Establishment of a plant-based system for identifying phytoeffectors and analysis of phytoeffector targets under abiotic stress in Arabidopsis

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To my parents

To Jan and Carli

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List of Abbreviations

3-AB	3-aminobenzamide
$[Ca^{2+}]_{cyt}$	cytosolic free Ca^{2+}
ABA	abscisic acid
ABRE	ABA-responsive *cis*-acting element
Aeq	Aequorin
AREB	ABA-responsive element-binding protein
At	*Arabidopsis thaliana*
bp	base pair
BSA	bovine serum albumin
CAB	chlorophyll a/b-binding protein
cADPR	cyclic ADP-ribose
CaMV	Cauliflower Mosaic Virus
CBL	calcineurin B-like protein
CCD	charge-coupled device
cm	centimeter
Col-0	Columbia-0
COR	cold-regulated
COS	constitutive expression of osmotically responsive genes
DMSO	dimethyl sulfoxide
dNTP	desoxy nucleoside triphosphate
DRE	dehydration-responsive element
DREB	dehydration-responsive element-binding protein
elf18	elongation factor18
EDTA	ethylene diamine tetraacetic acid
flg22	flagellin22
FMN	flavin mononucleotide
g	gram
GUS	*β*-glucuronidase
HOS	high expression of osmotically responsive genes
h	hour
JA	jasmonic acid
l	litre
LB	left border
LTI	low-temperature-induced
LOS	low expression of osmotically responsive genes
LOX	lipoxygenase
LUC	luciferase

M	molar
mg	milligram
min	minute
mM	millimolar
ml	millilitre
MS	Murashige and Skoog medium
NAD^+	nicotinamide adenine dinucleotide
ng	nanogram
PAL	phenylalanine ammonia lyase
PARG	poly(ADP-ribose) glycohydrolase
PARP	poly(ADP-ribose) polymerase
PCR	polymerase chain reaction
PEG	polyethylene glycol
RCD	radical-induced cell death
RD	dehydration-regulated
rh	relative humidity
RNAi	RNA interference
rpm	rotations per minute
ROS	reactive oxygen species
s	second
SAR	systemic acquired resistance
SDS	sodium dodecyl sulfate
SOS	salt overly sensitive
SRO	similar to RCD One
TF	transcription factor
TSAP	thermosensitive alkaline phosphatase
WT	wild type
µg	microgram
µM	micromolar
µl	microlitre

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1 Introduction

During their life cycle plants are frequently exposed to environmental stress. Abiotic stress factors, such as drought, cold, heat, salinity, nutrient deficiencies, ozone, high light intensity, or hypoxia lead to insecurity of a successful plant development and represent the main cause of yield losses of up to 50% worldwide on average (Bray *et al.*, 2000). The frequency and severity of such stress conditions are predicted to increase in the near future. Therefore, worldwide agriculture faces a major challenge in the coming years. The global water shortage, caused by a steadily growing world population that will reach more than 9 billion in 2050, and by the prevalent climate change, is becoming a more and more severe problem (Bray *et al.*, 2000; Golldack *et al.*, 2011; IPCC, 2007; Mittler and Blumwald, 2010).

As sessile organisms, plants depend on their own genetic potential to overcome such stress situations by activating appropriate defense mechanisms. Hence, there is an extensive body of research for suitable ways to improve the plants' performance in response to environmental stress in the field to satisfy a secure crop productivity and food supply. Many of the discussed adaptation strategies of crops to the changing global climate conditions have been insufficient, as many of the agronomic efforts, like crop rotation or preserving tillage, are inflexible and classical breeding approaches need a long time for realization. A very promising, but currently in many countries unacceptable methodology is the use of genetically modified plants (Savvides *et al.*, 2016). An alternative, so far little explored opportunity to adapt crops to abiotic stress is to activate the plant's own defense mechanisms through the application of chemical compounds that are able to activate the plant's endogenous defense machinery, often named as phytoeffectors.

The present work introduces a new plant-based approach for the identification of phytoeffectors that may be able to enhance the tolerance against abiotic stress and hence limit yield losses and improve yield stability under changing climatic conditions. Furthermore, the role of a corresponding target protein family to a defined class of potential phytoeffectors will be analyzed.

1.1 Abiotic stress – physiological aspects of drought and high salinity

Abiotic stress has a major impact on the utilization of the full genetic potential of a plant. Here, water stress is the biggest limiting factor, which includes both drought and salt stress. Plant responses to these stresses are closely related and also affected by environmental factors and the developmental stage of the plant (Bray *et al.*, 2000; Mittler and Blumwald, 2010).

