUDX2cR	GTCCAGATCCCTCCGGTTCA	
qPCRNUDX8eF	GTGGATCTAGCCGAGTTCGT	
qPCRNUDX8eR	GGGAGCATATTCGGTTCATC	
qpcrZmNUDX8fF	CTGAGGCCTGTGTCGAGTG	
qpcrZmNUDX8fR	GATGAAGGGCTGCTTCACGA	
SAND_F	AACTCTATGCAGCATTTGATCCACT	Housekeeping genes
SAND_R	TGATTGCATATCTTTATCGCCATC	
PP2A_F	TAACGTGGCCAAAATGATGC	
PP2A_R	GTTCTCCACAACCGCTTGGT	
YLS8_F	TTACTGTTTCGGTTGTTCTCCATTT	
YLS8_R	CACTGAATCATGTTCGAAGCAAGT	
SALK_046441_ Right	TTCGTTCATCAAGATTGCCTC	Salk lines genotyping
SALK_046441_ Left	TTTTGGTTTTTGGTTTTTCCC	
SALK_088538C_Right	CTGCAGCCTCCACAAGATTAG	
SALK_088538C_Left	TTAGGTTGTTCGTTTGATCGC	
SALK_046825_Right	GAAAAACCCACTCCTCTCCTG	
SALK_046825_Left	TCCATTTTGTTCTGACTTCCG	
LBb1.3	ATTTTGCCGATTTCGGAAC	

6.5 References

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CHAPTER 7

General discussion and future perspectives

7.1 Performance of NUDX and PARP genes in maize and Arabidopsis

Poly(ADP-ribosyl)ation pathway (PAR) has been implicated in different physiological processes and described as an important regulatory mechanism modulating plant responses to DNA damage, abiotic and biotic stresses (Amor et al., 1998; Doucet-Chabeaud et al., 2001; De Block et al., 2005; Vanderauwera et al., 2007; Ogawa et al., 2009; Ishikawa et al., 2009; Adams-Phillips et al., 2010; Li et al., 2011; Feng et al., 2015; Song et al., 2015; Zhang et al., 2015). A study of the currently published morphological and stress phenotypes on different poly(ADP-ribosl)ation pathway genotypes is summarised in Table 1. In our study, the role of the Poly(ADP-ribosyl)ation pathway in plant energy homeostasis and response to abiotic stresses was explored through genetic engineering approaches. The main objective of the study was to generate maize and Arabidopsis lines with an altered energy homeostasis to improve tolerance to drought, oxidative and genotoxic stresses through manipulation of the levels of two PAR paththway proteins: PARP and NUDX. We used the RNAi hairpin silencing and CRISPR/CAS9 gene editing strategies to downregulate/ knock-down PARP expression in maize and overexpressed the AtNUDX7 gene and its two maize homologues in maize and Arabidopsis. Many transgenic lines showing different levels of PARP and NUDX expression have been generated in maize and Arabidopsis. In addition, ZmNUDX Mutator transposon insertion lines and AtNUDX7 T-DNA insertion lines were obtained from the Maize genetics cooperation stock center and the Nottingham seed stock center respectively, characterised and used in this study. Functional analysis using oxidative stress, DNA damage and drought stress assays in addition to yield phenotyping experiments have been carried out on a number of the lines generated.

Overexpression of the AtNUDX7 gene in Arabidopsis using the Cauliflower mosaic virus promoter, p35S, conferred tolerance to paraquat-induced oxidative stress, showed a trend of tolerance to mild drought stress and resulted in improved seed yield phenotypes in addition to early flowering time (Table 2). In maize, overexpression of the AtNUDX7 gene using the strong constitutive *Brachypodium distachyon* promoter pBdEF1α, showed a strong tendency to water deficit stress tolerance but contrary to its phenotype in Arabidopsis resulted in lines showing sensitivity to paraquat-induced oxidative stress treatment (Table 2). Overexpression of the ZmNUDX2 and ZmNUDX8 genes in maize using the pBdEF1α promoter did not result in tolerance to water deficit nor paraquat-induced oxidative stress. However expressing these two maize genes, ZmNUDX2 and ZmNUDX8, in

