Functional analysis of Arabidopsis and maize transgenic lines overexpressing the ADP-ribose/NADH pyrophosphohydrolase, AtNUDX7

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ABSTRACT The conserved poly(ADP-ribosyl)ation (PAR) pathway consists of three genetic components that are potential targets to modulate the plant’s energy homeostasis upon stress with the aim to improve yield stability in crops and help secure food supply. We studied the role of the PAR pathway component ADP-ribose/NADH pyrophosphohydrolase (AtNUDX7) in yield and mild drought stress by using a transgenic approach in Arabidopsis thaliana and maize (Zea mays). Arabidopsis AtNUDX7 cDNA was overexpressed in Arabidopsis and maize by means of the constitutive Cauliflower Mosaic Virus 35S promoter and the strong constitutive Brachypodium distachyon pBdEF1a promoter, respectively. Overexpression of AtNUDX7 in Arabidopsis improved seed parameters that were measured by a novel, automated method, accelerated flowering and reduced inflorescence height. This combination of beneficial traits suggested that AtNUDX7 overexpression in Arabidopsis might enhance the ADP-ribose recycling step and maintain energy levels by supplying an ATP source in the poly(ADP-ribosyl)ation energy homeostasis pathway. Arabidopsis and maize lines with high, medium and low overexpression levels of the AtNUDX7 gene were analysed in automated platforms and the inhibition of several growth parameters was determined under mild drought stress conditions. The data showed that the constitutive overexpression of the Arabidopsis AtNUDX7 gene in Arabidopsis and maize at varying levels did not improve tolerance to mild drought stress, but knocking down AtNUDX7 expression did, however at the expense of general growth under normal conditions.

KEY WORDS: seed yield, flowering time, water deficit, mild drought stress, constitutive promoter

Introduction

The poly(ADP-ribosyl)ation (PAR) pathway (Fig. 1) is a post-translational protein modification process, activated upon single- or double-stranded DNA breaks, in which ADP-ribose subunits from nicotinamide adenine dinucleotide (NAD⁺) are covalently attached to target proteins mediated by the poly(ADP-ribose) polymerase enzyme (PARP). PARP activity can be reversed by a poly(ADP-ribose) glycohydrolase enzyme (PARG) generating free ADP-ribose molecules that can be degraded into adenosine monophosphate (AMP) and ribose-5-phosphate by an ADP-ribose-specific Nudix hydrolase enzyme (D’Amours et al., 1999). The AMP can be utilized to replenish the ATP and NAD⁺, leading to maintenance of cellular homeostasis (Rossi et al., 2002). 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 nonenzymatically, thereby altering or eliminating their function. Thus, the ADP-ribose py-
The PAR pathway has been broadly studied in animals; it plays a key role in DNA repair, genotoxic stress response, chromatin structure, transcription regulation, apoptosis, and cell cycle activities (D’Amours et al., 1999; Kim et al., 2005). In plants, PAR has been implicated in several physiological processes and described as an important regulatory mechanism modulating responses to abiotic and biotic stresses, such as oxidative stress (Amor et al., 1998; Ogawa et al., 2008; Ishikawa et al., 2009), DNA damage (Doucet-Chabeaud et al., 2001; Song et al., 2015), drought stress (De Block et al., 2005), osmotic stress (Li et al., 2011), immune response (Adams-Phillips et al., 2010; Ishikawa et al., 2010; Feng et al., 2015; Song et al., 2015), and also in growth (Schulz et al., 2012, 2014). PARP and PARG proteins (Fig. 1) are multifunctional in plants as well, and are involved in abiotic stress tolerance, DNA damage response, plant growth, and biotic stress response. Indeed, down-regulation of the PARP gene in Brassica napus (rapeseed) and Arabidopsis thaliana enhanced tolerance to a broad range of abiotic stresses (De Block et al., 2005). Arabidopsis parp mutants are hypersensitive to DNA damage induced by bleomycin and mytomycin (Song et al., 2015). Inhibition of Arabidopsis PARP enhanced plant growth by promoting the leaf cell number (Schulz et al., 2014), perturbed innate immune responses to microbe-associated molecular patterns, such as fl22 and elf18 (Adams-Phillips et al., 2010), and compromised basal defense responses (Feng et al., 2015; Song et al., 2015). The Arabidopsis parp1 mutants were more sensitive to cell damage under osmotic and oxidative stresses (Li et al., 2011), enhanced DNA damage and cell death upon treatment with bleomycin (Zhang et al., 2015), and accelerated the onset of disease symptoms upon infection with Botrytis cinerea (Adams-Phillips et al., 2010).