Drought and salt stress are manifested primarily as osmotic stress (Wang *et al.*, 2003). Soil salinization, causing high Na^+ and Cl^- concentrations in the soil solution, reduces the water potential in the root area of the plant, which affects water availability (Hasegawa *et al.*, 2000). The plant responses to drought and salt stress include morphological and developmental changes, such as inhibition of shoot growth and enhancement of root growth, but also alterations in ion transport and metabolic changes. Some of these reactions are primary induced by the stressor itself, whereas others are triggered by secondary factors caused by the primary stressor, such as phytohormones like abscisic acid (ABA), or reactive oxygen species (ROS). Generally, the plant responses to stress are of three kinds: maintenance of homeostasis, detoxification and recovery of growth (Xiong and Zhu, 2002).

A very early response of the plant to drought- or salt-induced osmotic stress is the closure of stomata, triggered by the phytohormone ABA, to prevent transpiration-based water loss (Blatt, 2000). The stress-induced turgor loss leads to the accumulation of compatible solutes, or osmolytes. Such solutes can be amino acids (e.g. proline), quaternary amines (e.g. glycine betaine) or sugars (e.g. trehalose). An increased accumulation of compatible solutes in transgenic plants can result in improved stress tolerance (Wang *et al.*, 2003). For example, transgenic tobacco (*Nicotiana tabacum*) overexpressing the *P5CS* gene, that encodes for an important synthase in the biosynthesis of proline, produced 10- to 18-fold more proline and exhibited better performance under salt stress (Kishor *et al.*, 1995).

In addition to the hyperosmotic stress, high NaCl concentrations also lead to ion toxicity (Hasegawa *et al.*, 2000). To counter this problem, plants have evolved numerous signaling and transport mechanisms. A prominent example

for this is the *SOS* (*Salt Overly Sensitive*) pathway. The excess of intra- or extracellular Na^+ elicits a cytosolic Ca^{2+} signal leading to the activation of the calcium-binding protein SOS3, which in turn interacts with and activates SOS2, a serine/threonine protein kinase. Together SOS3 and SOS2 regulate the expression and activity of SOS1, a plasma membrane Na^+/H^+ antiporter (Ishitani *et al.*, 2000; Liu *et al.*, 2000; Shi *et al.*, 2002; Zhu, 2002). Accordingly, overexpression of *SOS1* in Arabidopsis led to improved salt tolerance (Shi *et al.*, 2003). Additionally, an overexpression of the vacuolar Na^+/H^+ antiporter AtNHX1 in Arabidopsis plants also promoted salt tolerance (Apse *et al.*, 1999).

1.2 The transcriptional regulation of stress-responsive genes

In addition to functional genes that are up-regulated under abiotic stress, many genes with regulatory functions exist. Among them transcription factors (TFs) play important roles in multiple stress responses by regulating a broad spectrum of stress-responsive downstream genes. TFs are activated after stress perception and signal transduction by protein kinases or phosphatases, and they further bind to specific *cis*-acting elements in the promoters of stress-responsive genes in order to regulate their transcription (Wang *et al.*, 2016). TFs involved in abiotic stress responses, such as AP2/EREBP, MYB, WRKY, NAC, or bZIP proteins, may act either in an ABA-dependent or ABA-independent manner (Golldack *et al.*, 2011; Umezawa *et al.*, 2006).

The AP2/EREBP family is characterized by the presence of the highly conserved AP2/ERF (ethylene-responsive element-binding factor) DNA-binding domain, which interacts with GCC box and/or DRE/CRT (dehydration-responsive element/C-repeat element) *cis*-acting elements in the promoter of downstream genes (Riechmann and Meyerowitz, 1998). This TF family plays important roles in vegetative and reproductive development, in biotic and abiotic stress responses, and in plant hormone responses (Nakano *et al.*, 2006; Sharoni *et al.*, 2011). AP2/ERFBP members have been identified in many species, such as 145 in Arabidopsis (Riechmann and Meyerowitz, 1998). The AP2/EREBP family is grouped in four subfamilies. One of these subfamilies are the DREB (dehydration-responsive element-binding protein) TFs (Sharoni *et al.*, 2011), which have been extensively studied owing to their role under abiotic stress. They are able to regulate the expression of many dehydration/cold-

regulated (*RD*/*COR*) genes by binding to their *DRE*/*CRT cis*-acting elements (A/GCCGAC), such as *COR15A*, *RD29A/COR78*, and *COR6.6*. *DREB* TFs are further classified into two subgroups: *DREB1* and *DREB2*. *DREB1* genes are involved in cold stress responses, and *DREB2* genes respond to drought, high salinity and high temperatures (Liu *et al.*, 1998; Lucas *et al.*, 2011). Arabidopsis plants overexpressing the *DREB2A* gene exhibited a 75-83% higher survival rate upon drought stress compared to wild type plants (Sakuma *et al.*, 2006).

