Functional analysis of Arabidopsis poly(ADP-ribose) polymerases in stress response and seed germination

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1 General introduction

1.1 Poly(ADP-ribosy)lation and poly(ADP-ribose) polymerases in humans

Poly(ADP-ribosy)lation describes the rapid and transient posttranslational transfer of negatively charged ADP-ribose molecules onto target proteins (Fig. 1.1). First, ADP-ribose moieties are covalently attached to the target proteins. Subsequently, poly(ADP-ribose) chains of various length and branching complexity are synthesized forming O-glycosidic bonds between the ADP-ribose molecules (Hayashi *et al.*, 1983, Kiehlbauch *et al.*, 1993, D'Amours *et al.*, 1999, Gibson & Kraus, 2012). NAD^+ serves as substrate for poly(ADP-ribose) synthesis, and nicotinamide is formed as a concomitant product (Alvarez-Gonzalez & Mendoza-Alvarez, 1995, D'Amours *et al.*, 1999). In the following, the synthesized ADP-ribose polymers constitute a spatiotemporal interaction platform to modulate cellular responses. Several poly(ADP-ribosyl)ation site-containing proteins and poly(ADP-ribose) binding motif-containing proteins have been identified acting as poly(ADP-ribose) readers translating the poly(ADP-ribose) signal into cellular responses (Gupte *et al.*, 2017, Schuhwerk *et al.*, 2017). These proteins are components of essential cellular processes such as DNA repair, cell cycle checkpoint, chromatin remodeling, signaling, protein degradation, and cell death (Pleschke *et al.*, 2000, Mendoza-Alvarez & Alvarez-Gonzalez, 2001, Chang *et al.*, 2004, Haince *et al.*, 2007, Aguilar-Quesada *et al.*, 2007, Kanai *et al.*, 2007, Kedar *et al.*, 2008, Ahel *et al.*, 2008, Gagné *et al.*, 2008, Ahel *et al.*, 2009, Wang *et al.*, 2009, Kang *et al.*, 2011, Min *et al.*, 2013, Liu *et al.*, 2013, Aredia & Scovassi, 2014). The amino acids modified by poly(ADP-ribosyl)ation in the target proteins are predominantly glutamic acid and aspartic acid. Modification is performed via ester linkage (Tao *et al.*, 2009, Crawford *et al.*, 2018, Cohen & Chang, 2018). Recently, the modification of serine residues of target proteins by O-glycosidic bonds has been shown (Bonfiglio *et al.*, 2017). The enzymatic modification of lysine residues is currently a matter of debate (Cohen & Chang, 2018, Crawford *et al.*, 2018).

The enzymes catalyzing poly(ADP-ribosyl)ation are named poly(ADP-ribose) polymerases (PARPs). In the human genome 17 PARP genes have been identified (Amé *et al.*, 2004, Otto *et al.*, 2005, Hottiger *et al.*, 2010). They constitute a heterogeneous protein family with distinct structural domains, subcellular localizations, activities, and functions (Amé *et al.*, 2004). According to their structures and functions, the different PARP protein were classified as DNA-dependent PARPs (PARP1, PARP2, PARP3) which are activated upon DNA damage; Tankyrases (PARP5a, PARP5b) which are involved in telomere homeostasis, DNA repair, mitotic spindle formation, and cellular signaling; Cys-Cys-Cys-His zinc finger and WWE poly(ADP-ribose)-binding domain-containing PARPs (PARP7, PARP12, PARP13.1, PARP13.2); and poly(ADP-ribose)-binding macrodomain-containing PARPs (PARP9, PARP14, PARP15) (Amé *et al.*, 2004, Vyas *et al.*, 2013, Gupte *et al.*, 2017, Crawford *et al.*, 2018). The last two groups are defined according to their protein structure (Vyas *et al.*, 2013). So far, little is known about the function of their members (Vyas *et al.*, 2013, Gupte *et al.*, 2017). The remaining PARP proteins are grouped as unclassified PARPs as their domain architecture differs from each other and from the other groups (Vyas *et al.*, 2013). To catalyze poly(ADP-

ribosyl)ation, the catalytic triad motif H-Y-E within the catalytic PARP domain is essential but not sufficient (Rolli *et al.*, 1997, Vyas *et al.*, 2014). Therefore, only PARP1, PARP2, and the Tankyrases are *bona fide* PARPs. PARP3, PARP4, PARP6, PARP10, PARP14, PARP15, and PARP16 were found to exhibit mono(ADP-ribosyl)ation activity catalyzing the addition of a single ADP-ribose molecule onto target proteins. No catalytic activity has been found for PARP9 and PARP13 (Vyas *et al.*, 2014). PARP proteins were found in all kingdoms of life except yeast (Slade *et al.*, 2011, Perina *et al.*, 2014). In the following the DNA-dependent PARP proteins PARP1 through PARP3 will be introduced, as their *Arabidopsis* homologues are the subject of this work.

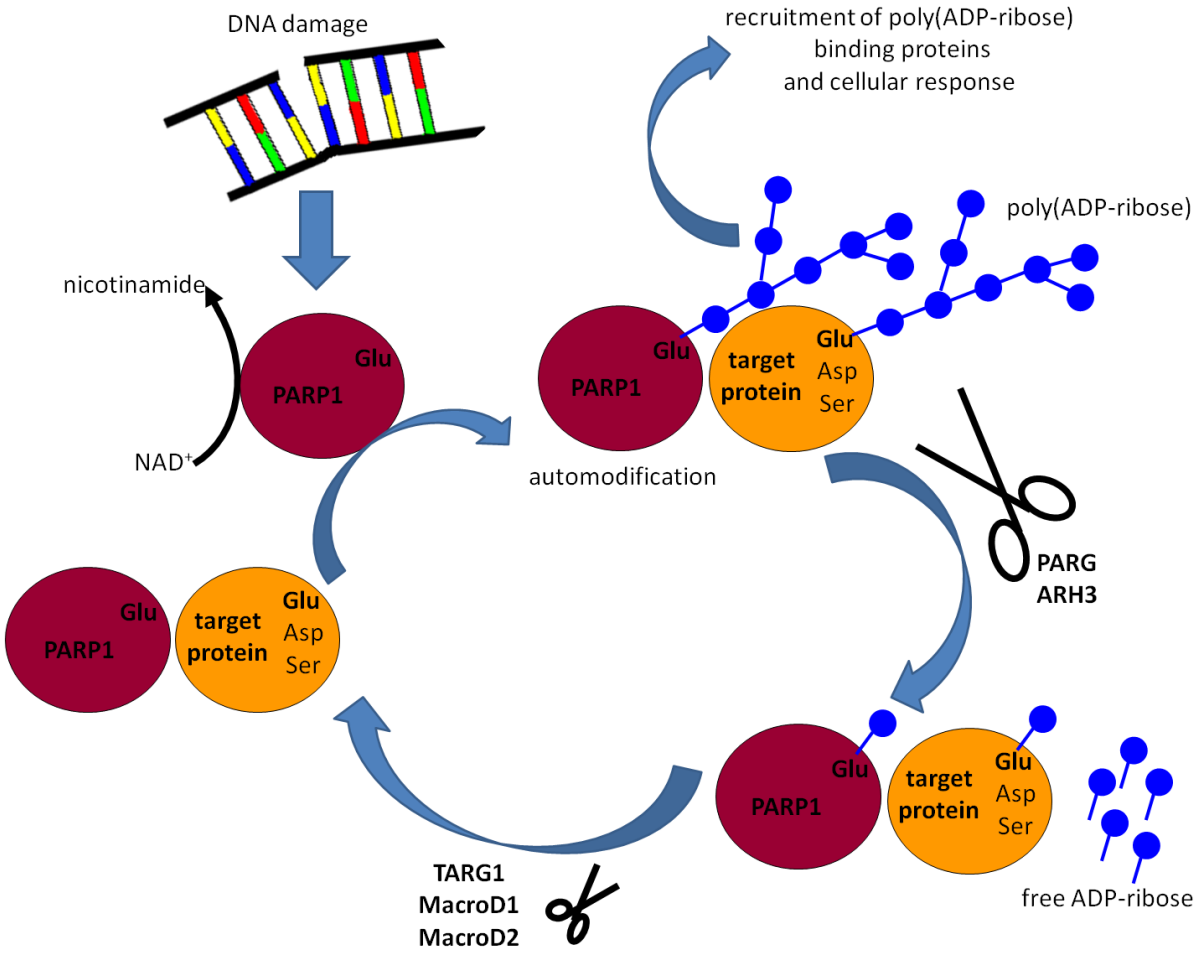

Figure 1.1 Cellular 'life-cycle' of ADP-ribose polymers. Activated upon DNA strand break, PARP1 and PARP2 catalyzes the transfer of ADP-ribose molecules onto itself and other target proteins. The generated ADP-ribose polymers serve as scaffolds recruiting proteins containing various poly(ADP-ribose)-binding domains, which initiates cellular responses. The ribose-ribose bonds are hydrolyzed by PARG, ARH3, TARG1, MacroD1, and MacroD2, allowing rapid poly(ADP-ribose) turnover and controlled cellular signaling processes.

1.1.1 PARP1, PARP2, and PARP3 are activated upon DNA damage

The best-studied PARP protein by now is the founding member of the protein family, PARP1. It is the most abundant PARP enzyme in mammalian cells and accounts for approximately 85% of poly(ADP-ribosyl)ation activity (Amé *et al.*, 2004). PARP1 is a 113 kDa protein with a well-defined modular architecture (Fig. 1.2) (Amé *et al.*, 2004). Overall, the PARP1 protein possesses an N-terminal DNA interaction domain, a central automodification domain and a C-terminal catalytic domain (Kraus & Lis, 2003, Amé *et al.*, 2004, Schuhwerk *et al.*, 2017). This catalytic region was found to be highly conserved in mammals, particularly the 50 amino acid-spanning so called "PARP signature" (Kraus & Lis, 2003). The "PARP signature" forms the active site of the PARP proteins and exhibits 100% conservation among vertebrates and 92% among all species (Kraus & Lis, 2003). Additionally, a WGR domain which is named after its repeating amino acid motif (W-G-R) is located in the catalytic region. Apart from the glutamic acid moieties that allow automodification of PARP1, the central automodification domain contains a BRCT (breast cancer susceptibility gene 1 C-terminus) domain that is known to be involved in protein-protein interactions (Rouleau *et al.*, 2010). This domain is commonly found in DNA damage response proteins (Rouleau *et al.*, 2010). Three zinc fingers, a bipartite nuclear localization signal, and a caspase (cysteine proteases cleaving at an aspartic acid) cleavage site form the DNA-binding domain. The two homologous zinc fingers Zn1 and Zn2 are able to bind to DNA single and double strand breaks and abnormal DNA structures (Ikejima *et al.*, 1990, Gradwohl *et al.*, 1990, Langelier *et al.*, 2012). The third zinc finger Zn3 is structurally unique and required for the activation of DNA-dependent catalytic activity of PARP1 (Langelier *et al.*, 2010). Upon association with damaged DNA, Zn3 and WGR domain refold to allow enhanced interdomain contacts and facilitate PARP1 catalytic activity (Langelier *et al.*, 2012). Additional conformational changes within PARP1 lead to further activation (Dawicki-McKenna *et al.*, 2015, Eustermann *et al.*, 2015, Steffen *et al.*, 2016, Gupte *et al.*, 2017).

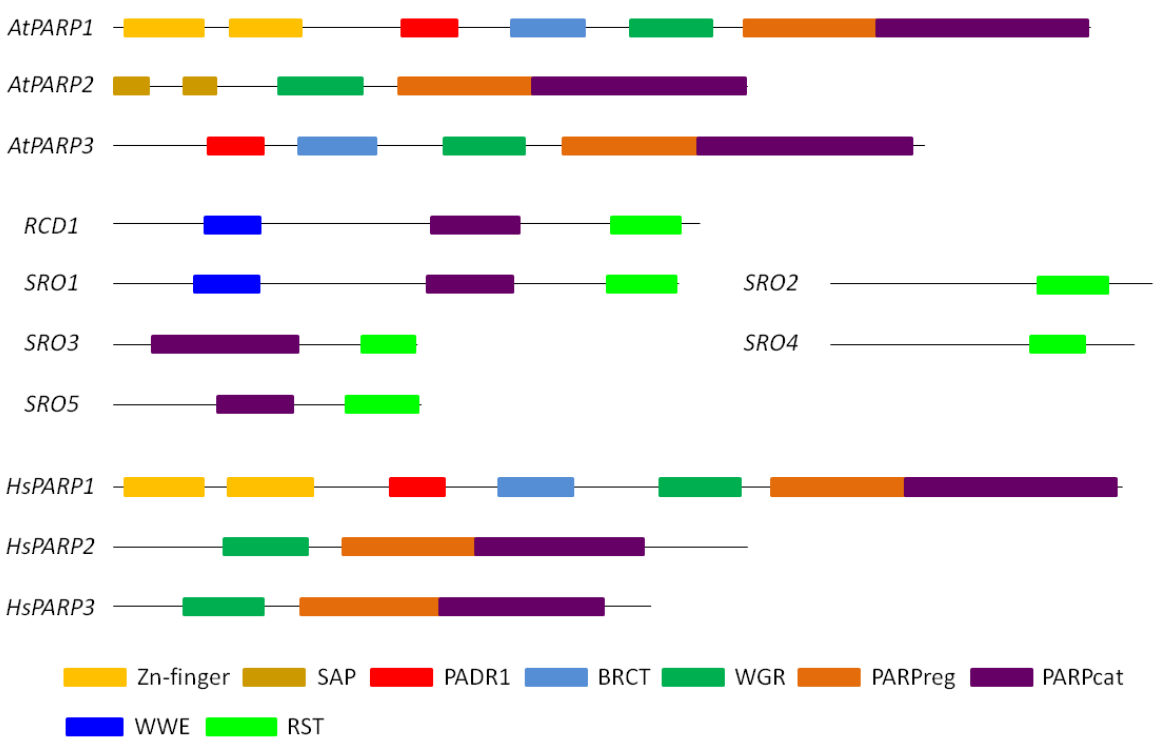

Figure 1.2 Schematic representation of domains in animal and plant PARP proteins. Domains were defined according to Pfam 27.0 and are displayed as colored boxes. ExPASY Prosite indicated the existence of PARPcat domains also in SRO2 and SRO4 which are absent in the Pfam analysis. Figure taken from Rissel et al., 2017b.

Upon activation, PARP1 automodification and poly(ADP-ribosyl)ation of other target proteins take place, recruiting DNA damage response proteins to the lesions (Fig. 1.1). The negative charges of automodified PARP1 proteins repulse the proteins from the DNA to allow access for the DNA damage repair machinery (Schuhwerk *et al.*, 2017). PARP1 has been found to be involved in virtually all DNA damage response pathways. Sensing DNA single strand breaks, PARP1 automodification recruits XRCC1 which scaffolds the assembly and activation of the base excision repair machinery and the subsequent repair of the small lesions caused by oxidation or alkylation (Masson *et al.*, 1998, D'Amours *et al.*, 1999, Okano *et al.*, 2003, El-Khamisy *et al.*, 2003). Additionally, PARP1 was implied to be involved in homologous recombination (HR) repair of DNA double strand breaks (DSB), since components of the HR machinery such as MRE11 (mitotic recombination 11) and ATM (ataxia telangiectasia-mutated) are rapidly recruited to DNA damage sites in a poly(ADP-ribose)-dependent manner (Haince *et al.*, 2007, Aguilar-Quesada *et al.*, 2007, Haince *et al.*, 2008). In line with this, PARP1 acts as a facilitator of HR repair at stalled DNA replication forks, as PARP1 binding to the stalled fork prevents the assembly of the non-homologous end joining (NHEJ) complex (Hochegger *et al.*, 2006, Beck *et al.*, 2014, Schuhwerk *et al.*, 2017). Contrastingly, PARP1 was also shown to interact with Ku70/Ku80 proteins which are known key players of NHEJ (Galande & Kohwi-Shigematso, 1999, Beck *et al.*, 2014). However, its precise role in NHEJ is intricate as classical NHEJ still proceeds in the absence of PARP1 (Beck *et al.*, 2014).

PARP1 competes with Ku proteins for DNA binding. Therefore, PARP1 appears not to be a core component of classical NHEJ but of an alternative NHEJ pathway (Wang *et al.*, 2006). In line with its function in DNA damage response, PARP1 also acts in chromosome remodeling, establishment and maintenance of heterochromatin, and transcriptional regulation (Frizzell *et al.*, 2009, Gibson & Kraus, 2012, Gupte *et al.*, 2017). In addition to its direct function in DNA damage response, PARP1 has also been implicated in various processes of cell death, such as apopotosis, parthanatos, programmed necrosis, and autophagy (Aredia & Scovassi, 2014). The common denominator of all these processes is an excessive formation of poly(ADP-ribose). During apoptosis, the increased PARP1 activity depletes the cellular energy pool, leading to an activation of caspases. The caspase-mediated cleavage of PARP1 is a hallmark of apoptosis, leading to the accomplishment of apoptotic cell death. Upon excessive activation of PARP1, poly(ADP-ribose) molecules appear to leave the nucleus and invade the mitochondria. Here, poly(ADP-ribose) is bound by AIF (apoptosis-inducing factor) which in turn is translocated to the nucleus where it promotes DNA fragmentation leading to parthanatos. During programmed necrosis and autophagy, PARP1 interacts with key components of both pathways, hence promoting cell death (Aredia & Scovassi, 2014).

PARP2 was identified in a study initiated to resolve the source of residual poly(ADP-ribose) formation in cells lacking PARP1 (Amé *et al.*, 1999). It accounts for approximately 15% of the overall cellular poly(ADP-ribose) synthesis (Amé *et al.*, 2004). The domain architecture of PARP2 differs from PARP1 (Fig. 1.2) (Amé *et al.*, 1999, Kutuzov *et al.*, 2014, Riccio *et al.*, 2016). PARP2 consists of an N-terminal region (NTR) containing a nuclear and a nucleolar localization signal and a caspase cleavage site, a WGR domain, and a catalytic PARP domain (Amé *et al.*, 1999, Kutuzov *et al.*, 2014, Riccio *et al.*, 2016). The NTR lacks the zinc finger domain found in PARP1 and is an intrinsically disordered protein region allowing flexible adaptation to various damaged DNA structures such as gaps, flaps, and recombination intermediates (Riccio *et al.*, 2016). However, the NTR does not localize to DNA damage sites on its own. The WGR and the catalytic domain are necessary and sufficient to direct PARP2 to DNA damage sites. PARP2 exhibits lower kinetics in recruiting to DNA damage sites than PARP1. Additionally the WGR domain is involved in the activation of poly(ADP-ribosyl)ation by PARP2 (Riccio *et al.*, 2016). The catalytic domains of PARP1 and PARP2 exhibit 69 % homology (Amé *et al.*, 1999). Additionally, the catalytic domain was found to contribute to the binding affinity and the localization to the DNA damage sites (Riccio *et al.*, 2016). Even though PARP2 lacks a conserved automodification domain, it is capable of automodification (Kutuzov *et al.*, 2014). PARP1 and PARP2 were found to form heterodimers and poly(ADP-ribosyl)ate each other (Schreiber *et al.*, 2002). Additionally, PARP2 has been shown to interact with an overlapping but distinct number of proteins compared to PARP1 (Isabelle *et al.*, 2010). In line with this, PARP2 was found to be an actor in response to DNA single strand break as PARP1 (Schreiber *et al.*, 2002, Hanzlikova *et al.*, 2017). Moreover, redundancy between PARP1 and PARP2 has been shown in cell survival and at stalled replication forks (de Murcia *et al.*, 2003, Bryant *et al.*, 2009). Moreover, PARP2 has also been found to be involved in the choice of DSB repair modes, channeling repair towards HR or an alternative

NHEJ (Fouquin *et al.*, 2017). The exact roles of PARP1 and PARP2 in DNA DSB response are still to be further elucidated.

To date, relatively little information is available for PARP3. Similar to PARP1 and PARP2, it was found to be activated upon DNA strand breaks (Gibson & Kraus, 2012). Subsequently, PARP3 confers mono(ADP-ribosyl)ation activity (Amé *et al.*, 2004). It was found to function in DNA DSB repair (Boehler *et al.*, 2011, Rulten *et al.*, 2011). Thereby, PARP3 is efficiently recruited to DNA damage sites and interacts with proteins of the classical NHEJ pathway (Boehler & Dantzer, 2011, Rouleau *et al.*, 2007). Additionally, it was shown to prevent DNA end resection and as a consequence promotes classical NHEJ (Beck *et al.*, 2014). Apart from this, PARP3 was found to mono(ADP-ribosyl)ate PARP1 and kick starts its activation (Loseva *et al.*, 2010).

1.1.2 The removal of poly(ADP-ribose) is a two-step process

So far, five enzymes have been identified to degrade poly(ADP-ribose) chains allowing rapid and dynamic poly(ADP-ribose) turnover and therefore tightly controlled cellular signaling (Fig. 1.1) (Gupte *et al.*, 2017, Schuhwerk *et al.*, 2017). The key enzyme among them is poly(ADP-ribose)glycohydrolase (PARG) (Lin *et al.*, 1997, Slade *et al.*, 2011, Barkauskaite *et al.*, 2013). PARG possesses exoglycosidic and endoglycosidic activity, hydrolyzing terminal and internal ribose-ribose linkages, respectively, releasing ADP-ribose oligomers (Thomassin *et al.*, 1992, Brochu *et al.*, 1994, Barkauskaite *et al.*, 2013). PARG enzymes, however, are not capable of cleaving the ester bond between the ADP-ribose molecule and the acceptor amino acids of the target protein. By now, only one PARG gene has been identified in mammals, but five protein isoforms resulting from alternative splicing have been found in different cellular compartments (Meyer-Ficca *et al.*, 2004, Meyer *et al.*, 2007). PARG protein function is essential, as genetic deletions of PARG in mice or drosophila are lethal (Koh *et al.*, 2004, Hanai *et al.*, 2004). Similar to PARG, ADP-ribosyl-hydrolase 3 (ARH3) was found to exhibit poly(ADP-ribose)-hydrolyzing activity in the nucleus, the cytosol and the mitochondrium (Fig. 1.1) (Oka *et al.*, 2006, Niere *et al.*, 2008). ARH3 shares only little structural similarity with PARG. It accounts for 10% of the poly(ADP-ribose)-hydrolyzing activity in the cell (Oka *et al.*, 2006, Niere *et al.*, 2008). The macrodomain-containing proteins terminal ADP-ribose protein glycohydrolase 1 (TARG1) and macrodomain-containing proteins D1 and D2 (MacroD1, MacroD2) possess the ability to hydrolyze the ester bond between the ribose and the acceptor amino acid (Fig. 1.1) (Jankevicius *et al.*, 2013, Rosenthal *et al.*, 2013, Sharifi *et al.*, 2013).

1.2 Poly(ADP-ribosyl)ation in plants

1.2.1 Three PARP proteins have been identified in the model plant *Arabidopsis thaliana*

In the late 1970s poly(ADP-ribosyl)ation activity was first shown in higher plants by the incorporation of [^{3}H]NAD into nuclei of onion and wheat embryo cells and onion meristematic root tissues (Payne & Bal, 1976, Whitby & Whish, 1977, Whitby & Whish, 1978, Whitby *et al.*, 1979). This incorporation was found to be an enzymatic reaction covalently linking poly(ADP-ribose) molecules to carboxyl groups of the target proteins (Willmitzer, 1979). Lysine-rich histones H1, H2A and H2B, but not arginine-rich histones H3 and H4 were identified as acceptor proteins for poly(ADP-ribose) molecules (Whitby *et al.*, 1979, Willmitzer, 1979). In addition, automodification of a 114 to 116 kDa protein was described in the early times of poly(ADP-ribose) research in plants (Willmitzer, 1979, Chen *et al.*, 1994).

The first *PARP* gene identified in plants was *Arabidopsis thaliana APP* (At4g02390), now renamed as *AtPARP2* (Lepiniec *et al.* 1995). The *APP* cDNA was identified due to its 62% similarity to the catalytic domain of human PARP1 during experiments carried out to identify *Arabidopsis* proteins that allow yeast cells to grow under different stress conditions. The PARP signature is conserved in APP. Apart from that, a nuclear localization signal and an automodification domain were found. In contrast to human PARP1, which possesses N-terminal zinc-finger domains, APP contains a N-terminal SAP domain (Fig. 1.2) (Lepiniec *et al.*, 1995). The SAP domain is a putative DNA-binding domain involved in nucleic acid metabolism, which has been named after three proteins known to contain it (SAF-A/B, Acinus and PIAS) (Aravind & Koonin, 2000). In total, the APP protein consists of 637 amino acids and has a size of 72 kDa. A single copy of the *APP* gene was mapped to chromosome 4 (Lepiniec *et al.*, 1995). Expression of *APP* in yeast cells revealed poly(ADP-ribosyl)ating activity. Polymers of up to 40 ADP-ribosyl residues were formed, while the main polymer size was 10 to 15 residues (Babiychuk *et al.*, 1998). APP expressed in yeast cells was found to be localized in the nucleus by using anti-APP antisera. The poly(ADP-ribosyl)ating activity was reduced by the application of the PARP inhibitors 3-aminobenzamide (3AB) and nicotinamide. In plants, APP peptide (Met^1 to Pro^{407})-GUS fusions were found to be active in cotyledons and roots. Due to the transparency of the root material, GUS activity could be localized to the nucleus (Babiychuk *et al.*, 1998). In the following, APP will be called AtPARP2 due to its structural similarities to human HsPARP2 (Fig. 1.2). Nuclear localization of AtPARP2 was confirmed by transient expression of a AtPARP2-GFP construct in *Nicotiana benthamiana* and in an *Arabidopsis* cell suspension culture (Song *et al.*, 2015, Pham *et al.*, 2015). In addition to its nuclear localization, AtPARP2 has also been suggested to be additionally localized in chloroplasts (Pham *et al.*, 2015). Promoter-GUS fusions and RNA *in situ* hybridization studies localized *AtPARP2* expression to imbibed seeds, the vegetative meristem of the shoot apex, stamen of open flowers, and late stages of embryo development (Pham *et al.*, 2015).

AtPARP1 (At2g31320) was identified in a screen for ionizing radiation-induced genes in *Arabidopsis thaliana* (Doucet-Chabeaud *et al.*, 2001). AtPARP1 consists of 983 amino acids and exhibits conserved structural motifs and invariant amino acids compared to HsPARP1. Similar to HsPARP1, AtPARP1 contains a conserved catalytic domain, zinc finger motifs, and a nuclear localization motif. The central automodification domain is less conserved, but glutamic acid residues are present allowing auto poly(ADP-ribosyl)ation. Apart from this, the BRCT-domain, allowing protein-protein interactions, is also less conserved. All in all, the structural similarities between AtPARP1 and HsPARP1 indicate functional similarities (Doucet-Chabeaud *et al.*, 2001). Like AtPARP2, AtPARP1-GFP was found to localize to the nucleus in *Nicotiana benthamiana* and in *Arabidopsis* cell suspension culture (Song *et al.*, 2015, Pham *et al.*, 2015). Additionally, PARP1-GFP was detected in chloroplasts and mitochondria when expressed in *Arabidopsis* protoplasts (Pham *et al.*, 2015). Fusion of the putative PARP1 promoter with *GUS* and RNA hybridization study revealed expression of *AtPARP1* in roots, the apices of inflorescences, the vegetative meristem, and during late stages of embryo development (Pham *et al.*, 2015).

In addition to the Arabidopsis PARP proteins, PARP1 and PARP2 proteins were characterized in maize (Mahajan & Zuo, 1998, Babiychuk *et al.*, 1998). Proteins similar to HsPARP1 and AtPARP1 have been identified simultaneously by two different groups. Mahajan and Zuo identified a protein that showed around 40% identity and 50% similarity to most vertebrate PARP proteins known at that time. This 115 kDa protein consists of a 988 amino acid sequence encoding for zinc fingers, a putative nuclear localization signal, and a NAD^+-binding domain (Mahajan & Zuo, 1998). The protein showed disulfide-mediated autodimerization; PARP activity was assessed by an activity blot. The formation of 45-60 poly(ADP-ribose) residues was demonstrated on histones, and protein activity was blocked by known inhibitors of mammalian PARP proteins, such as aminonaphtalimide, aminobenzamide, benzamide, 3-methoxybenzamide (3MB), and nicotinamide (Mahajan & Zuo, 1998). Two other proteins with 55% identity and 64% similarity to the human PARP signature were identified in two different maize varieties and called ZAP1 and ZAP2 (Babiychuk *et al.*, 1998). Both proteins differ from each other in the size of the N-terminal domain. Compared to ZAP2, ZAP1 lacks 11 amino acids, leading to a potentially non-functional Zn1 domain (Babiychuk *et al.*, 1998). The consequences of the divergent sequences have not been addressed so far. The *ZAP2* sequence is equal to ZmPARP1 identified by Mahajan and Zuo (1998). A ZmPARP2 protein was also identified by Babiychuk and collegues and called NAP. ZmPARP2 is 653 amino acids long and shows a similar structure as AtPARP2 (Babiychuk *et al.*, 1998).

So far, little is known about *AtPARP3* which was identified by *Arabidopsis* genome analysis. It was found to be mainly expressed in seeds, but also in seedlings and roots of adult plants (Hunt *et al.*, 2004, Feng *et al.*, 2015, Pham *et al.*, 2015). In addition, expression of *AtPARP3* was also found to be strongly induced in leaves by high levels of abiotic stress [i.e., 3 µM paraquat, 250 mM NaCl, high light (1600 µmol m^{-2} s^{-1}), or desiccation] (Ogawa *et al.* 2009). PARP3-GFP fusions were found to localize mainly to the nucleus, but also to the cytosol in

Arabidopsis cell suspension cultures and protoplasts (Pham *et al.*, 2015). The barley PARP3 homolog HvPARP3 was described to show a high level of sequence similarity with PARP3 proteins from *Arabidopsis*, rice, and human (Stolarek *et al.*, 2015).

Unfortunately, the nomenclature of *Arabidopsis PARP* genes has been inconsistent in the literature (Tab. 1.1). In this thesis, the following unified nomenclature is used, which we recently established (Rissel *et al.*, 2017): *AtPARP1* refers to the gene At2g31320, *AtPARP2* refers to At4g02390/*APP*, and *AtPARP3* refers to At5g22470.

Table 1.1 *PARP1* and *PARP2* nomenclature has been inconsistent in the literature. Table taken from Rissel et al., 2017b.

Reference	**At2g31320**	**At4g02390**
Zhang *et al*. (2015) Sci. Rep. 5:15892	AtPARP1	AtPARP2
Pham *et al*. (2015) Plant Mol. Biol., 89: 319-338	AtPARP2	AtPARP1
Song *et al*. (2015) PLoS Genet. 11(5): e1005200	AtPARP1	AtPARP2
Feng *et al*. (2015) PLoS Genet. 11(1): e1004936	AtPARP1	AtPARP2
Boltz *et al*. (2014) PLoS One, 9: e88872	AtPARP2	AtPARP1
Jia *et al*. (2013) Plant Mol. Biol., 82: 339-351	AtPARP1	AtPARP2
Schulz *et al.* (2012) PLoS One, 7 (5): e37287	AtPARP2	AtPARP1
Lamb *et al*. (2012) Cell Mol. Life Sci., 69: 175-189	AtPARP2	AtPARP1
Briggs and Bent (2011) Trends Plant Sci., 16: 372-380	AtPARP2	AtPARP1
Pellny *et al*. (2009) Molecular Plant, 2: 442-456	AtPARP1	AtPARP2
Ogawa *et al*. (2009) Plant J., 57: 289-301	AtPARP1	AtPARP2
Vanderauwera *et al*. (2007) PNAS, 104: 15150-15155	AtPARP2	AtPARP1
De Block *et al*. (2005) Plant J., 41: 95-106	AtPARP2	AtPARP1
Doucet-Chabeaud *et al.* (2001) Mol. Genet. Genomics 265: 954-963	AtPARP1	AtPARP2

1.2.2 In contrast to mammals, plants possess more than one *PARG* gene

In total, three genes with homology to human *PARG* have been identified in *Arabidopsis thaliana* (Hunt et al. 2004). For one of them no ESTs or cDNA could be found so far. Therefore, this gene is classified as a pseudo-gene. The other two genes, *PARG1* (At2g31870) and *PARG2* (At2g31865), are localized in tandem on chromosome 2 (Hunt *et al.*, 2004).