The Nudix-encoding (NUDX) genes (Fig. 1) might be an alternative for modulating energy homeostasis in plants as opposed to the PARP and PARG genes. Nudix hydrolases consist of a large family of conserved proteins in viruses, archaea, bacteria, and eukaryotes, characterized by the highly conserved Nudix box, GX5EX7REUXEEXGU, with U being a bulky, hydrophobic amino acid, usually Ile, Leu, or Val (Bessman et al., 1996). Almost all the major substrates for these enzymes are nucleoside diphosphates linked to some other moiety, x, hence the acronym “Nudix”. They have a broad substrate range, including: dinucleoside polyphosphates, ADP-ribose, NADH, nucleotide sugars, or ribo- and deoxyribonucleoside triphosphates, coenzyme A, mRNA cap, and FAD (Bessman et al., 1996; Dunn et al., 1999; Ogawa et al., 2005, 2008). Accumulation of these substrates is potentially toxic to the cell and their intracellular levels need to be precisely regulated. Therefore, a role for Nudix hydrolases in sanitizing or modulating the accumulation of these metabolites was postulated (Bessman et al., 1996).

The genome of the model plant Arabidopsis thaliana contains 28 genes coding for putative Nudix hydrolases (Yoshimura and Shigeoka, 2015). These proteins are classified into three types according to their predicted subcellular localization: cytosol, mitochondrion, and chloroplast. Arabidopsis Nudix hydrolases targeted to the cytosol include AtNUDX1 to AtNUDX11, AtNUDX25, and AtDCP2 (Ogawa et al., 2005; Yoshimura and Shigeoka, 2015). AtNUDX1 is the functional homolog of the Escherichia coli MutT (Ogawa et al., 2005) because it plays an important protective role against oxidative DNA and RNA damage in Arabidopsis cells through sanitization of their precursor pool in the cytosol (Yoshimura et al., 2007). However, the AtNUDX1 mutant plants...
did not exhibit any noticeable changes in their phenotype under normal or stressful conditions (Kraszewska, 2008); hence it remains to be shown whether the AtNUDX1 gene perturbations have any physiological impact on Arabidopsis plants.

Cytosolic AtNUDX2, AtNUDX6, AtNUDX7, and AtNUDX10 have a pyrophosphohydrolase activity toward both ADP-ribose and NADH (Ogawa et al., 2005). Overexpression of AtNUDX2 in Arabidopsis enhanced tolerance to oxidative stress due to maintenance of NAD+ and ATP levels by nucleotide recycling from free ADP-ribose molecules. However, AtNUDX2 is not the predominant ADP-ribose pyrophosphohydrolase in Arabidopsis, because its downregulation resulted only in a slight reduction (10%) of the ADP-ribose pyrophosphohydrolase activity in the transgenic plants, indicating that other enzymes with higher ADP-ribose pyrophosphohydrolase activity may exist in Arabidopsis cells (Ogawa et al., 2009). AtNUDX6 modulates NADH rather than ADP-ribose metabolism and significantly impacts the Arabidopsis plant immune response as a positive regulator of the NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1)-dependent salicylic acid signaling pathways (Ishikawa et al., 2010). AtNUDX7 is induced by multiple stresses and is involved in both biotic and abiotic stress responses (Bartsch et al., 2006; Jambunathan and Mahalingam, 2006; Ge et al., 2007, 2008; Adams-Phillips et al., 2008; Ishikawa et al., 2009; Jambunathan et al., 2010). AtNUDX7 showed a preferential activity for ADP-ribose and NADH when expressed in E.coli cells (Ge et al., 2007). AtNUDX7 has been proposed as the predominant ADP-ribose and NADH pyrophosphohydrolase in Arabidopsis cells, because AtNUDX7 loss-of-function mutant plants showed approximately 76.9% and 46.9% significantly reduced pyrophosphohydrolase activities toward ADP-ribose and NADH, respectively, in comparison to the levels in wild-type plants (Ishikawa et al., 2009). Overexpression of AtNUDX7 enhanced tolerance to paraquat-induced oxidative stress, due to the restoration of NAD+ and ATP levels upon activation of poly(ADP-ribosyl)ation reaction under oxidative stress, whereas knocking down AtNUDX7 led to the opposite observation. Hence, AtNUDX7 regulates the defense mechanisms against oxidative DNA damage via modulation of the PAR reaction (Ishikawa et al., 2009).

Here, AtNUDX7 was overexpressed in Arabidopsis and maize plants and analysed for seed yield, yield-associated parameters, and mild drought stress, which are highly desired.
traits in the light of the ongoing climate change and reduced arable land.