Arabidopsis using the p35S promoter showed a trend of tolerance to mild drought stress (Table 2). The results raised interest why AtNUDX7 expression in maize would induce drought stress tolerance phenotype while the overexpression of its maize homologues ZmNUDX genes did not result in the same phenotype in maize but only in Arabidopsis. One possible reason could be that the AtNUDX7 gene is induced endogenously upon drought stress perturbation and thus active in response to drought (Fig. 1) implying that all molecular components for its enzymatic activity are available. In contrast, the ZmNUDX genes are not induced endogenously upon drought stress perturbations as shown by experimental gene expression data upon mild and severe drought stress in maize (Chapter 5, Fig. 10 b-d) and also through Genevestigator metadata analysis (Fig. 1).

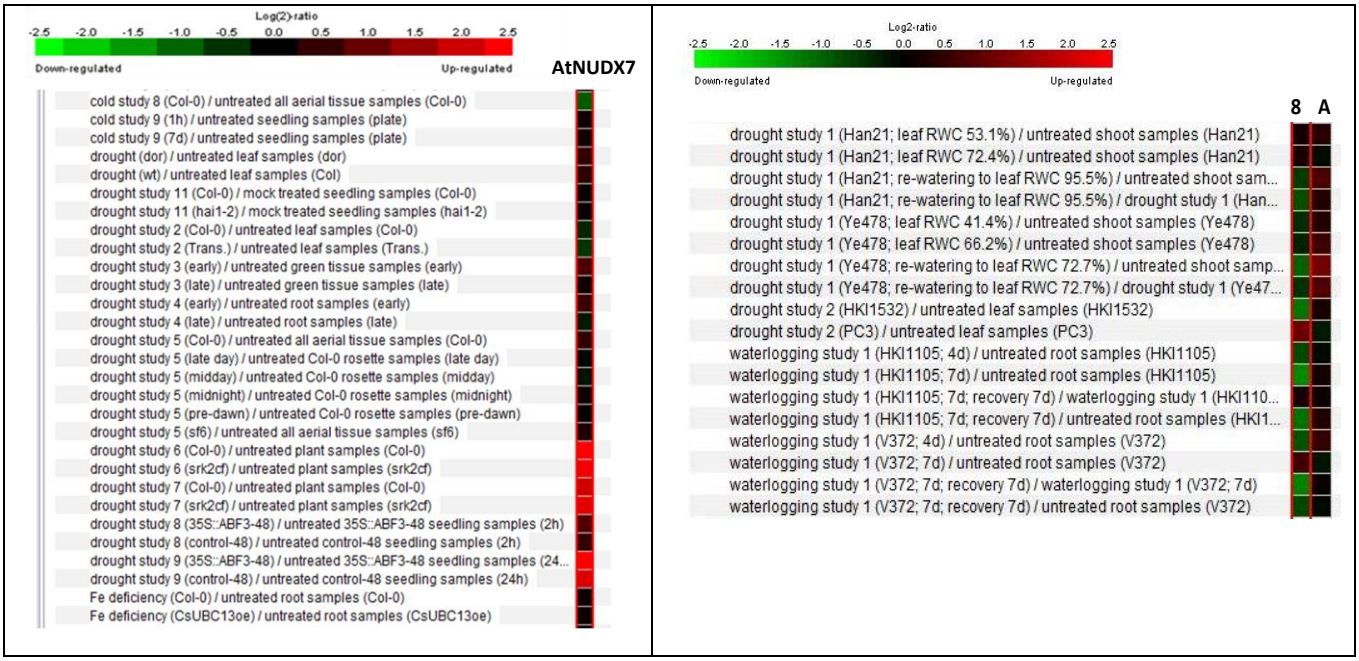

Fig. 1: Gene expression of AtNUDX7, ZmNUDX8 (8) and ACN26985 (A) under drought stress perturbations using Genevestigator metadata analysis software.

