AFRIKA FOCUS — Volume 30, Nr. 2, 2017 — pp. 66-76

Modulation of energy homeostasis in maize and *Arabidopsis* to develop lines tolerant to drought, genotoxic and oxidative stresses[1]

Elizabeth Njuguna (1,2), Griet Coussens (1,2), Stijn Aesaert (1,2), Pia Neyt (1,2), Sylvester Anami (3), Mieke Van Lijsebettens (1,2)—

(1) Department of Plant Biotechnology and Bioinformatics, Ghent University
(2) Center for Plant Systems Biology, VIB
(3) Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology, Kenya

Abiotic stresses cause crop losses worldwide that reduce the average yield by more than 50%. Due to the high energy consumed to enhance the respiration rates, the excessive reactive oxygen species release provokes cell death and, ultimately, whole plant decay. A metabolic engineering approach in maize (*Zea mays*) altered the expression of two poly(ADP-ribosyl)ation metabolic pathway proteins, poly(ADP-ribose) polymerase (*PARP*) and ADP-ribose-specific Nudix hydrolase (*NUDX*) genes that play a role in the maintenance of the energy homeostasis during stresses. By means of RNAi hairpin silencing and CRISPR/Cas9 gene editing strategies, the PARP expression in maize was downregulated or knocked down. The *Arabidopsis* NUDX7 gene and its two maize homologs, *ZmNUDX2* and *ZmNUDX8*, were overexpressed in maize and *Arabidopsis*. Novel phenotypes were observed, such as significant tolerance to oxidative stress and improved yield in *Arabidopsis* and a trend of tolerance to mild drought stress in maize and in *Arabidopsis*.

Key words: poly(ADP-ribose) polymerase, Nudix hydrolase, CRISPR/Cas9, maize, oxidative stress, drought stress

1 This is the report of PhD research carried out at Ghent University, under the supervision of Mieke Van Lijsebettens, Steven Runo, Dirk Inzé and Sylvester Anami.

Introduction

Maize (*Zea mays*), the staple food in Kenya, is consumed by approximately 96% of the population on a daily basis – lack of maize equals lack of food. A major constraint to maize production in East Africa is drought, because a significant proportion of the region is arid and semi-arid land: 75% in Kenya, 50% of Tanzania and Ethiopia, and 30% of Uganda (Jama and Zeila, 2005). In Sub-Saharan Africa, maize is the most important food crop, but drought destabilizes the yield perennially and irrigation is not feasible due to cost restraints.

Plants have to maintain high energy levels to grow and reproduce optimally. However, maintenance of this energy state is a daily challenge, because in their natural environment, plants must cope regularly with multiple mild or severe biotic and abiotic stresses that consume a lot of energy for the stress response mechanisms and for their survival struggle. The decrease in energy content in a cell and in whole plants can only be tolerated within a narrow range and a further drop in the energy content below a certain threshold results in cellular damage that eventually becomes irreversible and causes the death of the plant cells and, ultimately, of the whole plant (De Block and Van Lijsebettens, 2011). High energy consumption enhances the rate of respiration, resulting in the production of reactive oxygen species (ROS) (Rizhsky *et al.*, 2002; Tiwari *et al.*, 2002). Strong stresses in plants have been shown to induce poly(ADP-ribosyl)ation activity that breaks down the nicotinamide adenine dinucleotide (NAD+) pool and enhances the mitochondrial respiration (De Block *et al.*, 2005). The responsible enzyme, poly(ADP-ribose) polymerase (PARP), is activated by DNA damage caused by the free radicals of reactive oxygen species (Virág and Szabó, 2002). PARP uses NAD+ as a substrate to synthesize polymers of adenosine disphosphate (ADP)-ribose on a range of nuclear enzymes (Fig. 1). Stress-induced NAD+ depletion results, in turn, in energy decrease, because adenosine triphosphates (ATPs), the energy-carrying molecules, are required to resynthesize the depleted NAD+. ADP-ribose specific Nudix hydrolase (NUDX) hydrolyzes free ADP-ribose molecules produced during the reverse degradation of mono- or poly-(ADP-ribose), releasing adenosine monophosphate (AMP) and ribose-5-phosphate (Fig. 1). The production of AMP through degradation of ADP-ribose, generated by poly(ADP-ribose) glycohydrolase (PARG), has been reported to be an important pathway to reestablish utilizable energy units (Rossi *et al.*, 2002).