Results and Discussion

Overexpression of the AtNUDX7 gene in Arabidopsis and maize

The full-length cDNA of the AtNUDX7 gene (At4G12720) was overexpressed in Arabidopsis thaliana Columbia (Col-0) accession under the control of the constitutive Cauliflower Mosaic Virus 35S promoter using the plant Gateway expression vector pK2GW7 (Karimi et al., 2007), which carries a neomycin phosphotransferase II (nptII) selectable marker gene (Fig. 2A). After floral dip transformation, T0 transgenic Arabidopsis plants were selected on kanamycin-containing media at high density plating and, subsequently, T3 lines with a single-locus T-DNA insertion were identified (Table 1). Two-week-old T3 seedlings overexpression of p35S::AtNUDX7 lines and Col-0 control plants were used in a quantitative (q)PCR expression analysis. High, medium, and low overexpression levels of AtNUDX7 were observed in 10 independent transgenic p35S::AtNUDX7 lines in comparison to the Col-0 control, ranging between 2-fold to 50-fold (Fig. 2B, Table 1; supplementary Table S1). A loss-of-function mutant line, which we designated Atnudx7-1 (SALK-046441), with a T-DNA insertion in exon 1 of the AtNUDX7 gene, in the Col-0 background (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; Ogawa et al., 2016), was verified for its T-DNA insertion position, homozygous T-DNA insertion, AtNUDX7 gene expression, and was used as a negative control (Fig. 2C). A subset of the Arabidopsis lines overexpressing AtNUDX7 with higher overexpression levels, ranging between 17- and 54-fold (Table 1), values much higher than those in previously analysed lines (Ishikawa et al., 2009), and the Atnudx7-1 mutant line (Table 1) were subsequently used to study seed yield parameters, yield-associated parameters, flowering time, and inflorescence height.

The AtNUDX7 full-length cDNA was overexpressed in the maize B104 inbred line with the strong constitutive Brachypodium distachyon promoter, pBdEF1α (Coussens et al., 2012), or the constitutive maize ubiquitin promoter, pZmUBIL (Christensen et al., 1992), in the monocot multisite Gateway expression vector pBbm42GW7 (Karimi et al., 2013) that carries the bialaphos resistance (bar) selection maker gene (Fig. 2D). The AtNUDX7 overexpression construct was transformed with the EHA101 supervirulent Agrobacterium strain (Hood et al., 1986) and the Agrobacterium-mediated transformation method (Coussens et al., 2012; Anami et al., 2013). The transgenic T0 plants were backcrossed to the B104 control maize plants, generating T1 lines with a hemizygous T-DNA insertion. The T1 maize lines were analysed for bar gene segregation by means of a phosphinothricin acetyl transferase (PAT) assay and for T-DNA intactness with PCR; lines with an intact transgene and a functional assay

### TABLE 1

Summary of the Arabidopsis and Maize Transgenic Lines

| Arabidopsis genotype | T3 Arabidopsis line | T-DNA loci | Fold change | Functional assay |
|----------------------|---------------------|------------|-------------|-----------------|
| p35S::AtNUDX7        | AtNUDX7_OE-A1       | 1          | 54          | Seed yield/yield-associated parameters and mild drought stress |
|                      | AtNUDX7_OE-A2       | 1          | 50          |                  |
|                      | AtNUDX7_OE-A3       | 1          | 27          |                  |
|                      | AtNUDX7_OE-A4       | 1          | 31          |                  |
|                      | AtNUDX7_OE-A5       | 1          | 17          |                  |
|                      | AtNUDX7_OE-A6       | 1          | 2           |                  |
|                      | AtNUDX7_OE-A7       | 1          | 12          |                  |
|                      | AtNUDX7_OE-A8       | 1          | 50          |                  |
|                      | AtNUDX7_OE-A9       | 1          | 15          |                  |
|                      | AtNUDX7_OE-A10      | 1          | 3           |                  |
|                      | AtNUDX7             | 1          | -4          | Seed yield/yield-associated parameters and mild drought stress |

| Maize genotype       | T1 maize line       | T-DNA loci | AtNUDX7 expression level |
|----------------------|---------------------|------------|--------------------------|
| pBdEF1α::AtNUDX7     | AtNUDX7_OE-Zm1      | 1          | 0.9                      | Mild drought stress |
|                      | AtNUDX7_OE-Zm2      | 1          | 0.7                      |                  |
|                      | AtNUDX7_OE-Zm3      | 1          | 0.3                      |                  |
|                      | AtNUDX7_OE-Zm4      | 1          | 0.0005                   |                  |
|                      | AtNUDX7_OE-Zm5      | 1          | 0.0001                   |                  |
|                      | AtNUDX7_OE-Zm6      | 1          | 0.01                     |                  |
|                      | AtNUDX7_OE-Zm7      | 1          | 0.002                    |                  |
|                      | AtNUDX7_OE-Zm8      | 1          | 0.001                    |                  |
|                      | AtNUDX7_OE-Zm9      | 1          | 0.005                    |                  |
|                      | AtNUDX7_OE-Zm10     | 1          | 0.0008                   |                  |
|                      | AtNUDX7_OE-Zm11     | 1          | 0.003                    |                  |