In Arabidopsis, both PARG proteins are localized in the nucleus, the cytoplasm, and at the plasma membrane (Feng *et al.*, 2015, Zhang *et al.*, 2015). PARG1 (also known as TEJ) was first identified in a genetic screen for altered circadian period length in Arabidopsis (Panda *et al.*, 2002). Mutant plants carrying a G262E substitution in the PARG1 protein showed a prolonged free-running period concerning expression of circadian clock-controlled genes, and cotyledon and leaf movement compared to wild type plants. Additionally, these *tej* mutants flowered earlier. These phenotypes suggest a general clock defect, rendering PARG1 an important component that influences clock function in plants. Poly(ADP-ribose) polymer levels were found to be increased in the *tej* mutant plants suggesting that PARG1 is a *bona fide* PARG (Panda *et al.*, 2002). Poly(ADP-ribose)glycohydrolase activity of PARG1 has been validated *in vitro* and *in vivo* (Feng *et al.*, 2015). Western Blot and autoradiography of ^{32}P-NAD^{+} revealed that the recombinant PARG1 protein was able to remove poly(ADP-ribose) from automodified AtPARP2. Similarly, co-expression of *AtPARP2* and *AtPARG1* in Arabidopsis protoplasts led to a significant removal of poly(ADP-ribose) from automodified AtPARP2. In contrast to this, no poly(ADP-ribose) glycohydrolase activity towards automodified AtPARP2 or AtPARP1 was found for AtPARG2 *in vitro* and *in vivo*. This lack of activity could not be exclusively attributed to the presence of a polymorphism in the conserved PARG signature motif in PARG2, since a recombinant PARG2 protein carrying the conserved PARG motif did also not show any detectable poly(ADP-ribose) glycohydrolase activity. Therefore, additional deviations in the protein sequence of AtPARG1 and AtPARG2 were thought to account for the differences in enzyme activity as both sequences share only about 52% amino acid identity and 66% similarity (Feng *et al.*, 2015).

Proteins that possess the ability to hydrolyze the ester bond between the ribose and the acceptor amino acid in Arabidopsis have not been determined yet. However, the proteins encoded by the loci At1g63410, At1g69340, and At2g37710 show considerable homology to the human MacroD1 and MacroD2 proteins and are thus candidates for this function. No Arabidopsis proteins homologous to human ARH3 and TARG1 were found by NCBI BLASTP searches.

1.2.3 Plant PARPs play a role in DNA damage response and genome integrity

During about 40 years of work on poly(ADP-ribosyl)ation in plants, various studies showed that plant PARP proteins are components of DNA damage responses similar to their mammalian counterparts. The expression of *AtPARP1* and *AtPARP2* but not *AtPARP3* is induced by treatment with DNA-damaging agents, such as ionizing radiation, zeocin (a

radiometric drug that induces DSB) or cisplatin (an inhibitor of DNA replication by cross-linking of neighboring guanine bases) in *Arabidopsis* (Doucet-Chabeaud *et al.*, 2001, Boltz *et al.*, 2014, Yuan *et al.*, 2014). *AtPARP3* expression was only found to be induced in the absence of AtPARP1 or AtPARP2 (Boltz *et al.* 2014). In contrast, *HvPARP3* expression was induced in barley roots in response to bleomycin treatment (Stolarek *et al.*, 2015). Similar to *AtPARP* expression, PARP activity was found to be induced by DNA damaging agents such as zeocin and X-ray irradiation (Arena *et al.*, 2014, Liu *et al.*, 2017). Recombinant AtPARP1 and AtPARP2 are activated by nicked DNA as shown by automodification of the recombinant AtPARP proteins (Babiychuk *et al.*, 1998, Doucet-Chabeaud *et al.*, 2001, Feng *et al.*, 2015, Liu *et al.*, 2017). For AtPARP2, automodification was also shown *in vivo* (Feng *et al.*, 2015). Automodification of recombinant proteins and *in vivo* was blocked by the addition of the pharmacological PARP inhibitor 3AB (Babiychuk *et al.*, 1998, Liu *et al.*, 2017).

In agreement with increased gene expression and activity, the genetic inhibition of AtPARPs in *parp1* and *parp2* mutant plants enhanced the sensitivity of plant growth to methyl methane sulfonate [MMS, a DNA alkylation agent that induces N-alkyl lesions and single strand breaks (SSB)] and bleomycin (a glycopeptide that mainly induces DSB) (Jia *et al.*, 2013, Boltz *et al.*, 2014, Zhang *et al.*, 2015, Klemm *et al.*, 2017). Similarly, formation of true leaves was reduced in *parp2* seedlings grown on bleomycin and mitomycin C (a DNA cross-linking agent) (Song *et al.*, 2015). Some authors described that *parp2* mutants are more sensitive to DNA-damaging agents than *parp1* mutants (Boltz *et al.*, 2014, Song *et al.*, 2015). In line with this, poly(ADP-ribosyl)ation was strongly reduced in *parp2* but not in *parp1* mutants (Song *et al.*, 2015). Increased plant damages were observed in *parp1 parp2* double mutants compared to the corresponding single mutants, indicating that both *AtPARP* genes are involved in responses to DNA-damaging agents (Jia *et al.*, 2013, Boltz *et al.*, 2014, Zhang *et al.*, 2015, Song *et al.*, 2015). This notion is further supported by an enhanced expression of *AtPARP1* in *parp2* mutants and vice versa (Boltz *et al.*, 2014, Song *et al.*, 2015). Additionally, AtPARP1 and AtPARP2 were shown to physically interact with each other (Song *et al.*, 2015, Liu *et al.*, 2017). No exacerbation in the severity of plant damage was observed in *parp1 parp2 parp3* triple mutants, indicating that AtPARP3 is not active in DNA damage response in seedlings (Zhang *et al.*, 2015). However, in barley, *Hvparp3* mutation introduced by a TILLING approach led to an altered root growth response to bleomycin (Stolarek *et al.*, 2015). In summary, plant damage and reduced growth of *parp* mutants under genotoxic stress may be explained by aggravated DNA damage.

In addition to DNA-damaging agents, infection of plants with the bacterium *Pseudomonas syringae* pv. tomato (*Pst*) was shown to induce DNA damage (Song & Bent, 2014). In line with this, DNA damage was enhanced in *parp2* and *parp1 parp2* mutants in response to treatment with *Pst* (Song *et al.*, 2015).

Similar to *parp* mutant plants, Arabidopsis *parg1* mutant plants were found to exhibit enhanced sensitivity to mitomycin C and bleomycin (Adams-Phillips *et al.*, 2010). Mutant plants lacking AtPARG2 displayed no or only slightly increased sensitivity to DNA-damaging

agents, indicating that AtPARG1 is more important in the response to DNA damage caused by DNA-damaging agents (Adams-Phillips *et al.*, 2010, Zhang *et al.*, 2015). Notably, *parg1* mutations induced more severe plant damage than the lack of AtPARPs (Zhang *et al.*, 2015). This was attributed to the fact that free poly(ADP-ribose) is assumed to be toxic to plant cells, as it has been described for mammalian cells (Zhang *et al.*, 2015).

The exacerbated sensitivity of Arabidopsis *parp* mutant plants to various DNA-damaging agents showed that plant PARPs are important actors in DNA damage responses, similar to their human counterparts. In line with this, the ionizing radiation-mediated induction of *AtPARP2* was found to depend on the presence of ATM, as it was absent in *atm* mutant plants (De Schutter *et al.*, 2007). ATM is an initiator of various DNA damage repair pathways (Tuteja *et al.*, 2009). Yet, the involvement of plant PARP proteins in specific DNA repair pathways is still to be elucidated. For instance, the PARP inhibitor 3MB increased the number of recombination events in Arabidopsis and tobacco (Puchta *et al.* 1995). This indicates that PARPs negatively regulate DNA repair by HR. In line with this, expression of the HR components *AtRAD51* and *AtXRCC3* was enhanced by 3AB treatment (Ishikawa *et al.*, 2009). Moreover, expression of *AtPARP2* was induced in *mms21-1* mutant plants, while homologous recombination events were found to be reduced in this mutant (Yuan *et al.*, 2014). *AtMMS21* encodes a SUMO E3 ligase, a critical component of the SMC5/6 complex which fulfills a central role in genome stability maintenance (Yuan *et al.*, 2014). In contrast, expression of *AtXRCC2*, another component of HR, was found to be reduced upon 3AB treatment (Ishikawa *et al.*, 2009, Boltz *et al.*, 2014). These apparently contradicting findings are still to be elucidated. Another study indicated that PARPs are involved in an error-prone alternative pathway of NHEJ like their mammalian counterparts (Jia *et al.*, 2013). Triple mutant plants lacking AtPARP1, AtPARP2, and AtKu80, a component of classical NHEJ, are more sensitive to MMS than *parp1 parp2* and *ku80* mutants, indicating that different DNA damage response pathways are impaired in the *parp1 parp2 ku80* mutants (Jia *et al.*, 2013). Additionally, the extent of DNA damage was higher in the *parp1 parp2 ku80* mutant plants. A cell-free end-joining assay revealed a higher number of large deletions (>10 bp) at the ends of broken DNA strands in *ku80* and *parp1 parp2 ku80* than in *parp1 parp2* mutants. So, resection of nucleotides from the DNA ends has occurred mainly in the *ku80* and *parp1 parp2 ku80* mutants. An alternative NHEJ pathway in *ku80* mutants is microhomology-mediated end-joining (MMEJ). A higher level of MMEJ products were found in *ku* mutants compared to *parp1 parp2* or *parp1 parp2 ku80* mutants, indicating that PARPs are involved in MMEJ (Jia *et al.*, 2013). In contrast, Shen and colleagues suggested that PARPs act in alternative NHEJ independently of micro-homology (Shen *et al.*, 2017). Recently, one function of AtPARP1 in DNA damage response was further clarified. A *parp1rad5a* double mutant was found to be more sensitive to the DNA alkylating agent MMS than the corresponding single mutant plants (Klemm *et al.*, 2017). By contrast, *parp1rad5a* did not display enhanced sensitivity to the crosslinking agents cisplatin and mitomycin C compared to the *rad5a* mutant plants. No enhanced sensitivity to both agents was observed in the *parp1* mutant compared to wild type plants. These findings prompted the authors to

conclude that AtPARP1 is involved in the repair of base alkylations in a pathway parallel to that involving RAD5a which possibly corresponds to base excision repair (BER) and HR-independent single strand break repair (Klemm *et al.*, 2017). In line with this, 3AB repressed paraquat-induced *XRCC1* expression (Ishikawa *et al.*, 2009). XRCC1 is a component of the gap-filling and nick-sealing step of BER (Spampinato, 2017). *AtPARP2* expression is induced in the absence of Ligase1, another component of BER, indicating that AtPARP2 also interacts with BER (Babiychuk et al., 1998). In addition to transcriptionally regulated proteins, first poly(ADP-ribosyl)ation targets of AtPARP2 were recently identified *in vitro* (Feng *et al.*, 2016). These proteins mostly localize to the nucleus and associate with DNA and RNA metabolism, response to stresses, response to biotic and abiotic stimuli, and transcription. Within this study, *in vivo* poly(ADP-ribosyl)ation was also shown for four proteins (Feng *et al.*, 2016).

In adult Arabidopsis plants, AtPARP3 was suggested to be either inactive or not involved in DNA damage responses (Zhang *et al.*, 2015). In contrast, strong expression was found in seeds (Hunt *et al.*, 2004, Feng *et al.*, 2015). This expression pattern was found to correlate with the expression of *Nicotinamidase 2* (*NIC2*) (Hunt *et al.*, 2007). NIC2 is a component of the pathway recycling the PARP feedback inhibitor nicotinamide to NAD^+. Germination of *nic2* mutant seeds was found to be hypersensitive to MMS, potentially due to reduced levels of poly(ADP-ribosyl)ation which are, in turn, due to reduced nicotinamide degradation(Hunt *et al.*, 2007, Hunt & Gray, 2009). In addition, poly(ADP-ribose) levels did not correlate with the depth of seed dormancy in different ecotypes of Arabidopsis but with their sensitivity to MMS. Hence, AtPARP3 was suggested to be involved in protecting the plant embryo from DNA damage in the seed (Hunt *et al.*, 2007, Hunt & Gray, 2009).

Similar to PARPs, the role of PARGs in DNA damage responses of plants is still to be elucidated. Enhanced expression of HR components (i.e. *SMC6A*, *SMC6B*, *RAD17*, *RAD51*, *RAD54*, *REV7*) and NHEJ components (i.e. *LIG4*, *Ku70*, *Ku80*) was found in *parg1* mutants (Zhang *et al.*, 2015). Contrastingly, *RAD51* and *SWI* expression induced by the bacterial elicitor flg22 are disrupted in *parg1* mutants (Briggs *et al.*, 2017). Apart from that, induction of At*PARG1* expression is attenuated in *atm* and *atr* mutant plants and *vice versa* (Zhang *et al.*, 2015). Similar to ATM, ATR is an initiator of various DNA damage repair pathways (Tuteja *et al.*, 2009). Hence, PARGs, like PARPs, appear to act as a switch between different DNA repair pathways *in planta*. Yet, their exact function is still ambiguous.

In line with a role in plant DNA damage response, PARPs were shown to have a poly(ADP-ribosyl)ating activity on chromosomal proteins. AtPARP1 and AtPARP2 associate with chromosomes in dividing cells via their N-terminal domains (Babiychuk *et al.*, 2001). Thereby, both proteins co-localize and probably compete for suggested heterochromatin association sites (Babiychuk *et al.*, 2001). Histones H1, H2A, and H2B in wheat and tobacco and histones H1.1 and H1.3 in Arabidopsis were found to be targeted by poly(ADP-ribosyl)ation, putatively creating a chromatin structure more accessible to RNA polymerase II, as found for human PARP1 (Whitby *et al.*, 1979, Willmitzer, 1979, Feng *et al.*, 2015). A

potential transcriptional regulation by PARP proteins is indicated by the findings of Storozhenko and colleagues (Storozhenko *et al.*, 2001). AtPARP1 was found to interact with DIP1 and DIP2, two proteins homologous to the transcriptional coactivator ALY, via its DNA-binding domain *in vitro* and in yeast (Storozhenko *et al.*, 2001). Expression of At*PARP1* and At*PARP2* but not At*PARP3* is increased in response to telomerase dysfunction (Cifuentes-Rojas *et al.*, 2012, Boltz *et al.*, 2014). However, in contrast to their human counterparts, AtPARP1 and AtPARP2 do not stimulate telomerase activity. Apart from this, both Arabidopsis PARPs are not involved in telomere end protection and telomere length protection in seedlings and flowers (Boltz *et al.*, 2014). In contrast, HvPARP3 was suggested to be involved in telomere length maintenance in barley seedlings (Stolarek *et al.*, 2015).

Mammalian PARP proteins are regulators of various facets of cell death (Aredia & Scovassi, 2014). Comparable functions have also been found for plant PARPs (Amor *et al.*, 1998, Tian *et al.*, 2000). Thus, treatment with the PARP inhibitors 3AB and nicotinamide blocked heat shock- and H_2O_2-induced PCD in cultured tobacco and soybean cells (Amor *et al.*, 1998, Tian *et al.*, 2000). The PARP inhibitor-mediated protection from H_2O_2-induced PCD was found to be most effective during the first 30 min after H_2O_2 treatment. At that point, a sharp drop in NAD^+ levels in the soybean cells indicated the onset of PARP activity. Overexpression of *AtPARP2* in soybean cell culture resulted in reduced cell death upon low concentrations of H_2O_2 (mild oxidative stress), but a dramatically increased cell death upon high H_2O_2 concentrations (severe oxidative stress). In addition, *AtPARP2* expression reduced the amount of nicked DNA under both mild and severe oxidative stress (Amor *et al.*, 1998). Additionally, cleavage of AtPARP proteins by Caspase-3, a central component of programmed cell death, was demonstrated in tobacco cells directly after PCD-inducing heat shock treatment (Tian *et al.*, 2000). These findings suggest that plant PARPs fulfill similar functions as their mammalian counterparts: They act as a switch between DNA damage repair under mild stress conditions and PCD under severe stress conditions.

Apart from DNA damage and PCD, plant PARPs were also found to be involved in plant development. The formation of tracheary elements in artichoke cell cultures, artichoke tubers, and pea root explants was inhibited by addition of the PARP inhibitor, 3AB (Phillips & Hawkins, 1985). In addition to this, *AtPARP1* and *AtPARP2* expression and their activity increased in Arabidopsis cell cultures during the exponential growth phase (Pellny *et al.*, 2009). This increase in expression and activity was temporally linked to an increase in marker gene expression for S to G2 phase transition in the cell cycle. Simultaneously, there was a correlation between the increase in PARP activity and an increase in the glutathione pool during exponential growth of the cell culture (Pellny *et al.*, 2009). Hence, PARP activity is linked to cell cycle progression and redox regulation, further supporting a regulatory function of AtPARPs in plant development. In line with this, seed germination was found to be altered in *parp1*, *parp2*, and *parp3* mutant plants (Pham *et al.*, 2015). Under non-stressed conditions, *parp3* plants were found to germinate faster than the wild type, while *parp1* and *parp2* exhibited slower and partially reduced germination rates (Pham *et al.*, 2015). In addition to germination, plant growth regulation appears to involve poly(ADP-ribosyl)ation,

since the PARP inhibitor 3MB has been shown to improve Arabidopsis growth under non-stressed conditions in different culture systems (Schulz *et al.*, 2012, Schulz *et al.*, 2014). Enhanced growth by 3MB was attributed to higher leaf cell numbers due to a shortened cell division cycle, and a higher number of stomata resulting in an increased overall leaf size (Schulz *et al.*, 2014). Moreover, 3MB treatment altered gene expression in Arabidopsis plants under unstressed conditions, affecting components of plant responses to external and internal stimuli and abiotic stress response, circadian rhythm, plant growth, energy metabolism and photosynthesis, and primary and secondary metabolism (Schulz *et al.*, 2012, Schulz *et al.*, 2014, Briggs *et al.*, 2017). For the PARP inhibitor 3AB, contrasting effects have been reported, depending on the 3AB concentration used. Weight of Arabidopsis plants was reduced upon treatment with 2.5 mM 3AB, although plants appeared healthy (Adams-Phillips *et al.*, 2008, Briggs *et al.*, 2017). Similarly, 5 mM 3AB reduced root and shoot growth in Arabidopsis plants (Boltz *et al.*, 2014). In contrast, 1 mM 3AB promoted plant biomass and root system development, resulting in more lateral roots, formation of secondary order lateral roots, increased lateral root length, and increased primary root length compared to the control plants (Liu *et al.*, 2017). 3AB-mediated enhanced lateral root growth was shown to be not due to enhanced lateral root initiation but due to faster growth after initiation (Liu *et al.*, 2017). Similar, although weaker, effects were observed for another PARP inhibitor, 6(5H)-phenanthridinone. Similar to pharmacological PARP inhibition, genetic AtPARP inhibition in *parp1*, *parp2*, and *parp3* mutant plants led to higher root elongation (Pham *et al.*, 2015). The absence of AtPARP1 and AtPARP2 in *parp1 parp2* double mutant plants led to the formation of a larger root system compared to the wild type (Liu *et al.*, 2017). However, this phenotype was weaker than that elicited by the inhibitors. Nevertheless, several authors stated that *parp1*, *parp2*, *parp1 parp2*, or *parp1 parp2 parp3* mutants did not display noticeable developmental defects indicating that, in contrast to their mammalian counterparts, plant PARPs are not essential for plant development (Song *et al.*, 2015, Zhang *et al.*, 2015, Liu *et al.*, 2017). Similarly, *parg* mutant plants grew normally under control conditions (Zhang *et al.*, 2015), indicating that AtPARGs are also not essential for normal plant growth (Zhang *et al.*, 2015).

As poly(ADP-ribosyl)ation of nuclear proteins consumes NAD^+, Pham and colleagues assumed that plant PARP proteins also affect the plant primary metabolism (Pham *et al.*, 2015). Indeed, primary metabolites such as amino acids, sugars, carboxylates were found to differ between wild type, *parp1-5*, *parp2-2*, and *parp3-2* mutant plants under unstressed conditions (Pham *et al.*, 2015). Interestingly, the photosynthetic machinery was not compromised by the lack of AtPARPs. Taken together, these data suggest that PARP proteins also fulfill non-redundant physiological functions *in planta* (Pham *et al.*, 2015).

1.2.4 Poly(ADP-ribosyl)ation in plant responses to abiotic stress and biotic attack

In line with their role in DNA damage responses, Arabidopsis PARP proteins were found to be part of plant responses to external stressors. Expression of *AtPARP* and *AtPARG* genes

was altered in response to abiotic stresses such as salinity, desiccation, high light, or oxidative stress (Doucet-Chabeaud *et al.*, 2001, Ogawa *et al.*, 2009). Additionally, *PARP1* or *PARP2* knockdown in oilseed rape and Arabidopsis by *PARP* hairpin constructs enhanced the stress tolerance to desiccation, short-term paraquat treatment, and high light (De Block *et al.*, 2005). Thereby, the *AtPARP1* knockdown was found to be more effective than *AtPARP2* knockdown to enhance overall abiotic stress tolerance. In plant lines exhibiting knockdown of both *AtPARP1* and *AtPARP2*, stress tolerance was further enhanced. This enhancement was associated with a reduced PARP activity in the different plant lines under stress (De Block *et al.*, 2005). Accordingly, energy overconsumption, which has been described as a response to PARP activation following severe stress in mammalian cells, was prevented in *AtPARP* knockdown lines. Energy homeostasis and normal levels of mitochondrial respiration were found to be maintained, and ROS production was kept low (De Block *et al.*, 2005). Hence, energy preservation under stress was suggested as a cause of the observed enhanced abiotic stress tolerance of *AtPARP* knockdown lines. Additional explanations were provided by Vanderauwera and colleagues performing a transcriptomic study on plants with reduced *AtPARP1* expression (Vanderauwera *et al.*, 2007). Under high light stress, *AtPARP1* knockdown led to attenuated expression of temperature-responsive genes and oxidative stress-dependent genes. Furthermore, genes involved in cellular transport and energy metabolism were repressed, further supporting the hypothesis that reduced PARP activity enhanced plant stress tolerance by reducing oxidative stress and preserving energy homeostasis. In contrast, genes responsive to ABA, dehydration, and cold were hyperinduced by *AtPARP1* knockdown under high light stress. Simultaneously, ABA levels were found to be higher in the plants exhibiting reduced *AtPARP1* expression compared to wild type plants upon high light stress. ABA-independent stress-responsive gene expression was not affected by the reduced PARP1 activity. Additionally, expression of starch metabolism and flavonoid biosynthesis genes was found to be enhanced in the *AtPARP1* knockdown plants under high light stress. Taken together, these findings point to a role of AtPARP1 as a negative transcriptional regulator of plant stress responses in an ABA-dependent way (Vanderauwera *et al.*, 2007). Based on those transcriptomics data and the previous findings by De Block and colleagues, an additional hypothesis explaining the enhanced overall abiotic stress tolerance due to reduced PARP activity in Arabidopsis and *Brassica napus* plants was proposed. This model linked the enhanced NAD^+ levels to the enhanced ABA levels and the enhanced expression of ABA-responsive genes via cyclic ADP-ribose (cADPR) (Vanderauwera *et al.*, 2007). This signaling molecule is synthesized by ADP-ribose cyclase using NAD^+ as a substrate and has been shown to be involved in eliciting Ca^{2+} signaling pathways in ABA signal transduction pathways (Xiong & Zhu, 2003, Berger *et al.*, 2004, Hunt *et al.*, 2004, Sanchez *et al.*, 2004). According to this, Vanderauwera and colleagues propose the following model: Reduction of At*PARP1* gene expression leads to preservation of the NAD^+ pools under stress, thereby promoting cADPR synthesis. Consequently, cADPR triggers the release of Ca^{2+} from internal stores, which then induces ABA production and subsequent ABA-responsive gene expression, eventually resulting in enhanced stress tolerance.

The enhanced stress tolerance by PARP inhibition is further supported by work on a knockout mutant of the PARP antagonist AtPARG1, *parg1-3*, which exhibits enhanced sensitivity to osmotic stress, desiccation, and oxidative stress by paraquat as compared to wild type plants (Li *et al.*, 2011). Additionally, *AtAOX1* and *AtAPX2*, two oxidative stress-responsive genes, were found to be down-regulated in those *parg1-3* mutant plants. These results show that AtPARG1 is required for stress tolerance, indicating that a balance of PARP and PARG action is critical for the activation of abiotic stress responses. This is of particular importance, as free ADP-ribose is known as a cell death signal in mammalian cells (Andrabi *et al.*, 2006). Surprisingly, *PARG1* overexpressors in the *parg1-3* mutant background did not differ from the wild type in their response to the stress treatments. The authors speculate that the excess of transcript is not translated into protein (Li *et al.*, 2011), which may represent an additional checkpoint to balance ADP-ribose levels in plant.

Apart from genetic PARP inhibition, pharmacological PARP inhibition has been used as a tool to elucidate the role of PARP proteins in plants in several studies (De Block *et al.*, 2005, Ishikawa *et al.*, 2009, Geissler & Wessjohann, 2011, Schulz *et al.*, 2012, Schulz *et al.*, 2014). Similar to genetic PARP inhibition, pharmacological PARP inhibition by 3MB improved energy homeostasis in plants. While the expression of genes related to photosynthesis, the effective photosynthetic quantum yield, and the electron transport rate were induced, low-energy-status marker genes were unaltered in Arabidopsis plants grown under unstressed conditions (Schulz *et al.*, 2014). NAD^+ content was found to be increased under both unstressed and oxidative stress conditions (Schulz *et al.*, 2012). Notably, 3MB treatment de-regulated gene expression of components of the phenylpropanoid pathway under unstressed and oxidative stress conditions (Schulz *et al.*, 2012, Schulz *et al.*, 2014). Additionally, relative abundance of metabolites from the phenylpropanoid pathway such as flavonols and lignins decreased upon 3MB treatment (Schulz *et al.*, 2014). These findings can explain reduced leaf pigmentation, reduced anthocyanin accumulation, and enhanced plant growth upon 3MB treatment under oxidative stress caused by paraquat (Schulz *et al.*, 2012). Reduction of anthocyanin accumulation was not specific to 3MB, as the PARP inhibitors 3-methylbenzamide and 3-aminophtalhydrazide acted similarly. Additionally, other stress-related metabolites, such as galactinol or myo-inositol were reduced by the inhibitors. Apart from its interference in oxidative stress responses, 3MB was also shown to enhance plant growth in response to salt, heat, and high-light stress (Schulz *et al.*, 2012). Additionally, 3MB and nicotinamide were shown to enhance the tolerance of *Brassica napus* hypocotyl explants to oxidative stress elicited by acetyl salicylic acid (De Block *et al.*, 2005). In another study, the PARP inhibitor 4-amino-1,8-naphthalamide enhanced growth rates of *Lemna minor* facing osmotic stress by polyethylene glycol treatment (-0.3 MPa) and oxidative stress tolerance of an Arabidopsis cell culture to H_2O_2 (Geissler & Wessjohann, 2011). In contrast to the finding that the pharmacological inhibition of PARP reduced plant sensitivity to paraquat treatment (De Block *et al.*, 2005), other authors showed that 3-AB treatment of Arabidopsis seedlings *enhanced* the sensitivity to long-term paraquat treatment (Ishikawa *et al.*, 2009).

This discrepancy might be explained by the different levels of stress and the resulting extent of PARP activation.

Besides being challenged by abiotic stresses, plants also face biotic attacks. Microbe-associated molecular patterns (MAMP) are structural or functional units of a microbe that are recognized by the plant immune system and elicit defense responses. The N-terminally conserved 22 amino acids of flagellin (flg22) from *Pseudomonas* bacteria and the N-terminal 18 amino acids of EF-Tu (elf18) from *Escherichia coli* are such MAMPs (Felix *et al.*, 1999, Kunze *et al.*, 2004). They elicit immune responses such as oxidative burst, cell wall reinforcements by callose and lignin, and transcriptional induction of defense genes. The PARP inhibitor 3AB has been shown to block flg22- and elf18-induced callose deposition in Arabidopsis seedlings (Adams-Phillips *et al.*, 2008, Adams-Phillips *et al.*, 2010). In line with this, the combined treatment of 3AB and flg22 was deleterious to Arabidopsis plants (Adams-Phillips *et al.*, 2008). In contrast, wounding-induced callose deposition was not affected. Similarly, no alterations were found in early MAMP responses, such as enhanced ROS production and activation of response genes (Adams-Phillips *et al.*, 2010). It is still unclear at which step 3AB interacts with pathways required for callose deposition, but salicylic acid can bypass the blockage exhibited by 3AB (Adams-Phillips *et al.*, 2010). The PARP inhibitor 3MB has been shown to negatively influence the phenylpropanoid pathway (Schulz *et al.*, 2012, Schulz *et al.*, 2014). Similar observations were also made for 3AB, which was shown to inhibit the phenylalanine ammonium lyase, a component of the phenylpropanoid pathway (Berglund *et al.*, 1996). Additionally, the elf18-induced guaiacyl-lignin accumulation was blocked upon 3AB treatment (Adams-Phillips *et al.*, 2010). Transcriptomic analyses revealed that pharmacological PARP inhibition by 3AB de-regulated MAMP-induced transcriptional responses in Arabidopsis, assigning PARPs a role as a transcriptional regulators of MAMP responses and further supporting the findings above (Briggs *et al.*, 2017). However, in contrast to pharmacological PARP inhibition, genetic inhibition of AtPARP1 or AtPARP2 in T-DNA single knockout lines did not alter flg22-induced callose deposition, whereas *parp1 parp2* double mutant lines exhibited enhanced or reduced callose deposition, depending on the plant age and/or flg22 concentration applied (Song *et al.*, 2015, Feng *et al.*, 2016): The transfer of 5-day-old seedlings to liquid medium containing 1 µM flg22 enhanced callose deposition, while infiltration of leaves from 4-week-old plants with 0.5 µM flg22 reduced this response (Song *et al.*, 2015, Feng *et al.*, 2016). The flg22-induced ROS burst was not altered in the mutant plants (Song *et al.*, 2015). Nevertheless, *parp2-1* mutant plants and *parp1 parp2* double mutant plants displayed enhanced sensitivity towards the *Pst* strain DC3000 (Song *et al.*, 2015, Feng *et al.*, 2015). This strain also induced an activation of PARP in Arabidopsis plants, as demonstrated by reduced cellular NAD^+ levels and increased leaf poly(ADP-ribose) content (Adams-Phillips *et al.*, 2010). Taken together, these findings emphasize the role of PARP proteins and particularly PARP2 as regulatory components of the basal immune response.