We investigated the divergence of the catalytic domain and N-terminal ends that might explain differences in the NUDX catalytic activity or substrate affinity as an alternative explanation for the difference in activity of the AtNUDX7 versus ZmNUDX transgenic lines. The AtNUDX7 and its ZmNUDXs homologues belong to the Nudix hydrolase superfamily and have a highly conserved 23 residue Nudix motif represented by $GX_5EX_7REVXEEXGU$ in which U depicts and aliphatic hydrophobic residue such as isoleucine, leucine or valine and X is any amino acid. The Nudix motif functions as the catalytic site and metal binding site (Mn^{2+} and Mg^{2+}). Amino acid sequence alignment of the AtNUDX7, ZmNUDX2 and ZmNUDX8 shows that the key amino acids of the NUDX motif are conserved except for the last amino acid of ZmNUDX8 in which isoleucine is replaced with another hydrophobic amino acid, valine. Hence, the NUDX box is highly conserved between AtNUDX7,

ZmNUDX2 and ZmNUDX8 and will not account for the difference in water deficit tolerance. However, their N-terminal region is variable which may possibly account for differences in substrate specificity or affinity (Fig. 2). Thus the ZmNUDX genes with longer N-terminal extension may have high affinity for a different substrate more than ADP-ribose and overexpression of the ZmNUDX genes in maize may only result in enhancing the activity of pathways different from PAR. This would account for the non-response to water deficit perturbation by overexpression of ZmNUDX genes in maize but tolerance attained by overexpression of the AtNUDX7 gene in maize and Arabidopsis, proposed to be achieved via the PAR pathway.

Fig. 2: Amino acid sequence alignment of ZmNUDX2 (ACG43116), ZmNUDX8 (ACG41054) and AtNUDX7 (AEE83169) using COBALT softwarein NCBI. Red box indicates the conserved NUDX motif $GX_5EX_7REVXEEXGU$ where U is Ile, Leu or Val and X is any amino acid.

Overexpression of the AtNUDX7 gene in Arabidopsis using the p35S, resulted in lines showing significant increase in seed yield phenotypes such as total seed weight, seed number, seed size and mass per seed in addition to early flowering time, reduced rosette leaf number and inflorescent height in comparison to wildtype. One line showed a remarkable consistence in the significant increase in three seed yield parameters: seed number, seed size and mass per seed and a reduction in flowering time, rosette leaf number and inflorescent height in comparison to wildtype. The early flowering going together with increased yield parameters is a remarkable combination of beneficial traits. Our result, to the best of our knowledge, is the first report showing the involvement of the AtNUDX7 in seed yield and yield-contributing parameters in Arabidopsis and indicates a versatile function of NUDX beyond its reported role in abiotic and biotic stress responses as a candidate target for yield improvement. The improved seed yield phenotype in OE AtNUDX7 lines can be attributed to maintenance of high energy levels through enhancing the recycling step of ADP-ribose

and re-establishing the energy levels by supplying an ATP source in the PAR energy homeostasis pathway. Modulation of the PAR pathway through PARP inhibition has previously been reported in controlling plant growth by promoting leaf cell number under non-stress conditions (Schulz et al., 2014) and in repressing the accumulation of stress protective agents such as anthocyanin, and of ascorbate under stress conditions that was correlated with enhanced biomass production and growth of Arabidopsis plants (Schulz et al., 2012). Hence, increased yield in the AtNUDX7 lines in our study could also be the result of yield stability under suboptimal green-house conditions.