Shaded lines were functionally analysed.
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A direct correlation between early flowering time and reduced rosette leaf number had previously been reported (Alonso-Blanco et al., 1998). Seed number and inflorescence height were significantly lower in the Atnudx7-1 mutant line than those in Col-0, whereas seed size, mass per seed, and flowering time were similar as in the Col-0 control. The reduced size of Atnudx7-1 mutant plants under normal conditions had also previously been observed (Bartsch et al., 2015).

**Improved seed yield parameters and early flowering time upon overexpression of AtNUDX7 in Arabidopsis**

The Arabidopsis NUDX7 protein restores the NAD\(^+\) and ATP levels upon activation of the PAR reaction under abiotic and biotic stresses (Ishikawa et al., 2009; Ogawa et al., 2016), which might improve seed yield stability upon expression modulation. Hence, we investigated seed yield parameters in the two high overexpression (OE) lines, AtNUDX7_OE-At1 and AtNUDX7_OE-At2, the medium OE line, AtNUDX7_OE-At3, the low OE line Atnudx7_OE-At5, the At nudx7-1 mutant line (Salk-046441_1), and the Col-0 control line (Table 1). Total seed weight, seed number per 10 siliques, seed size, mass per seed, in addition to the yield-associated parameters, flowering time, number of leaves at bolting, and inflorescence height were determined according to Van Daele et al. (2012). The seed yield and yield-associated parameters determined for the p35S::AtNUDX7 OE lines, the Atnudx7-1 mutant line, and the Col-0 control line are presented and summarized (Figs. 3, 4; supplementary Table S2).

The high OE line AtNUDX7_OE-At2 had a significantly increased total seed weight per plant and a significant increase in number of seeds per 10 siliques (seed number) when compared to Col-0, with a seed mass and size comparable to those of Col-0. A significant increase in seed number, seed size, and mass per seed was visible in the moderate OE line, AtNUDX7_OE-At3, but not in the total seed weight. The total seed weight was determined by weighing the total seed harvested when fully mature and dried, whereas imaging was used for determination of seed number and seed size. Mass per seed was calculated as described (Materials and Methods). The difference in methodology to obtain the seed parameters might be the reason for the lack in increase in the total seed weight in spite of the increase in seed number, seed size, and mass per seed in AtNUDX7_OE-At3, but not in the total seed weight. The total seed weight was determined by weighing the total seed harvested when fully mature and dried, whereas imaging was used for determination of seed number and seed size. Mass per seed was calculated as described (Materials and Methods).

In the low OE line AtNUDX7_OE-At5, the total seed weight per plant had also significantly increased, whereas seed number, seed size, and mass per seed remained unchanged (Figs 3 and 4; supplementary Table S2). In a replicate experiment, improved seed yield parameters were measured in the same three AtNUDX7 OE_At1, AtNUDX7 OE At3, and AtNUDX7 OE_At5 lines, but were more pronounced in the high and medium OE lines than in the low OE line.

The high OE lines AtNUDX7_OE-At1 and AtNUDX7_OE-At2, and the moderate OE line AtNUDX7_OE-At3 were significantly early flowering, had a reduced number of leaves at bolting, and
Thus, the water deficit treatment (final shoot area between the standard error of the mean (reduction upon the water deficit treatment is indicated per genotype. Error bars mark the plant development or during subtle environmental fluctuations, resulting in greenhouse conditions, maintenance of energy homeostasis by overexpression of the ADP-ribose recycling step and maintenance of the energy levels by Student’s test.

Fig. 4. Heat map of seed yield and flowering time parameters in four T3 p35S::AtNUDX7 overexpression lines and the Atnudx7-1 mutant line (SALK-046441_1). Percentage increase or reduction in the parameters compared to the wild-type (wt) control is indicated. Significant differences determined with the Student’s t-test.