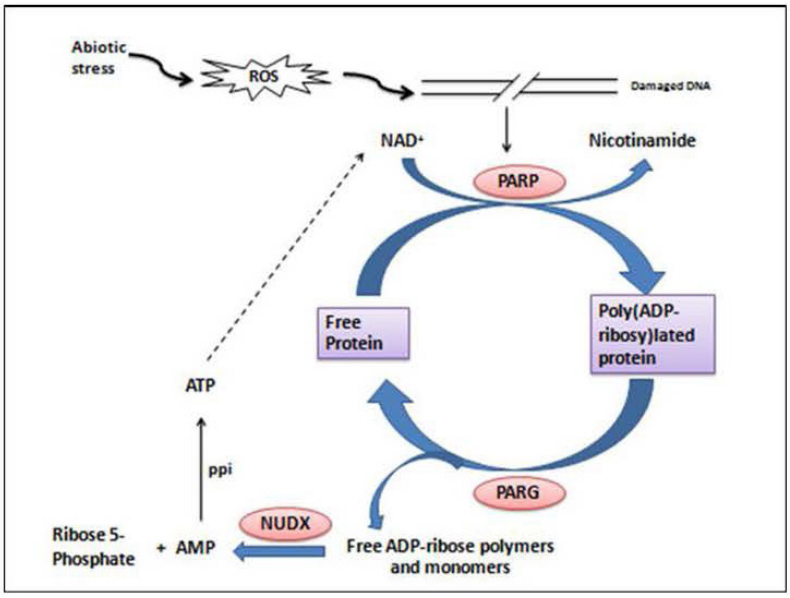

Figure. 1: Role of the poly(ADP-ribosyl)ation pathway in stress response and energy homeostasis. Reactive oxygen species (ROS) produced during abiotic stress may lead to a single- or double-stranded break in the DNA, triggering poly(ADP-ribose) polymerase (PARP) activity. PARP catalyzes the formation of a poly(ADP-ribose) chain on free proteins by sequential addition of ADP-ribose molecules from nicotinamide adenine dinucleotide (NAD+). Poly(ADP-ribose) glycohydrolase (PARG) catalyzes the catabolism of the poly(ADP-ribose) chain into free ADP-ribose monomers and polymers that are hydrolyzed to adenosine monophosphate (AMP) and ribose 5-phosphate by the activity of the ADP-ribose--specific Nudix hydrolase (NUDX) enzyme. AMP is an available precursor of adenosine triphosphate (ATP) that can be used to replenish the NAD+ pool.

Many strategies to improve tolerance to drought stress have been developed in plants, starting from conventional breeding methods to marker-assisted breeding to obtain desirable traits, such as stress tolerance, but generally they are time consuming 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 the plant's performance under adverse environmental conditions and, hence, the yield. Indeed, plants have been genetically modified (i) to overproduce detoxification enzymes, such as superoxide dismutases that scavenge ROS, (ii) to accumulate osmoprotectants, such as glycine betaine or proline, under water deficit or salt stress conditions, and (iii) 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; Anami *et al.*, 2009). In this PhD thesis, we focused on the role of the poly(ADP-ribosyl)ation (PAR) pathway in the energy homeostasis, its function in stress responses, and its potential in generating stress tolerance in plants. The main aim of the study was to generate maize and *Arabidopsis thaliana* lines with an altered energy homeostasis to im-

prove the tolerance to drought, oxidative, and genotoxic (DNA damage) stresses through engineering of the levels of two PAR pathway proteins, namely PARP and NUDX. Previously, down-regulation of the *PARP* gene in *Arabidopsis* and *Brassica napus* (rapeseed) by RNA interference (RNAi) gene silencing had been shown to result in plants with reduced NAD+ depletion and ATP consumption and tolerance to a broad range of abiotic stresses, such as high light, drought, and heat (De Block *et al.*, 2005). Additionally, overexpression of the *NUDX* gene in *Arabidopsis* was reported to confer tolerance to paraquat-induced oxidative stress (Ogawa *et al.*, 2009; Ishikawa *et al.*, 2009). The increased energy use efficiency avoids the need for a too intense mitochondrial respiration and, consequently, reduces the formation of ROS. We investigated whether genetic engineering for high energy use efficiency under stress conditions is a valuable approach to enhance the overall stress tolerance in maize, as a model for cereal crops. Moreover, a novel role for NUDX in promoting yield improvement was demonstrated in *Arabidopsis*.