Two approaches were used to downregulate the PARP1 and PARP2 gene expression in maize, RNAi hairpin silencing and CRISPR gene editing technology. RNAi hairpin silencing of the ZmPARP1 gene resulted in maize lines showing a tendency to sensitivity to hydroxyurea-induced DNA damage in spite of 5 to 6 fold down regulation levels of the endogenous ZmPARP1 gene expression possibly due to the residual activity of ZmPARP2 gene. Indeed Arabidopsis PARP1 and PARP2 have been reported to play an important role in DNA damage response by binding to nicked DNA and facilitating the DNA repair process (Doucet-Chabeaud et al., 2001; Jia et al., 2013). However, recent studies shows that in contrast to animals, PARP2 is the predominant poly(ADP-ribose) polymerase in Arabidopsis DNA damage in response to bleomycin, mitomycin C or gamma radiation (Song et al., 2015). We thus changed approach and used CRISPR/ CAS9 gene editing system to develop lines with a mutation in the ZmPARP catalytic domain. We successfully developed ZmPARP2_CRISPR lines with more than 200bp deletion in the catalytic domain, however, so far only T1 lines with segregating Cas9-induced deletion phenotypes have been generated. We also developed ZmPARP1_CRISPR lines with single nucleotide polymorphism or few base pair deletions. Owing to guide RNAs failure, we did not obtain CRISPR maize lines with mutation in both ZmPARP1 and ZmPARP2. Evaluation of the DNA damage response in the T1 ZmPARP2_CRISPR maize lines resulted in mixed phenotypes with some lines showing a trend of tolerance and others a trend of sensitivity to hydroxyurea-induced DNA damage. DNA damage analysis on T2 lines having a stable and uniform ZmPARP2 CRISPR-induced mutation are planned in the future experiments. In addition, for the purpose of studying PARP gene response to DNA damage, we propose the use of bleomycin which induced more severe double stranded breaks (Povirk, 1996) thus can trigger higher PARP enzyme activity resulting in NAD^+ depletion and activation of programmed cell death. The use of hydroxyurea limits dNTPs availability for the DNA polymerase thus arresting the replication fork (Koç et al., 2004) resulting in site specific DNA damage and single stranded DNA breaks which may in turn result in activation of the DNA repair process.

RNAi hairpin silencing of the ZmPARP1 gene resulted in maize lines showing a tendency to sensitivity to water deficit stress. In addition, RNAi hairpin silencing of AtPARP2 gene resulted in Arabidopsis

lines showing a tendency to sensitivity to mild drought stress. These results are in contrast to a previous report in which RNAi silencing of the AtPARP1 or AtPARP2 genes enhanced drought stress tolerance in Arabidopsis thaliana (De Block et al., 2005). However the type of drought stress assays (water deficit) applied in our study was quite different from the one in the report and it is thus a challenge to compare the outcome. In our study, water deficit was fully conducted in automated platforms where the irrigation of plants was based on the daily measurement of the gravimetric soil water content and its adjustment to preset values according to the requirements of the well watered or water deficit treatment. The complete experiment took a period of 21 days and approximately one month for Arabidopsis and maize lines respectively. In De Block et al. (2005), the plants were grown for 7-8 days *in vitro* then transferred to soil and 8-9 days after transfer water was withheld for 6 days after which they were watered once and 7-10 days later scored when control plants turned yellow. Addtionally the contradictory observation could be caused by instability of RNAi due to environmental conditions such as temperatures. The RNAi instability can result in a loss of the silencing or complete silencing after some generation. Also noteworthy, the level of downregulation of the PARP gene expression is very important to give the desired phenotype: too much downregulation can be detrimental while too low down-regulation may result in no altered phenotype (Marc De Block, personal communication). Interestingly, the T1 ZmPARP2_CRISPR edited maize lines showed a tendency to water deficit stress tolerance and thus a repeat of this experiment with T2 lines having a stable and uniform ZmPARP2 CRISPR-induced mutation is anticipated.