Fig. 5. Final shoot area of five T3 p35S::AtNUDX7 Arabidopsis overexpression lines, the Atnudx7-1 mutant line (SALK-046441_1), and the Col-0 control line under well-watered and water deficit conditions. The percentage of the final shoot area reduction upon the water deficit treatment is indicated per genotype. Error bars mark standard error of the mean (n = 16). The asterisk indicates significant difference in the final shoot area between the AtNUDX7-1 mutant line and the Col-0 control line upon the water deficit treatment (P = 0.0244, two-way analysis of variance with custom hypothesis Wald tests, corrected for multiple testing).

Evaluation of the AtNUDX7 overexpression Arabidopsis lines under mild drought stress

Mild drought stress treatment has been proposed to be a better test for superior growth performance during water deficit conditions than severe drought stress treatment that activates water saving and plant survival mechanisms (Skirycz et al., 2011). Thus, a mild drought stress experiment was set up on an automated weighing, imaging and watering (WIWAM) high-throughput phenotyping platform according to established protocols (Skirycz et al., 2011; Clauw et al., 2015). Two irrigation conditions were selected in the experiment, namely a well-watered control and a mild soil water deficit treatment, for which plants were watered up to a set soil water content based on daily target weight calculations using a gravimetric method. Seven Arabidopsis genotypes, comprising the two high overexpression lines, AtNUDX7_OE-At1 and AtNUDX7_OE-At2, the two medium overexpression lines AtNUDX7_OE-At3 and AtNUDX7_OE-At4, the low overexpression line AtNUDX7_OE-At5, the AtNUDX7-1 mutant line (SALK-046441_1), and the Col-0 control line were analysed (Table 1). The final projected shoot area of the well-watered and water deficit-treated plants was determined on the last day of the experiment (Fig. 5).

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, rosette area reduction was used as an indicator of mild drought stress response. A 20% to 40% reduction in shoot area was observed in plants growing under mild drought stress conditions when compared to the well-watered plants at the end of the experiment. Although the reduction in the final shoot area varied in the OE AtNUDX7 lines in comparison to that of the Col-0 control upon water deficit, none of the differences was statistically significant (Fig. 5). The AtNUDX7-1 mutant line had a significantly lower reduction in shoot area of 10% compared to Col-0 control plants under water deficit treatment (P = 0.0244, two-way analysis of variance with custom
hypothesis Wald tests, corrected for multiple testing with Sidak step-down), indicating tolerance to mild drought stress (asterisk, Fig. 5). However, the Atnudx7-1 mutant plants were smaller than the wild type under normal conditions (Fig. 5).

Previously, modulation of the PAR pathway via downregulation of the PARP gene expression in Arabidopsis and rapeseed had been found to give rise to plants with tolerance to a broad range of abiotic stresses, including drought (De Block et al., 2005). The drought stress treatment in that report was more severe than in this study; the plants were grown for 7 to 8 days in vitro, were then transferred to soil, and 8 to 9 days after transfer water was withheld for 6 days, whereafter they were rewatered once, and finally scored 7 to 10 days later, when control plants turned yellow. Metadata analysis with the Genevestigator software (Zimmermann et al., 2004) revealed that the AtNUDX7 gene is induced in several severe drought stress studies in Arabidopsis. Mild and severe drought stress responses are regulated by different mechanisms: whereas during mild drought stress plants maintain growth despite the reduced resources, during severe drought stress, survival mechanisms are triggered, 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 (Skirycz et al., 2011; Claeyts and Inzé, 2013). Thus, we speculate that the mild water deficit conditions used in our study investigates a trait different to that tested under the more severe drought stress conditions (De Block et al.), 2012).

**Evaluation of the AtNUDX7 overexpression maize lines under mild drought stress**

Previously, we had shown that an Arabidopsis full-length cDNA can be functional in maize and, instead of looking for its ortholog, it might be used to modulate a conserved pathway (Nelissen et al., 2012). Hence, the full-length cDNA of the AtNUDX7 gene was cloned behind the Brachypodium distachyon pBdEF1α promoter, transformed in maize, and high, medium, and low overexpression lines were analysed for their response to mild drought stress in an automated platform. 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 treatments: well-watered control and soil water deficit. The length of the 4th leaf was measured daily from the base of the plant to the leaf tip and from its appearance until maturity and was used to determine the leaf growth rate. As soon as the 4th leaf stopped growing, its blade weight, blade and sheath weights, blade width, total leaf area, and also fresh and dry weights of the seedlings were measured. Three T3 homozygous maize lines, AtNUDX7_OE-Zm1, AtNUDX7_OE-Zm2, and AtNUDX7_OE-Zm3, with a high, medium and low overexpression level of the AtNUDX7 gene, respectively, and the B104 control line were analysed. Upon water deficit, the