The bZIP (basic leucine zipper) TF family members are also involved in the response to various abiotic stresses, such as drought and salinity (Jakoby *et al.*, 2002; Wang *et al.*, 2016). 75 members of this family have been identified in Arabidopsis. These TFs are characterized by the presence of a basic region that binds DNA and by a leucine zipper dimerization motif. They are classified into 10 groups, whereby the members of group A are involved in ABA or stress signaling (Jakoby *et al.*, 2002). This group consists of seven members, including the AREB (ABA-responsive element-binding protein) TFs. Most studies on their role in abiotic stress responses showed that their expression is induced by ABA and that they regulate the transcription of stress-responsive genes via an ABA-dependent pathway by binding to the ABRE (ABA-responsive *cis*-acting element; ACGTGG/TC) element in the promoter region (Jakoby *et al.*, 2002; Narusaka *et al.*, 2003; Uno *et al.*, 2000). An overexpression of *AREB1* in Arabidopsis leads to an increased drought tolerance, reflected by an enhanced ability to recover from drought and by increased survival rates after dehydration (Fujita *et al.*, 2005).

Some functional genes involved in abiotic stress responses are induced by both DREBs and AREBs due to the existence of both types of *cis*-acting elements in their promoter region (Fujita *et al.*, 2009). Examples for such genes are *RD29A*, *KIN2* or *RAB18.* Therefore, these kinds of stress-related genes can be activated via the ABA-dependent and the ABA-independent pathway, respectively (Fig 1.1) (Narusaka *et al.*, 2003).

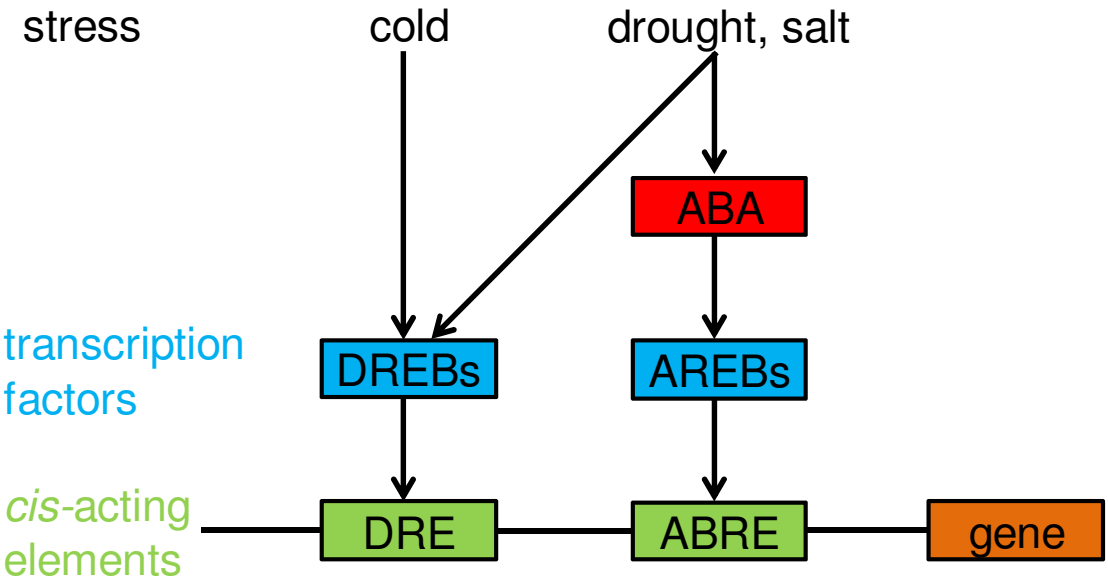

Figure 1.1 Simple model of the transcriptional stress response in plants.