Tolerance to severe drought stress and the ability of plants to continue to grow under mild stress conditions are different traits. Under severe drought stress, plants respond by activating survival mechanisms such as stomatal closure to limit water loss, reduction of shoot growth, diversion of carbon and energy to storage and biosynthesis of protective compounds all of which lead to a penalty in plant growth and yield. These drought stress tolerance or avoidance mechanism are mostly attributed to increasing abscisic acid (ABA) levels that result in stomatal closure, induction of drought stress-responsive gene expression and metabolic changes (Seki et al., 2007). Plants in actual field conditions have to adapt to continuously fluctuating environmental conditions but rarely have to cope with extremes such as flooding or severe drought except in arid conditions. Frequently, plants experience mild drought stress that depending on developmental stage causes yield losses to some degree. Unfortunately, the response to mild drought stress is poorly understood compared with severe water deficit. Imposing mild drought stress requires a precise and well-monitored experimental setup, including a tight control of the soil water content and defining the precise timing of the drought onset (Skirycz et al., 2010; Clauw et al., 2015). Interestingly, most of the genes

identified with a role in stress tolerance in mature tissues under severe stress conditions seem to have little effect on growth inhibition in mild drought conditions (Skirycz et al., 2011). Superior growth performance and biomass yield gain is better determined under mild drought stress condition in which the plant through several adaptations such as reprogramming of energy metabolism and osmotic adjustment can balance survival and continued growth depending on the stress level (Skirycz et al., 2011; Claeys and Inzé, 2013). We thus speculate that the mild water deficit testing used in our study is testing a different trait as compared to the more severe drought stress testing reported in De Block et al., (2005) and thus explaining the differences in the results.

An interesting consideration is the relationship between water deficit and oxidative stress, however both are broad topics and analyzed in very different ways, even when the study is on the relationship between reactive oxygen species and drought. Most studies come to the conclusion that drought induces accumulation of reactive oxygen species but the parameters analyzed, the intensity and duration of the treatment, the plant developmental stages or tissue material used is very diverse. In some experiment, water deficit is induced using polyethylene glycol (PEG) while others limit the water given to plants but the terms “moderate” and “severe stress” used are very subjective. Also when drought treatment is imposed on intact plants where different organs cooperate as a whole, the response obtained will be quite different if it is applied to cut leaves. Oxidative stress is determined in different ways with many studies assessing the accumulation of different antioxidants and others inducing oxidative stress using reagents such as paraquat. There is a great need to standardize drought and oxidative stress treatments in order to compare the responses. The seemingly contradictory results we obtained as compared to published work on NUDX in oxidative stress tolerance might be explained by different molecular pathways tested in the used assays despite an assumed common basis in ROS activation.

Table 1: Currently published morphological and stress phenotypes on different poly(ADP-ribosl)ation pathway genotypes.

At Genotype	Promoter /Mutant	Morphology	Abiotic	Biotic	Oxidative	Genotoxic	Osmotic	References
OE-AtNUDX2	CaMV 35S promoter	Wt			√			Ogawa et al., 2009
RNAi-AtNUDX2	CaMV 35S promoter	Wt			Wt			Ogawa et al., 2009
OE-AtNUDX6	CaMV 35S promoter	Wt		√	Wt			Ishikawa et al., 2010
KO-nudx6		Wt		√				Ishikawa et al., 2010; Wang et al., 2013
OE-AtNUDX7	CaMV 35S promoter				√			Ishikawa et al., 2009
KO-nudx7	Salk_046441 (nudt7-1)	Stunted growth, differs per growing environments	√	√	√			Bartsch et al., 2006; Jambunathan and Mahalingam, 2006; Ge et al., 2007; Adams-Phillips et al., 2008; Ishikawa et al., 2009; Straus et al.,2010; Jambunathan et al., 2010; Wang et al., 2013
KO-nudx6 / nudx7	SALK_084842 (nudt6-2) / Salk_046441 (nudt7-1)	Extremely stunted, curly & deformed leaves	√	√				Wang et al., 2013
hpAtparp1	CaMV 35S promoter		√		√			De Block et al., 2005
hpAtparp2	CaMV 35S promoter		√		√			De Block et al., 2005; Vanderauwera et al., 2007
hpAtparp2 (signature)	CaMV 35S promoter		√					De Block et al., 2005
KO-parp1	GABI_380E06 (parp1-2)			Wt		√		Song et al., 2015
KO-parp2	GABI_420G03 (PARP2-1)			√		√		Song et al., 2015
KO-parp1 / parp2	GABI_380E06 (parp1) / GABI_420G03 (parp2)/ SALK_ 140400 (parp2)	Wt		√		√		Song et al., 2015; Boltz et al., 2014; Feng et al., 2015
KO-parp3	Salk_108092	Delayed germination						Rissel et al., 2014
OE-AtPARG1	CaMV 35S promoter	Wt	Wt		Wt		Wt	Li et al., 2011
KO-parg1	SALK_147805 (parg1-1), SALK_116088 (parg1-2), FLAG315E11 (parg1-3), (parg1-4)	Wt	√	√	√	√	√	Adams-Phillips et al., 2010; Li et al., 2011; Zhang et al., 2015, Feng et al., 2015
KO-parg2	GABI_072B04			√		√		Adams-Phillips et al., 2008; Adams-Phillips et al.,2010; Zhang et al., 2015