Fig. 6. Endpoint parameters measured in the mild drought stress experiment to compare the homozygous pBdEF1α:AtNUDX7 overexpression T3 maize lines, AtNUDX7_OE-Zm1, AtNUDX7_OE-Zm2, and AtNUDX7_OE-Zm3 with the B104 control maize under well-watered and water deficit conditions. The percentage reduction of each parameter upon the water deficit treatment is indicated per genotype. The asterisks mark significantly higher reductions of leaf 4 blade weight, blade and sheath weight, blade width, and total area of the AtNUDX7_OE-Zm2 line upon water deficit stress in comparison to the B104 control ($P = 3.17E-03, 5.55E-04, 8.65E-03, and 7.93E-04$ respectively, two-way analysis of variance with custom hypothesis Wald tests, corrected for multiple testing). Error bars indicate standard deviation ($n = 12$).
TABLE 2

PERCENTAGE OF REDUCTION IN LEAF 4 GROWTH OF T3 MAIZE LINES TRANSGENIC FOR pbU7E1a::AtNUDX7 UPON WATER DEFICIT TREATMENT

| T3 maize lines | Reduction in leaf 4 growth |
|----------------|--------------------------|
| AtNUDX7_OE-Zm1 | 19.6%                    |
| AtNUDX7_OE-Zm2 | 25.2%                    |
| AtNUDX7_OE-Zm3 | 22.6%                    |
| Wh-B104        | 20.3%                    |

high and low overexpression maize lines, AtNUDX7_OE-Zm1 and AtNUDX7_OE-Zm3, respectively, had a higher reduction percentage in all the parameters measured than the B104 wild type, although not statistically significant. However, the leaf 4 blade weight, blade and sheath weights, blade width, and total leaf area of the medium overexpression line AtNUDX7_OE-Zm2 were respectively 15%, 16%, 9% and 15% significantly more reduced under water deficit conditions than those of the B104 control (asterisks, Fig. 6), whereas the reduction in plant biomass and plant dry weight under water deficit of the AtNUDX7_OE-Zm2 line was not statistically different from that in the B104 maize control (Fig. 6). Additionally, the reduction percentage in leaf 4 growth for the three AtNUDX7 overexpression maize lines did not significantly differ from that of the B104 control upon the water deficit treatment (Table 2). The water deficit experiment on the automated platform was done in three repeats; in the previous two experiments, with fewer individuals per genotype, most parameters were not significantly different from the B104 maize control.

Therefore, our data indicate that overexpression of the AtNUDX7 gene in maize does not confer tolerance to mild drought stress. The use of a drought-stress-inducible promoter for AtNUDX7 overexpression might be more appropriate than the strong constitutive promoter used, which would allow the modulation of the PAR energy salvage pathway only on a need basis. In addition, the Arabidopsis-derived AtNUDX7 gene might not function properly in maize, because it diverges from its close maize homologs, GRMZM2G101693 and GRMZM2G175816, that have longer N-terminal extensions on their protein sequence, possibly affecting their ADP-ribose substrate affinity (supplementary Fig. S1). Preliminary experiments indicated that overexpression of the maize homologs of AtNUDX7 in maize and Arabidopsis did not confer tolerance to mild drought stress, suggesting that they probably do not participate in the mild drought stress response.

In conclusion, the different levels of constitutive overexpression of the Arabidopsis AtNUDX7 gene in Arabidopsis and also in maize did not result in a mild drought stress tolerance phenotype. However, downregulation of AtNUDX7 resulted in mild drought stress tolerance under water deficit but growth under normal conditions was reduced. We hypothesise that the AtNUDX7 component of the PAR pathway might only be involved in severe drought response mechanisms, in analogy with the PARP component (De Block et al., 2005), and that it might be worthwhile to test it in future experiments.

Materials and Methods

Plant material and growth conditions

Transgenic lines and a loss-of-function mutant line (SALK-046441) were derived from Arabidopsis thaliana (L.) Heynh. accession Col-0 and were grown either in tissue culture rooms, growth rooms, or under greenhouse conditions. Tissue culture room conditions were 21°C temperature, 16 h light/8 h darkness, and 80 μmol m⁻² s⁻¹ light intensity, whereas the growth room conditions were 22°C temperature, 55% relative humidity, 100 μmol m⁻² s⁻¹ light intensity, and 16-h light/8-h dark regime, and greenhouse conditions were 21°C temperature, 55%-60% relative humidity, 100 μmol m⁻² s⁻¹ light intensity, and 16-h light/8-h dark regime. In vitro plants in the tissue culture room were grown on full-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) sucrose, whereas plants in the growth room and greenhouse were cultured on trays containing jiffy soil (sphagnum peat moss).