An actor in PARP-mediated plant immunity has been identified recently (Feng *et al.*, 2016). Mutant plants lacking the AtPARP2-poly(ADP-ribosyl)ated protein DWADLE (DDL) exhibited

an exacerbated sensitivity to *Pst* DC3000, *P. syringae pv. maculicola*, non-pathogenic *Pst* DC3000 hrcC (a type III secretion mutant of *Pst* DC3000), and non-adaptive pathogen *P. syringae pv. phaseolicola* (Feng *et al.*, 2016). Similarly, *ddl* mutants showed reduced calllose deposition in response to flg22 and *Pst* DC3000 hrcC treatment. In contrast to pharmacological PARP inhibition, early MAMP-induced gene expression was found to be reduced in *parp1 parp2* mutants, but not in *ddl* (Feng *et al.*, 2015, Feng *et al.*, 2016); late transcriptional responses to MAMP treatment were reduced in both *parp1 parp2* and *ddl* (Feng *et al.*, 2016). These partially overlapping phenotypes of *parp1 parp2* and *ddl* further indicated an interaction of these proteins in response to MAMPs. In fact, an interaction of AtPARP2 and DDL was confirmed by immunoprecipitation and bimolecular fluorescence complementation assays, and found to be enhanced by flg22. The direct interaction of both proteins was markedly stronger upon poly(ADP-ribosylation). In addition to AtPARP2, AtPARP1 was also shown to poly(ADP-ribosyl)ate DDL, but to a lesser extent than AtPARP2. A DDL protein lacking its poly(ADP-ribosyl)ation sites was unable to complement the susceptibility to MAMP treatment, indicating that poly(ADP-ribosyl)ation of DDL is essential for proper plant immune response. As DDL infers with late MAMP-induced gene expression, and as it was suggested to interact with histone acetyltransferases in Arabidopsis, Feng and colleagues concluded that DDL is involved in poly(ADP-ribosyl)ation-mediated chromatin remodeling to allow access to target gene promoters during plant immunity (Feng *et al.*, 2016).

Since PARGs antagonize PARP action, *parg* mutants should also display altered responses to biotic attack. Accordingly, *parg1* but not *parg2* mutants displayed a more severe growth inhibition upon elf18 treatment accompanied by an enhanced pigment accumulation (Adams-Phillips *et al.*, 2010). Mature *parg1* plants also displayed enhanced flg22-induced callose deposition and altered expression of flg22-regulated genes (Feng *et al.*, 2015). Similar to *parp* mutant plants and pharmacological PARP inhibition, the early MAMP-induced ROS production was not altered in the *parg1* mutant plants. Nevertheless, this mutant displayed transcriptional changes in defense gene expression (Briggs *et al.*, 2017). These changes could not be assigned to specific pathogen-induced signaling pathways, leading to the conclusion that AtPARG1 acts as a regulatory element at response pathways junctures (Briggs *et al.*, 2017). Apart from this, the onset of symptoms of *B. cinerea* infection was accelerated in both *parg1* and *parg2* mutant plants (Adams-Phillips *et al.*, 2010). Hence, particularly AtPARG1 can be described as a positive regulator of biotic stress responses. Against this background, it is surprising that *AtPARG2* expression is up-regulated in response to *Pst* and *Botrytis cinerea* infection and flg22 treatment, while *AtPARG1* was only transiently induced by treatment with flg22 and elf18 (Adams-Phillips *et al.*, 2008, Adams-Phillips *et al.*, 2010, Song *et al.*, 2015). This discrepancy is still to be elucidated.

Summarizing the role of PARPs and PARGs in plant responses to abiotic stresses and biotic attack, it can be stated that PARPs appear to be negative regulators of abiotic stress responses, while they may act positively in response to biotic attack. In contrast, PARGs appear to act as positive regulators in response to both, abiotic stresses and biotic attack.

1.2.5 PARP-like proteins also act in plant stress responses

In addition to the canonical PARP proteins, members of the SRO protein family have been found to possess a highly conserved catalytical PARP domain (Ahlfors *et al.*, 2004, Jaspers *et al.*, 2010). Therefore, SRO proteins can be regarded as part of the plant PARP protein family. SRO proteins were found to be present in all sequenced land plant genomes with considerable variation concerning their composition between the sequenced plant species (Jaspers *et al.*, 2010, You *et al.*, 2014). The SRO protein family comprises the proteins Radical-induced cell death1 (RCD1) and its homologues Similar to RCD-One1 (SRO1) through SRO5 (Belles-Boix *et al.*, 2000) (Jaspers *et al.*, 2010). In contrast to the canonical PARP proteins, the close homologues RCD1 and SRO1 contain a central catalytic PARP domain which is flanked by an N-terminal WWE domain and a C-terminal RST domain, while SRO2 through SRO5 lack the WWE domain (Ahlfors *et al.*, 2004, Jaspers *et al.*, 2009). Radical-induced cell death 1 (RCD1) is the founding member of this protein family. It was initially identified during screenings for ozone sensitivity in Arabidopsis and oxidative stress regulators in yeast (Overmyer *et al.*, 2000, Belles-Boix *et al.*, 2000). Accordingly, Arabidopsis *rcd1* mutant plants were found to be hypersensitive to ozone treatment and the resulting apoplastically produced ROS (Overmyer *et al.*, 2000, Ahlfors *et al.*, 2004). In contrast, *rcd1* plants were resistant to ROS formed in the chloroplasts upon paraquat treatment (Ahlfors *et al.*, 2004, Fujibe *et al.*, 2004). *RCD1* is expressed ubiquitously and constitutively throughout Arabidopsis plants (Jaspers *et al.*, 2009, Teotia & Lamb, 2009). It was found to localize predominantly to the nucleus (Fujibe *et al.*, 2006, Katiyar-Agarwal *et al.*, 2006) but upon salt and oxidative stress, RCD1 was also localized in the cytoplasm (Katiyar-Agarwal *et al.*, 2006). RCD1 was shown to be involved in responses to salt stress and to the fungal pathogen *Hyaloperonospora arabidopsis*, in the regulation of stomatal conductance, in UV-B responses, in PCD, and in plant hormone signaling (Fujibe *et al.*, 2004, Ahlfors *et al.*, 2004, Overmyer *et al.*, 2005, Katiyar-Agarwal *et al.*, 2006, Jiang *et al.*, 2009, Morales *et al.*, 2015, Wirthmueller *et al.*, 2018). Additionally, *rcd1* mutant plants show severe developmental defects such as stunted growth, altered rosette and leaf morphology, early flowering time, and a high degree of differentially regulated genes (Ahlfors *et al.*, 2004, Jaspers *et al.*, 2009, Teotia & Lamb, 2009). Moreover, RCD1 was found to physically interact with the Na^+/H^+ antiporter SOS1 and particularly with transcription factors from various families, such as AP2/ERF, NAC, and basic helix–loop–helix (Belles-Boix *et al.*, 2000, Katiyar-Agarwal *et al.*, 2006, Jaspers *et al.*, 2009, You *et al.*, 2013). The most prominent transcription factor RCD1 was found to interact with is DREB2A (Belles-Boix *et al.*, 2000, Jaspers *et al.*, 2009, Vainonen *et al.*, 2012). This transcription factor has been shown to be a transcriptional regulator of genes involved in the responses to various stresses, such as drought, salinity, and heat, and it is also involved in plant senescence (Sakuma *et al.*, 2006, Vainonen *et al.*, 2012). Summarizing all those findings about the PARP-like protein RCD1, several authors have proposed it to be a central hub in plant stress response, ROS homeostasis, plant hormone signaling, and PCD (Ahlfors *et al.*, 2004, Overmyer *et al.*, 2005, Jiang *et al.*, 2009).

The PARP-like protein SRO1 shares 76% similarity with RCD1 (Teotia & Lamb, 2009). Both proteins were identified to be paralogs likely arising from gene duplication (Teotia & Lamb, 2009). They localize both to the nucleus and are both expressed ubiquitously throughout Arabidopsis plants (Jaspers *et al.*, 2009, Teotia & Lamb, 2009) (Teotia & Lamb, 2011), whereby expression levels of *SRO1* are generally lower than those of *RCD1* (Teotia & Lamb, 2009). In contrast to *rcd1* mutant plants, *sro1* plants did not exhibit sensitivity to ozone and salt, and grew normally under unstressed conditions (Jaspers *et al.*, 2009, Teotia & Lamb, 2009). Similar to RCD1, SRO1 was shown to interact with transcription factors, but only with a smaller subset compared to RCD1 (Jaspers *et al.*, 2009). Double mutants lacking both RCD1 and SRO1 showed detrimental developmental defects (Jaspers *et al.*, 2009, Teotia & Lamb, 2009, Teotia & Lamb, 2011). Collectively, RCD1 and SRO1 appear to be unequally redundant proteins (Jaspers *et al.*, 2009).

The third member of the SRO family characterized so far is SRO5 (Borsani *et al.*, 2005, Babajani *et al.*, 2009, Jaspers *et al.*, 2010). In contrast to *RCD1* and *SRO1*, which showed hardly any changes in their expression upon stress, *SRO5* transcript levels changed upon salt, ozone, light, wounding, anoxia, and bacterial elicitors (Babajani *et al.*, 2009, Jaspers *et al.*, 2010). Additionally, SRO5 was shown to be involved in the response to salt and oxidative stress in a very peculiar way (Borsani *et al.*, 2005). Upon salt stress, SRO5 transcripts form siRNAs with an overlapping gene in antisense orientation, P5CDH (1-pyrroline-5-carboxylate dehydrogenase), thus regulating it on post-transcriptional level. SRO5 itself is apparently involved in ROS regulation (Borsani *et al.*, 2005). Surprisingly, expression of the *P5CDH* gene was not enhanced in *sro5* mutant plants (Babajani *et al.*, 2009, Jaspers *et al.*, 2010). Apart from this, *SRO5* and *P5CDH* were not found to overlap in *Arabidopsis lyrata*, grapevine, and poplar (Jaspers *et al.*, 2010). It was therefore proposed that the regulation of *P5CDH* is not a primary function of the *SRO5* gene (Babajani *et al.*, 2009, Jaspers *et al.*, 2010). Similar to RCD1 and SRO1, SRO5 was found to localize to the nucleus and to interact with transcription factors including DREB2A (Jaspers *et al.*, 2010). This may be an alternative explanation for SRO5 action *in planta*. Similar to *SRO5*, *SRO2* and *SRO3* showed changes in their transcript levels in response to light, salt and ozone (Jaspers *et al.*, 2010).

1.3 Aims of this thesis

The presently available data supports the notion that plant PARPs and PARP-like proteins function as key hubs with regulatory function in DNA damage and stress responses. Nevertheless, several assumptions concerning the function of PARP proteins *in planta* are still based on homology and structural conservation between plant PARPs and their mammalian counterparts. Even though poly(ADP-ribosyl)ation has been demonstrated in plants, it is to be clarified if plant PARP proteins exhibit functional similarities or even identities to mammalian PARP proteins. Additionally, the exact function of the individual plant PARP proteins in plant DNA damage and abiotic and biotic stress responses are not yet fully understood.

Therefore, studies were conducted to survey the functional similarity of Arabidopsis PARPs to HsPARP1 in a yeast cell growth-based assay (Publication 1). In this set-up, the ability of known PARP inhibitors to inhibit the PARP proteins was also to be monitored.

Unlike AtPARP1 and AtPARP2, no function for AtPARP3 had been described so far. However, its expression in seeds suggested a role during storage and/or germination. This hypothesis was to be tested by a functional characterization, including subcellular localization, a refined expression analysis, and the analysis of its role in seed germination and storability (Publication 2).

The mechanisms by which the canonical Arabidopsis PARP proteins act in plant responses to abiotic and biotic stress were to be further elucidated by phenotypical analyses of knockout mutants lacking the *PARP* genes (Publication 3). Surprisingly, in the course of these studies, mutant lines were found not to display any phenotypical differences to the wild type in their response to various stresses. Therefore, the stress responses of *parp* double and triple mutant plants were to be characterized. The absent effects of *parp* mutations on stress responses prompted an *in silico* analysis of alternative targets of PARP inhibitors that had been described to alter plant stress tolerance.

2 Publication 1

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A yeast growth assay to characterize plant poly(ADP-ribose) polymerase (PARP) proteins and inhibitors

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ABSTRACT

Poly(ADP-ribose) polymerases (PARPs) have been implicated in responses of plants to DNA damage and numerous stresses, whereby the mechanistic basis of the interference is often unclear. Therefore, the identification of specific inhibitors and potential interactors of plant PARPs is desirable. For this purpose, we established an assay based on heterologous expression of *PARP* genes from the model plant *Arabidopsis thaliana* in yeast. Expression of *AtPARP*s caused an inhibition of yeast growth to different extent, which was alleviated by inhibitors targeted at human PARPs. This assay provides a fast and simple means to identify target proteins and pharmacological inhibitors of AtPARP1.

Heterologous expression of genes in yeast has proven to be a powerful tool to study their function. In line with this, several authors have described the characterization of human *Poly(ADP-ribose) polymerases* (*HsPARPs*) in yeast [1–5]. Poly(ADP-ribosyl)ation (PARylation) is a posttranslational protein modification, adding poly(ADP-ribose) moieties to nuclear proteins and thereby recruiting DNA repair components to DNA damage sites. Hence, PARPs are key elements of cellular genome integrity, and as such, PARPs have been attributed functions in diseases, such as cancer [6].

Expression of *HsPARP1* and *HsPARP2* results in a growth arrest of yeast cells [1–5], which has been attributed to the production of poly(ADP-ribose) moieties [3,4]. The presence of a DNA-binding domain was shown to be indispensable for HsPARP1-induced yeast growth inhibition [1]. The expression of the PARP antagonist *Poly(ADP-ribose) glycohydrolase* (*PARG*), which removes ADP-ribose moieties from target proteins of PARylation, as well as the application of PARP inhibitors can revert the growth inhibition [1,3,4]. This action of known PARP inhibitors has been exploited to establish a screening procedure to identify novel HsPARP inhibitors [3]. In addition, such a growth assay provides a means to study potential targets of poly(ADP-ribosyl)ation.

Recently, PARP proteins have gained an increased interest in plant science. As in animals, they have been linked to plant DNA damage responses [7–9], but, in addition, they were also considered to be regulators of plant stress responses [10,11]. However, the mechanistic basis of many presumed plant PARP functions has not been fully resolved yet [12]. To study functional similarities of HsPARP1 and the Arabidopsis PARP proteins, and to develop a screening procedure for potential pharmacological inhibitors, we expressed the three canonical *AtPARP*s and *HsPARP1* heterologously in *Saccharomyces cerevisiae*. To this end, *HsPARP1* and *AtPARP1* (At2g31320) cDNAs were PCR-amplified from plasmids IRATp970E1051D (imaGENES, Germany) and U19185 (ABRC), respectively. cDNAs of *AtPARP2* (At4g02390) and *AtPARP3* (At5g24470) were amplified as described previously [9]. Primers used to amplify the cDNA sequences are listed in Table 1 in Supplementary Material. The resulting cDNAs were cloned into the yeast expression vector pYES2 by homologous recombination performed in the yeast

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wild type strain W303-1A as described elsewhere [3]. The pYES2 expression vector contains a galactose (Gal)-inducible promoter allowing a temporally controlled expression, which is required because *PARP* expression was supposed to inhibit yeast cell growth. Recombinant plasmids were purified and employed for yeast transformation. Transformed cells were transferred to liquid synthetic complete (LSC) medium (Formedium, UK) without uracil (Ura) containing 2% glucose (Glc) as carbon source and incubated at 30 °C overnight. 150 μL of liquid yeast culture were mixed with 850 μL of sterile glycerol, aliquoted, and frozen at −80 °C to produce cryo-starter aliquods. Those starter cultures ensured identical starting conditions in all experiments.

For yeast growth assays, an overnight culture in LSC-Ura + Glc was started from a 50 μL cryo-starter aliquod. After *c.* 24 h of growth, the culture was diluted with LSC-Ura + Glc to yield 1×10^6 cells mL^{-1} next morning. The cell suspension was washed with distilled water, diluted to 1×10^6 cells mL^{-1} in LSC medium containing 2% Gal to induce *PARP* gene expression, and 100 μL of cell suspension were transferred into wells of a 96-well plate. All incubations were carried out at 30 °C. OD_{600} was measured at the indicated time points using a microplate reader (Mithras LB940S3, Berthold Technologies, Germany). As determined by RT-PCR analysis, all *PARP* genes were expressed under inducing conditions.

As expected, induction of Hs*PARP1* expression in W303-1A yeast cells resulted in growth inhibition compared to the control cells carrying the empty vector (Fig. 1A). The same was observed for At*PARP1*-expressing cells, where OD_{600} increased only slightly within a 43 h growth period. The expression of At*PARP2* only partially delayed yeast cell growth, and At*PARP3* expression did not affect cell growth consistently (Fig. 1A). In contrast to the zinc-finger DNA-binding domains of HsPARP1 and AtPARP1, AtPARP2 has a SAP domain involved in DNA binding [13]. AtPARP3 apparently lacks any DNA binding domain [12]. Since a truncation of the DNA binding site has been shown before to repress the HsPARP1-induced growth inhibition in yeast cells [1], these differences in the structure of the N-terminal region of AtPARP2 and AtPARP3 are likely to be the reason for the absence of full yeast growth inhibition.

To analyze if growth inhibition of yeast cells expressing At*PARP1* is related to poly(ADP-ribosyl)ation activity, PARylation of yeast protein was determined. To this end, *PARP* expression was induced in LSC-Ura + Gal medium for 6 h. Approx. 100 mg yeast cells were harvested and frozen at −80 °C. 500 μL protein extraction buffer [25 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1% protease inhibitor cocktail (Sigma-Aldrich)] and 600 μL acid-washed glass beads (425–600 μm, Sigma-Aldrich) were added to the frozen yeast cells, and cells were lysed in a tissue lyser (Qiagen) at 30 Hz for 5 min. The lysate was centrifuged in a microfuge for 3 min at max. speed and 4 °C. The supernatant was transferred to a new tube, and total protein concentration was determined by Bradford assay. 8 μg protein lysate were mixed with 1 volume of 40% methanol and spotted onto a nitrocellulose membrane using a dot blot apparatus (Biometra, Germany). Protein spots on the nitrocellulose membrane were excised by a hole-punch and transferred to a 96-well plate. PAR residues were determined using a PAR monoclonal antibody (10H) (Enzo Life Sciences, Farmingdale, USA) and a secondary anti-mouse antibody coupled to horseradish peroxidase. ECL reagent (250 mg L^{-1} luminol, 0.1 M Tris-HCl pH 8.6, 1% DMSO, 1 g L^{-1} para-coumaric acid) was added, and luminescence was quantified by using a microplate reader (Mithras LB940S3). Yeast cells expressing *HsPARP1* exhibited PARylation activity (Fig. 1B) as described previously [1,3–5]. Similarly high PAR modification was also observed in At*PARP1*-expressing yeast cells (Fig. 1B). Hence, AtPARP1 shares cellular functions with HsPARP1 in yeast. Previous authors used the activity of HsPARP1 in yeast to identify potential poly(ADP-ribose) acceptor proteins homologous to human nuclear proteins [5]. As yeast and plants also share homologous genes, the identification of AtPARP1 target proteins in yeast cells can provide a shortcut to identify putative AtPARP target proteins *in planta*.

Pharmacological inhibition of Arabidopsis PARP proteins *in planta* has been widely used to study their function, but the results are not unequivocal [12]. We modified the yeast growth assay to determine the activity of known human PARP inhibitors to AtPARP1, i.e., 3-aminobenzamide (3-AB), 6-(5H)-phenantridinone (PHE), and 4-amino-1,8-naphtalimde (4-ANI), and to establish a method to identify new inhibitors of AtPARP1. Since yeast cells lacking efflux pumps exhibit an increased drug sensitivity, the yeast

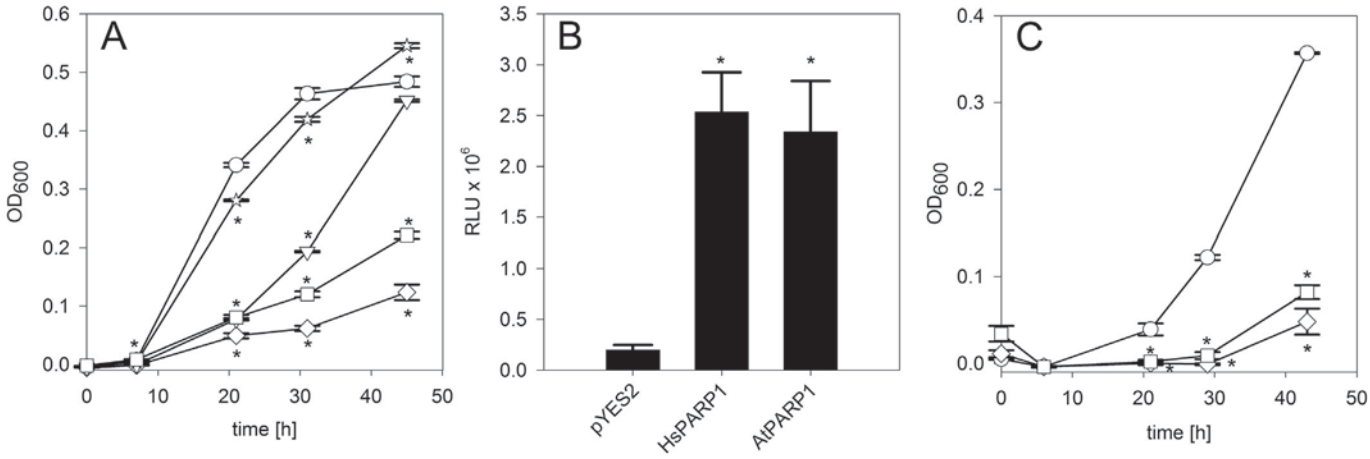

Fig. 1. Expression of Hs*PARP1* and At*PARP1* genes in yeast cells inhibits growth. (A) Growth of the W303-1A strain transformed with pYES2 empty vector (circles), or pYES2 carrying Hs*PARP1* (diamonds), At*PARP1* (squares), At*PARP2* (triangles), or At*PARP3* (stars). *PARP* expression was induced by the addition of galactose to the medium. Growth was scored by determining OD_{600} at the indicated time points. (B) Protein PARylation in W303-1A yeast cells as determined by PAR antibody after 6 h of PARP gene expression. (C) Growth of the AD1234567 strain transformed with pYES2 empty vector (circles), or pYES2 carrying Hs*PARP1* (diamonds) or At*PARP1* (squares). *PARP* expression was induced by the addition of galactose to the medium. Growth was scored by determining OD_{600} at the indicated time points. (A–C) Error bars represent SE of three replicates. An asterisk indicates that the mean of the yeast strain transformed with the *PARP* gene is significantly different from the mean of the pYES2-transformed strain according to two-sample two-sided Welch T-test at significance level 0.05. Experiments were repeated twice with similar results.

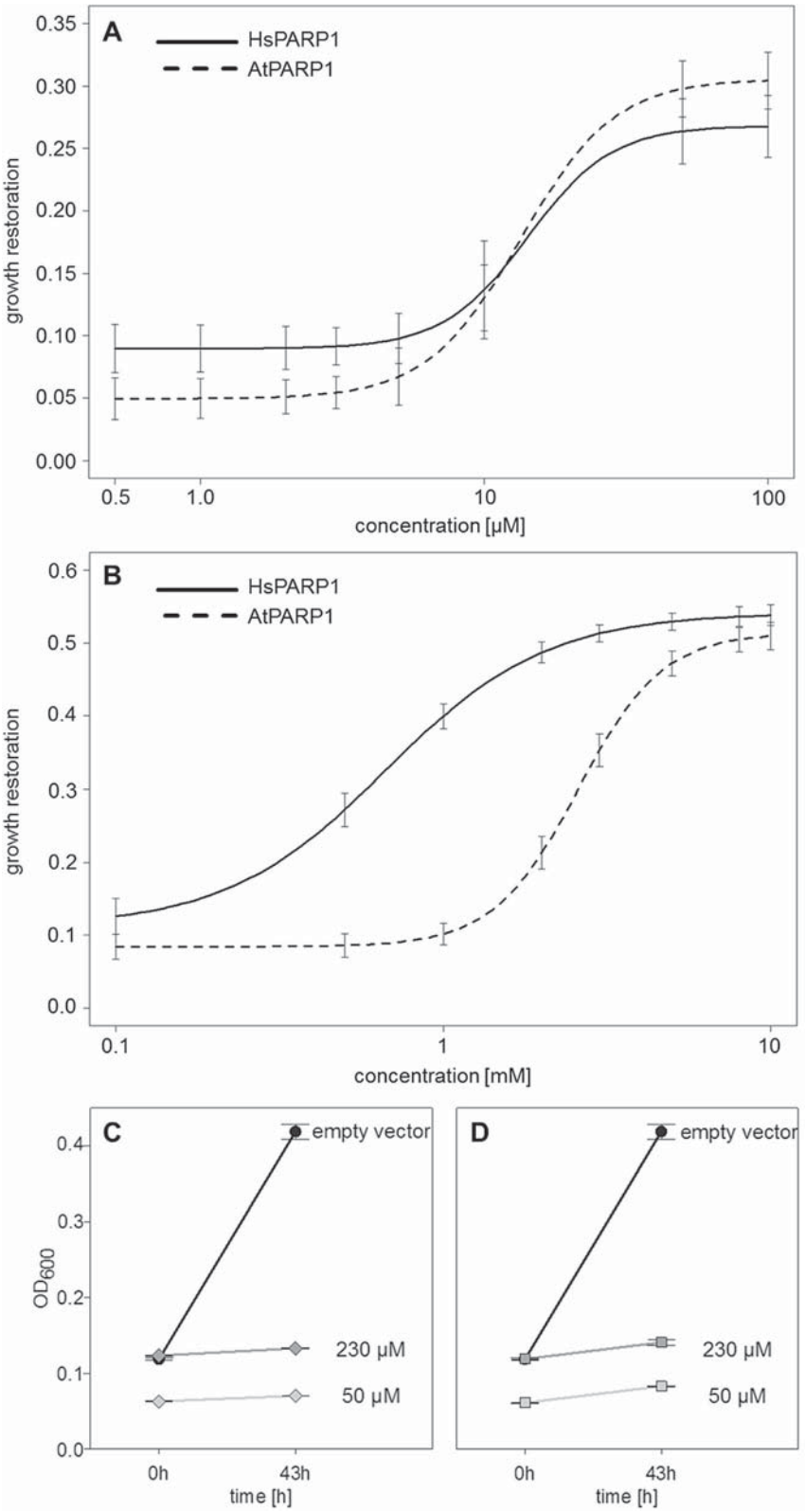

Fig. 2. The human PARP inhibitors PHE and 3-AB, but not 4-ANI, restore yeast cell growth inhibited by Hs*PARP1* and At*PARP1* expression. (A, B) The PARP inhibitors PHE (A) and 3-AB (B) restored growth in a dose-dependent manner. Dose-response curves of one out of two independent experiments are shown. (C, D) 4-ANI did not restore growth of yeast expressing Hs*PARP1* (C) or At*PARP1* (D). (A–D) In all experiments, OD_{600} of the cultures was measured at the beginning of the experiment and after 43 h of growth. For 4-ANI, only the lowest and the highest tested concentrations of PARP inhibitors are shown. Error bars represent SE of three replicates per yeast strain and inhibitor concentration. Experiments were repeated twice with similar results.

strain AD1234567 (*MATα; PDR1-3, ura3, his1, yor1Δ::hisG, snq2Δ::hisG, pdr5Δ::hisG, pdr10Δ::hisG, pdr11Δ::hisG, ycf1Δ::hisG, pdr3Δ::hisG*) [14], which lacks multiple ABC transporters, was employed for pharmacological assays. Again, induction of Hs*PARP1* and At*PARP1* expression led to an inhibition of yeast growth (Fig. 1C). To test their potential to restore growth, human PARP inhibitors were added as 10-fold stock solutions to yeast cell cultures (90 μL) in a 96-well microplate. OD_{600} was measured at 0 h and 43 h of growth. To quantify the potential of the inhibitors to restore yeast growth, dose-response curves were calculated using the statistical software R (version 3.3.2) [15] and a four-parameter log-logistic model as implemented in the drc package [16]. Two independent experiments were performed for each inhibitor. Weighed means and the corresponding standard errors were calculated as described by Jones and colleagues [17]. As expected, the addition of PHE to the growth medium alleviated the growth inhibition induced by Hs*PARP1* expression (Fig. 2A); an IC_{50} value of 13.5 ± 1.9 μM was determined, confirming a previous report [3]. Growth restoration was also observed for 3-AB (Fig. 2B). The calculation of the dose-response relationship resulted in an IC_{50} of 2.59 ± 0.1 mM, which is more than a 100 times that obtained for PHE. This difference is in agreement with inhibitor assays on the HsPARP1 protein itself, for which IC_{50} values of 0.3 and 30 μM have been determined for PHE and 3-AB, respectively [18–20]. The addition of PHE and 3-AB also reverted the growth arrest caused by expression of the plant gene At*PARP1* (Fig. 2A and B). Here, IC_{50} values of 9.4 ± 1.5 μM and 0.70 ± 0.05 mM were determined for PHE and 3-AB, respectively. Hence, the assay demonstrated that the human PARP inhibitors PHE and 3-AB block AtPARP1 activity. Surprisingly, the established human PARP inhibitor 4-ANI did not restore cell growth of yeast expressing HsPARP1 (Fig. 2C) or AtPARP1 (Fig. 2D). This ineffectiveness is in contrast to the high inhibitory capacity of 4-ANI on the HsPARP1 protein, where IC_{50} values of 0.15–0.18 μM have been reported [18,20]. A possible reason for this discrepancy may lie in an inability of 4-ANI to permeate the cell wall or the plasma membrane of the yeast cell. This notion is supported by the fact that 3-AB and PHE, but not 4-ANI, interfered with a biotic stress response in Arabidopsis, which also requires the permeation of cell wall and plasma membrane [12].

In this study, an assay was developed that demonstrated the functional similarity of HsPARP1 and AtPARP1 in yeast cells. This assay allows the screening for pharmacological inhibitors to AtPARP1 that are effective *in planta*. In addition, the assay may serve to identify target proteins of AtPARP1.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2017.04.002.

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3 Publication 2

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RESEARCH PAPER

The nuclear protein Poly(ADP-ribose) polymerase 3 (AtPARP3) is required for seed storability in *Arabidopsis thaliana*

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Keywords

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ABSTRACT

The deterioration of seeds during prolonged storage results in a reduction of viability and germination rate. DNA damage is one of the major cellular defects associated with seed deterioration. It is provoked by the formation of reactive oxygen species (ROS) even in the quiescent state of the desiccated seed. In contrast to other stages of seed life, DNA repair during storage is hindered through the low seed water content; thereby DNA lesions can accumulate. To allow subsequent seedling development, DNA repair has thus to be initiated immediately upon imbibition. Poly(ADP-ribose) polymerases (PARPs) are important components in the DNA damage response in humans. *Arabidopsis thaliana* contains three homologues to the human HsPARP1 protein. Of these three, only *AtPARP3* was very highly expressed in seeds. Histochemical GUS staining of embryos and endosperm layers revealed strong promoter activity of *AtPARP3* during all steps of germination. This coincided with high ROS activity and indicated a role of the nuclear-localised AtPARP3 in DNA repair during germination. Accordingly, stored *parp3-1* mutant seeds lacking *AtPARP3* expression displayed a delay in germination as compared to Col-0 wild-type seeds. A controlled deterioration test showed that the mutant seeds were hypersensitive to unfavourable storage conditions. The results demonstrate that AtPARP3 is an important component of seed storability and viability.

INTRODUCTION

Seed viability is an important agronomic trait; only viable seeds can produce healthy and productive offspring. Reduced seed viability as a result of seed deterioration is associated with the loss of membrane integrity, DNA damage and retarded metabolic activity (Priestley 1986). All these processes are linked to the action of reactive oxygen species (ROS). The production of ROS, and ensuing DNA damage, occur at different stages of a seed's life: during desiccation, storage and germination (Weitbrecht *et al.* 2011). During desiccation, ROS formation mainly results from respiration. At this stage of seed development DNA repair pathways are still active until the water content is limited (Bailly *et al.* 2008). Due to the low water content in dry seeds, metabolic activity is highly restricted; the seed remains in a quiescent stage. During this stage, metabolic ROS production is very unlikely. Here, non-enzymatic processes such as lipid peroxidation and Amadori and Maillard reactions lead to the formation of ROS (Sun & Leopold 1995; Kranner *et al.* 2010). The operation of enzymatic DNA repair mechanisms is also limited. Hence, DNA damage can accumulate in stored seeds, especially when seeds are stored under unfavourable conditions, such as high temperature and moisture (Weitbrecht *et al.* 2011). Prolonged seed storage can be simulated using artificial ageing experiments. Such controlled deterioration tests are a well-described means to study seed viability, longevity and storability (Tesnier *et al.* 2002; Rajjou *et al.* 2008).