Table 2: Yield and stress phenotypes on different poly(ADP-ribosl)ation pathway genotypes obtained in our study. WW indicates Well watered, WIWAM and SHRIMPY are the automated robots in which water deficit experiment was performed)

Arabidopsis ID	Promoter /Mutant	Morphology / Yield	Mild Drought	PQ Oxidative	DNA damage
OE-AtNUDX7	CaMV p35S	Increased total seed weight, seed number, seed size and mass per seed. Reduced flowering time, rosette leaves number and inflorescent height	Trend to tolerance	Tolerant	
KO-nudx7	Salk_046441	Reduced seed number and inflorescent height		Wildtype	
OE-ZmNUDX2	CaMV p35S	Normal in WW WIWAM	Trend to tolerance		
OE-ZmNUDX8	CaMV p35S	Normal in WW WIWAM	Trend to tolerance		
RNAi hpAtparp2	CaMV p35S	Normal in WW WIWAM	Trend to Sensitivity		
Maize ID	**Promoter /Mutant**	**Morphorlogy**	**Water deficit**	**PQ Oxidative**	**DNA damage**
OE-AtNUDX7	pBdEF1α	Normal in WW SHRIMPY	Tolerant	Sensitive	
OE ZmNUDX2	pBdEF1α	Normal in WW SHRIMPY	Wt	Sensitive	
OE ZmNUDX8	pBdEF1α	Normal in WW SHRIMPY	Wt		
RNAi hpZmPARP1	pUBIL	Normal in WW SHRIMPY	Trend to sensitivity		Trend to Sensitivity
CRISPR_ZmPARP2	Cas9 del	Normal in WW SHRIMPY	Trend to tolerance		Trend to Sensitivity or Tolerance

7.2 Next steps for NUDX / PARP Maize and Arabidopsis lines

Overall, our study shows that overexpression of AtNUDX7 gene expression in Arabidopsis enhanced tolerance to paraquat induced-oxidative stress and resulted in a tendency to mild drought stress tolerance in addition to showing improved seed yield phenotypes pointing to a possible role of AtNUDX7 in crop yield improvement. Also overexpression of ZmNUDX2 and ZmNUDX8 gene in Arabidopsis showed a trend of tolerance to mild drought stress. It would be interesting to also test the OE ZmNUDX in Arabidopsis lines for their response to paraquat-induced oxidative stress and for seed yield and yield related parameters. In addition, our study shows that overexpression of AtNUDX7 gene in maize resulted in a line showing significant tolerance to water deficit stress in one experiment and a strong tendency towards water deficit stress tolerance in a second experiment carried out in an automated platform. We propose a larger scale and longer time span study of this AtNUDX7 line for water deficit tolerance and also yield parameters in the Phenovision platform at PSB-VIB-UGent. We noted the importance of developing or optimising other maize oxidative stress