Methodology

The poly(ADP-ribosyl)ation pathway was altered through engineering of the expression levels of the *PARP* and *NUDX* genes in maize and in *Arabidopsis*. The *PARP* gene activity in maize was downregulated and knocked-out by means of the RNAi hairpin silencing approach and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) gene editing system, respectively. The *Arabidopsis AtNUDX7* and its two maize homologs, *ZmNUDX2* and *ZmNUDX8*, were overexpressed in the B104 maize genotype with the *Brachypodium distachyon pBdEF1α* promoter (Coussens *et al.*, 2012) and the maize ubiquitin p*ZmUBIL* promoter (Christensen *et al.*, 1992). The maize B104 inbred line was transformed with *Agrobacterium tumefaciens* cocultivation of immature embryos (Karimi *et al.*, 2013; Coussens *et al.*, 2012). In addition, *AtNUDX7* and its two maize homologs, *ZmNUDX2* and *ZmNUDX8*, were also overexpressed in the *Arabidopsis* wild type (accession Columbia-0 [Col-0]) for complementation studies by means of the floral dip transformation method (Clough and Bent, 1998). Maize and *Arabidopsis* transgenic lines showing different expression levels of *NUDX* or *PARP* genes 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 Arabidopsis Seed Stock Center, respectively. The mutant lines were characterized to confirm knockdown or knockout of their respective genes and used as control lines in functional assays. The generated transgenic lines of maize and *Arabidopsis* were analyzed functionally with a hydroxyurea-induced-DNA damage assay, a paraquat-induced oxidative stress assay, and mild drought stress experiments in automated platforms. Yield improvement in the *Arabidopsis* lines was determined by using several parameters, such as total seed weight, seed size, seed number, mass per seed, flowering time, rosette leaf number, and inflorescence height.

Results

Perturbation of the maize PARP gene expression by using the RNAi hairpin silencing and the CRISPR/Cas9 gene editing system

An RNAi hairpin construct was designed with a 650-bp inverted repeat cDNA sequence from the ZmPARP1 catalytic domain of the B73 inbred maize line separated by a rice *Waxy* intron. The construct was cloned into a pMCG1005 RNAi expression vector that was recloned into the EHA101 supervirulent *Agrobacterium* strain and transformed into the B104 maize genotype. Nineteen independent events were obtained and analyzed for their *bar* marker gene segregation. Subsequently, 10 T1 lines with one or two T-DNA loci were analyzed for their ZmPARP1 and ZmPARP2 gene expression levels with the quantitative polymerase chain reaction (qPCR) technique. Seven of the RNAi hairpin lines showed significant downregulation of the ZmPARP1 gene expression in a range of 2.8- to 6-fold. No significant downregulation of the ZmPARP2 gene expression was observed in the RNAi hairpin lines carrying a ZmPARP1 catalytic domain sequence in the hairpin construct, thus indicating that regions with 100% identity between the ZmPARP1 and ZmPARP2 DNA sequences are necessary for efficient RNAi hairpin silencing. AtPARP2 is the predominant poly(ADP-ribose) polymerase in *Arabidopsis* DNA damage and drought stress tolerance (Song *et al.*, 2015; Block *et al.*, 2005). Hence, we adopted the revolutionary CRISPR/Cas9 gene editing technology to develop three new constructs targeting (i) a large deletion in the ZmPARP2 catalytic domain, (ii) a large deletion in the ZmPARP1 catalytic domain, and (iii) a frameshift mutation in the catalytic domain of both ZmPARP genes. A pair of guide RNAs (gRNA) targeting different regions of the PARP catalytic domain were used to generate the large deletions (approximately 200 bp), whereas single gRNAs per catalytic domain were used to generate the frameshift mutations. The gRNAs were cloned with the Golden Gate system into the pBUN411-Sp expression vector (Xing *et al.*, 2014) that carries a Cas9 nuclease protein, recloned into the EHA101 supervirulent *Agrobacterium* vector, and then transformed into the B104 maize genotype. Eight out of 20 T0 events obtained from the ZmPARP2 construct had homozygous deletions ranging between 201 bp to 233 bp in the ZmPARP2 catalytic domain and all the 11 T0 events obtained from the ZmPARP1 construct had either a homozygous single nucleotide insertion or a homozygous 4-bp deletion. Unexpectedly, all the nine T0 events of the third construct had a wild-type genotype, indicating a failure in both gRNAs in directing the Cas9 protein to the target sites. Genotyping of T1 progenies obtained by self-fertilization of the T0 parents with a large homozygous deletion in the ZmPARP2 catalytic domain resulted in lines showing a segregation of the Cas9-induced mutation. Many lines maintained the large deletion, but a few lines had either a single nucleotide insertion or a few basepair deletions, possibly due to a masked wild-type allele in some T0 parents with new mutations upon the Cas9 activity as a consequence. Preliminary DNA damage assays were carried out on these T1 lines with large deletion or small insertions or deletions (inDels) in the ZmPARP2 catalytic domain. The RNAi and CRISPR lines were also tested for water deficit tolerance in an automated platform at the VIB Center for Plant Systems Biology (see below).