The germination process starts with the uptake of water into the seed. This is associated with high levels of oxidative stress due to the restart of respiration and metabolic activity (Waterworth *et al.* 2011). The DNA repair machinery now has to cope with damage accumulated during storage and that arising from the start of metabolic activity. Double strand breaks are considered to be the most critical DNA lesions since they can cause chromosomal fragmentation and rearrangement (Waterworth *et al.* 2011; Ventura *et al.* 2012). In eukaryotes, DNA double strand breaks are repaired through two different pathways: homologous recombination (HR) and non-homologous-end-joining (NHEJ). In plants, homologues to components of animal HR and NHEJ have been identified (Waterworth *et al.* 2011).

In humans poly(ADP-ribose) polymerases (PARPs) have been described as important components of the DNA damage response. Activated upon DNA strand breaks, HsPARP1 uses NAD^+ as a substrate to transfer ADP-ribose moieties onto nuclear proteins involved in the DNA strand break response. Thereby poly(ADP-ribose) chains are formed, recruiting proteins involved in DNA repair. Human PARP1 has been described to be involved in both DNA single and double strand break responses (De Vos *et al.* 2012; Pines *et al.* 2013). It is the founding member of the PARP family, comprising 17 members, all showing structural homology to the catalytic domain of HsPARP1. Apart from HsPARP1, only HsPARP2 and HsPARP3 show DNA-dependent activity (De Vos *et al.* 2012; Pines *et al.* 2013). Human PARP2 is also involved in DNA

Table 1. Primers used in this study.

PCR screening and RT-PCR	*parp3-1*_for	AAAGCCTGAAACGATGACGG
	*parp3-1*_rev	AAGGCACAGTTATACAAGAGTCCAT
	Salk_LBa	GGTTCACGTAGTGGCCATCG
	Salk_LBb	GCGTGGACCGCTTGCTGCAACT
PARP3-EYFP	PARP3-EYFP_for	AAAAAACCCGGGATGAAGGTTCACGAGACAAGATCT
	PARP3-EYFP_rev	AAAAAACCCGGGCTCTGGTTCGACATCGACTATCTC
Pr*PARP1*-GUS	Pr*PARP1*-GUS_for	AAAAAAGGATCCTCTCTGCTTCTCCTTCTTCTTGAG
	Pr*PARP1*-GUS_rev	AAAAAAGGATCCTTCTCCGGTAAGAGACAATTACACA
Pr*PARP2*-GUS	Pr*PARP2*-GUS_for	AAAAAAGGATCCGTAAATGGTTTCACAACTTGGTTCC
	Pr*PARP2*-GUS_rev	AAAAAAGGATCCTTTCGTCTTCTTCTTCAGGAGAA
Pr*PARP3*-GUS	Pr*PARP3*-GUS_for	AAAAAACCCGGGAGCATTGTCTCTATCAACCCC
	Pr*PARP3*-GUS_rev	AAAAAACCCGGGTGAGCAAACTCTTTGAACTGTATGA

single strand break repair (Pines *et al.* 2013). Action of HsPARP3 was studied only recently; it was found to act as a component of NHEJ. One possible interaction partner of HsPARP3 is the Ku70/Ku80 complex (Rouleau *et al.* 2007). Additionally, an interaction with the XRCC4/Lig4 complex has been described (Rulten *et al.* 2011).

In the genome of *Arabidopsis thaliana* three *PARP* genes have been identified, *AtPARP1*, *AtPARP2* and *AtPARP3* (Lepiniec *et al.* 1995; Babiychuk *et al.* 1998; Hunt *et al.* 2004). Protein sequences of AtPARP1 and AtPARP2 show strong homology to the catalytic domain of HsPARP1 (Lepiniec *et al.* 1995; Babiychuk *et al.* 1998). Similar to HsPARPs, AtPARP1 and AtPARP2 have been described to interact with DNA and are involved in DNA damage and strand break responses and in cell cycle progression (Amor *et al.* 1998; Babiychuk *et al.* 2001; Doucet-Chabeaud *et al.* 2001; Storozhenko *et al.* 2001; Jia *et al.* 2013). *AtPARP3* is expressed in seed tissue, but has not been characterised functionally (Hunt *et al.* 2007).

To further elucidate the role of AtPARP3 during seed germination, histochemical staining methods were employed to study *AtPARP3* promoter activity and ROS accumulation, and seed germination of a *parp3* mutant was scored. Our results indicate that AtPARP3 is an important component for the maintenance of seed viability during storage.

MATERIAL AND METHODS

Phylogenetic analysis

To identify PARP proteins in *Populus trichocarpa*, *Oryza sativa* and *Physcomitrella patens* BLAST searches were performed using the NCBI database (http://blast.ncbi.nlm.nih.gov). Phylogenetic analysis was performed using Clustal W2 (www.ebi.ac.uk./Tools/msa/clustalw2/). A phylogenetic tree was generated using TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Plant material and identification of homozygous *parp3* mutant plants

A T-DNA insertion line for *PARP3* (SALK_108092) was obtained from the European Arabidopsis Stock Centre (NASC; Alonso *et al.* 2003; Ülker *et al.* 2008). To validate the T-DNA insertion by PCR, the left border primer SALK_LBa and gene-specific primers spanning the predicted insertion site were used (Table 1). The amplicon was sequenced using the SALK_LBb primer. To confirm gene knockout, RNA was extracted from dry seeds using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, Traufkirchen, Germany). cDNA synthesis was performed using Superscript II reverse transcriptase (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. RT-PCR was done with gene-specific primers spanning the T-DNA insertion site (Table 1). *AtACT2* served as a positive control.

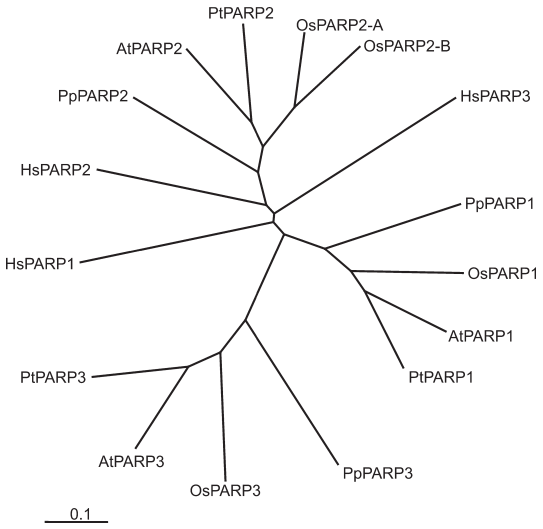

Fig. 1. PARP proteins are found in diverse plant species. A phylogenetic analysis was performed using the distance-based neighbour-joining method with Clustal W2. The tree was visualised with TreeView software. Full-length sequences of the PARP proteins were used for sequence alignment and aligned sequences were used to generate the phylogenetic tree. The scale bar of 0.1 represents 10% sequence divergence. The accession numbers of the sequences available in the NCBI database are as follows: *Populus trichocarpa*: PtPARP1 (XP_002302058), PtPARP2(XP_006375453), PtPARP3 (XP_002313672); *Oryza sativa*: OsPARP1 (NP_001059453), OsPARP2-A (Q5Z8Q9), OsPARP2-B (QOJMY1), OsPARP3 (NP_001047021); *Physcomitrella patens*: PpPARP1 (XP_001769471), PpPARP2 (XP_001782209), PpPARP3 (XP_001763226); *Homo sapiens*: HsPARP1 (NP_001609.2), HsPARP2 (NP_05475), HsPARP3 (NP001003931.2); *Arabidopsis thaliana*: AtPARP1 (NP_850165), AtPARP2 (NP_192148), AtPARP3 (NP_197639).

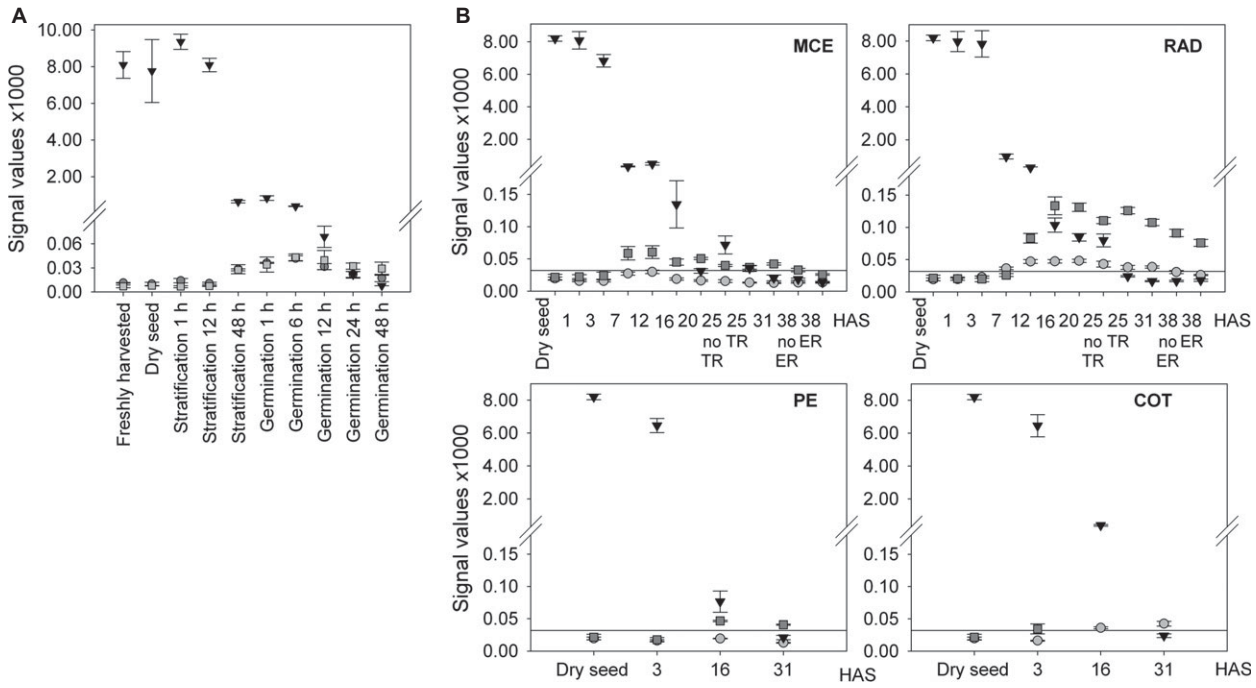

Fig. 2. Publicly available microarray data reveal high *AtPARP3* transcript levels in dry, imbibed and germinating seeds. A: Expression of *AtPARP1* (circles), *AtPARP2* (squares) and *AtPARP3* (triangles) during different stages of seed imbibition and germination. Data were retrieved from an assay described in Narsai *et al.* (2011). Data points marked with asterisks indicate an absent call ($P > 0.06$). B: Expression of *AtPARP1* (circles), *AtPARP2* (squares) and *AtPARP3* (triangles) in the micropylar and chalazal endosperm (MCE), the peripheral endosperm (PE), the radical and hypocotyl (RAD) and the cotyledons (COT). Gene expression was determined at different time points (hours after sowing, HAS) of seed germination. At 25 HAS, gene expression level was determined in both seeds with a ruptured testa (TR) and a non-ruptured testa (no TR). At 38 HAS, transcript level was determined prior to endosperm rupture (ER) and after endosperm rupture (no ER). Data were retrieved from an assay described in Dekkers *et al.* (2013).

Cloning of GUS and EYFP vectors

To generate the pBI101-PrPARPx-GUS vectors, the sequences including the 5′UTR and promoter of the three *AtPARP* genes were cloned from *Arabidopsis* Col-0 genomic DNA. For *AtPARP1* a sequence of 2034 bp and for *AtPARP2* a sequence of 2041 bp upstream of the ATG was PCR-amplified using *BamHI* restriction site-containing primers. To amplify *AtPARP3, XmaI* restriction site-containing primers were used, amplifying a sequence of 1961 bp upstream of the ATG (Table 1). All sequences were cloned upstream of the *uidA* gene into the binary vector pBI101.3 (Jefferson *et al.* 1987). *Arabidopsis* Col-0 plants were stably transformed with *Agrobacterium tumefaciens* strain GV3101 using the floral dip method (Clough & Bent 1998). Transformed plants were selected on half-strength MS agar plates containing 50 $mg \cdot l^{-1}$ kanamycin.

For the construction of an EYFP localisation vector, *AtPARP3* cDNA was obtained from 16-day-old *Arabidopsis* Col-0 plants, which were treated for 2 days with 250 mM NaCl to induce *AtPARP3* expression (Ogawa *et al.* 2009). Primers containing *XmaI* restriction sites (Table 1) were used to amplify *AtPARP3* without the stop codon. *AtPARP3* was cloned into the *XmaI* restriction site of the pART7 vector containing *EYFP* ligated into the *BamHI* restriction site of the plasmid (Peiter *et al.* 2007).

Germination and controlled seed deterioration assays

Seeds were collected from mutant and wild-type plants grown side-by-side in a controlled environment greenhouse. To analyse seed germination, seeds were surface-sterilised with 4% NaOCl (33.3% bleach) and 0.02% Triton X-100. Approximately 150 seeds per genotype were sown onto three half-strength MS agar plates (adjusted to pH 5.8 with 2.5 mM MES-KOH). Seeds were stratified for 2 days at 4 °C and plates were placed horizontally into a plant growth cabinet (AR-75; Percival Scientific, Perry, IA, USA) set to 16 h/8 h day/night, 22/18 °C, 65% RH, and a light intensity of 130 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Seed germination was scored under a stereomicroscope (SteREO Discovery V.20; Carl Zeiss, Jena, Germany) at the indicated time points. Experiments were repeated three times. Statistically significant differences were determined using the *t*-test tool of the SigmaPlot10.0 (Systat Software, San Jose, CA, USA) software package.

A controlled deterioration assay was performed as described in Bentsink *et al.* (2000). In brief, seeds were equilibrated for 4 days at 15 °C and 85% RH. Control seeds were re-dried for 3 days at 20 °C and ambient humidity. Seed ageing was performed for 4 days at 40 °C and 85% RH, followed by re-drying as above. Seeds were stored at 4 °C. Germination assays were performed immediately after deterioration.

Histochemical estimation of *PARP* gene expression and ROS activity

To analyse *PARP* gene expression during germination, PrPARP1-GUS, PrPARP2-GUS and PrPARP3-GUS seeds were subjected to a germination assay. At indicated stages of germination, seeds were collected from agar plates and seed coats

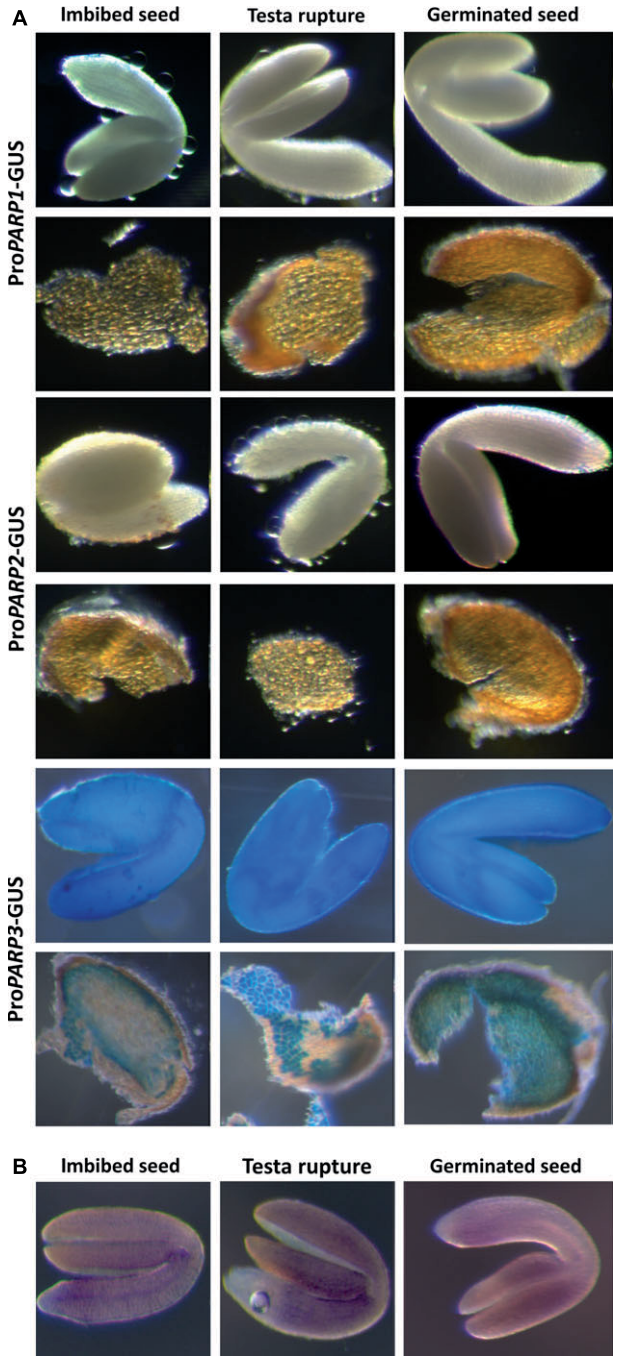

Fig. 3. *AtPARP3* expression and ROS activity coincide during seed germination. A: *AtPARP* promoter activity in embryo and endosperm tissues during different stages of seed germination. Gene expression was visualised with GUS staining. The seed coats were removed before staining. B: ROS activity during different stages of seed germination. ROS activity was visualised with NBT staining. The seed coats were removed before staining.

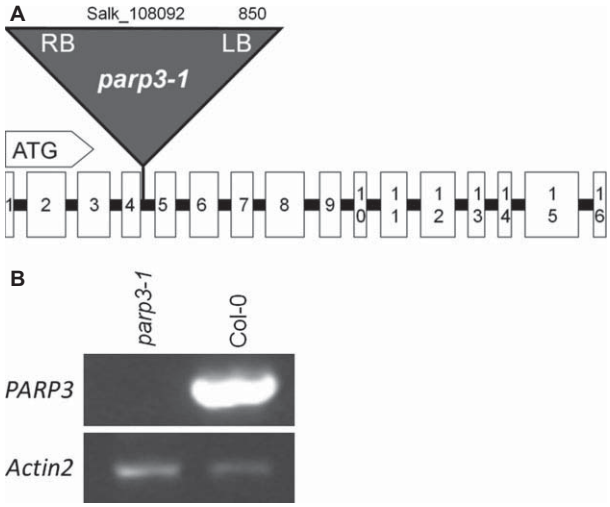

Fig. 4. Identification of a T-DNA insertion line for *AtPARP3*. A: Model of the *AtPARP3* genomic region and the T-DNA insertion. Coding regions are presented as white boxes; introns are shown with a line. The triangle indicates the site of the T-DNA insertion. B: RT-PCR analysis on RNA isolated from seeds showing the absence of the full-length *AtPARP3* transcript in the mutant line. *AtACT2* served as a control.

were removed under a stereomicroscope. Embryos and seed coats were transferred to GUS staining solution (100 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 3 mM $K_4[Fe(CN)_6]$, 0.5 mM $K_3[Fe(CN)_6]$, 0.1% Triton X-100, 2 mM X-Gluc in DMSO). The staining solution was vacuum-infiltrated twice for 5 min, and PrPARP3-GUS tissues were further incubated for 30 min at 37 °C. PrPARP1-GUS and PrPARP2-GUS tissues were incubated for at least 16 h. Stained embryos were stored in 80% ethanol. To determine ROS activity in embryos of germinating *Arabidopsis* Col-0 seeds, embryos were incubated in 0.5 $mg \cdot ml^{-1}$ nitroblue tetrazolium (NBT) in 10 mM potassium phosphate buffer (pH 7.8) for 30 min at room temperature in the dark. Subsequently, seeds were stored in 70% glycerol. Pictures of GUS and ROS staining were taken using an Axiocam HRc digital camera (Carl Zeiss) mounted on a SteREO Discovery V.20 stereomicroscope.

Localisation of AtPARP3 in mesophyll protoplasts

Mesophyll protoplasts were transformed with the pART7-PARP3-EYFP plasmid as described before (Peiter *et al.* 2005). After transformation, protoplasts were incubated for 20–24 h at 23 °C in the dark. EYFP fluorescence was visualised with confocal microscopy using a LSM 510META (Carl Zeiss).

RESULTS

Phylogenetic analysis

Homologues to the three *Arabidopsis* PARP proteins were present in all other plant species that we examined (Fig. 1). The moss *Physcomitrella patens* and the deciduous tree *Populus trichocarpa* possess one orthologue of each PARP, while the graminaceous crop rice (*Oryza sativa)* possesses one orthologue of PARP1 and PARP3, but two orthologues of PARP2.

Publicly accessible microarray data reveal high *AtPARP3* transcript levels in dry, imbibed and germinating seeds

A survey of publicly available microarray data showed a specific pattern of *PARP* gene expression in seeds. Data obtained by Narsai *et al.* (2011) revealed particularly high *AtPARP3*

expression in freshly harvested and dry seeds. During seed stratification and subsequent germination the expression level of *AtPARP3* decreased. At the time point of 48 h of germination, *AtPARP3* expression was absent from seed tissue (Fig. 2A). *AtPARP1* and *AtPARP2* transcripts were absent until 48 h of stratification. Subsequently, transcript levels slightly increased (Fig. 2A). Data from a high-resolution transcriptome analysis of Dekkers *et al.* (2013) confirmed this pattern of *PARP* gene expression during the germination process (Fig. 2B). *AtPARP1* and *AtPARP2* transcripts were absent in dry seeds and during early phases of imbibition, and remained low in subsequent phases. *AtPARP3* expression level was high in dry seeds, remained at that level during the first hours of imbibition, and decreased thereafter. This expression pattern was similar in micropylar and chalazal endosperm, peripheral endosperm, radicle and hypocotyl, and in cotyledons (Fig. 2B).

Expression of *AtPARP3* and ROS activity coincide in germinating embryos

Promoter-GUS analysis was employed to validate *AtPARP3* gene expression in germinating seeds and to obtain spatial information on promoter activity. During all stages of germination, high levels of promoter activity were visualised throughout the embryo and the endosperm (Fig. 3A). Particularly strong expression was found in the radical tip. This supported the notion of a role for *AtPARP3* during seed germination. *AtPARP1* and *AtPARP2* promoter activity in germinating seeds could not be shown in promoter-GUS analysis, supporting the very low transcript levels in the *in silico* data sets (Fig. 2).

Activity of ROS in embryos of germinating seeds was visualised with NBT staining. Similar to the GUS staining, ROS activity was evenly distributed in the germinating embryo during all stages (Fig. 3B). Hence, during germination *AtPARP3* gene expression and ROS activity coincided.

A knockout line was identified for *AtPARP3*

To analyse the function of *AtPARP3* in seeds, we identified a T-DNA insertion line, *parp3-1*, in the SALK collection. The *AtPARP3* gene consists of 16 exons and 15 introns. The left border of the T-DNA was found to localise in the fourth intron, 850 bp downstream of the ATG (Fig. 4A). According to PCR analysis, the right border is located upstream of the left border, towards the 5′-end of the gene. RT-PCR on RNA from dry seeds confirmed that no *AtPARP3* transcript was present in this T-DNA line (Fig. 4B).

Seeds of *parp3-1* are affected in storability

Seeds of the *parp3-1* mutant and its Col-0 wild type that had been stored for 8 months were subjected to a germination assay. The *parp3-1* mutant showed a clear delay in seed germination compared to the Col-0 seeds (Fig. 5A). To analyse whether this delay was linked to enhanced ABA sensitivity or reduced seed storability, germination of freshly harvested seeds was studied. Under control conditions, freshly harvested *parp3-1* mutant seeds did not show the germination delay (Fig. 5B). Treatment of seeds with 5 μM ABA prolonged the germination process in both genotypes and reduced the germi-

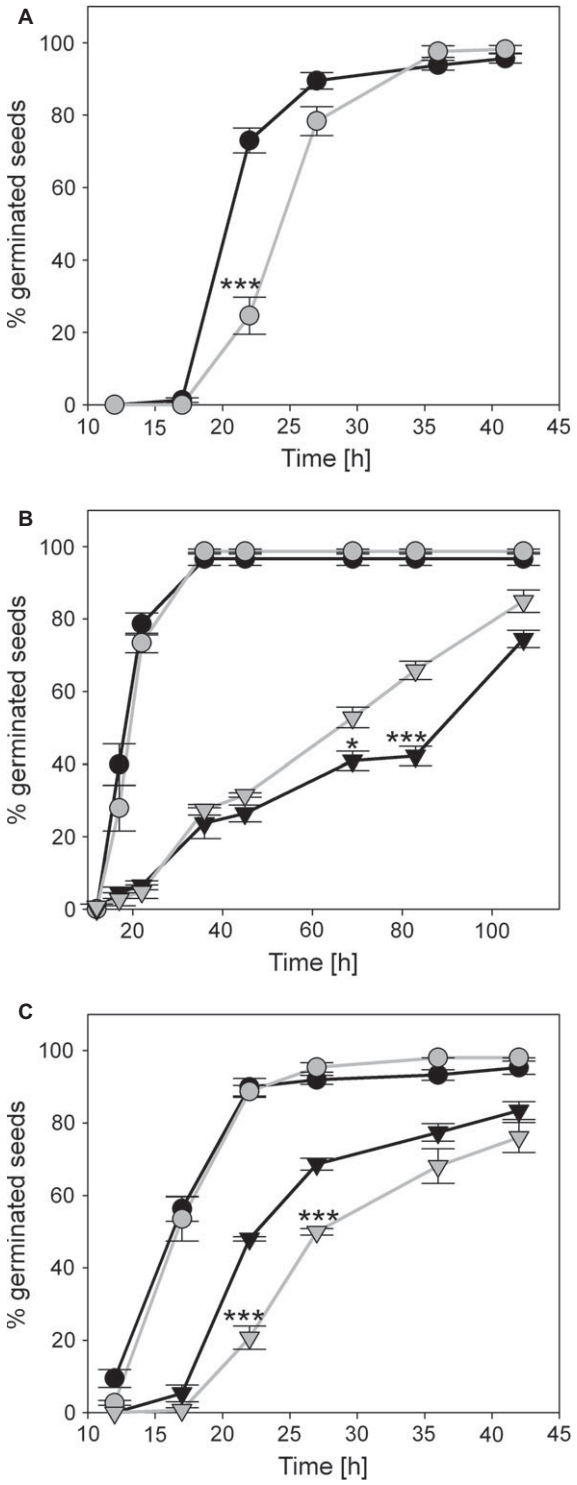

Fig. 5. Stored *parp3-1* mutant seeds show a delay in germination, which is due to accelerated deterioration. A: Germination of stored Col-0 (black) and *parp3-1* (grey) seeds on half-strength MS medium. B: Germination of Col-0 (black) and *parp3-1* (grey) seeds on half-strength MS medium (circles) or on half-strength MS medium supplemented with 5 μM ABA (triangles). C: Germination of freshly harvested Col-0 (black) and *parp3-1* (grey) seeds after equilibration (circles) or after artificial seed ageing (triangles). Data are means ± SE of 150 seeds. The experiments were repeated three times with comparable results. The asterisks mark significant differences (***$P < 0.005$, *$P < 0.05$).

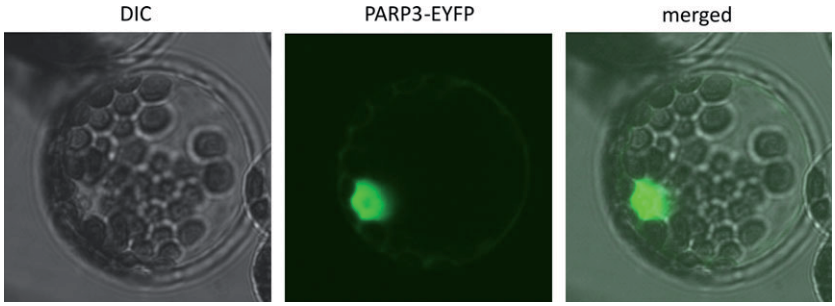

Fig. 6. Confocal image of an *Arabidopsis* mesophyll protoplast transiently expressing a *PARP3-EYFP* fusion construct.

nation rate to approx. 80%. Surprisingly, *parp3-1* mutant seeds even showed slightly accelerated germination compared to Col-0 seeds (Fig. 5B). The freshly harvested seeds underwent a controlled deterioration treatment. Thereby seeds were first equilibrated at relatively low temperature and high humidity, followed by artificial ageing under high temperature and high humidity conditions. Equilibration did not affect the germination of freshly harvested *parp3-1* and Col-0 seeds (Fig. 5C), whereas artificial seed ageing reduced the germination of both genotypes. However, this reduction was more pronounced in the *parp3-1* mutant seeds.

The *AtPARP3* protein is localised to the nucleus

The localisation of plant PARP3 proteins has not been determined previously. As the observed germination phenotype is in line with a role of the protein in genome stability, it was localised in transiently transformed mesophyll protoplasts using an AtPARP3-EYFP fusion construct. Similar to AtPARP1 and AtPARP2, AtPARP3 was localised in the nucleus, supporting a function in DNA repair (Fig. 6).

DISCUSSION

Recently, AtPARP3 has been hypothesised to be involved in DNA damage repair in stored seeds, but such a role in maintaining viability has not been demonstrated hitherto (Hunt *et al.* 2007; Hunt & Gray 2009). In this study, we showed high *AtPARP3* promoter activity throughout the plant embryo coinciding with ROS activity during germination. Promoter activities of *AtPARP1* and *AtPARP2* were below the threshold of detection with histochemical GUS staining, albeit in publicly available microarray data sets their expression was shown to increase slightly above the background during germination (Figs 2 and 3). This indicated an important and specific role of *AtPARP3* in the repair of ROS-induced DNA damage, which accumulates during seed storage and/or occurs during germination. This hypothesis is further supported by the fact that we identified a nuclear localisation signal in the PARP3 protein sequence from NLSMapper prediction (Kosugi *et al.* 2009). In support of nuclear localisation, the PARP3-EYFP fusion construct was localised in the nucleus of mesophyll cells (Fig. 6).

Interestingly, PARP3 is present in all plant species that we examined (Fig. 1). An expression of this gene during the very early stages of germination, similar to the pattern we observed in *Arabidopsis*, has also been demonstrated for the rice orthologue (Howell *et al.* 2009). This indicates that the role of PARP3 during germination may be widely conserved throughout the plant kingdom.

Like HsPARP1, *AtPARP1* contains two Zn-finger domains, which are responsible for the sensing of DNA single strand nicks (Eustermann *et al.* 2011). According to ClustalW alignment, *AtPARP3* shows 31% sequence identity to AtPARP1, and both proteins share several functional domains, *i.e.* PADR1, BRCT and WGR, as well as the regulatory and catalytic PARP domains. However, *AtPARP3* lacks the two DNA-binding Zn-finger domains of *AtPARP1*. Similarly, HsPARP3, which was attributed a specific role in double strand break repair *via* acceleration of the NHEJ pathway (Rouleau *et al.* 2007; Rulten *et al.* 2011), also lacks Zn-finger domains. It is therefore tempting to infer that AtPARP3 plays an important role in DNA double strand break repair in seeds.