The B104 maize genotypes (Hallauer et al., 1997) were grown either in growth rooms or under greenhouse conditions. The maize growth room conditions were 24°C temperature, 55% relative humidity, 230 μE m⁻² s⁻¹ light intensity, and 16-h light/8-h dark regime, whereas the greenhouse conditions were 22-26°C temperature, 45% relative humidity, 300 μE m⁻² s⁻¹ light intensity, and 16 h light/8-h dark regime. Maize seeds were sown on trays containing jiffy soil (sphagnum peat moss) and placed in the maize growth room, where the seedlings grew for 2 to 3 weeks, whereafter they were transferred to larger soil pots and placed in the greenhouse until maturity.

Arabidopsis and maize transformation, PAT assay, and T-DNA integrity check

Arabidopsis plants were transformed with the AtNUDX7 overexpression construct by means of the Agrobacterium tumefaciens floral dip transformation method (Clough and Bent, 1998). Immature embryos of the B104 maize inbred line were transformed with the AtNUDX7 overexpression construct according to Coussens et al. (2012), with the exception that 2,4-D had been replaced by dicamba (3.32 mg/l). The T-DNA intactness was determined by PCR analysis with forward primers binding to either the pBdEF1x or the pZmUBIL promoter (Coussens et al., 2012) and reverse primers binding to the T33S terminator region to confirm that a complete AtNUDX7 gene had been inserted. Transgenic plant materials were selected with the bar marker gene, of which the activity was identified by detection of the PAT protein with the PAT assay kit (AgraStrip®LL Strip test kit; Romer Labs®, Union, MO, USA), according to the manufacturer’s instructions.

qPCR expression analysis

RNA was isolated from 2-week-old Arabidopsis T3 seedlings (consisting of four pools of five seedlings for the AtNUDX7 overexpression lines and three pools of five seedlings for the Atnudx7-1 mutant line) and from 10- to 12-day-old division zone tissue of the 4th leaf of T1 maize (consisting of five pools of three transgenic (+) and the same for the azygous (-) maize seedlings) with the RNeasy Plant Mini Kit (Qiagen) and the cDNA prepared with the SuperScript III First-Strand Synthesis System for reverse-transcription PCR (Invitrogen), according to the manufacturers’ protocols. qPCR experiments were carried out in a LightCycler®480 Real-Time SYBR Green PCR System (Roche) and all reactions were done in three technical replicates. For the Arabidopsis samples, the expression levels were normalized to the reference genes SAND (AT2G28390), PP2A (AT1G13320), and YLS8 (AT5G08290), whereas for the maize samples, the expression levels were normalized to the reference genes 18SrRNA and EF1x (GenBank accession X00794.1 and NM_001112117.1, respectively).

Measurement of seed yield and yield-associated parameters

Seed yield parameters were measured as described (Van Daele et al., 2012). To determine the total seed weight, 25 plants per genotype were grown for approximately 3.5 months under greenhouse conditions until the seeds were fully mature and dried; all the seeds were harvested, cleaned, and weighed. The mean seed weight per plant was then established and indicated as the total seed weight per plant. For the seed size, the seed area of 200-400 seeds per plant of 10 plants per genotype was measured by applying an image analysis macro (supplementary Fig. S2).
on the ImageJ software (http://imagej.nih.gov/ij/). To determine whether an increase or decrease in the seed size was accompanied by an increase or decrease in mass, the mass per seed of the genotypes was assessed by dividing the mass of seeds by their total number. More precisely, the mass of 200-400 seeds per plant and 10 plants per genotype was obtained by weighing the seeds on a scale and the respective number of seeds counted through the image analysis macro on the ImageJ software. First, the scale of the pictures was manually set in the ImageJ software. With a single macro (supplementary Fig. S2), all pictures with a JPG file extension in a selected folder were automatically opened and cropped. Next, the background was removed by adjusting the Brightness/Contrast to a minimum of 0 and a maximum of 72. Subsequently, the images were saved and processed to binary values to measure the projected seed area with a size from 0.02 to infinity and a circularity of 0.00-1.00. Hereafter, the number of seeds per 10 siliques, termed seed number, was counted from 16 plants per genotype grown under greenhouse conditions for 2 months until the plants had reached maturity. Seeds from 10 yellow or brown unopened siliques from the middle of the main inflorescence of each plant were harvested and counted by means of the image analysis macro on the ImageJ software as described above. To determine the flowering time and number of leaves at bolting, 25 plants per genotype were grown under growth room conditions. Flowering time was calculated as the difference between the first day of appearance of the flower bud and the day of sowing and indicated as days after sowing (DAS) as unit. The number of leaves (excluding the cotyledons) at bolting was counted at the first day of flower bud appearance. To determine the inflorescence height, the length of a fully stretched primary inflorescence was recorded of 16 plants per genotype, grown under greenhouse conditions for 2 months until the plants had reached maturity and no increase in length was observed anymore.