Functional analysis of altered NUDX and PARP gene expression in maize for drought stress tolerance

AtNUDX7 has been proposed as a predominant ADP-ribose pyrophosphatase in *Arabidopsis* cells (Ishikawa *et al.*, 2009). *AtNUDX7* and its homologs, *ZmNUDX2* and *ZmNUDX8*, were overexpressed individually in the B104 maize genotype with the strongly constitutive *Brachypodium distachyon pBdEF1α* promoter and also the maize ubiquitin *pZmUBIL* promoter. Cloning was carried out with the Gateway system (pBbm42GW7 monocot multisite vectors; Karimi *et al.*, 2013) and the constructs were transformed into the B104 genotype. T1 lines were characterized for the *bar* marker gene segregation and T-DNA intactness by PCR analysis. A number of lines with one or two T-DNA locus insertions per construct were selected for qPCR expression analysis. Lines carrying the *pZmUBIL* promoter had generally very low expression levels in comparison to lines with the *pBdEF1α* promoter that had varyingly high, medium, and low *NUDX* gene expression levels in a range of 2– to 42– fold, suitable for functional analysis. These lines were upscaled to generate T3 lines with homozygous T-DNA insertion and used in the drought stress studies. A *Mutator* (*Mu*) transposon insert in the first exon of *ZmNUDX8* (McCarty *et al.*, 2005) successfully disrupted the *ZmNUDX8* gene transcription, resulting in a 5– to 6-fold downregulation of its expression and, hence, was used as mutant line control. Eighteen maize genotypes, consisting of *AtNUDX7*/*ZmNUDX* overexpression (OE) lines, *ZmNUDX8* Mu insert line, *ZmPARP1* RNAi-silenced lines, *ZmPARP2* CRISPR knockout lines, and their respective wild-type maize lines were evaluated for water deficit stress (mild drought stress) responses. A mild drought stress experiment was set up in an automated high-throughput phenotyping platform for maize seedlings at the VIB Center for Plant Systems Biology (Nelissen *et al.*, 2017). We observed that overexpression of the *Arabidopsis AtNUDX7* gene in maize by using the *Brachypodium distachyon pBdEF1α* promoter resulted in a significant tolerance to mild drought stress in a high OE line and a strong tendency to mild drought stress tolerance in the same line in a second experiment. The *ZmNUDX2* and *ZmNUDX8* OE lines showed a wild-type phenotype, whereas the *ZmPARP1* RNAi-silenced lines had a tendency to mild drought stress sensitivity. Interestingly, the T1 *ZmPARP2* CRISPR knockout lines had a tendency to mild drought stress tolerance. Hence, a repeat of this experiment with T2 lines with a stable and uniform *ZmPARP2* Cas9-induced mutation is anticipated.