The DNA double strand break repair by HsPARP3 is conferred *via* an interaction with the Ku70/Ku80 heterodimer and recruitment of the XRCC4/DNA ligase4 complex (Rouleau *et al.* 2007; Rulten *et al.* 2011). The Ku70/Ku80 complex may also be an interaction partner for AtPARP3. Seeds of *ku70* mutant *Arabidopsis* plants were shown to exhibit reduced germination potential on MMS, an alkylating agent inducing single strand breaks (Riha *et al.* 2002). During DNA replication these single strand breaks are converted into double strand breaks. These are then repaired by NHEJ before cell cycle progression from G1 to S phase (Riha *et al.* 2002). Moreover, *AtPARP3* possibly also recruits the DNA ligases AtLIG4 and AtLIG6 to sites of DNA double strand breaks. DNA ligase LIG4 has been described to play a specific role in NHEJ in humans (Rulten *et al.* 2011). LIG6 is a plant-specific enzyme. Seeds carrying mutations for either or both genes are affected in germination, similar to *parp3-1* seeds (Waterworth *et al.* 2010).

The stored *parp3-1* seeds showed delayed germination compared to Col-0 seeds (Fig. 5A). It was tested whether this phenotype might be linked to enhanced ABA sensitivity or to reduced seed viability as a consequence of seed storage. Freshly harvested *parp3-1* seeds exhibited accelerated germination as compared to the wild type on 5 μM ABA (Fig. 5B). In contrast, artificial ageing of the *parp3-1* seeds evoked a similar phenotype as seed storage (Fig. 5C). Seed storage and artificial seed ageing are known to provoke DNA damage, which can finally result in programmed cell death. In *parp3-1* mutant seeds, the initiation of DNA double strand break repair is likely to be restricted, which would affect cell cycle progression and hence lead to the observed delay in seed germination and reduced seed viability.

Seed viability strongly depends on DNA integrity. During storage, DNA damage accumulates and has to be repaired during

germination. Structural homology to AtPARP1 and HsPARP3 suggests a role for AtPARP3 in DNA double strand break repair *via* NHEJ. Further research is now necessary to better understand the DNA double strand break response in germinating seeds. In particular, possible interactions between AtPARP3 and components of the NHEJ pathway need to be determined.

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4 Publication 3

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No Silver Bullet – Canonical Poly(ADP-Ribose) Polymerases (PARPs) Are No Universal Factors of Abiotic and Biotic Stress Resistance of *Arabidopsis thaliana*

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Abiotic and biotic stress can have a detrimental impact on plant growth and productivity. Hence, there is a substantial demand for key factors of stress responses to improve yield stability of crops. Members of the poly(ADP-ribose)polymerase (PARP) protein family, which post-translationally modify (PARylate) nuclear proteins, have been suggested as such universal determinants of plant stress responses. A role under abiotic stress has been inferred from studies in which a genetic or, more commonly, pharmacological inhibition of PARP activity improved the performance of stressed plants. To further elucidate the role of PARP proteins under stress, T-DNA knockout mutants for the three *Arabidopsis thaliana* PARP genes were subjected to drought, osmotic, salt, and oxidative stress. To exclude a functional redundancy, which was indicated by a transcriptional upregulation of the remaining *parp* genes, a *parp* triple mutant was generated. Surprisingly, *parp* mutant plants did not differ from wild type plants in any of these stress experiments, independent from the number of *PARP* genes mutated. The *parp* triple mutant was also analyzed for callose formation in response to the pathogen-associated molecular pattern flg22. Unexpectedly, callose formation was unaltered in the mutant, albeit pharmacological PARP inhibition robustly blocked this immune response, confirming previous reports. Evidently, pharmacological inhibition appears to be more robust than the abolition of all *PARP* genes, indicating the presence of so-far undescribed proteins with PARP activity. This was supported by the finding that protein PARylation was not absent, but even increased in the *parp* triple mutant. Candidates for novel PARP-inhibitor targets may be found in the SRO protein family. These proteins harbor a catalytic PARP-like domain and are centrally involved in stress responses. Molecular modeling analyses, employing animal PARPs as templates, indeed indicated a capability of the SRO proteins RCD1 and SRO1 to bind nicotinamide-derived inhibitors.

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Collectively, the results of our study suggest that the stress-related phenotypes of *parp* mutants are highly conditional, and they call for a reconsideration of PARP inhibitor studies. In the context of this study, we also propose a unifying nomenclature of *PARP* genes and *parp* mutants, which is currently highly inconsistent and redundant.

Keywords: abiotic stress, drought stress, flg22, plant immunity, pharmacological inhibition, poly(ADP-ribose) polymerases, salt stress, SRO proteins

INTRODUCTION

The frequency and severity of abiotic stress conditions, such as drought or heat waves, are prospected to increase markedly in the near future due to the prevalent climate change. These incidences, which also exacerbate disease pressure, are difficult to predict and can occur during sensitive stages of the cropping season, with a potentially detrimental impact on crop yield. To safeguard crop productivity and food security, it is necessary to find ways to improve the plants' performance under such conditions in the field. For this reason, there has been an intense search for key regulators in the plant's genetic set-up that have robust and consistent effects on stress tolerance. Members of the Poly(ADP-Ribose) Polymerase (PARP) protein family *sensu stricto* have been presumed to possess this property, and the interference with PARP activity -pharmacologically or genetically- has been suggested to improve plant stress responses (De Block et al., 2005; Jansen et al., 2009; Geissler and Wessjohann, 2011; Schulz et al., 2012).

Proteins of the PARP family are present in all eukaryotes except yeast. They are characterized by a PARP domain (Karlberg et al., 2013). The best-studied member of this protein family is its founding member human PARP1 (HsPARP1). Activated upon DNA strand breaks, HsPARP1 forms poly(ADP-ribose) chains by attaching ADP-ribose molecules to nuclear proteins, including itself, using NAD^+ as substrate. This fast and transient protein modification activates the DNA repair machinery (Pines et al., 2013). In humans, the PARP family comprises 17 members of which not all have PARP activity (Karlberg et al., 2013; Pines et al., 2013). In the model plant *Arabidopsis thaliana* three canonical PARP proteins have been identified, PARP1, PARP2, and PARP3 (Lepiniec et al., 1995; Babiychuk et al., 1998; Doucet-Chabeaud et al., 2001; Hunt et al., 2004). Unfortunately, the nomenclature of those Arabidopsis PARP proteins has been inconsistent in the past, with PARP1 and PARP2 being interchanged (Supplementary Table 1). In the following, PARP1 stands for the protein with the highest similarity to HsPARP1, encoded by At2g31320, while PARP2 is the protein encoded by At4g02390. Similar to the inconsistent gene nomenclature, the denomination of mutants of those genes is currently redundant and not co-ordinated. In this paper, we propose a unified mutant nomenclature, as described in the "Results" section.

Similar to their human counterparts, Arabidopsis PARP proteins play a role in DNA damage responses and the maintenance of DNA integrity under a range of circumstances. Thus, they mediate DNA repair, but also trigger programmed cell death, in response to oxidative genome stress (Amor et al., 1998), and the expression of *PARP1* and *PARP2* is induced by ionizing radiation (Doucet-Chabeaud et al., 2001). Consequently, knockout mutants for both genes are hypersensitive to DNA-damaging agents (Jia et al., 2013; Boltz et al., 2014; Song et al., 2015; Zhang et al., 2015). Both proteins have been shown to be associated with chromatin (Babiychuk et al., 2001) and to be involved in an alternative non-homologous DNA end joining pathway (Jia et al., 2013). Poly(ADP-ribosyl)ating activity of PARP1 and PARP2 has been demonstrated, confirming the presumed enzymatic action of the proteins (Babiychuk et al., 1998; Feng et al., 2015). Thereby, PARP2 was found to be the main contributor to PARP activity in plants.

Aside from their positive role in DNA repair, early inhibitor experiments indicated an involvement of PARPs in oxidative stress responses (Berglund et al., 1996). This association was also apparent in experiments with *Brassica napus* calli, in which chemical PARP inhibition improved growth under oxidative stress (De Block et al., 2005). In the same study, knockdown of *PARP* gene expression in Arabidopsis by RNAi constructs led to an increased tolerance to methyl viologen (paraquat). Those transgenic lines also showed an improved performance under drought stress (De Block et al., 2005). This obviously negative effect of PARPs on abiotic stress tolerance was explained by the load of NAD^+-consuming PARP activity on the plant's energy status. Alternatively, transcriptome analyses indicated that *PARP* effects on stress tolerance may be due to an interference in transcriptional and hormonal responses (Vanderauwera et al., 2007). In that study, high-light stress trigged decreased transcriptional oxidative stress responses, but increased levels of abscisic acid (ABA) and ABA-responsive gene expression, in *PARP1* RNAi plants as compared to the wild type. Chemical PARP inhibition similarly improved growth under stress, but also under control conditions (Schulz et al., 2012).

Besides those reports on a likely involvement of PARPs in abiotic stress responses, there is evidence that this protein modification also interferes with pathogen responses. The bacterial peptides flg22 and elf18 trigger cellular signaling networks that eventually lead to the launch of defense responses, such as the deposition of callose or lignin and the accumulation of pigments. These stress responses were blocked in Arabidopsis seedlings treated with PARP inhibitors (Adams-Phillips et al., 2008, 2010). In addition, *parp1 parp2* double mutants were slightly more susceptible to Pseudomonas bacteria (Feng et al., 2015).

In addition to the three canonical PARP proteins, members of another protein family, SRO (Similar to RCD One), also contain the catalytic core of the PARP domain, but not the

regulatory PARP domain (Jaspers et al., 2010b). This family comprises its founding member RCD1 (Radical-induced Cell Death 1) and its homologs SRO1 through SRO5. So far, RCD1 and SRO1 have been functionally characterized most extensively. RCD1 has initially been identified as a positive regulator of the tolerance to ozone and apoplastic superoxide, and *rcd1* mutants are hypersensitive to those stresses (Overmyer et al., 2000). Conversely, *rcd1* mutants are more resistant to methyl viologen, which triggers chloroplastic superoxide generation (Ahlfors et al., 2004; Fujibe et al., 2004). They are also more tolerant to freezing and UV-B radiation (Fujibe et al., 2004), but less salt-tolerant, which has been related to its interaction with the Na^+/H^+-antiporter SOS1 (Katiyar-Agarwal et al., 2006). The homeostasis of hormone signaling pathways, such as ABA, ethylene, salicylic acid, and jasmonate, is altered in *rcd1* mutants, and hence, RCD1 has been suggested to function as integrative node in hormonal signaling networks (Ahlfors et al., 2004; Overmyer et al., 2005). RCD1 interacts with numerous other proteins, many of which are transcription factors involved in stress responses (Jaspers et al., 2009; Vainonen et al., 2012). The protein most closely related to RCD1, SRO1, has partially redundant functions to RCD1 in development and stress responses (Jaspers et al., 2009; Teotia and Lamb, 2009). Taken together, SRO proteins are centrally involved in stress responses, redox regulation, hormonal signaling, and transcriptional networks.

All hitherto analyzed PARP-domain proteins (i.e., PARPs and SROs) have been suggested to act in various stress responses, whereby their involvement in different types of oxidative stress has been studied most extensively. In this context, SROs have positive or negative effects, depending on the nature of the stress. In contrast, canonical PARPs have been suggested as generally negative factors of abiotic stress tolerance, either by posing a load on energy status or by affecting transcriptional stress responses. However, there is only a very limited number of studies in support of such an effect of canonical PARPs, most of them based on pharmacological inhibition, which of course may not be selective to PARP targets but may also affect other proteins not looked at in these studies. Importantly, the degree of functional redundancy of the three *PARP* genes in stress responses is largely unclear. For this reason, we analyzed the response of Arabidopsis single, double, and triple *parp* knockout lines to various abiotic stresses and to a biotic cue. Surprisingly, in contrast to previous reports, plant performance was not altered in any of the mutant lines. Protein homology modeling indicated that the previously reported interferences of PARP inhibitors in responses to abiotic and biotic stress may have been caused by off-site effects on SRO family proteins. Such a more complex picture was supported by our finding that the knockout of all *PARP* genes leads to a constitutive activation of cellular PARP activity, possibly mediated by SRO proteins.

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana T-DNA insertional mutant lines for *PARP1* and *PARP2* were obtained from Nottingham Arabidopsis Stock Centre (NASC) and are shown in Supplementary Table 2. The lines are part of the GABI-Kat (Rosso et al., 2003) and SAIL (Sessions et al., 2002) collections. To validate T-DNA insertions, PCR reactions were performed, using the GABI-Kat left border primer 8409 or the SAIL left border primer LB1-short and gene-specific primers spanning the predicted T-DNA insertion site (Supplementary Table 3) (Ülker et al., 2008). For detailed mapping, the PCR products were sequenced. To confirm gene knockout, RNA was extracted from leaves of 14-day-old plants using the Spectrum Plant Total RNA Kit (Sigma). cDNA synthesis was performed using Superscript II reverse transcriptase (Life technologies) according to manufacturer's instructions. RT-PCR was done with gene-specific primers spanning the T-DNA insertion site. *ACT2* served as a housekeeping reference gene. A homozygous T-DNA knockout line for *PARP3*, *parp3-1* (SALK_108092) has been genotypically analyzed previously (Rissel et al., 2014). *parp* double mutant plants were generated by crossing *parp2-1* with *parp1-1* or *parp3-1*, and *parp3-1* with *parp1-1*. The *parp* triple mutant originated from a cross of *parp2-1 parp1-1* with *parp3-1 parp1-1*.

Quantification of *PARP* Gene Expression by qRT-PCR

PARP gene expression was analyzed on leaves and roots of 14-day-old plate-grown plants. RNA extraction and cDNA synthesis were performed as described above. Gene expression was determined by qRT-PCR as described previously (Lange et al., 2014), running a denaturation step at 95°C for 10 min followed by 40 amplification cycles (95°C for 15 s, 60°C for 1 min). *UBQ10* (At4g05320) was used as housekeeping reference gene (Peiter et al., 2007). Primers are listed in Supplementary Table 3.

Determination of Stomatal Conductance

Plants were grown in 40 g of a mixture of soil substrate (Tonsubstrat ED 73, Einheitserde Werkverband) and vermiculite in the ratio 2:1. To prevent sciarid infection, Biomükk (BioFA, Germany) was added to the mixture. The pots were covered with a nylon mesh to avoid loss of soil and contamination of leaves. After 5 weeks, plants of similar size for all genotypes were selected. Plant culture was performed in a growth room under short-day conditions (10 h light at 21°C, 14 h dark at 18°C, 130 μmol m^{-2} s^{-1}, 65% RH). The 10th, 11th, and 12th leaves of the plants were labeled with a thread. Experiments were conducted on 6-week-old plants. Pots were watered to identical weights until the evening before onset of measurements. Subsequently, water was withheld. Stomatal conductance was measured by using a porometer (AP4, Delta-T Devices, Cambridge, UK) at 11 am for the next 8–10 days. Experiments were performed in triplicate.

Root Growth Assays

To measure root elongation, surface-sterilized seeds were sown onto 1/2 Murashige and Skoog (MS) agar plates (pH 5.8). Seeds were stratified for 2 days at 4°C. Then agar plates were placed near-vertically into a plant growth cabinet (AR-75, Percival

Scientific, Perry, IA, USA) set to long-day conditions (16 h light at 22°C, 8 h dark at 18°C, 130 μmol m^{-2} s^{-1}, 65% RH). After 5 days of pre-culture, seedlings were transferred to 1/2 MS agar plates containing the indicated treatment. Root tip position was marked with a felt pen on the plate, and main root length was measured every 2–3 days. After 13–15 days plants were harvested and shoot fresh weight was determined.

Visualization of Callose Deposition

Callose deposition was determined according to Adams-Phillips et al. (2010). Surface-sterilized seeds were sown onto 1/2 MS agar plates (pH 5.8) containing 2% sucrose. After seed stratification at 4°C for 2 days, plates were placed near-vertically in a plant growth cabinet (ATC-26, Conviron, Winnipeg, MB, Canada) set to short-day conditions (10 h light at 22°C, 14 h dark at 18°C, 130 μmol m^{-2} s^{-1}, 65% RH), and plants were grown for 5 days. Thereafter, plants were transferred to liquid 1/2 MS medium containing 1.5% sucrose in 24-well microtiter plates and grown for another 24 h under the same conditions. Subsequently, 1 μM flg22 was added to the liquid medium, and plants were incubated for another 24 h. PARP inhibitors in DMSO or DMSO only were added at indicated time points and concentrations. For fixation, plants were transferred to FAA (formaldehyde, acetic acid, alcohol) solution and incubated for 24 h. Fixed seedlings were stored in 100% ethanol. Before staining, plants were washed in 50% ethanol and 67 mM KH_2PO_4 (pH 12). Subsequently, plants were stained in 0.01% aniline blue [in 67 mM KH_2PO_4 (pH 12)] for 1 h in the dark. To visualize callose deposition, plants were mounted onto slides in 70% glycerol and 30% staining solution. Six to twelve cotyledons per treatment were visualized under a fluorescence microscope (Axioskop, Carl Zeiss, Jena, Germany) equipped with a UV filter set (No. 9, Zeiss) and photographed with a digital camera (Axiocam MRc, Zeiss) driven by the AxioVision 4.7 software (Zeiss).

Determination of Poly(ADP-ribosyl)ation

Seeds were sown as a lawn onto the soil substrate-vermiculite mixture described above. After stratification, plants were cultured in a plant growth cabinet (AR-75, Percival Scientific) under long-day conditions (16 h light at 22°C, 8 h dark at 18°C, 130 μmol m^{-2} s^{-1}, 65% RH) for 32 days. Then, control plant leaves were cut and frozen in liquid nitrogen. To induce DNA damage and stimulate poly(ADP-ribosyl)ation, plants were treated with 1000 J m^{-2} UV-C light (254 nm) using a UV crosslinker (HL-2000, HybriLinker System, UVP, USA). Leaves were harvested 2 h after UV treatment and frozen in liquid nitrogen. After grinding in liquid nitrogen, nuclear protein was extracted as described by Xia et al. (1997). In brief, 2 g of frozen ground material was homogenized in 4 mL Honda buffer [2.5% Ficoll 400, 5% dextran T40, 400 mM sucrose, 25 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, protease inhibitor cocktail (P9599, Sigma–Aldrich)]. The homogenate was filtrated through a 70 μm (pore size) nylon net by centrifuging at 30 × *g* and 4°C. The tube was washed with 2 mL Honda buffer. Triton X-100 was added to a final concentration of 0.5%, and samples were incubated on ice for 15 min. Afterward, samples were centrifuged for 5 min at 1500 × *g* and 4°C, and the pellet was washed with Honda buffer containing 0.1 % Triton X-100. The pellet was resuspended in Honda buffer and centrifuged for 5 min at 100 × *g* and 4°C to pellet starch and cellular debris. The nuclei in the supernatant were centrifuged for 10 min at 1800 × *g* and 4°C. The pellet was resuspended in 150 μl Honda buffer. Subsequently, 3 μg protein sample were spotted in triplicate onto a nitrocellulose membrane using a dot blot 96 apparatus (Biometra, Göttingen, Germany) according to manufacturer's instructions. Equal protein loading was confirmed by Ponceau staining (0.2% Ponceau S in 0.5% acetic acid). Staining was fixed in 0.5% acetic acid. The membrane was washed in PBS and blocked with BSA (Carl Roth). Poly(ADP-ribosyl)ation was visualized using a monoclonal poly(ADP-ribose) antibody (10H, Enzo Life Sciences). After addition of a secondary anti-mouse antibody coupled to a horseradish peroxidase, ECL reagent (250 mg L^{-1} luminol, 0.1 M Tris-HCl pH 8.6, 1% DMSO, 1 g L^{-1} para-coumaric acid) was added. Luminescence was detected and quantified using a photon-counting camera (HRPCS218, Photek, St. Leonards on Sea, UK). The experiment was performed twice with similar results.

Molecular Modeling of RCD1 and SRO1

The PARP domains of Arabidopsis SRO1 (At2g35510, residues 245–463, according to NCBI) and RCD1 (At1g32230, residues 248–469, according to NCBI) were modeled using the catalytic domains of HsPARP10 [PDB entry 3HKV, Karlberg et al. (unpublished)], GgPARP1 [PDB entry 2PAX, Ruf et al. (1998)], or HsPARP14 [PDB entry 3SE2, Wahlberg et al. (2012)] as template structures. The templates were selected according to their co-crystallized inhibitors 3-aminobenzamide (3-AB), 4-amino-1,8-naphthalimide (4-ANI), and 6-(5H)-phenanthridinone (PHE), respectively. Using YASARA software [YASARA Structure, version 12.11.25, Krieger et al. (2002)], the three-dimensional structures of AtRCD1 and AtSRO1 were built. Since in YASARA template inhibitors are automatically transferred onto the target structure, each homology model includes the corresponding template inhibitor in the target active site. The models were finally refined by the YASARA module md-refinement which performs 20 steps of simulated annealing molecular dynamics simulations.

Statistical Analysis

In **Figures 4**, **5**, **7**, and **8** and Supplementary Figures 3–5, comparisons of two sample means were performed with two-sided two-sample Welch *t*-tests (Welch, 1947). In **Figures 6** and **11,** one-sided two-sample Welch *t*-tests were performed because an increase in gene expression and photon counts, respectively, was presumed. To compare more than two sample means (**Figure 3**; Supplementary Figure 2), one-factorial analysis of variance was performed at significance level of $\alpha = 0.05$, followed by a *post hoc* Tukey HSD test (Tukey, 1949), if significant differences were detected. In all figures, an asterisk indicates that the sample mean of the mutant line is significantly different from the sample mean of the wild type for the same treatment and time point ($P < 0.05$). Statistical analysis was performed in R software (version 3.3.2; R Core Team, 2016). Experiments were repeated two to three times with similar results.

RESULTS

Expression of *PARP* Genes Is Mostly Unresponsive to Drought, Osmotic, and Salt Stress

Canonical *PARP* genes have been suggested to act as regulators of abiotic stress resistance. Such a role is likely to be reflected in a transcriptional regulation under those conditions. To test this notion, we analyzed a number of microarray experiments in which plants were subjected to drought, osmotic, or salt stress (Kilian et al., 2007; Perera et al., 2008; Zhang et al., 2008; Mizoguchi et al., 2010; Chan et al., 2011; Bhaskara et al., 2012; Kinoshita et al., 2012). The experimental layouts and stress intensities varied substantially between those studies. Nevertheless, *PARP1* and *PARP2* were not notably up or downregulated in any of those experiments (**Figure 1**). *PARP3* gave a similar picture in most cases, albeit the upregulation was more pronounced in few instances, reaching up to 80-fold in one drought stress study. However, *PARP3* expression is nearly undetectable under control conditions (Rissel et al., 2014), so that its expression level is very low even under inducing conditions. This general unresponsiveness of *PARP* gene expression to abiotic stress was surprising, considering their presumed involvement in stress responses.

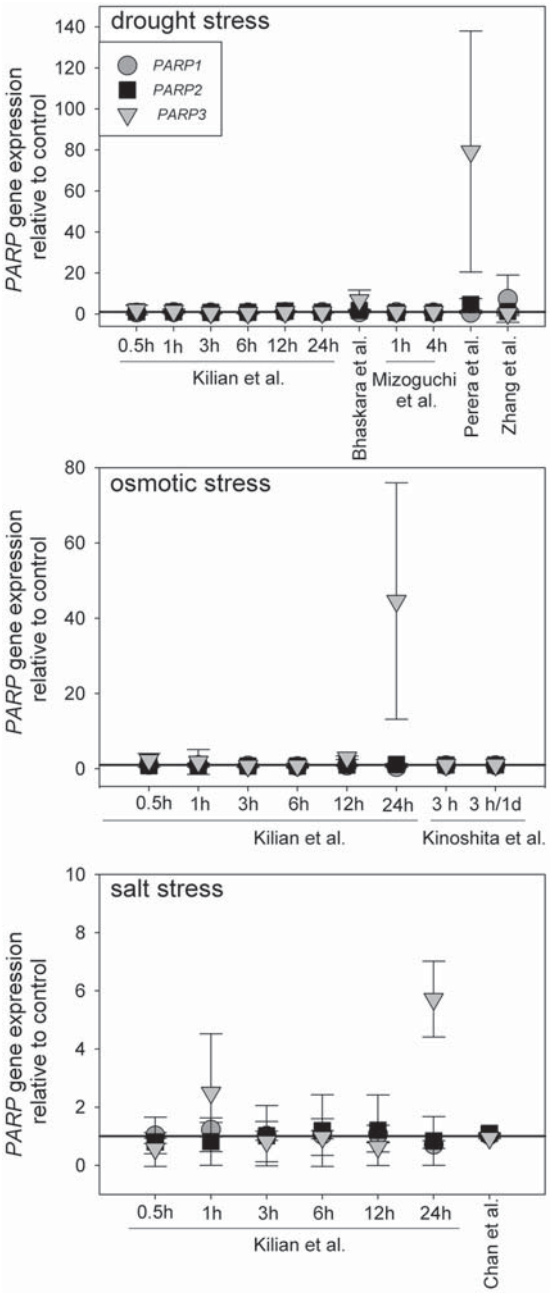

FIGURE 1 | Publicly available microarray data show a lack of stress-responsiveness of *PARP1* (circles), *PARP2* (squares), and *PARP3* (triangles). Data were retrieved from drought, osmotic, and salt stress studies (Kilian et al., 2007; Perera et al., 2008; Zhang et al., 2008; Mizoguchi et al., 2010; Chan et al., 2011; Bhaskara et al., 2012; Kinoshita et al., 2012). Expression levels are relative to control. Error bars represent standard deviation.

Identification of T-DNA Insertional Knockout Mutants for *PARP1* and *PARP2* Genes

To elucidate the involvement of PARP proteins in plant stress responses we searched publicly available T-DNA mutant collections for mutant lines for *PARP1* and *PARP2*. A mutant line for *PARP3* was identified previously (Rissel et al., 2014). In total, seven *parp1* mutant lines were identified carrying T-DNA insertions either in the promoter region of the gene or in its exons (**Figure 2A**; Supplementary Figure 1A). For *PARP2,* five mutant lines were identified with T-DNA insertions showing intron or exon localization (**Figure 2C**; Supplementary Figure 1B). The exact location of T-DNA borders, as determined by sequencing of genomic DNA, can be found in **Figure 2** and Supplementary Figure 1. Since an exon-localized T-DNA insertion is most promising to prevent full-length gene transcription, mutant lines carrying such an insertion were further characterized. The *PARP1* gene consists of 19 exons (**Figure 2A**). The T-DNA insertions in the *parp1-1*, *parp1-2*, and *parp1-3* mutants are located in exon 10, 8, and 14, respectively. The *PARP2* gene consists of 18 exons (**Figure 2C**). The T-DNA insertions in the *parp2-1* and *parp2-2* mutants are located in exon 16 and 15, respectively. Semi-quantitative RT-PCR analysis on leaves of 2-week-old plants confirmed the lack of *PARP1* and *PARP2* transcripts in those *parp1* and *parp2* T-DNA lines, respectively (**Figures 2B,D**).

A Unified Nomenclature for Arabidopsis *PARP* Genes and *parp* Mutants

Some of the T-DNA lines shown in **Figure 2** and Supplementary Figure 1 have been employed in previous analyses, but their nomenclature has been redundant and inconsistent so far. In combination with the above-mentioned inconsistency of the gene nomenclature (Supplementary Table 1), this complicates the integration and discussion of published experimental data. We have therefore compiled all publications involving *parp* mutants

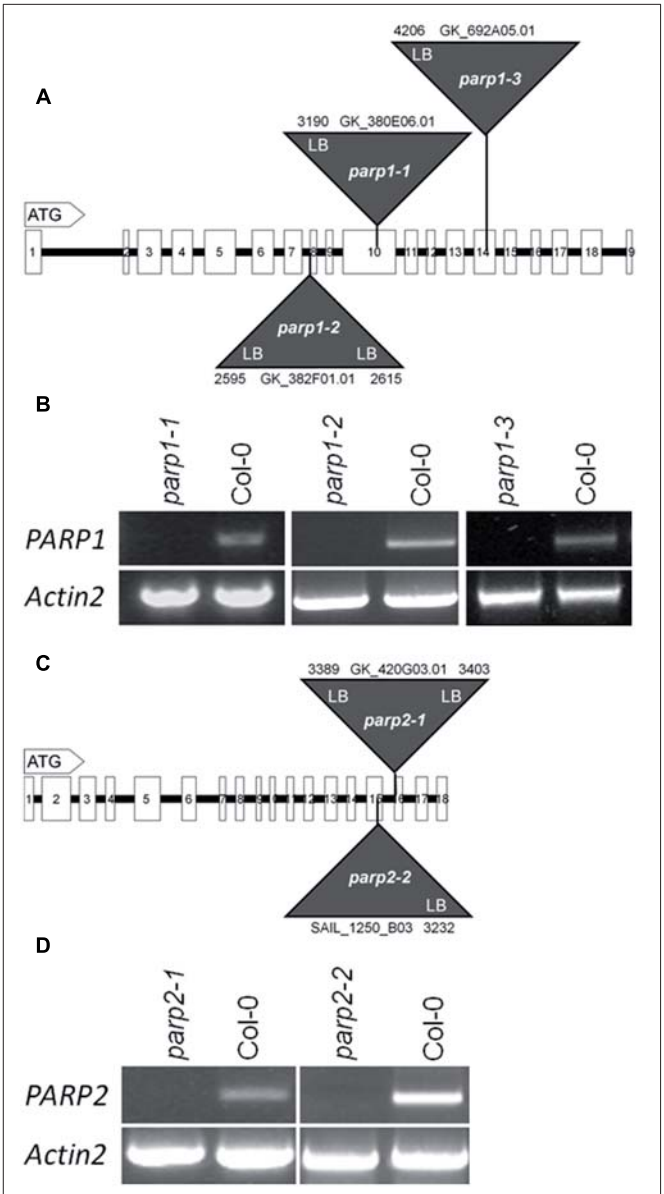

FIGURE 2 | T-DNA insertion lines were identified for *Arabidopsis thaliana PARP1* and *PARP2*. (A,C) Model of the genomic regions and the T-DNA insertions in *PARP1* **(A)** and *PARP2* **(C)**. Coding regions are presented by white boxes; introns are shown by a line. Triangles indicate the sites of T-DNA insertions. The numbers indicate the last nucleotide before and the first nucleotide after the insertion, counting from the start condon. LB and RB indicate the left and right border of the T-DNA, as determined by sequencing. **(B,D)** RT-PCR analysis of leaf RNA showing the absence of full-length transcript of the respective *PARP* gene in the mutant lines. *Actin2* served as a control.

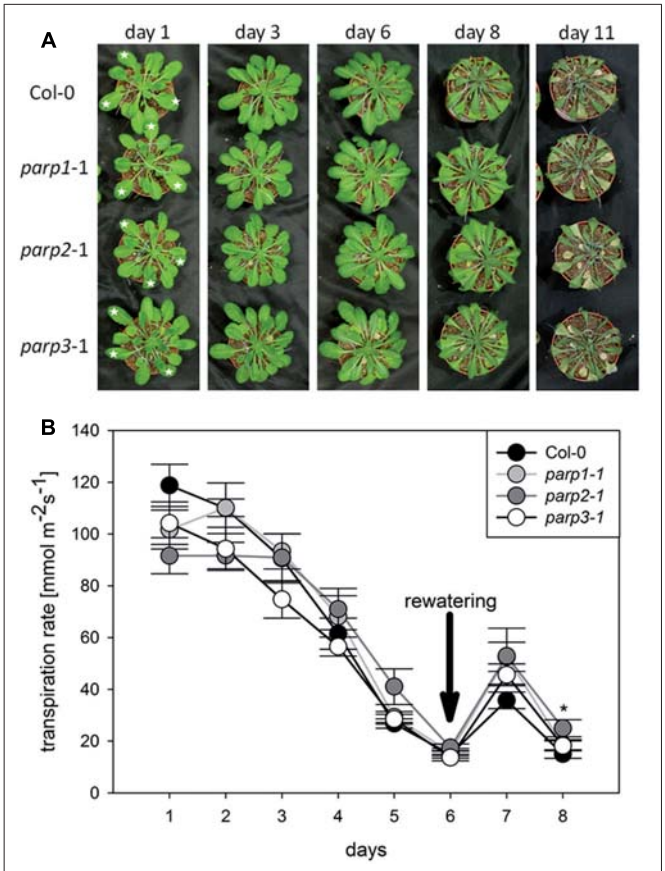

FIGURE 3 | Desiccation tolerance and stomatal conductance are not altered in *parp* mutant plants compared to the wild type. (A) Images of soil-grown Col-0 wild type and *parp* mutant plants at different stages of desiccation. **(B)** Transpiration during desiccation determined by porometry. Three leaves of a similar developmental stage were measured [marked by stars in **(A)**]. After 6 days plants were re-watered with 20 mL water. Data represent the sample means ± SE of 3–4 plants per line and three leaves per plant. Data for individual leaves are shown in Supplementary Figure 2.

and suggest a unified mutant nomenclature, which is shown in Supplementary Table 2. This nomenclature is consistent with the annotation in the TAIR[1] and Araport[2] databases.