assays in addition to leaf disk assay for complementary testing and verifying the results with methods such as spraying the maize plants with paraquat reagent and quantifying the chlorophyll contents and the fresh weight. We propose further oxidative stress and water deficit stress analysis in T2 ZmPARP2_CRISPR maize lines having a stable and uniform mutation that also showed a trend of tolerance to water deficit treatment in their T1 generation. Similar to the ZmNUDX lines, it will be interesting to analyse the CRISPR edited ZmPARP lines for their yield related phenotype to establish if the yield phenotype obtained in OE AtNUDX7 Arabidopsis lines and attributed to PAR energy homeostasis pathway can be translated to maize.

Analysis of the Poly(ADP-ribose) glycohydrolase (PARG), an enzyme that hydrolyses the poly(ADP-ribose) polymer producing free ADP-ribose monomers and polymers, was not in the scope of this study. For a more complete study of the PAR pathway, we propose an OE of the PARG gene in maize and Arabidopsis and carrying out of similar functional analysis to confirm the role of PAR pathway in water deficit and oxidative stress response. We further propose carrying out enzymatic assays on the transgenic lines to see the effect of OE or downregulation of NUDX and PARP respectively on their substrate. Table 3 shows a summary of the currently published enzymatic assays in Arabidopsis NUDX and PARP mutants.

Table 3: Currently published enzymatic assays for analysis of AtNUDX and AtPARP mutant lines. A tick indicates positive phenotype and Wt represents wildtype phenotype

NUDX Enzyme Assays	**KO-nudx7**	**OE-AtNUDX7**	**OE-AtNUDX2**	**RNAi-AtNUDX2**	**KO-nudx6**	**OE-AtNUDX6**	
ATP determination	√	√	√				Ogawa et al., 2009; Ishikawa et al., 2010; Ishikawa et al., 2009
NAD+/ NADH determination	√	√	Wt	Wt	√	√	
NADH pyrohposphatase	√	√	Wt	Wt	√	√	
ADP-ribose determination	√	√	√	√	Wt	Wt	
ADP-ribose pyrohposphatase	√	√	√	√	Wt	Wt	
PARP Enzyme Assays	**hpAtparp1**	**hpAtparp2**	**hpAtparp2 (signature)**	**KO-parp1**	**KO-parp2**	**KO-parp1 / parp2**	
Poly(ADP-ribose activity/ light	√	√	√				De Block et al., 2005
NAD+ - ATP content / light			√				
Bleomycin				√	√	√	Song et al., 2015
Mitomycin C				√	√	√	Song et al., 2015
Methly methane Sulphonate						√	Boltz et al., 2014

Several challenges were faced on using the Mu insertion lines from the Uniform Mu collection as control lines in the functional analysis of OE ZmNUDX lines in maize. First since the Uniform Mu collection is developed in W22 maize inbred background, it was difficult to compare its functional analysis results with transgenic lines developed in B104 background. In addition, Mu transposon insertions disrupts only one gene at a time, ZmNUDX8 in our study, we did not find a Mu insertion in the closely related ZmNUDX2 gene that could be used to generate double mutant. Thus ZmNUDX2 remained active in the plant and would possibly compensate for the ZmNUDX8 knocked down activity. In addition, we had to screen a large number of samples and cross heterozygous lines to obtain homozygous Mu insertion, a process that is laborious and time consuming especially in maize. We propose developing KO ZmNUDX control lines using the CRISPR gene editing technology in the future targeting both ZmNUDX2 and ZmNUDX8 genes or targeting the conserved Nudix motif in the B104 genotype in order to complete the functional analysis of these genes. Alternatively, overexpression trans-dominant negative mutants with mutations in the conserved Nudix motif would result in a stable downregulation of ZmNUDX enzymatic activity and suitable control lines for ZmNUDX functional analysis.

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