Functional analysis of maize and Arabidopsis NUDX genes in Arabidopsis for seed yield, oxidative, and mild drought stress responses

The *AtNUDX7* gene and its maize homologs *ZmNUDX2* and *ZmNUDX8* were overexpressed individually in *Arabidopsis* Col-0 accession by means of the cauliflower mosaic virus *pCaMV35S* promoter. The cloning was carried out with the Gateway system (pK2GW7 plant expression vector; Karimi *et al.*, 2007) and transformation with the floral dip method (Clough and Bent, 1998). Transgenic seedlings were selected through high-density plating on selective medium. Subsequently, the segregation analysis was done through selection on kanamycin to generate T3 homozygous lines with one T-DNA locus. A mutant line with a T-DNA insertion in exon 1 of the *AtNUDX7* gene was obtained from the Nottingham

Arabidopsis Seed stock Center, characterized for homozygous T-DNA insertion and used as control line in the experiments. Lines showing varying high, medium, and low expression levels of the different NUDX genes were obtained upon qPCR analysis. The OE *AtNUDX7* in the Col-0 lines were analyzed for their seed yield and yield-related parameters. Several phenotypes were observed, such as significant increase in total seed weight, seed number, seed size, and mass per seed, in addition to reduced flowering time, rosette leaf number, and inflorescence height when compared to the wild type. One line showed a remarkable combination of phenotypes, including the significant increase in the three seed yield parameters, seed number, seed size and mass per seed, and a reduction in flowering time, rosette leaf number, and inflorescence height in comparison to the wild type. The rosette area of OE *AtNUDX7* in the Col-0 lines under paraquat-induced oxidative stress was evaluated and was significantly larger in a number of lines than that of the wild type, indicating that overexpression of *AtNUDX7* conferred oxidative stress tolerance to the *Arabidopsis* plants. Twelve genotypes, consisting of the OE *AtNUDX7*, *ZmNUDX2*, or *ZmNUDX8* in the Col-0 lines, the *Arabidopsis AtPARP2* RNAi hairpin-silenced line, and their respective wild-type control lines, were evaluated for their mild drought stress response in the automated high-throughput phenotyping platform for *Arabidopsis* plants according to established protocols (Skirycz *et al.*, 2011; Clauw *et al.*, 2015) at the VIB Center for Plant Systems Biology. Analysis of the final shoot area of the plants after 21 days in the automated platform indicated that overexpression of *AtNUDX7*, *ZmNUDX2*, or *ZmNUDX8* in *Arabidopsis* showed a trend of tolerance to mild drought stress, whereas on the contrary, the *AtPARP2* RNAi-silenced line had a tendency to mild drought stress sensitivity.

Discussion

Overexpression of the *AtNUDX7* gene in maize by means of the strong constitutive Brachypodium distachyon promoter p*BdEF1α* resulted in lines showing a significant or strong tendency to mild drought stress tolerance. Similarly, overexpression of the same *AtNUDX7* gene in *Arabidopsis* with the cauliflower mosaic virus promoter p*CaMV35S* resulted in a trend of tolerance to mild drought stress, in addition to conferring significant tolerance to paraquat-induced oxidative stress. These oxidative stress tolerance results support previous studies in which modulation of the *AtNUDX2* or *AtNUDX7* genes conferred enhanced tolerance to oxidative stress in *Arabidopsis*, as assessed by the leaf phenotype, survival rates, and chlorophyll content (Ogawa *et al.*, 2009; Ishikawa *et al.*, 2009). In addition, our findings indicate that the ADP-ribose-specific *NUDX* genes play a role in plant response to mild drought stress. ADP-ribose-specific NUDXs are involved in nucleotide recycling in the PAR pathway by hydrolyzing free ADP-ribose molecules into ribose-5-phosphate and AMP, a ready precursor for ATP synthesis (Rossi *et al.*, 2002). Upon drought stress, the ROS production increases (Cruz de Carvalho, 2008) that induces the PARP protein activity (Virág and Szabó, 2002) and, thus, the PAR pathway. Thus, the PAR pathway activation will trigger the nucleotide recycling action of the ADP-ribose-specific NUDXs, reestablishing the energy levels by supplying an ATP source. We propose that overexpression of *AtNUDX7* enhances the mild drought and oxidative stress tolerance through enhancing the ADP-ribose recycling step in the PAR energy homeostasis pathway.