[1]http://www.arabidopsis.org

[2]http://www.araport.org

Mutation of Individual *PARP* Genes Does Not Alter Performance of Plants Exposed to Various Abiotic Stresses

To analyze the link between PARPs and drought responses, we performed a soil desiccation experiment comparing 6-week-old wild type (Col-0) and *parp1-1*, *parp2-1*, and *parp3-1* mutant plants. Surprisingly, all three *parp* mutant lines did not show a visibly enhanced tolerance to this stress as compared to the Col-0 plants (**Figure 3A**). Stomatal conductance of the 10th, 11th, and 12th leaf was measured during the desiccation period using porometry (Supplementary Figure 2). Since transpirational water loss for the three leaves was similar, their mean values were calculated. The three *parp* mutant lines showed a similar transpiration rate as the wild type (**Figure 3B**).

Drought and osmotic stress affect not only shoot growth and transpiration, but also primary root elongation. To monitor root and shoot growth in response to abiotic stresses, *parp* mutants and wild type plants were grown on agar plates. To

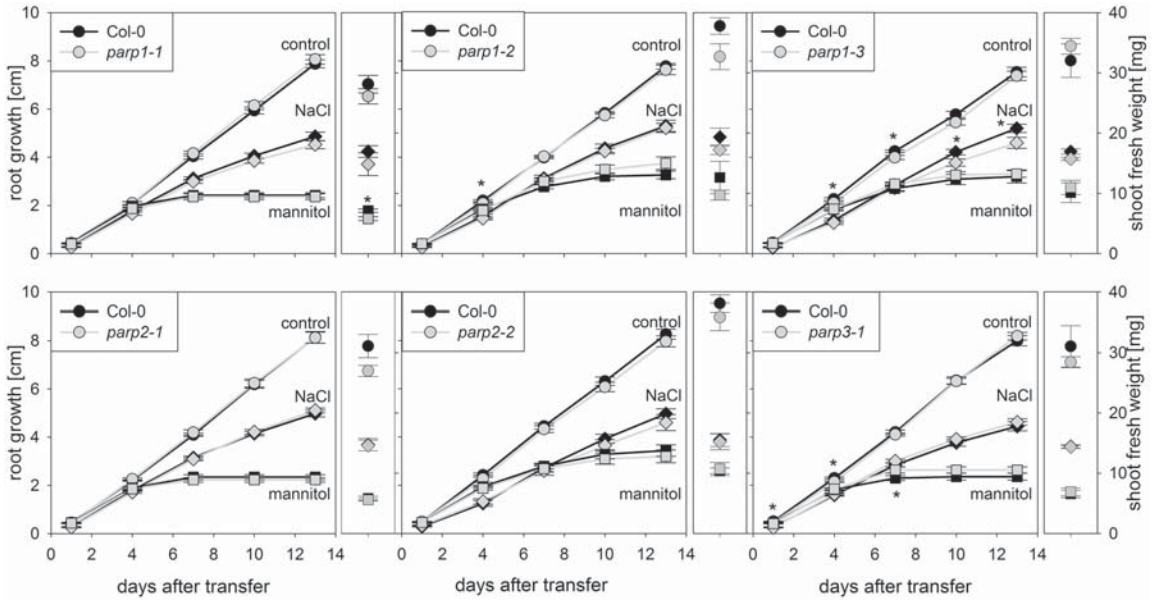

FIGURE 4 | Root growth of *parp* mutant seedlings subjected to salt or osmotic stress is not altered compared to the wild type. Root growth (large panels) of Col-0 and *parp* mutants on control plates (circles), and plates supplemented with 100 mM NaCl (diamonds) or 100 mM mannitol (squares). Shoot fresh weight was determined at the end of the experiment (small panels). Data represent the sample means ± SE of 15 plants per line.

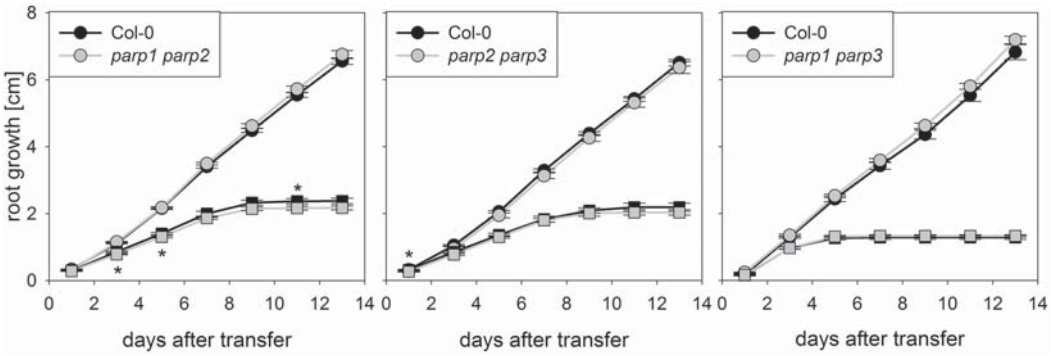

FIGURE 5 | Root growth of *parp* double mutant seedlings subjected to osmotic stress is not altered compared to the wild type. Root growth of Col-0 and *parp* double mutants on control treatment (circles) or 100 mM mannitol (squares). Data represent the sample means ± SE of 15 plants per line.

mimic drought stress, mannitol was applied as osmoticum. Furthermore, NaCl and H_2O_2 were applied as abiotic stress factors. Under control conditions, all plant genotypes showed similar root growth rates (**Figure 4**; Supplementary Figure 3). Mannitol (100 mM), NaCl (100 mM), and H_2O_2 (0.5 mM) treatments reduced root growth and shoot fresh weight. Unexpectedly, *parp* mutants did not show any pronounced and consistent differences to the Col-0 plants in root growth and shoot weight in response to the applied stress treatments.

The *parp* mutant plants did not display the hypertolerance to abiotic stress that we expected from previous studies which mostly employed PARP inhibitors and knockdown lines. A possible reason for this might be a functional redundancy of the three PARP proteins. To further elucidate this, *parp1-1*, *parp2-1*, and *parp3-1* mutants were crossed with each other to generate double mutant lines, which were subjected to an osmotic stress assay. On agar plates containing 100 mM mannitol, root growth and shoot fresh weight of the double mutants was not different from that of the wild type (**Figure 5**).

parp Triple Knockout Does Not Alter Plant Response to Various Abiotic Stresses

To determine whether expression of the residual third *PARP* gene may be upregulated in the double mutants, its transcript level was determined in 2-week-old plants. In *parp2-1 parp3-1* mutant plants, *PARP1* expression was doubled in shoots, while the expression in roots was similar between wild type and double mutant (**Figure 6**). The *parp1-1 parp3-1* double mutation also led to a tendentially increased expression of *PARP2* in shoots

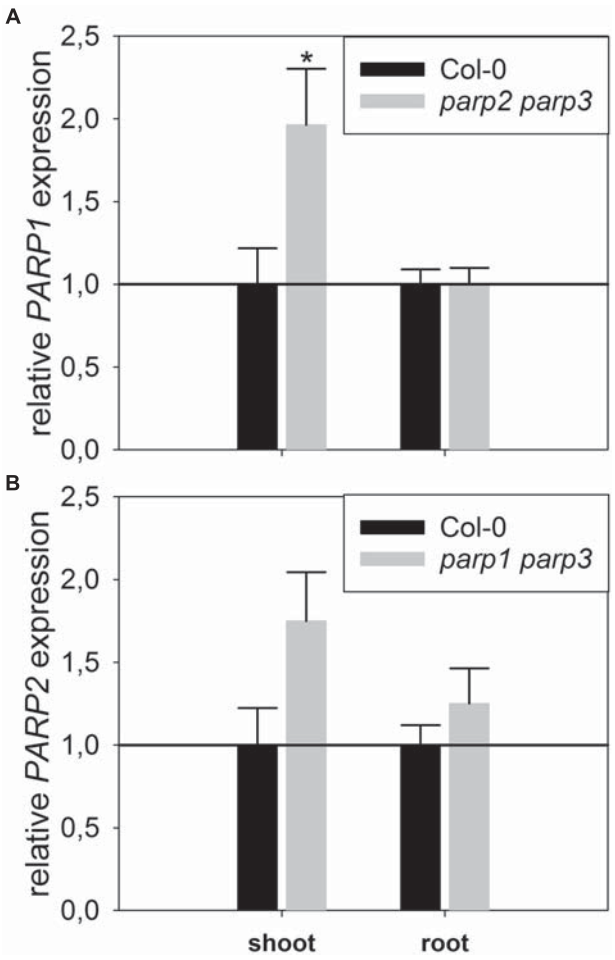

FIGURE 6 | Expression of *PARP1* and *PARP2* is induced in *parp* double mutants lacking the other two *PARP* genes. A qRT-PCR was performed on shoots of plate-grown seedlings of *parp2-1 parp3-1* **(A)** and *parp1-1 parp3-1* **(B)** using *AtUBI10* as reference gene. *PARP3* gene expression was below detection limit. Data represent the sample means ± SE of four plants per line.

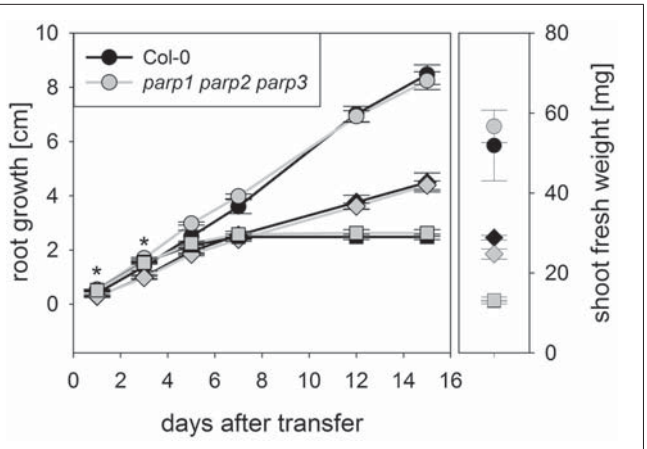

FIGURE 7 | Root growth of *parp1-1 parp2-1 parp3-1* mutant plants exposed to salt or osmotic stress is not altered compared to the wild type. Root growth (large panel) of Col-0 and *parp1-1 parp2-1 parp3-1* mutants on control plates and on plates supplemented with 100 mM NaCl (diamonds) or 100 mM mannitol (squares). Shoot fresh weight was determined at the end of the experiment (small panel). Data represent the sample means ± SE of 15 plants per line.

(1.7 fold; $P = 0.059$). *PARP3* expression was found to be below the detection level in leaves of the *parp1-1 parp2-1* mutant, as it was in the wild type. These data indicate that, at least in the double mutants involving *parp3*, the residual *PARP* gene may at least partially compensate for the knocked-out ones. In addition, since PARPs have been described to be post-translationally activated upon stress, it could not be fully excluded that PARP3 activity is induced in the *parp1-1 parp2-1* double mutants (Bürkle and Virag, 2013). Therefore, *parp1-1 parp2-1* and *parp1-1 parp3-1* double mutant plants were crossed to generate a *parp* triple mutant. This line was subjected to osmotic, salt, and oxidative stress assays as described above. Like the *parp* single and double mutant plants, *parp1-1 parp2-1 parp3-1* plants did not show an enhanced performance compared to the wild type under any of those conditions (**Figure 7**; Supplementary Figure 4).

We analyzed if the lack of all three *PARP* genes had an impact on the response of soil-grown adult Arabidopsis plants to drought stress. Triple mutants were subjected to desiccation as described above for the single mutant plants, and plant phenotype and transpiration were monitored. As before, triple mutants did not show a visibly enhanced stress tolerance (**Figure 8A**). Also, both genotypes showed similar transpiration rates (**Figure 8B**; Supplementary Figure 5). Hence, it could not be confirmed in any of our experiments that abiotic stress tolerance is improved by an absence of functional *PARP* genes.

Pharmacological PARP Inhibition but Not Genetic Knockout Blocks flg22-Induced Callose Deposition

Apart from abiotic stress, PARP action has been linked to biotic stress responses. Previously, PARP inhibition by the PARP inhibitor 3-AB was shown to block flg22-induced, but not wounding-induced, callose deposition in cotyledons of Col-0 seedlings (Adams-Phillips et al., 2010). Thus, PARP proteins seem to specifically interact with the flg22-triggered defense pathway. To confirm this, we first tested other known PARP inhibitors for their potential to block flg22-induced callose deposition. Similar to 3-AB, 6-(5H)-phenanthridinone blocked the callose deposition in Col-0 cotyledons (**Figure 9**). Very bright fluorescent spots which appeared on the edges of the cotyledons after phenanthridinone treatment were due to precipitation of the inhibitor. Interestingly, 4-ANI, another PARP inhibitor, did not prevent callose deposition in response to flg22 treatment (**Figure 9**). In summary, two different known PARP inhibitors were effective in blocking callose deposition, which may indeed point to a role of PARPs in plant response to bacterial attack. A similar effect was therefore expected for the *parp* triple mutant. Surprisingly, the pattern of callose deposition was not altered in cotyledons of this line, as compared to the wild type (**Figure 9**). The application of 3-AB to flg22-treated *parp* triple mutant

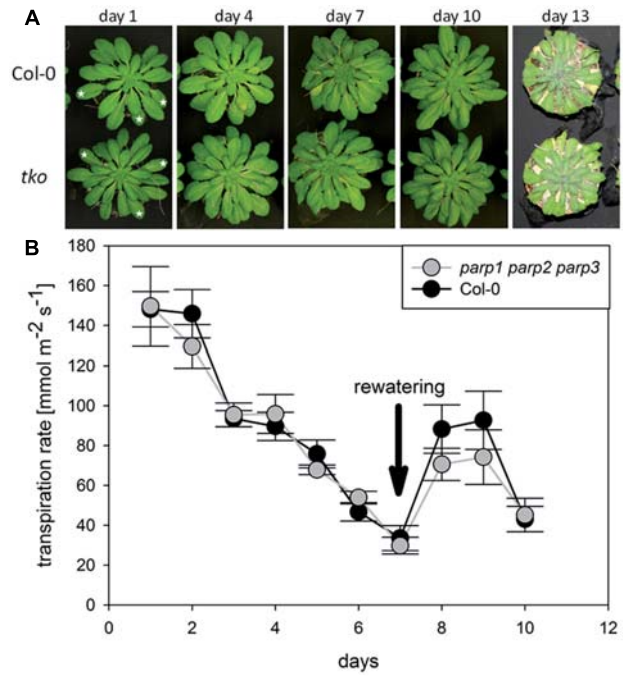

FIGURE 8 | Desiccation tolerance and stomatal conductance are not altered in *parp1-1 parp2-1 parp3-1* mutant plants compared to the wild type. (A) Images of soil-grown Col-0 wild type and *parp1-1 parp2-1 parp3-1* mutant plants at different stages of desiccation. **(B)** Transpiration during desiccation determined by porometry. Three leaves of a similar developmental stage were measured [marked by stars in **(A)**]. After 7 days plants were re-watered with 20 mL water. Data represent the sample means ± SE of three plants per line and three leaves per plant. Data for individual leaves are shown in Supplementary Figure 5.

seedlings evoked the expected blocking of callose deposition. These data indicate that the employed inhibitors act on targets other than or in addition to classical PARPs, affirming a similar assumption based on previous studies with PARP inhibitors (Geissler and Wessjohann, 2011).

PARP Inhibitors Are Likely to Interact with Other Plant Proteins

Proteins of the RCD1/SRO family contain a presumed catalytic PARP domain but not the regulatory one [Jaspers et al. (2010b); Supplementary Figure 6]. To analyze if pharmacological PARP inhibitors, commonly employed to infer roles of PARPs in plants, potentially interact with these proteins, the PARP domains of RCD1 and SRO1 were modeled, and their active sites were analyzed with respect to the ability to bind 3-AB, 4-ANI, and 6-(5H)-phenanthridinone (**Figure 10**). The structures of the six homology models can be inspected in detail on the pdb files included in the Supplementary Material. Despite low overall sequence identities between the templates and RCD1 or SRO1 (between 15.8 and 21.6%, depending on target and alignment), active site inspections confirmed that all three inhibitors could be bound via the same type of interactions that are observed in X-ray structures of ADP ribosyltransferase-type PARPs, e.g., HsPARP10 (including 3-AB), GgPARP1 (including 4-ANI), and HsPARP14 (including 6-(5H)-phenanthridinone). In HsPARP10 or GgPARP1, the nicotinamide moiety of inhibitors is recognized by two hydrogen bonds of a glycine residue. Further stabilization is mediated through stacking between hydrophobic tyrosine side chains. In RCD1 and SRO1, despite a three-dimensional conservation of the active site, both polar and non-polar interaction patterns are disrupted by exchanges in primary sequence. In RCD1 and SRO1, the conserved glycine of animal PARPs is exchanged by a proline (Pro_{334} and Pro_{330}, respectively). This results in only one possible hydrogen bond between RCD1 or SRO1 and the inhibitor (mediated by the proline backbone oxygen atom). Alternatively, after performing the md-refinement simulations, 3-AB adopts a pose in AtRCD1 with preferred hydrophobic interactions between proline and the phenyl moiety of 3-AB. The only amino acid in the classical PARP motif (Ferraris, 2010) that is conserved in RCD1 and SRO1 is a tyrosine (Tyr_{378} and Tyr_{372}, respectively), suggested to be responsible for π–π interactions between the inhibitor and the receptor. The same interaction pattern resulted also for the binding pose of 4-ANI and phenanthridinone. Another tyrosine which is conserved in animal PARPs is replaced by a histidine in plant RCD1 or SRO1 (His_{365} and His_{361}, respectively), which still allows the inhibitors to be stacked between two residues in the same way as it is in animal PARPs. Furthermore, in animal PARPs there is a conserved histidine (e.g., His_{862} in HsPARP1) in close proximity to the binding site of the inhibitors which is necessary for specific activity (Marsischky et al., 1995). This amino acid is replaced by a Leu_{333} in AtRCD1 and Val_{329} in AtSRO1. However, these replacements do not influence the putative binding of the inhibitors. In all the models the binding site is accessible for the inhibitors to penetrate.

In summary, although we do not exclude that some slightly different docking poses of the ligands in the binding site may occur, it could be shown that in principle all these ligands may act as inhibitors for AtSRO1 and AtRCD1 as well. The possibility to bind the inhibitors does not necessarily imply that the proteins have an activity as PARP enzymes. Even if RCD1 and SRO1 would act merely as non-enzymatic scaffolding proteins, the binding of an inhibitor may disturb protein-protein interactions and, hence, protein function.

PARP Activity Is Constitutively Upregulated in a *parp* Triple Knockout Mutant

The application of PARP inhibitors has been frequently demonstrated to modulate plant responses to biotic and abiotic cues. This differs from our findings on *parp* mutants, and our modeling analysis indicated that inhibitors may also target non-PARP proteins. Because inhibitor effects are still likely to be caused by an interference with enzymatic activity, e.g., a reduction in protein poly(ADP-ribosyl)ation, we tested whether this activity is completely abolished by genetic deletion of all three classical *PARP*s. To this end, we performed a dot-blot assay employing a monoclonal poly(ADP-ribose) antibody (**Figure 11**). Since equal protein concentrations were spotted

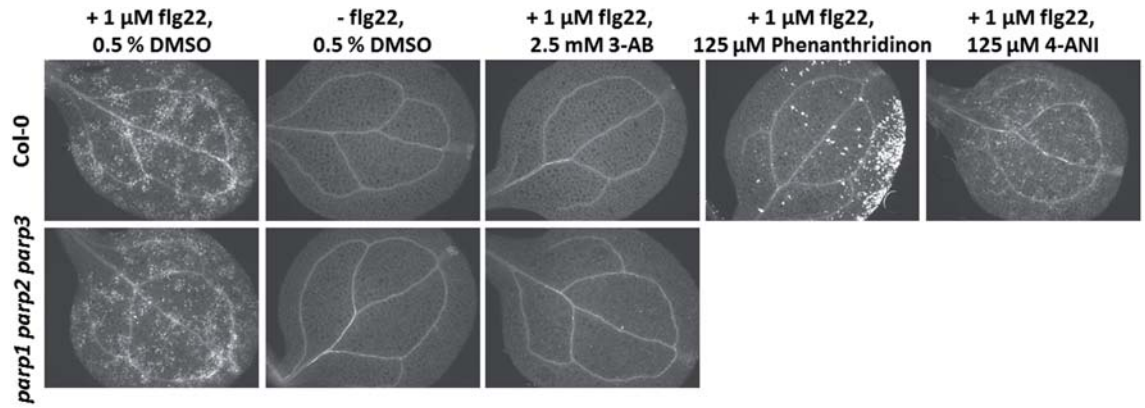

FIGURE 9 | Pharmacological PARP inhibition but not *PARP* gene knockout blocks flg22-induced callose deposition. Callose deposition in 7-day-old Col-0 or *parp1-1 parp2-1 parp3-1* mutant seedlings was determined 24 h after the indicated treatment. Images are representative of a minimum of six cotyledons per treatment and genotype.

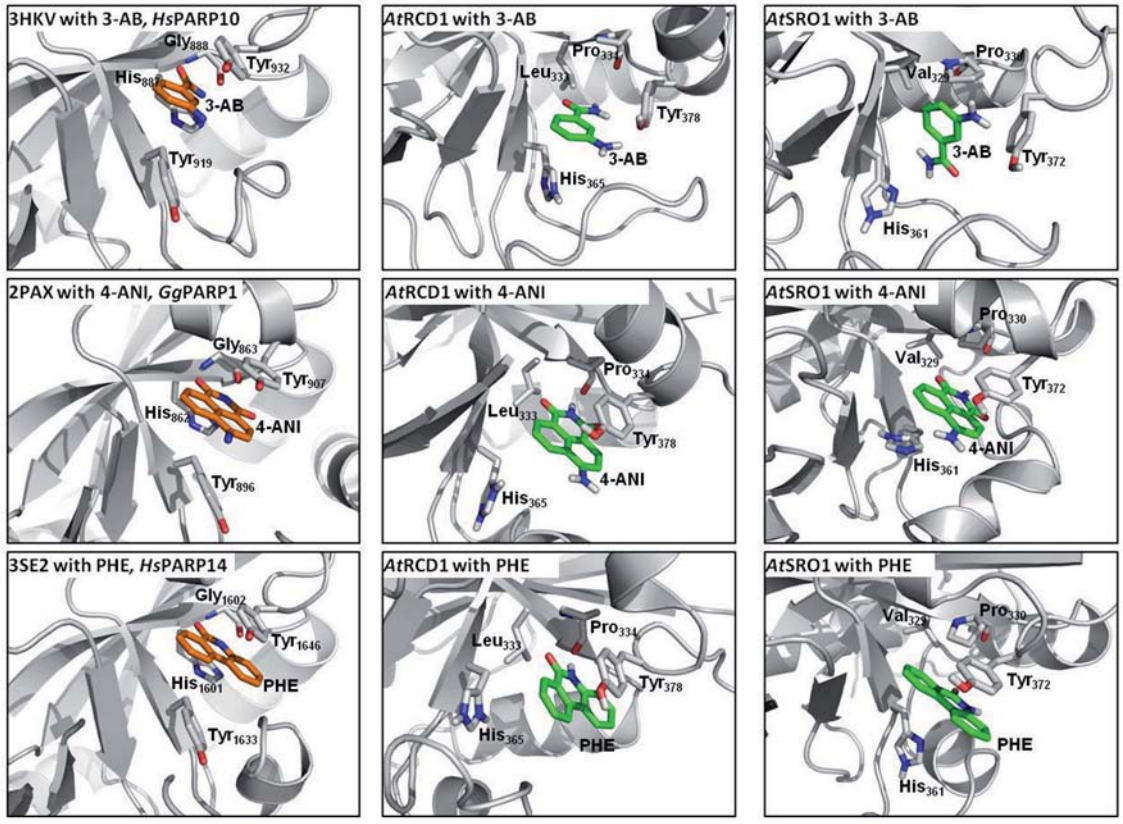

FIGURE 10 | Comparisons of active sites and inhibitor binding in crystal structures of animal ADP ribosyltransferase proteins, AtRCD1, and AtSRO1. Left panels show crystal structures of HsPARP10, GgPARP1, and HsPARP14 with co-crystallized inhibitors 3-aminobenzamide (3-AB), 4-amino-1,8-naphthalimide (4-ANI), and 6-(5H)-phenanthridinone (PHE), respectively. Center and right panels show homology models of AtRCD1 and AtSRO1 with bound inhibitors. Despite different amino acid motifs, the inhibitors can be bound in the same manner as in the template structures.

onto the nitrocellulose membrane and samples were processed identically, the background signal from the antibody is expected to be similar in all samples. In wild type plants, protein poly(ADP-ribosyl)ation was induced by UV light stress, which is expected from its role in DNA damage repair. Most surprisingly, under unstressed conditions, poly(ADP-ribosyl)ation was not found to be abolished, but to be even increased in the triple *parp* mutant as compared to the wild type. This activity was

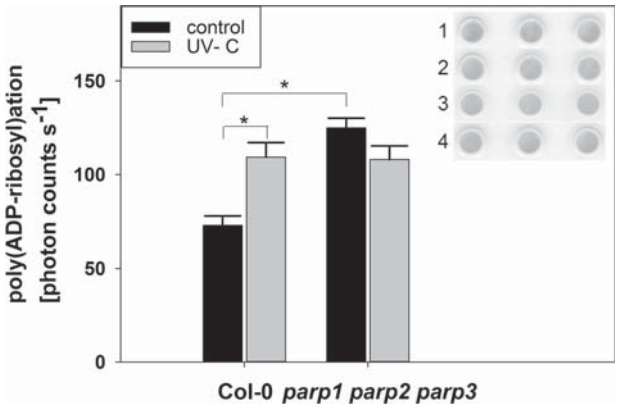

FIGURE 11 | Poly(ADP-ribosyl)ation is enhanced by UV radiation and in *parp* triple knockout plants. PARylation of nuclear proteins was analyzed in 32-day-old leaf material using a poly(ADP-ribose) monoclonal antibody. The insert shows Ponceau staining of protein loaded onto the nitrocellulose membrane (1: Col-0 control, 2: *parp1-1 parp2-1 parp3-1* control, 3: Col-0 UV-treated, 4: *parp1-1 parp2-1 parp3-1* UV-treated). Error bars represent standard error ($N = 3$).

not further stimulated by UV illumination. This result further supports the presence of additional proteins with PARP activity in Arabidopsis, whose activity is increased by the knockout of the classical *PARP* genes.

DISCUSSION

PARP Genes Do Not Play a Universal Role in Growth under Abiotic Stress Conditions

Under the conditions that we tested in this study, *parp* T-DNA insertion mutants did not exhibit altered stress responses compared to wild type plants (**Figures 3** and **4**). This was also the case in all double mutant combinations (**Figure 5**) and in a triple mutant (**Figures 7** and **8**) and was therefore not due to functional redundancy, although our expression analysis pointed to some degree of transcriptional feedback (**Figure 6**). These findings apparently disagree with previous studies employing plants with genetically downregulated PARP activity, from which a negative role of this gene family in abiotic stress resistance was inferred (De Block et al., 2005; Jansen et al., 2009; Schulz et al., 2012). This discrepancy may be explained by different experimental conditions and/or plant genotypes and indicates that the role of PARPs as factors of growth and stress responses is less universal than commonly assumed. For example, De Block et al. (2005) worked with lines derived from the *Arabidopsis thaliana* C24 ecotype in their desiccation experiments, whereas in the present study *A. thaliana* mutants in the Col-0 background were used. General differences in stress tolerance between both genotypes are not unlikely, since the C24 ecotype has been described to be more susceptible to cold stress and UV-B irradiation as compared to Col-0 (Klotke et al., 2004; Kalbina and Strid, 2006). Apart from that, different methodological approaches to alter *PARP* gene expression were employed. In the present study, we analyzed T-DNA insertion mutants, while plants carrying hairpin constructs have been used in other studies (De Block et al., 2005). Expression of the target gene is fully blocked in insertional T-DNA knockout mutants, whereas RNAi-mediating hairpin constructs lead to a partial knockdown and insert randomly into the plant genome, which may potentially affect other genes. Hence, ecotype and genetic modification may explain some of the discrepancies between previous reports and the results we obtained. In addition, growth conditions and age varied between the different studies showing an effect or no effect of *PARP* interference. However, we employed two very different systems, growing the plants on agar plates and on soil, without detecting a role of this gene family. In conclusion, enhanced stress response by a repressed *PARP* expression appears to be a conditional phenotype. This notion is supported by a general unresponsiveness of *PARP* gene expression to osmotic, drought, or salt stress (**Figure 1**).

Pharmacological PARP Inhibitors May Have Off-Target Effects

Apart from the genetic interference with *PARP* genes, pharmacological inhibition has been used in the past to elucidate the role of plant PARP proteins in stress responses. In those studies, PARP inhibitors known to be potent in human cells were employed. Positive effects of pharmacological PARP inhibition on plant performance under stress have been described for several plant species, various developmental stages, and different stress factors, such as oxidative stress, osmotic stress, or salt stress (De Block et al., 2005; Geissler and Wessjohann, 2011; Schulz et al., 2012). Conversely, pharmacological PARP inhibition negatively interfered with plant immune responses to pathogen-associated molecular patterns, such as flg22 or elf18 (Adams-Phillips et al., 2010). This was confirmed in the present study; two PARP inhibitors blocked flg22-induced callose deposition (**Figure 9**). However, the genetic abolition of all three *PARP* genes did not provoke this effect. These findings indicate that pharmacological PARP inhibition is more effective than genetic reduction of PARP activity, which points to the existence of other or additional proteins targeted by pharmacological PARP inhibitors. This is in agreement with a previous study of PARP inhibitor action on plants by some of us, which casted a first doubt on PARP inhibition as a cause for drought stress tolerance (Geissler and Wessjohann, 2011). In the current study, this notion is supported by both, experimental evidence and computer modeling. Experimentally, we made the surprising observation that PARP activity is not abolished, but instead constitutively induced in a *parp* triple knockout line (**Figure 11**). Hence, there are bound to be further proteins with PARP activity that may be targeted by the employed inhibitors. Possible candidates are members of the SRO protein family, which have been assigned key roles in stress responses of Arabidopsis, wheat, and rice (Katiyar-Agarwal et al., 2006; Teotia and Lamb, 2009; Liu et al., 2014; You et al., 2014). SRO proteins contain a presumed catalytic PARP domain, albeit *in vitro* assays failed to show any enzymatic activity (Jaspers et al., 2010b). Although overall

protein sequence similarities to crystallized PARP proteins were low, homology modeling of the catalytic domain of SRO proteins was possible (**Figure 10**). Those PARP inhibitor modeling studies showed that 3-AB, 4-ANI, and phenanthridinone should be able to bind to the binding pocket of RCD1 and SRO1, the best-characterized members of the SRO protein family with partially redundant functions.