Mild drought stress experiment in an automated platform for Arabidopsis

The experiment was set up on an automated WIWAM XY platform (www.wiwam.com) for high-throughput phenotyping according to established protocols (Skirycz et al., 2011; Clauw et al., 2015). The WIWAM system is placed in an Arabidopsis growth room with 21°C temperature, 55% relative humidity, 16-h day/8-h night regime, and 100 μmol m⁻² s⁻¹ light intensity. Seeds were stratified for 2 days before sowing in pots containing 80 to 90 g soil. Seeds of the same age were used for all genotypes and watering was carried out daily at the same time to avoid biases. Sixteen seedlings per genotype were grown for the well-watered treatment and 16 seedlings per genotype for the water deficit treatment. Soil water content of the well-watered control plants was set at a constant value of 2.19 g water per g dry soil during the entire experiment. For the mild drought stress treatment, plants were grown for 9 days under well-watered conditions; then the daily target soil water content was reduced and maintained at 1.19 g water per g dry soil until the end of the experiment (21 days after sowing). Pots were randomized on the WIWAM platform on a daily basis. On the last day, the final shoot area was determined by processing the rosette images.

Mild drought stress experiment in an automated platform for maize

The AtNUDX7 overexpression maize lines were analysed in an automated platform for their response to mild drought stress by daily weighing and watering of the soil pots. A soil water content of 2.40 g and 1.00 g water per g dry soil was chosen for the well-watered treatment and the soil water deficit treatment, respectively, corresponding to a soil water potential of -0.01 MPa and -6 MPa, respectively. Plants were randomised on the automated platform on a daily basis. Seeds of the same age were used for all genotypes and watering was carried out daily at the same time to avoid biases. Per genotype, 18 seedlings were grown in the well-watered and the water deficit treatments. The plants were allowed to develop for approximately 1 month and harvested when leaf 4 was fully mature and no longer increased in length. Several parameters were determined from leaf 4, which is the first leaf growing autonomously by photosynthesis assimilation and independently of kernel storage. The few plants with more than a 5-day delay in leaf 4 appearance were not used or did not germinate, hence, 12 to 18 plants per genotype and treatment were analysed. The length of leaf 4 was measured daily from the base of the plant to the leaf tip and from its appearance until its harvest to determine the leaf growth rate (expressed in mm/h) as (L5-L1)/(Tp5-Tp1), where L1 and L5 are the lengths of leaf 4 measured in mm at time point (T) 1 and 5, respectively. To compare the growth performance between the genotypes, the reduction percentage in the growth rate of leaf 4 under the water deficit condition was determined per genotype as follows: (average leaf growth rate under well-watered condition - average leaf growth rate under water deficit condition) / (average leaf growth rate under well-watered condition) *100. The endpoint parameters measured upon harvesting include final blade weight, final blade and sheath weight, final blade width and total area of leaf 4 and the total plant biomass and the total plant dry weight.

Data analysis

Seed yield and yield-associated parameters

Statistical data analysis for the seed yield and yield-associated parameters was carried out with the Student’s t-test. P-values were corrected for multiple testing with the Bonferroni correction (supplementary Table S2).

Arabidopsis mild drought stress experiment

Statistical data analysis was carried out for the final shoot areas measured in the Arabidopsis mild drought stress experiment on the WIWAM automated platform with the aim to determine the different effects upon mild drought stress of each transgenic line when compared to the 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% significance 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 with Sidak step-down as implemented in the SAS software (Version 9.4 of the SAS System for Windows 7 64bit; Copyright © 2002-2012 SAS Institute Inc. Cary, NC, USA; www.sas.com). The analysis was done with the GLM procedure and correction for multiple testing of the interaction effect with the MULTTEST procedure.

Maize mild drought stress experiment

All the endpoint growth parameters measured in the mild drought stress experiments were analysed statistically with the aim to determine the different effects upon water deficit of each transgenic line compared to the control line as described for Arabidopsis except that family-wise error rates were calculated based on the maxT procedure as implemented in SAS.

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