In contrast, overexpression of the *ZmNUDX2* and *ZmNUDX8* genes in maize by means of the pBdEF1α promoter did not result in tolerance to mild drought stress. However, these two maize genes expressed in *Arabidopsis* with the p*CaMV35S* promoter showed a trend of tolerance to mild drought stress. The results raised the question of why the *AtNUDX7* expression in maize would induce the mild drought stress tolerance phenotype, whereas the overexpression of its maize homologs did not result in the same phenotype in maize, but only in *Arabidopsis*. One possible explanation could be obtained from metadata analysis that showed that the *AtNUDX7* gene is induced endogenously upon drought stress perturbation, implying that all molecular components for its enzymatic activity are available, and that the *ZmNUDX* genes are not. Secondly, the *ZmNUDX* proteins have longer N-terminal regions than the *AtNUDX7* protein, which may affect the ADP-ribose substrate affinity or specificity. More experiments confirming our results would be a prerequisite to make conclusive remarks on the differences in the *ZmNUDX* and *AtNUDX* gene activity in maize and *Arabidopsis*.

Overexpression of the *AtNUDX7* gene in *Arabidopsis* resulted in lines with an improved seed yield and yield-related parameters, such as increased total seed weight, increased seed number without a trade-off in seed size, increased mass per seed, early flowering time, and reduced plant height. The early flowering and reduced plant height together with increased seed yield parameters is a remarkable combination of beneficial traits. This result, to the best of our knowledge, is the first that shows the involvement of the *AtNUDX7* in seed yield and yield-contributing parameters in *Arabidopsis* and that hints at a versatile NUDX function 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 enhancement of the ADP-ribose recycling step and reestablishment of the energy levels by supplying an ATP source in the PAR energy homeostasis pathway. Previously, modulation of the PAR pathway through PARP inhibition has been reported to control plant growth by increasing the leaf cell numbers under nonstress conditions (Schulz *et al.*, 2014) and to repress the accumulation of stress-protective agents, such as anthocyanin, and of ascorbate under stress conditions, correlating 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 greenhouse conditions.

NUDX overexpression might be a more promising approach to obtain yield stability upon water deficit/drought stress in cereal crops as compared to *PARP* downregulation. Modulation of the ADP-ribose-specific NUDX protein may have fewer pleiotropic effects than modulation of the stress-responsive PARP proteins that are involved either in DNA damage detection and repair or apoptosis. The extent of the PARP activity 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, 2001; Amor *et al.*, 1998). In drought stress assays, the level of downregulation of PARP might be very critical, because preliminary results indicated either tolerance or sensitivity in CRISPR or RNAi lines with downregulated

PARP expression. In our study, the OE *NUDX* phenotypes, such as significant tolerance to oxidative stress, improved seed yield parameters trends of mild drought stress tolerance in maize and *Arabidopsis*, support this working hypothesis.