In addition to the catalytic PARP domain, both proteins also contain an N-terminal WWE domain and a C-terminal RST domain (Supplementary Figure 6), which are known to mediate protein-protein interactions. Prominent interaction partners of RCD1 and SRO1 are DREB2-type transcription factors (Jaspers et al., 2009), central regulators of drought, salt, and heat stress responses. DREB2A is regulated by protein stability (Sakuma et al., 2006), and there is substantial evidence that binding of RCD1 to DREB2A designates the protein to degradation (Vainonen et al., 2012). Hence, RCD1 is a negative regulator of DREB2A. A similar role for SRO1 has not yet been investigated, but may be assumed from its interaction with DREB2A and its partial functional redundancy with RCD1 (Jaspers et al., 2009).

The PARP domain has been suggested to facilitate complex formation of SRO proteins with their interaction partners (Jaspers et al., 2010a). This domain would hence be required for the designation of DREB2A to degradation by binding to RCD1 and possibly SRO1. This, in turn, means that occupation of the PARP domain by pharmacological compounds is likely to increase the stability of DREB2A by blocking its interaction with RCD1. Therefore, one potential effect of PARP inhibitors may be an increased DREB2A activity, leading to the commonly observed increased stress resistance. However, in addition to altered stress responses, *rcd1* knockout mutants show severe developmental defects (Fujibe et al., 2004; Jaspers et al., 2009; Teotia and Lamb, 2009; Hiltscher et al., 2014), which are not induced by PARP inhibitors (De Block et al., 2005; Adams-Phillips et al., 2010; Geissler and Wessjohann, 2011; Schulz et al., 2012). This discrepancy may be explained by the fact that, in contrast to a genetic knockout of *RCD1*, the RST domain is still present in the PARP-inhibitor-complexed RCD1. Hence, interactions with transcription factors involved in plant development may still be possible.

CONCLUSION

The lack of stress-related phenotypes in *parp* mutants, the higher effectiveness of pharmacological PARP inhibition, and the PARP activity in a *parp* triple knockout mutant indicate that additional proteins are affected by the inhibitors. We identified RCD1 and SRO1 as possible candidates. Further research is required to investigate this likely interaction, which may eventually be harnessed to improve the performance of field crops under stress conditions.

AUTHOR CONTRIBUTIONS

DR performed experiments; PH, LW, and WB conducted modeling analyses; DR, PH, KT, WB, LW, and EP designed and oversaw the research and analyzed data; DR and EP wrote the article with contributions of PH, KT, LW, and WB.

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SUPPLEMENTARY MATERIAL

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5 General Discussion

In contrast to their mammalian counterparts, still little is known about the functions of plant poly(ADP-ribose)polymerases. The studies presented in this thesis were performed to further elucidate PARP action *in planta*.

To study potential functional similarities to the so far best-characterized PARP protein, HsPARP1, and to infer putative functions in plant cells, the three canonical Arabidopsis PARP proteins were expressed in yeast. In those experiments, *AtPARP1* expression inhibited yeast cell growth similarly to *HsPARP1* expression (Fig. 2.1A and C). This growth inhibition was reverted by the addition of the PARP inhibitors 3AB and phenanthridinone (Fig. 2.2A and B), leading to the conclusion that HsPARP1 and AtPARP1 exhibit functional similarities in yeast. On the contrary, *AtPARP2* expression inhibited yeast growth only partially, while *AtPARP3* expression did not affect growth (Fig. 2.2C and D). This can be attributed to differences in or the lack of the DNA-binding domain in AtPARP2 and AtPARP3. Similarly, the DNA-binding domain of HsPARP2 is structurally different from HsPARP1. However, *HsPARP2* expression is able to inhibit yeast growth (Perkins *et al.*, 2001). Therefore, on one hand the yeast growth inhibition assay revealed that Arabidopsis and human PARPs share structural or functional features. On the other hand, the results also indicated plant-specific functions for AtPARP2 and AtPARP3.

One of these plant-specific functions is shown in publication 2 of this thesis. A screening of publically available microarray datasets and histochemical GUS staining revealed an accumulation of *AtPARP3* transcripts specifically in the embryo and the endosperm of dry seeds, during imbibition, and during seed germination (Figs. 3.2 and 3.3A). A similar expression pattern was also found for the *PARP3* orthologue in rice (Howell *et al.*, 2009). *AtPARP3* transcript accumulation coincided with ROS activity in the embryo, pointing to a role of AtPARP3 in ROS-induced DNA damage responses during seed storage and germination (Fig. 3.3B). As AtPARP3 shows structural homologies to AtPARP1 and human PARP3, similar functions can be assumed. Human PARP3 is involved in non-homologous end join (NHEJ) repair of DNA damage (Rouleau *et al.*, 2007, Rulten *et al.*, 2011). Recently, similar results were found for PARP3 in *Hordeum vulgare*. Expression of *HvPARP3* was enhanced in young barley roots 36 h after germination. This induction was further enhanced by the addition of the DNA double strand break (DSB)-inducing agent bleomycin (Stolarek *et al.*, 2015). These findings further support a function of plant PARP3 proteins in DSB repair via NHEJ. Database searches revealed the presence of sequences homologous to *AtPARP3* in other plant species such as *Populus trichocarpa*, *Physcomitrella patens*, *Oryza sativa*, *Brachipodium distachyon*, *Sorghum bicolor*, *Zea mays*, and *Hordeum vulgare* (Fig. 3.1) (Howell *et al.*, 2009, Stolarek *et al.*, 2015). This leads to the conclusion that the function of PARP3 is conserved throughout the plant kingdom.

Apart from AtPARP3's function in seed storability and germination, PARP proteins and poly(ADP-ribosyl)ation have been attributed an important role in plant development

(Vainonen *et al.*, 2016). For instance, tracheary element differentiation was found to be inhibited upon 3AB treatment in cultured explants of peas and artichokes (Phillips & Hawkins, 1985). Moreover, *AtPARP1* and *AtPARP2* expression and activity were found to be enhanced at a time of high cell cycle activity during the exponential growth phase of Arabidopsis cell cultures. This enhancement was accompanied by an increase in the glutathione pool, linking PARP activity to cellular redox homeostasis (Pellny *et al.*, 2009). In addition, constant poly(ADP-ribosyl)ation due to the lack of the poly(ADP-ribose)-degrading PARG1 in the *tej* mutant accelerated flowering compared to the wild type (Panda *et al.*, 2002). In contrast, in the present thesis, all examined *parp* knockout mutant lines, including a *parp1 parp2 parp3* triple mutant, did not display any observable developmental defects under standard growth conditions (Figs. 4.4, 4.5, 4.7). This is in line with findings obtained by other authors (Song *et al.*, 2015, Zhang *et al.*, 2015, Liu *et al.*, 2017), suggesting that PARPs are not essential for plant development. Moreover, a recent study showed that the poly(ADP-ribosyl)ation of the AtPARP2 target protein DAWDLE (DDL) is not essential to restore developmental defects caused by *ddl* knockout (Feng *et al.*, 2016). Similar to the plant mutants lacking PARP1, PARP2, and/or PARP3, untreated mice lacking PARP2 or PARP3 show normal development (de Murcia *et al.*, 2003, Boehler *et al.*, 2011). Similarly, mice carrying an insertion in exon 1 of *PARP1* did not show developmental defects, while an insertion in exon 4 led to a reduced size of the animals and an insertion in exon 2 caused spontaneous skin disease (De Murcia *et al.*, 1997, Wang *et al.*, 1995, Masutani *et al.*, 1999). In contrast to plants, the lack of both PARP1 and PARP2 proteins is embryonically lethal to mice highlighting the enormous developmental impact of PARP in mammals (de Murcia *et al.*, 2003). Hence, plant PARP proteins seem to be necessary but not essential for correct development. Therefore, further studies are required to elucidate the role of plant PARP proteins in plant development.

In addition to their function in DNA damage responses, plant PARPs, particularly AtPARP1 and AtPARP2, have been suggested as negative regulators of plant abiotic stress responses (De Block *et al.*, 2005). Arabidopsis plants with reduced PARP activity caused by the expression of *PARP* hairpin constructs were shown to exhibit enhanced tolerance to desiccation and oxidative stress (De Block *et al.*, 2005). In stark contrast, in the present study, Arabidopsis wild type and *parp* T-DNA insertional knockout mutant plants responded similarly to desiccation, salt, osmotic, and oxidative stress (Figs. 4.3, 4.4, 4.5, 4.7, 4.8). These opposing results led to the conclusion that the enhanced stress tolerance phenotype is conditional. Moreover, flg22-induced callose deposition was not found to be altered in *parp1-1 parp2-1 parp3-1* triple knockout seedlings in the present study (Fig. 4.9). These findings contrast with a reduction of callose deposition in a *parp1-3 parp2-3* double mutant produced by other authors (Feng *et al.*, 2016). That *parp1-3 parp2-3* mutant resulted from a crossing of *parp* single mutants different from the mutants used to generate the *parp1-1 parp2-1 parp3-1* mutant in the present study. Additionally, the Arabidopsis plants differed in their developmental stage at the time of flg22 application. Hence, these contrasting findings further support the notion of a conditionality of the observed phenotype, which remains to

be tested experimentally. Conditional phenotypes occur frequently in loss-of-function mutants, as it is likely that plants have evolved many adaptive traits that allow them to cope with changes in their environment (Bouche & Bouchez, 2001). Factors accounting for conditional phenotypes are light, temperature, and nutritional status, and interactions among them are possible (Bouche & Bouchez, 2001). Accordingly, a large number of descriptions of conditional phenotypes can be found in literature. For instance, Lloyd and Meinke have identified 522 phenotypes as conditional in a dataset of 2400 loss-of-function mutant phenotypes analyzed (Lloyd & Meinke, 2012). Conditional phenotypes have been found in all aspects of plant development. Some examples are presented in the following: The conditional root expansion mutant *quill* shows similar root growth as the wild type on 0.5% sucrose, but dramatically reduced root growth on 4.5% sucrose medium. Under these conditions root growth of wild type plants is even pronounced (Hauser *et al.*, 1995). Similarly, the *petit1* mutant shows reduced hypocotyl elongation on sucrose-containing medium but not on sucrose-free medium (Kurata & Yamamoto, 1998). The photoperiod-insensitive early-flowering 3 mutant *elf3* shows rhythmic leaf movement in the dark and under several light/dark regimes, but not under constant light (Hicks *et al.*, 1996). Not only the light regime but also the light intensity can affect plant phenotypes. Consequently, *vad1* (*vascular associated cell death1*) mutants show hypersensitive response-induced lesions under high but not under low light intensities (Lorrain *et al.*, 2004). By contrast, plants lacking the MYB domain-containing proteins MYB33 and MYB65 exhibit male sterility specifically under low light conditions (Millar & Gubler, 2005). These examples illustrate the wide range of conditional phenotypes identified so far in plant research using loss-of-function mutant lines.

In contrast to the genetic knockout of *PARP* genes, known pharmacological PARP inhibitors were able to block flg22-induced plant defense responses consistently in the present work and in previous studies (Fig. 4.9) (Adams-Phillips *et al.*, 2010). No callose deposition in response to flg22 was observed after treatment with the known PARP inhibitors 3AB and phenanthridinone, albeit, in the present study, the PARP inhibitor 4ANI did not block this defense response (Fig. 4.9). Interestingly, this finding corresponds to the results obtained in the yeast growth assay, where the addition of 4ANI was not able to reverse HsPARP1- or AtPARP1-induced growth arrest (Fig. 2.2C and D). Since 4ANI has been described as a potent inhibitor of human PARP enzymes (Banasik *et al.*, 1992, Putt & Hergenrother, 2004), the lack of activity on HsPARP1 and AtPARP1 might be explained by the inhibitor's inability to permeate the cell wall. This inability to reach its target may also be the reason why 4ANI did not block flg22-induced callose deposition.

In principle, pharmacological PARP inhibition appears to modify plant responses to stress more consistently than genetic *PARP* knockout (De Block *et al.*, 2005, Geissler & Wessjohann, 2011, Schulz *et al.*, 2012). Additionally, pharmacological PARP inhibition was shown to affect plant growth and development under unstressed conditions (Schulz *et al.*, 2014). This suggests the idea that pharmacological PARP inhibitors do not only affect the activity of canonical PARPs, but also have off-target effects *in planta*. That idea was

supported by the finding that protein poly(ADP-ribosyl)ation was not absent, but even increased in the *parp* triple mutant (Fig. 4.11), which may be caused by other enzymes with this activity. In this thesis, proteins belonging to the SRO family are proposed as such potential alternative targets since they contain a catalytic PARP domain. In *silico* analysis revealed that the PARP inhibitors 3AB and phenanthridinone are indeed likely to bind to this domain (Fig. 4.10). In contrast, a recent study indicated that phenanthridinone does not bind to the catalytic PARP domain of RCD1 in the way it binds to human PARP1 (Wirthmueller *et al.*, 2018). Nevertheless, phenanthridinone may have a disruptive role in RCD1 function and a yet undefined interaction with the catalytic PARP domain is still conceivable. The unequally redundant proteins, RCD1 and SRO1, have been reported repeatedly to be important actors in plant stress responses (Overmyer *et al.*, 2000, Ahlfors *et al.*, 2004, Fujibe *et al.*, 2004, Katiyar-Agarwal *et al.*, 2006, Jaspers *et al.*, 2009, Jiang *et al.*, 2009, Vainonen *et al.*, 2012, Morales *et al.*, 2015, Wirthmueller *et al.*, 2018). Additionally, SRO5 has been linked to stress responses (Borsani *et al.*, 2005, Babajani *et al.*, 2009, Jaspers *et al.*, 2010). The complex involvement of SRO proteins in stress responses matches with the commonly observed alterations of stress responses by pharmacological PARP inhibitors. Due to the potential off-site effects on SRO or other proteins, the use of pharmacological PARP inhibitors to infer PARP function in plants has to be reconsidered. The notion that PARP inhibitors have off-site effects is further supported by a recent transcriptomics study in which, under standard growth conditions, a treatment with the established PARP inhibitors 3AB and 3MB altered the expression of 228 and 3935 genes, respectively (Briggs *et al.*, 2017). This difference by one order of magnitude makes it highly unlikely that the effects are caused by the inhibition of canonical PARP proteins alone. It is an important future task to experimentally determine the potential off-site targets of PARP inhibitors, as this may allow the design of specific inhibitors to those proteins. Pull-down experiments using the pharmacological "PARP inhibitors" as bait may be a suitable approach to identify such proteins.

In their natural habitats, plants are under constant threat of biotic and abiotic stressors either simultaneously or sequentially (Ramegowda & Senthil-Kumar, 2015, Pandey *et al.*, 2017, Zhang & Sonnewald, 2017). Global warming is proposed to increase this threat (IPCC, 2014). Hence, future food safety is in danger. The inhibition of PARP by genetic and pharmacological means has been suggested as a promising tool to sustain crop yields and hence food safety, since PARP inhibition has been found to improve plant abiotic stress responses (De Block *et al.*, 2005, Vanderauwera *et al.*, 2007, Schulz *et al.*, 2012). Similarly, callose deposition induced by the bacterial MAMP flg22 was found to be increase in mutant plants lacking AtPARP1 and AtPARP2 (Song *et al.*, 2015). However, in the present study flg22-induced callose deposition was blocked by pharmacological PARP inhibition (Fig. 4.9), which agrees with results obtained by other authors (Adams-Phillips *et al.*, 2008, Feng *et al.*, 2016, Adams-Phillips *et al.*, 2010). Moreover, *parp1 parp2* mutant plants exhibited increased susceptibility to *Pst* infection (Song *et al.*, 2015, Feng *et al.*, 2015). Therefore, the currently available data suggest that PARP inhibition may not be a suitable tool to stabilize food security, as it apparently has opposing effects on abiotic and biotic stress resistance, which

are, in addition to this, of a conditional nature. However, simultaneous or sequential occurrence of abiotic and biotic stress has been shown to elicit tailored subsets of physiological and transcriptional responses that differ from those to single stressors (Ramegowda & Senthil-Kumar, 2015, Pandey *et al.*, 2017, Zhang & Sonnewald, 2017). Accordingly, abiotic and biotic stressors have been reported numerously to have either additive effects, i.e. increasing plant stress level and symptoms, or antagonizing effects, i.e. increasing resistance to one of the stressors (Ramegowda & Senthil-Kumar, 2015, Pandey *et al.*, 2017, Zhang & Sonnewald, 2017). For their general roles in genome stability and stress responses, canonical PARPs and SROs may be determinants in the coordination of plant responses to multiple stresses. To follow up this idea, the response to the combined appearance of abiotic and biotic stressors needs to be studied in plants with reduced PARP activity.

The present study has opened new avenues for research on PARPs in plants. The expression of AtPARP3 in seeds was confirmed and a function in DNA DSB was inferred from the simultaneous expression of AtPARP3 and ROS activity, the impaired seed storability, and the function of human PARP3. Yet, its exact role in DNA DSB repair is to be elucidated. Therefore, germination of *parp3* mutant seeds has to be scored in the presence of DNA DSB-inducing agents such as zeocin or bleomycin. Additionally, crossings of *parp3* mutant plants with *ku70* and *ku80* mutants will provide evidence whether AtPARP3 acts in NHEJ as its mammalian counterpart. Since direct evidence of poly(ADP-ribosyl)ation activity of AtPARP3 is lacking so far, PARP activity has to be determined in seeds of *parp3*. Aberrant poly(ADP-ribose) levels will indicate whether AtPARP3 is capable of poly(ADP-ribosyl)ation. However, a mono(ADP-ribosyl)ation activity by this enzyme is also conceivable, similar to its human counterpart. New tools recently have become available to detect poly and mono(ADP-ribose) in cells using natural readers of bound ADP-ribose in mammalian cells (Gupte *et al.*, 2017). These tools include the macrodomain-containing protein Af1521, detecting mono(ADP-ribosyl)ation, and poly(ADP-ribose)-binding proteins fused to GFP or GST-tags. Those assays allow a detailed analysis of mono and poly(ADP-ribose) levels and the tracking of cellular ADP-ribosylation activity (Gupte *et al.*, 2017). As such readers have not yet been identified in plants, it has to be tested whether the mammalian tools are suitable to be applied in plants. If they are applicable, these tools will aid the further understanding of the function of plant PARPs, in particular of AtPARP3, in DNA damage repair.

The present study furthermore revealed that the stress-related phenotypes of *parp* knockout mutants are of a conditional nature. In contrast to previous findings by some other authors, genetic PARP inhibition did not alter plant responses to abiotic or biotic stressors. This discrepancy calls for an extensive systematic analysis of the effect of growth conditions and developmental state of the plant on PARP-induced alterations of stress responses. Furthermore, the putative alternative targets of pharmacological PARP inhibitors have to be identified, since pharmacological PARP inhibition has been shown to consistently affect plant stress responses. Finally, to better understand the role of PARPs in plant responses to stress

and DNA damage, poly(ADP-ribosyl)ated proteins and poly(ADP-ribose)-binding proteins need to be identified.

6 Summary

Poly(ADP-ribosyl)ation is a rapid and transient posttranslational protein modification that was described first in mammalian cells. Activated by sensing of DNA strand breaks, poly(ADP-ribose)polymerase1 (PARP1) transfers ADP-ribose units onto itself and other target proteins using NAD^+ as a substrate. Subsequently, DNA damage response and other cellular responses are initiated. In plants, PARPs have also been implicated in responses to DNA damage. Additionally, they have been linked to plant responses to numerous stresses, whereby the mechanistic basis of the interference is often unclear, and reports have been inconsistent. This thesis thus aimed (1) to establish a yeast-based assay to analyse the function of Arabidopsis PARPs and the effectiveness of PARP inhibitors, (2) to determine a role of the enigmatic AtPARP3 in Arabidopsis, and (3) to elucidate the involvement of AtPARP1, AtPARP2, and AtPARP3 in abiotic and biotic stress reponses.

To allow the identification of specific inhibitors and potential interactors of plant PARPs, an assay based on heterologous expression of *PARP* genes from Arabidopsis in yeast was established. Expression of *AtPARP*s caused an inhibition of yeast growth to different extent, which was alleviated by inhibitors targeted at human PARPs. This assay provides a fast and simple means to identify target proteins and pharmacological inhibitors of AtPARP1. Moreover, it revealed functional similarity of human PARP1 and AtPARP1 and suggested plant-specific functions for AtPARP2 and AtPARP3.

Prior to the present work, *AtPARP3* has only been known to be strongly expressed in seeds, but a functional characterization of the gene had not been pursued. The deterioration of seeds during prolonged storage results in a reduction of viability and germination rate. DNA damage is one of the major cellular defects associated with seed deterioration. It is provoked by the formation of reactive oxygen species (ROS) even in the quiescent state of the desiccated seed. In contrast to other stages of seed life, DNA repair during storage is hindered through the low seed water content; thereby DNA lesions can accumulate. To allow subsequent seedling development, DNA repair has thus to be initiated immediately upon imbibition. Histochemical GUS staining of embryos and endosperm layers revealed strong promoter activity of *AtPARP3* during all steps of germination. This coincided with high ROS activity and indicated a role of AtPARP3 in DNA repair during germination. This was supported by the nuclear localization of AtPARP3 and the fact that stored *parp3-1* mutant seeds lacking *AtPARP3* expression displayed a delay in germination as compared to Col-0 wild-type seeds. A controlled deterioration test showed that the mutant seeds were hypersensitive to unfavourable storage conditions. The results demonstrate that AtPARP3 is an important component of seed storability and viability.

In addition to their involvment in DNA damage responses, plant PARP proteins have also been suggested to be universal determinants of plant responses to abiotic and biotic stresses. A role under abiotic stress has been inferred from studies in which a genetic or, more commonly, pharmacological inhibition of PARP activity improved the performance of stressed plants. To further elucidate the role of PARP proteins under stress, T-DNA knockout

mutants for the three *AtPARP* genes were subjected to drought, osmotic, salt, and oxidative stress. To exclude a functional redundancy, which was indicated by a transcriptional upregulation of the remaining *AtPARP* genes, a *parp* triple mutant was generated. Surprisingly, *parp* mutant plants did not differ from wild type plants in any of these stress experiments, independent from the number of *AtPARP* genes mutated. The *parp* triple mutant was also analyzed for callose formation in response to the pathogen-associated molecular pattern flg22. Unexpectedly, callose formation was unaltered in the mutant, albeit pharmacological PARP inhibition robustly blocked this immune response, confirming previous reports. Evidently, pharmacological inhibition appears to be more robust than the abolition of all *AtPARP* genes, indicating the presence of so-far undescribed proteins with PARP activity. This was supported by the finding that protein poly(ADP-ribosyl)ation was not absent, but even increased in the *parp* triple mutant. Candidates for novel PARP inhibitor targets may be found in the SRO protein family. These proteins harbor a catalytic PARP-like domain and are centrally involved in stress responses. Molecular modeling analyses, employing animal PARPs as templates, indeed indicated a capability of the SRO proteins RCD1 and SRO1 to interact with nicotinamide-derived inhibitors. Collectively, these results suggest that the stress-related phenotypes of *parp* mutants are highly conditional, and they call for a reconsideration of PARP inhibitor studies on plants due to potential off-target effects.

7 Zusammenfassung

Poly(ADP-ribosyl)ierung ist eine schnelle und transiente posttranslationale Proteinmodifikation, die zuerst in Säugetierzellen beschrieben wurde. Aktiviert durch DNA-Strangbrüche überträgt Poly(ADP-ribose) Polymerase 1 (PARP1) ADP-Ribose-Einheiten auf sich selbst und auf andere Zielproteine. NAD^+ fungiert dabei als Substrat. Daraufhin werden DNA-Reparaturmechanismen und andere zelluläre Stressantworten eingeleitet. In Pflanzen wurde PARP-Proteinen ebenfalls eine Rolle in der Aktivierung von DNA-Reparaturprozessen zugeschrieben. Zudem wurden sie mit der pflanzlichen Antwort auf verschiedene Stressoren in Verbindung gebracht. Dabei sind die Mechanismen, wie PARPs an der pflanzlichen DNA-Reparatur und der pflanzlichen Stressantwort beteiligt sind, jedoch oft unklar und die Berichte darüber inkonsistent. Diese Arbeit zielte daher darauf ab, (1) ein Hefe-basiertes Testsystem zu entwickeln, um die Funktion der PARP-Proteine aus Arabidopsis und die Wirksamkeit bekannter PARP-Inhibitoren gegenüber diesen Proteinen zu analysieren, (2) eine Funktion des bisher wenig untersuchten AtPARP3 in Arabidopsis zu ermitteln und (3) die Beteiligung von AtPARP1, AtPARP2 und AtPARP3 an der abiotischen und biotischen Stressantwort von Pflanzen aufzuklären.

Um spezifische Inhibitoren und mögliche Interaktoren pflanzlicher PARPs zu identifizieren, wurde ein Testsystem entwickelt, dass auf der heterologen Expression der Arabidopsis *PARP*-Gene in Hefezellen basiert. Durch die Expression der verschiedenen *AtPARP*s wurde das Hefewachstum unterschiedlich stark gehemmt. Diese Hemmung konnte durch Inhibitoren, die zur Hemmung des menschlichen PARP-Proteins entwickelt wurden, wieder aufgehoben werden. Das entwickelte Testsystem stellt eine schnelle und einfache Methode zur Identifizierung von Zielproteinen und Inhibitoren von AtPARP1 dar. Außerdem zeigte es funktionelle Ähnlichkeiten zwischen dem menschlichen PARP1 und PARP1 aus Arabidopsis auf und legt spezifische Funktionen für PARP2 und PARP3 in Pflanzen nahe.

Vor dieser Arbeit war über *AtPARP3* nur bekannt, dass es in Samen stark exprimiert wird, eine funktionale Charakterisierung des Gens wurde bislang aber nicht durchgeführt. Die Alterung von Samen während einer ausgedehnten Lagerung verringert deren Lebensfähigkeit und Keimrate. Eine der wichtigsten zellulären Folgen der Alterung ist die Schädigung der DNA. Sie wird durch die Produktion reaktiver Sauerstoffspezies (ROS) auch während der Samenruhe trockener Samen hervorgerufen. Im Gegensatz zu anderen Stadien, wird die DNA-Reparatur im trockenen Samen durch den geringen Wassergehalt verhindert, so dass DNA-Schäden akkumulieren können. Um eine weitere Entwicklung des Samens zu ermöglichen, muss die Reparatur der DNA-Schäden direkt nach der Flüssigkeitsaufnahme in den Samen einsetzen können. Histochemische GUS-Färbungen des Embryos und der Endosperm-Schicht zeigten eine starke Aktivität des *AtPARP3*-Promotors während aller Keimungsstadien. Gleichzeitig konnte auch eine hohe ROS-Aktivität festgestellt werden. Dies führte zu dem Schluss, dass AtPARP3 an der DNA-Reparatur während der Samenkeimung beteiligt sein könnte. Diese Annahme wurde unterstützt durch die nukleäre Lokolisation von AtPARP3 und die Tatsache, dass gelagerte *parp3-1*-Samen, denen das intakte *AtPARP3*-Gen

fehlt, eine verzögerte Keimung im Vergleich zum Wildtyp zeigten. Ein Versuch zur künstlichen Samenalterung zeigte eine Hypersensitivität von Samen der Mutante gegenüber ungünstigen Lagerbedingungen. Diese Ergebnisse zeigen, dass AtPARP3 eine wichtige Rolle in der Lebens- und Lagerfähigkeit von Samen spielt.

Zusätzlich zu ihrer Beteiliung an der DNA-Reparatur wurde den pflanzlichen PARP-Proteinen auch eine Funktion als Schlüsselregulatoren in der pflanzlichen Antwort auf abiotischen und biotischen Stress zugesprochen. Eine generelle Funktion der PARP-Proteine in der abiotischen Stressantwort wurde aus Studien abgeleitet, in denen die genetische, vor allem aber die pharmakologische Hemmung von PARPs zu einer Verbesserung der pflanzlichen Stressantwort führten. Um die Funktion der pflanzlichen PARPs während der Stressantwort weiter aufzuklären, wurden T-DNA-Insertionsmutanten aller drei Arabidopsis *AtPARP*-Gene einer Untersuchung ihrer Sensitivität gegenüber Trockenheit, osmotischem Stress, Salzstress und oxidativem Stress unterzogen. Da eine transkriptionelle Induktion der verbleibenden *AtPARP*-Gene auf eine funktionalle Redundanz deutete, wurde eine *parp*-Dreifachmutante erzeugt. Überraschenderweise unterschied sich keine der *parp*-Mutanten in ihrer Stressantwort vom Wildtyp, unabhängig von der Anzahl fehlender *AtPARP*-Gene. Callose-Ablagerungen sind eine pflanzliche Antwort auf die den bakteriellen Elizitor flg22. Die pharmakologische PARP-Hemmung verhinderte diese Callose-Ablagerungen, was vorherige Studien bestätigte. Unerwarteterweise zeigte jedoch die *parp*-Dreifachmutante auch hier keinen Unterschied zum Wildtyp. Offenbar verhindert eine pharmakologische PARP-Hemmung die Bildung von Callose-Ablagerungen stabiler als das Ausschalten aller *AtPARP*-Gene. Dies führte zu der Schlussfolgerung, dass es bisher nicht beschriebene Proteine mit einer PARP-Aktivität gibt. Diese Schlussfolgerung wurde dadurch untermauert, dass sich in der *parp*-Dreifachmutante keine Verringerung, sondern eine Erhöhung der Poly(ADP-ribosyl)ierungs-Aktivität zeigte. Mögliche alternative Zielproteine für die pharmakologische PARP-Hemmung finden sich in der SRO-Proteinfamilie. Diese Proteine besitzen eine katalytische PARP-Domäne und spielen eine zentrale Rolle in der pflanzlichen Stressantwort. Molekulare Modellierungen, die tierische PARP-Proteine als Vorlage nutzten, zeigten, dass die SRO-Proteine RCD1 und SRO1 mit Nikotinamid-basierten PARP-Inhibitoren interagieren könnten. Zusammengenommen deuten diese Ergebnisse darauf hin, dass die Stressphänotypen von *parp*-Mutanten hochgradig konditionaler Natur sind und die Studien mit PARP-Inhibitoren in Pflanzen aufgrund wahrscheinlicher Off-Target-Effekte einer Neubewertung bedürfen.

8 Appendix

8.1 Supplementary material for Publication 1

A yeast growth assay to characterize plant poly(ADP-ribose) polymerase (PARP) proteins and inhibitors

Dagmar Rissel, Peter Paul Heym, Edgar Peiter

Supplementary Material

Table 1. Primers used to amplify the cDNA sequences of *HsPARP1*, *AtPARP1*, *AtPARP2*, and *AtPARP3* for their expression in yeast cells.