Conclusion and perspectives

Overall, our study shows that the *AtNUDX7* gene overexpression in *Arabidopsis* enhanced tolerance to paraquat-induced oxidative stress, resulted in a trend of tolerance to mild drought stress, and showed improved seed yield phenotypes, pointing to a putative role of the *AtNUDX7* gene in crop yield improvement. Overexpression of the maize genes *ZmNUDX2* and *ZmNUDX8* in *Arabidopsis* also hinted at a trend of tolerance to mild drought stress. Testing of the latter *Arabidopsis* lines overexpressing the maize *NUDX* genes for their response to oxidative stress and for yield improvement might add supportive data. In addition, our research indicated that overexpression of the *AtNUDX7* gene in maize resulted in a line showing a strong tendency to mild drought stress tolerance in an automated platform. We propose an increased scale and extended time span to study this *AtNUDX7* maize line for drought stress tolerance and oxidative stress response, as well as yield performance in a phenotyping platform, such as the Phenovision at the VIB Center for Plant Systems Biology. Knockdown of the *ZmPARP2* gene with the CRISPR/Cas9 gene editing system resulted in T1 maize lines that had a tendency to tolerance to mild drought stress. Further confirmation will be carried out in T2 *ZmPARP2* CRISPR lines with a stable and uniform mutation. Generation of *parp1parp2* double mutant lines by crossing the *ZmPARP1* and *ZmPARP2* CRISPR-edited lines obtained in this work will produce superior lines for DNA damage stress response analysis. Also interesting would be to analyze the CRISPR-edited *ZmPARP* maize lines for their yield performance to establish whether the improved yield phenotype obtained by overexpressing *AtNUDX7* in *Arabidopsis* and attributed to the PAR energy homeostasis pathway can be translated to maize. Pyramiding of the functional overexpression *NUDX* and knock-out *PARP* lines in maize and *Arabidopsis* will be a useful strategy to further this research. Beyond the scope of this thesis was the analysis of PARG, a key PAR pathway protein that hydrolyzes the poly(ADP-ribose) polymer that produces free ADP-ribose monomers and polymers. For a more complete study of the PAR pathway, we propose an overexpression of the PARG gene in maize and *Arabidopsis* and similar functional analyses to confirm the role of the PAR pathway in drought stress tolerance, oxidative stress response, and yield improvement. We further suggest to carry out enzymatic assays, such as ATP and NAD+ determination on the transgenic lines generated, to see the effect of overexpression and knockdown/downregulation of *NUDX* and *PARP*, respectively, under stress and nonstress conditions at the protein level.

Acknowledgements

The authors would like to thank Martine De Cock for critical reading and editing the manuscript and to acknowledge the contributions of Linus Paul, Yolaine Van Haver, Thomas Eekhout, Hilde Nelissen, Kirin De Muynck, Hannes Van Haeren, Stijn Dhondt, Nathalie Wuyts, Valerie Van Ruyskensvelde, and Veronique Storme. This work was funded through the Belgian Development Corporation, Vlaamse Interuniversitaire Raad, VLIR-UOS ICP-PhD scholarship.

References

Amor, Y., Babiychuk, E., Inzé, D., & Levine, A. (1998). *The involvement of poly(ADP-ribose) polymerase in the oxidative stress responses in plants.* FEBS Letters, 440(1), 1-7.

Anami, S., De Block, M., Machuka, J., & Van Lijsebettens, M. (2009). *Molecular improvement of tropical maize for drought stress tolerance in sub-Saharan Africa.* Critical Reviews in Plant Sciences, 28(1-2), 16-35.

Bürkle, A. (2001). *Poly(APD-ribosyl)ation, a DNA damage-driven protein modification and regulator of genomic instability.* Cancer Letters, 163(1), 1-5.

Christensen, A. H., Sharrock, R. A., & Quail, P. H. (1992). Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Molecular Biology, 18(4), 675-689.

Clauw, P., Coppens, F., De Beuf, K., Dhondt, S., Van Daele, T., Maleux, K., Inzé, D. (2015). *Leaf responses to mild drought stress in natural variants of Arabidopsis.* Plant Physiology, 167(3), 800-816.

Clough, S. J., & Bent, A. F. (1998). *Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana.* The Plant Journal, 16(6), 735-743.

Coussens, G., Aesaert, S., Verelst, W., Demeulenaere, M., De Buck, S., Njuguna, E., Van Lijsebettens, M. (2012). *Brachypodium distachyon promoters as efficient building blocks for transgenic research in maize.* Journal of Experimental Botany, 63(11), 4263.

Cruz de Carvalho, M. H. (2008). Drought stress and reactive oxygen species: production, scavenging and signaling. Plant Signaling & Behavior, 3(3), 156-165.

De Block, M., & Van Lijsebettens, M. (2011). *Energy efficiency and energy homeostasis as genetic and epigenetic components of plant performance and crop productivity.* Current Opinion in Plant Biology, 14(3), 275-282.

De Block, M., Verduyn, C., Brouwer, D. D., & Cornelissen, M. (2005). *Poly(ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance.* The Plant Journal, 41(1), 95-106.

Ishikawa, K., Ogawa, T., Hirosue, E., Nakayama, Y., Harada, K., Fukusaki, E., Shigeoka, S. (2009). *Modulation of the poly(ADP-ribosyl)ation reaction via the Arabidopsis ADP-ribose/NADH pyrophosphohydrolase, AtNUDX7, is involved in the response to oxidative stress.* Plant Physiology, 151(2), 741-754.

Jama, B., & Zeila, A. (2005). Agroforestry in the drylands of eastern Africa: a call to action: World Agroforestry Centre, Eastern and Central Africa Regional Programme.

Karimi, M., Depicker, A., & Hilson, P. (2007). *Recombinational cloning with plant Gateway vectors.* Plant Physiology, 145(4), 1144-1154.

Karimi, M., Inzé, D., Van Lijsebettens, M., & Hilson, P. (2013). *Gateway vectors for transformation of cereals.* Trends in Plant Science, 18(1), 1-4.

McCarty, D. R., Mark Settles, A., Suzuki, M., Tan, B. C., Latshaw, S., Porch, T., Lai, J. (2005). *Steady-state transposon mutagenesis in inbred maize.* The Plant Journal, 44(1), 52-61.

Nelissen, H., Sun, X. H., Rymen, B., Jikumaru, Y., Kojima, M., Takebayashi, Y., Vuylsteke, M. (2017). *The reduction in maize leaf growth under mild drought affects the transition between cell division and cell expansion and cannot be restored by elevated gibberellic acid levels.* Plant Biotechnology Journal, in press (doi: 10.1111/pbi.12801).

Ogawa, T., Ishikawa, K., Harada, K., Fukusaki, E., Yoshimura, K., & Shigeoka, S. (2009). *Overexpression of an ADP-ribose pyrophosphatase, AtNUDX2, confers enhanced tolerance to oxidative stress in Arabidopsis plants.* The Plant Journal, 57(2), 289-301.

Rizhsky, L., Liang, H., & Mittler, R. (2002). *The combined effect of drought stress and heat shock on gene expression in tobacco.* Plant Physiology, 130(3), 1143-1151.

Rossi, L., Denegri, M., Torti, M., Poirier, G. G., & Scovassi, A. I. (2002). *Poly(ADP-ribose) degradation by post-nuclear extracts from human cells.* Biochimie, 84(12), 1227-1233.

Schulz, P., Jansseune, K., Degenkolbe, T., Méret, M., Claeys, H., Skirycz, A., Hannah, M. A. (2014). *Poly(ADP-ribose) polymerase activity controls plant growth by promoting leaf cell number.* PLoS ONE, 9(2), e90322.

Schulz, P., Neukermans, J., Van Der Kelen, K., Mühlenbock, P., Van Breusegem, F., Noctor, G., Hannah, M. A. (2012). *Chemical PARP inhibition enhances growth of Arabidopsis and reduces anthocyanin accumulation and the activation of stress protective mechanisms.* PLoS ONE, 7(5), e37287.

Skirycz, A., Vandenbroucke, K., Clauw, P., Maleux, K., De Meyer, B., Dhondt, S., Tognetti, V. B. (2011). *Survival and growth of Arabidopsis plants given limited water are not equal.* Nature Biotechnology, 29(3), 212-214.

Song, J., Keppler, B. D., Wise, R. R., & Bent, A. F. (2015). *PARP2 is the predominant poly(ADP-ribose) polymerase in Arabidopsis DNA damage and immune responses.* PLoS Genetics, 11(5), e1005200.

Tiwari, B. S., Belenghi, B., & Levine, A. (2002). *Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death.* Plant Physiology, 128(4), 1271-1281.

Virág, L., & Szabó, C. (2002). *The therapeutic potential of poly(ADP-ribose) polymerase inhibitors.* Pharmacological Reviews, 54(3), 375-429.

Xing, H.-L., Dong, L., Wang, Z.-P., Zhang, H.-Y., Han, C.-Y., Liu, B., Chen, Q.-J. (2014). *A CRISPR/Cas9 toolkit for multiplex genome editing in plants.* BMC Plant Biology, 14(1), 327.

Yang, S., Vanderbeld, B., Wan, J., & Huang, Y. (2010). *Narrowing down the targets: towards successful genetic engineering of drought-tolerant crops.* Molecular Plant, 3(3), 469-490.