Gene	Primer Sequence	Source
HsPARP1	GTTAATATACCTCTATACTTTAACGTCAAGGAGAAAAAACGGGAGGATGGCGGAGTCTTCGGATAAG	Perkins et al., 2001
	TGAATGTAAGCGTGACATAACTAATTACATGATGCGGCCCTCCTCTCCCAATTACCACAGGGAGGTC	
AtPARP1	GTTAATATACCTCTATACTTTAACGTCAAGGAGAAAAAACCGGAGAAATGGCAAGCCCACATAAGCCGTG	this study
	TGAATGTAAGCGTGACATAACTAATTACATGATGCGGCCCTCTTCAGGCTCATCTCTTGTGCTTAAACCTTACT	
AtPARP2	GTTAATATACCTCTATACTTTAACGTCAAGGAGAAAAAACGACGAAAATGGCGAACAAGCTCAAAGTC	this study
	TGAATGTAAGCGTGACATAACTAATTACATGATGCGGCCCTCATAAGTTTTAGTGCTTGTAGTTGAATTTGACTTGG	
AtPARP3	GTTAATATACCTCTATACTTTAACGTCAAGGAGAAAAAACTTGCTCAATGAAGGTTCACGAGACAAGATC	this study
	TGAATGTAAGCGTGACATAACTAATTACATGATGCGGCCCTCCTGTAATCTACTCTGGTTCGACATCGACTATC	

8.2 Supplementary material for Publication 3

All supplementary material is available under
https://www.frontiersin.org/article/10.3389/fpls.2017.00059/full#supplementary-material

Supplementary Material

No silver bullet - Canonical Poly(ADP-Ribose) Polymerases (PARPs) are no universal factors of abiotic and biotic stress responses of *Arabidopsis thaliana*

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Supplementary Presentations. Zip archive containing pdb files of protein models shown in Figure 10. (AtRCD1_3AB.pdb; AtRCD1_4ANI.pdb; AtRCD1_PHE.pdb; AtSRO1_3AB.pdb; AtSRO1_4ANI.pdb; AtSRO1_PHE.pdb)

Supplementary Table 1. The nomenclature of *Arabidopsis thaliana PARP1* and *PARP2* has been inconsistent in the literature.

Reference	**At2g31320**	**At4g02390**
this work	PARP1	PARP2
Zhang et al. (2015) Sci. Rep. 5:15892	PARP1	PARP2
Pham et al. (2015) Plant Mol. Biol., 89: 319-338	PARP2	PARP1
Song et al. (2015) PLOS Genet. 11: e1005200	PARP1	PARP2
Feng et al. (2015) PLOS Genet. 11: e1004936	PARP1	PARP2
Boltz et al. (2014) PLOS ONE 9: e88872	PARP2	PARP1
Jia et al. (2013) Plant Mol. Biol. 82: 339-351	PARP1	PARP2
Schulz et al. (2012) PLOS ONE 7: e37287	PARP2	PARP1
Lamb et al. (2012) Cell Mol. Life Sci. 69: 175-189	PARP2	PARP1
Briggs and Bent (2011) Trends Plant Sci. 16: 372-380	PARP2	PARP1
Pellny et al. (2009) Mol. Plant 2: 442-456	PARP1	PARP2
Ogawa et al. (2009) Plant J. 57: 289-301	PARP1	PARP2
Vanderauwera et al. (2007) PNAS 104: 15150-15155	PARP2	PARP1
De Block et al. (2005) Plant J. 41: 95-106	PARP2	PARP1
Doucet-Chabeaud et al. (2001) Mol. Genet. Genom. 265: 954-963	PARP1	PARP2

Supplementary Table 2. A unified nomenclature of Arabidopsis *parp* mutants.

PARP1 (At2g31320)		
Mutant	**Collection**	**previously published as**
parp1-1	GABI_380E06	*parp2* [1]; *parp1-1* [7]
parp1-2	GABI_382F01	*parp1-2* [7]
parp1-3	GABI_692A05	*atparp1* [2]; *parp1* [3]; *parp1* [8]
parp1-4	SALK_145153	*parp2* [6]
parp1-5	SALK_111410	*parp-2* [4]
parp1-6	SALK_109413	
parp1-7	SALK_141560	
PARP2 (At4g02390)		
Mutant	**Collection**	**previously published as**
parp2-1	GABI_420G03	*parp2-1* [7]
parp2-2	SAIL_1250_B03	*parp-3* [4]
parp2-3	SALK_140400	*parp1* [1]; *atparp2* [2]; *parp2* [3]; *parp2* [8]
parp2-4	SALK_097261	*parp1* [6]
parp2-5	SAIL_683_F10	
PARP3 (At5g22470)		
Mutant	**Collection**	**previously published as**
parp3-1	SALK_108092	*parp3-1* [5]; *parp3* [6]; *parp3* [8]
parp3-2	SAIL_632_D07	*parp-1* [4]

[1] Boltz et al. (2014), PLOS ONE, 9: e88872

[2] Feng et al. (2015), PLOS Genet. 11(1): e1004936

[3] Jia et al. (2013), Plant Mol. Biol. 82: 339-351

[4] Pham et al. (2015), Plant Mol. Biol. 89: 319-338

[5] Rissel et al. (2014), Plant Biol. 16: 1058-1064

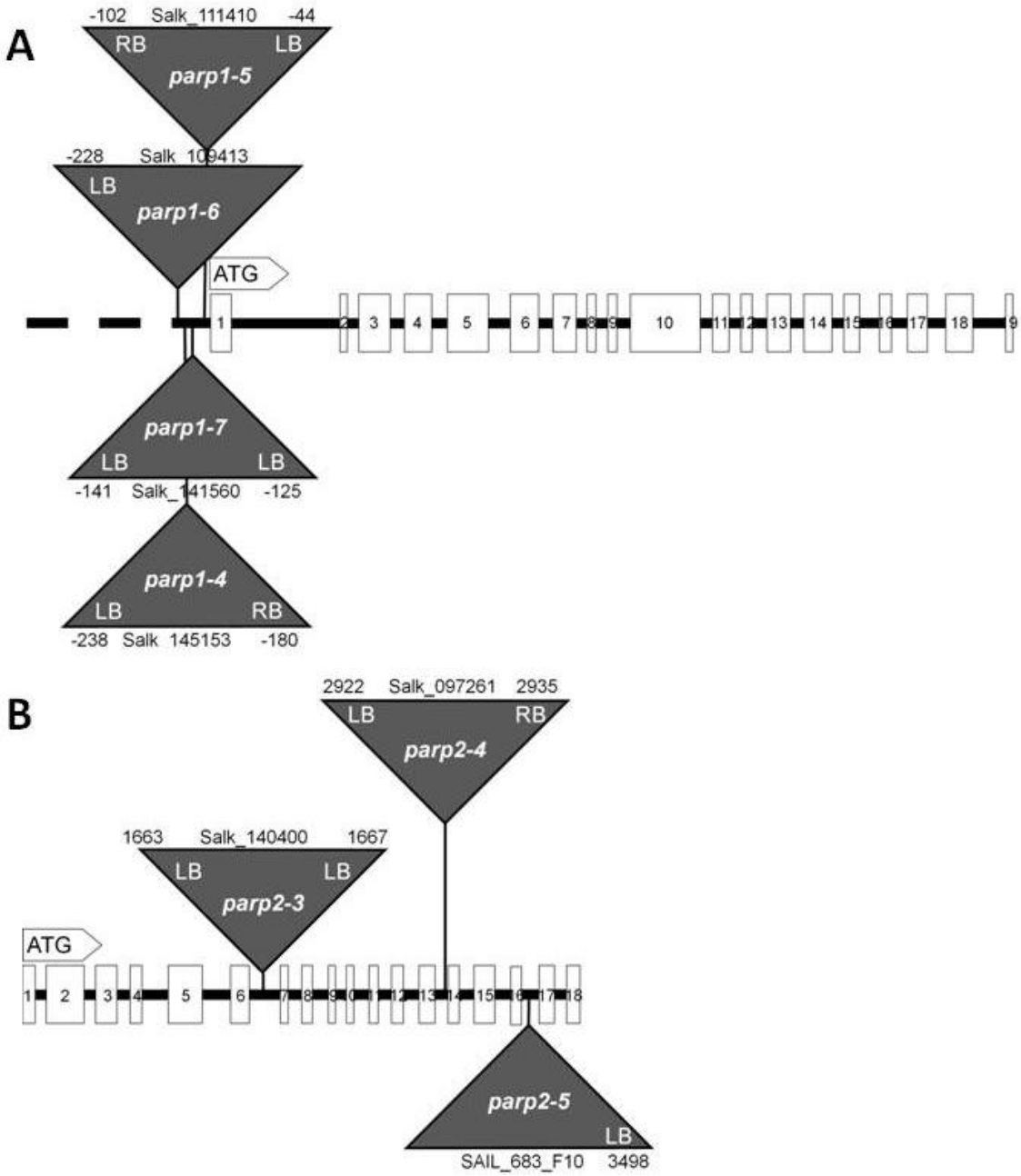
[6] Schulz et al. (2012), PLOS ONE 7: e37287

[7] Song et al. (2015), PLOS Genet. 11: e1005200

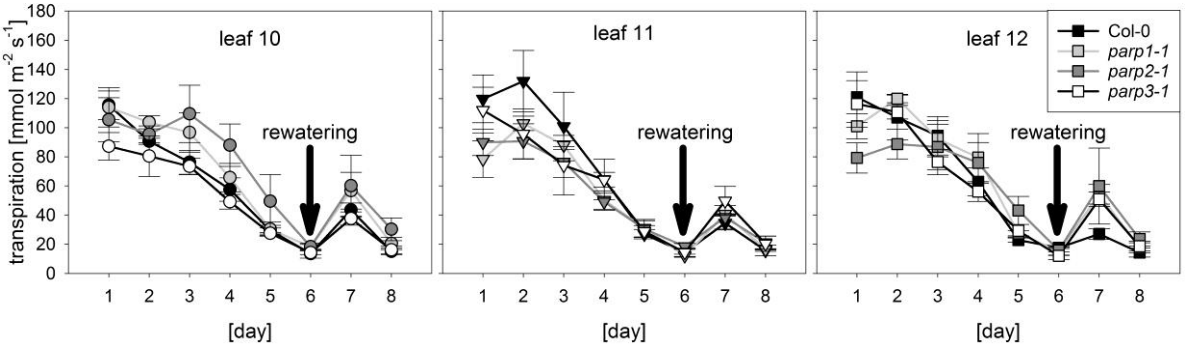
[8] Zhang et al. (2015), Sci. Rep. 5:15892

Supplementary Table 3. Primers used in this work.

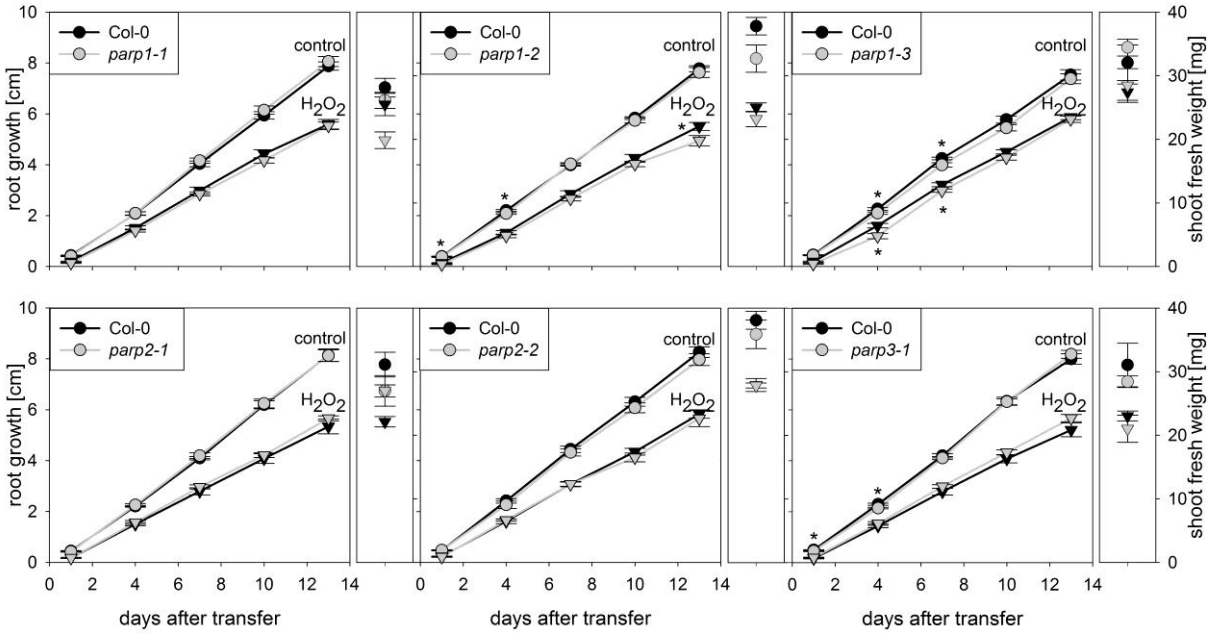
PCR screening and RT-PCR	*parp1-1*_for	ACTCCTCAAGGAGTGAAAGGC
	*parp1-1*_rev	ATCTCGAACTCCATCATTGC
	*parp1-2*_for	TGGAGCAAATGTTCTCATTCC
	*parp1-2*_rev	GATGCTTACAATGTCCAACGG
	*parp1-3*_for	TTGAGGCATTGACGGAGATAC
	*parp1-3*_rev	TTTCTCCCAATGCAACTTCAC
	GABI_8409	ATATTGACCATCATACTCATTGC
gene expression	*PARP1*_rt_for	GAAATACTAAGGAAAGGCAACCAT
	*PARP1*_rt_rev	TGTCAGTCCACAAACAACCAAA
PCR screening and RT-PCR	*parp2-1*_for	AGAACACTCATGCAAAGACGC
	*parp2-1*_rev	ACGCATCTTGATTTGTTCCAC
	*parp2-2*_for	AGAACACTCATGCAAAGACGC
	*parp2-2*_rev	AAGTGGAACAACAACACCGTC
	GABI_8409	ATATTGACCATCATACTCATTGC
	SAIL_LB1_short	CAGAAATGGATAAATAGCCTTGCTTC
	SAIL_LB3_short	GCATCTGAATTTCATAACCAATC
gene expression	*PARP2*_rt_for	GGCAAGATAAGCAAGTCCACA
	*PARP2*_rt_rev	ACTCAGTTCCTCAAGCCTCGT
reference genes	*ACT2*_rt_for	TCCCTCAGCACATTCCAGCAGAT
	*ACT2*_rt_rev	AACGATTCCTGGACCTGCCTCATC
	*UBQ10*_rt_for	CACACTCCACTTGGTCTTGCGT
	*UBQ10*_rt_rev	TGGTCTTTCCGGTGAGAGTCTTCA



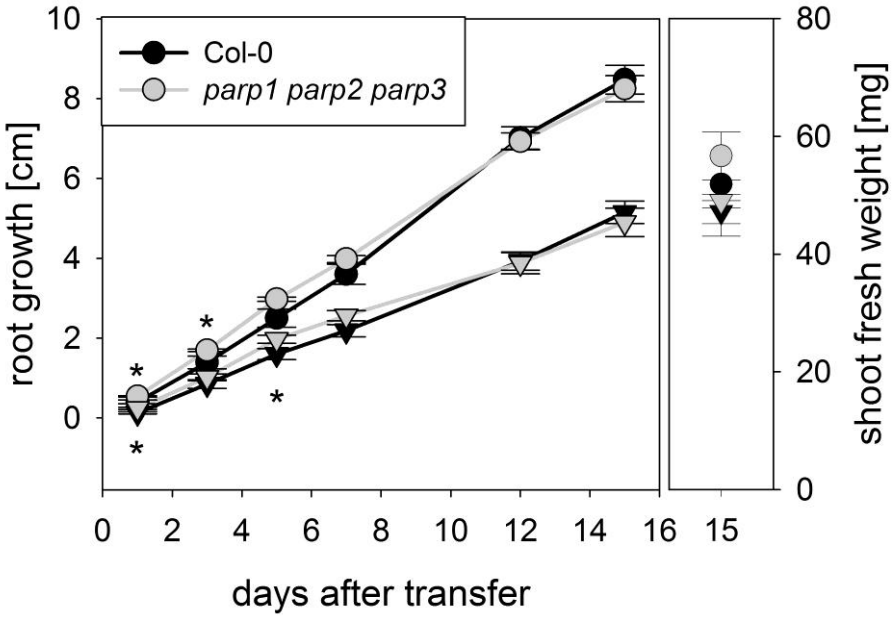
Supplementary Figure 1. Additional T-DNA insertion lines identified for *PARP1* and *PARP2*. Model of the genomic regions and the T-DNA insertions of *PARP1* (A) and *PARP2* (B). Coding regions are presented by white boxes, introns are shown by a line. Triangles indicate the sites of T-DNA insertion. The insertion lines originated from the SALK and the SAIL collections. The numbers indicate the last nucleotide before and the first nucleotide after the insertion, counting from the start codon. LB and RB indicate the left and right border of the T-DNA, as determined by sequencing.



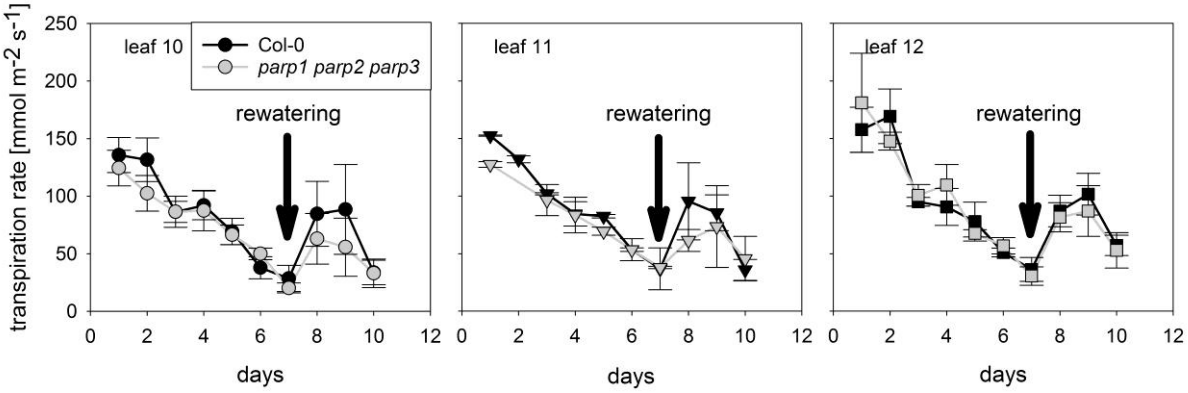
Supplementary Figure 2. Stomatal conductance is not altered in *parp* mutant plants compared to the wild type. Transpiration during desiccation was determined by porometry on leaves 10, 11, and 12. After 6 days plants were re-watered with 20 mL water. Data represent the means ±SE of 3-4 plants per line.



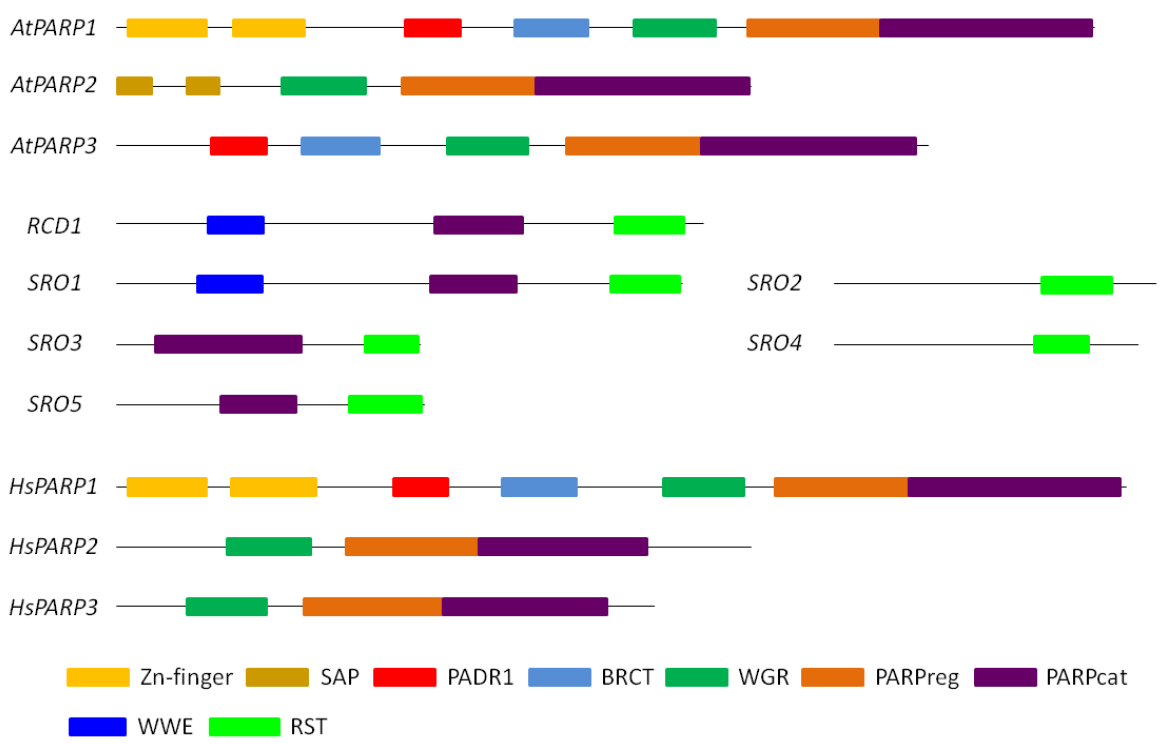
Supplementary Figure 3. Growth of *parp* mutant plants subjected to oxidative stress is not altered compared to the wild type. Root growth (large panels) of Col-0 and *parp* mutants on control plates (circles) or on plates containing 0.5 mM H_2O_2 (triangles). Shoot fresh weight was determined at the end of the experiment (small panels). The H_2O_2 treatment was contained in the experiment displayed in Figure 4. Control values shown in Figure 4 are included for comparison. Data represent the means ±SE of 15 plants per line.



Supplementary Figure 4. Growth of *parp1-1 parp2-1 parp3-1* mutant plants subjected to oxidative stress is not altered compared to the wild type. Root growth (large panel) of Col-0 and *parp1-1 parp2-1 parp3-1* mutants on control plates (circles) or on plates containing 0.5 mM H_2O_2 (triangles). Shoot fresh weight was determined at the end of the experiment (small panel). The H_2O_2 treatment was contained in the experiment displayed in Figure 7. Control values shown in Figure 7 are included for comparison. Data represent the means ±SE of 15 plants per line.



Supplementary Figure 5. Stomatal conductance is not altered in *parp1-1 parp2-1 parp3-1* mutant plants compared to the wild type. Transpiration during desiccation was determined by porometry on leaves 10, 11, and 12. After 7 days plants were re-watered with 20 mL water. Data represent the means ±SE of 3 plants per line.



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10 List of Abbreviations

°C	Degree celsius
µE	MicroEinstein
µg	Microgram
µl	Microliter
µM	Micromolar
µm	Micrometer
µmol	Micromole
½ MS	Half-strength Murashige & Skoog medium
3AB	3-aminobenzamide
ACT2	Actin2
ABA	Abscisic acid
ADP-ribose	Adenosine diphosphate ribose
ALY	Ally of AML-1 and LEF-1
4-ANI	4-amino-1,8-naphtalimde
Arabidopsis	*Arabidopsis thaliana* L. Heyn
Aox1	Alternative oxidase 1
AP2/ERF	APETALA2/ethylene response factor
APP	*Arabidopsis thaliana* homologue of PARP
Apx1	Ascorbate peroxidase 2
ARH3	ADP-ribose hydrolase 3
Asp	Asparagine
At	*Arabidopsis thaliana* (in genes and proteins)
ATM	Ataxia telangiectasia-mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related protein
BER	Base excision repair
bp	Base pairs
BRCT	Breast cancer susceptibility gene 1 C-terminus
BSA	Bovine serum albumin
c.	Circa
C-terminus	Carboxy terminus
Ca^{2+}	Calcium
cADPR	Cyclic ADP-ribose
cDNA	Complementary DNA
Col-0	Columbia-0
COT	Cotyledons
d	Day
Δ	Greek capital letter Delta (≙ deletion)
DIP1/2	DNA-binding domain interacting protein 1/2
DMSO	Dimethyl sulfoxide

DNA	Desoxyribonucleic acid
DREB2A	Dehydration-responsive element/C-repeat-binding proteins 2A
DSB	DNA double strand break
DAWDLE/DDL	Gene/protein named after prolonged growth period of its loss of function mutant
E	Glutamic acid
ECL	Enhanced chemical luminescence
elf18	N-terminal 18 amino acids of EF-Tu
ER	Endosperm rupture
EST	Expressed sequence tag
EYFP	Enhanced yellow fluorescent protein
FAA	Formaldehyde, acetic acid, alcohol
flg22	N-terminally conserved 22 amino acids of flagellin
FRK1	Flg22-induced receptor-like kinase
g	Gram
Gal	Galactose
GFP	Green fluorescent protein
G	Glycine
Gg	*Gallus gallus*
Glc	Glucose
Glu	Glutamate
GUS	β-glucoronidase
h	Hour
HCl	Hydrochloric acid
H	Histidine
H^+	Proton
HAS	Hours after sowing
H_2O_2	Hydrogen peroxide
HR	Homologous recombination
Hs	Human
Hv	*Hordeum vulgare* (in genes and proteins)
Hz	Hertz
i.e.	Id est
kDa	Kilodalton
KH_2PO_4	Potassium dihydrogen phosphate
Ku70/Ku80	Protein complex involved in DNA damage response
L	Liter
LB	Left border
Leu	Leucine
LIG4	Ligase4
LSC	Liquid synthetic complete
m	Meter

MAMP	Microbe-associated molecular pattern
M	Molar
MacroD1	Macrodomain-containing protein D1
MacroD2	Macrodomain-containing protein D1
3MB	3-methoxybenzamide
MCE	Micropylar and chalazal endosperm
MES	2-(N-morpholino) ethanesulfonic acid
Met	Methionine
$MgCl_2$	Magnesium chloride
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MMEJ	Micro-homology-mediated end joining
MMS21	Methyl methanesulfonate sensitivity gene 21
MMS	Methyl methanesulfonate
MRE11	Mitotic recombination 11
MYB15	Myeloblastosis transcription factor 15
Na^+	Sodium
NAC	Acronym derived from three genes initially discovered to contain the NAC domain: NAM, ATAF1 and −2, and CUC2
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NaOCl	Sodium hypochlorite
NAP	Non-classical poly(ADP-ribose)polymerase
NHEJ	Non-homologous end joining
N-terminus	Amino terminus
NBT	Nitroblue tetrazolium
NIC2	Nicotinamidase2
no TR	Non-ruptured testa
NTR	N-terminal region
OD	Optical density
Os	*Oryza sativa* (in genes and proteins)
P	Phosphorus
PAR	Poly(ADP-ribose)
PARG	Poly(ADP-ribose)glycohydrolase
PARP	Poly(ADP-ribose)polymerase
PCD	Programmed cell death
P5CDH	1-pyrroline-5-carboxylate dehydrogenase
PCR	Polymerase chain reaction
PE	Peripheral endosperm
pH	Negative decadic logarithm of proton activity

PHEN	6-(5H)-phenantridinone
Pp	*Physcomitrella patens* (in genes and proteins)
Pr	Promoter
Pro	Proline
Pst	*Pseudomonas syringae* pv. *tomato*
Pt	*Populus trichocarpa* (in genes and proteins)
Pv	Pathovar
qRT-PCR	Quantitative reverse transcription PCR
RAD	Radical and hypocotyl
RAD5a	Double-stranded DNA repair protein
RAD51	Double-stranded DNA repair protein
RB	Right border
RCD1	Radical-induced cell death1
REV7	Protein putatively involved in translesion synthesis
RH	Relative humidity
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
RST domain	RCD1-SRO-TAF4 protein domain
RT-PCR	Reverse transcription PCR
s	Second
SAP domain	Protein domain named after SAF-A/B, Acinus, and PIAS
S. cerevisiae	*Saccharomyces cerevisiae*
SE	Standard error
Ser	Serine
SMC	Structural maintenance of chromosomes proteins
SOS1	Salt overly sensitive 1
SRO	Similar to Radical-induced cell death one
SUMO E3 ligase	Small ubiquitin-like modifier E3 ligase
SSB	DNA single strand break
SWI	Protein involved in involved in sister chromatid cohesion and chromosome organization
TARG1	Terminal ADP-ribose glycohydrolase 1
TR	Ruptured testa
Tris	Tris(hydroxymethyl) aminomethan
Tyr	Tyrosine
UBI10	Ubiquitin 10
Ura	Uracil
Val	Valine
WGR domain	Protein domain named after the most conserved central motif of the domain
WWE domain	Protein domain named after three of its conserved residues

x-Gluc	5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid
XRCC	X-ray repair cross-complementing protein
ZAP1/2	Zinc-finger poly(ADP-ribose)polymerase 1/2
Zm	*Zea mays* (in genes and proteins)
Zn	Zinc

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13 Bibliography

13.1 Publications

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Ulber, L., Rissel, D. (2017) Farmer's awareness of resistant weeds and perception of resistance management strategies: Results from a German survey. Global Herbicide Resistance Challange 2017, 14-18 May 2017, Denver

Rissel D., Ulber L. (2016) Evolution of herbicide resistance in *Apera spica-venti* as a consequence of repeated herbicide applications. 7th International Weed Science Congress, 19-25 June 2016, Prague

Ulber, L., Rissel, D. (2016) Impact of weed control strategies on resistance evolution in *Alopecurus myosuroides* – a long term field trial. 27th German Conference on Weed Biology and Weed Control, 23 - 25 February 2016

Rissel D., Rosenhauer M., Ulber L., Petersen J. (2015) Monitoring of resistance development against ALS inhibiting herbicides in dicotyledonous weeds in Germany. 17th European Weed Research Society Symposium, 22-26 June, Montpellier

Rissel D., Peter K., Thor K., Peiter E. (2012) Poly(ADP-ribose) polymerases in plant stress tolerance – key regulators or overrated? International Conference of the German Society of Plant Nutrition, 5- 8 September 2012, Bonn

Poster presentations:

Rissel, D., Ulber, L. (2018) Studying cytochrome P450-based non-target site resistance in *Apera spica-venti*. 61st German Conference on Plant Protection, 11-14 September 2018, Stuttgart

Rissel, D., Ulber, L. (2018) Studying cytochrome P450-based non-target site resistance in *Apera spica-venti*. 18th European Weed Research Society Symposium, 17-21 June 2018, Ljubljana

Rissel D., Ulber L. (2017) Non-target-site resistance evolution in *Apera spica-venti*. Global Herbicide Resistance Challange 2017, 14-18 May, Denver

Ulber L., Rissel D. (2015) Selecting for reduced herbicide sensitivity in *Apera spica-venti*. Resistance 2015, 14-16 September 20015, Rothamsted Research, Harpenden

Rissel D., Losch J., Peter K., Peiter E. (2013) Poly(ADP-ribose)polymerases - regulators in plant stress response? National Meeting of the German Society of Plant Nutrition, 9-10 May 2011, Freising-Weihenstephan

Rissel D., Peter K., Thor K., Peiter E. (2011) Exploring the role of poly(ADP-ribose)polymerases in plants. National Meeting of the German Society of Plant Nutrition, 27-29 September 2011, Kiel

Rissel D., Seelmann M., Thor K., Peiter E. (2010) Plant poly(ADP-ribose)polymerases – modulators of stress tolerance? International Conference of the German Society of Plant Nutrition, 30 September - 2 October 2010, Hannover

13.2 Curriculum vitae

Name:	Dipl. agr.-biol. Dagmar Rissel
Date/Place of Birth:	17 January 1984, Dinslaken
Nationality:	German

January 2014 – now	Research Associate at the Julius Kühn Institute, Institute for Plant Protection in Field Crops and Grassland
November 2009 – September 2014	Doctoral studies at the Martin Luther University Halle-Wittenberg, Institute of Agricultural and Natural Sciences
January 2012 – February 2013	Research Fellowship granted by Agrochemisches Institut Piesteritz e.V.
November 2008 – December 2011	Research Associate at Agrochemisches Institut Piesteritz e.V. and Martin Luther University Halle-Wittenberg
October 2003 – October 2008	Studies of agricultural biology at the University of Hohenheim
1992 – June 2003	School education completed with Abitur

14 Declaration under Oath

Eidesstattliche Erklärung / Declaration under Oath*

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

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Datum / *Date* Unterschrift des Antragstellers / *Signature of the applicant*