ABA, abscisic acid; DREB, dehydration-responsive element binding protein; AREB, ABA-responsive element binding protein; DRE, dehydration-responsive element; ABRE, ABA-responsive element

1.3 The use of phytoeffectors in abiotic stress tolerance research

Plant stress responses are very complex and regulated by a multi-gene network. This complicates the analysis of individual genes in order to enhance plant stress tolerance (Mittler and Blumwald, 2010; Varshney *et al.*, 2011). Although the functionality of these networks is not completely understood, it has been shown that some exogenously applied chemical agents can have a positive influence on stress tolerance. Those chemical compounds, here referred to as “phytoeffectors”, interfere in the plant signaling network and, depending on their mode of action, cause specific molecular and physiological changes in the plant organism.

The use of phytoeffectors in plant abiotic stress research is part of the so called “chemical genetics” approach. The intention of chemical genetics is to employ chemical compounds to investigate biological questions in a similar manner to mutational analysis. This means that biological material is systemically screened against a large set of chemical substances for phenotypes of interest (McCourt and Desveaux, 2010). The chemical compounds, usually small molecules, are able to alter protein function and

thereby reveal the biological role of these target proteins (Zheng *et al.*, 2004). Compared to classical genetic approaches, the use of small molecules comprises some advantages, like generating different and often complementary information, as well as overcoming lethality, genetic redundancy, and pleiotropic effects (Tóth and Van der Hoorn, 2010). A very prominent example of using chemical genetics was the identification of the long-sought ABA receptor *PYR1* by using pyrabactin as a highly specific ABA agonist (Park *et al.*, 2009).

In abiotic stress tolerance research, several chemical substances have been identified as positive regulators. These have been found to enhance tolerance against various abiotic stresses and may be used as priming agents (reviewed in Savvides *et al.*, 2016). For example, sodium nitroprusside (SNP), an inorganic compound that is used as NO donor, was used as a priming agent for salt-stressed rice plants, leading to increased expression of stress-related genes like the above-mentioned *P5CS* (Uchida *et al.*, 2002). Another compound, melatonin, which acts as a growth regulator in plants, enhanced the expression of *CBF* and *DREB* transcription factors in Arabidopsis plants in response to chilling (Bajwa *et al.*, 2014).

For the identification of new phytoeffectors able to promote plant performance under abiotic stress, the experimental procedure involves three stages, as commonly performed in a chemical genetics project (Blackwell and Zhao, 2003): (1) The development of a suitable screening assay, preferentially in a small volume like a microtiter plate format, (2) the screen of candidate compounds followed by a secondary screen to verify the hits, and (3) the phenotypic characterization and target identification.

1.4 Arabidopsis as a model system

Many of the functional and regulatory genes involved in abiotic stress have been characterized in the model plant *Arabidopsis thaliana* L., commonly named Arabidopsis. *A. thaliana*, a small plant of the *Brassicaceae* family, is a very popular model organism, which has been used in many laboratories for solving a broad spectrum of questions regarding plants genetics and physiology. The most important advantages of this plant are: (1) Its small genome, which contains 25,498 genes arranged on five chromosomes, (2) its small size, allowing its cultivation on a small area, and (3) its short life cycle of

about six weeks from germination to the production of mature seeds (Arabidopsis Genome Initiative, 2000; Meinke *et al.*, 1998). Furthermore, thousands of loss-of-function mutants harboring random T-DNA insertions throughout the genome are available (Meinke *et al.*, 1998). By the year 2000 the whole genome of *A. thaliana* was sequenced (Arabidopsis Genome Initiative, 2000). The available information on specific genes has tremdously increased and can be found in various databases, such as TAIR (The Arabidopsis Information Resource, www.arabidopsis.org), Genevestigator (www.genevestigator.com/gv), eFP (Electronic Fluorescent Pictograph) Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), and the plant membrane protein database ARAMEMNON (aramemnon.botanik.uni-koeln.de).

1.5 Aims of this thesis

Abiotic stress, especially drought and high salinity, has a major impact on worldwide agriculture. To maintain productivity under stress, the use of chemical genetic approaches, such as the application of phytoeffectors, offers a promising alternative to classical genetics. It has been shown that an upregulation, either genetically or pharmacologically, of both *DREB* and *AREB* transcription factors leads to an increased tolerance of plants to various kinds of abiotic stress (Bajwa *et al.*, 2014; Fujita *et al.*, 2005; Kasuga *et al.*, 1999). The promoter activity of their target genes may thus be a suitable screening feature to identify new phytoeffectors. In particular, the promoter of their target gene *RD29A* has been used to investigate plant stress responses (Xiao *et al.*, 2006; Quist *et al.*, 2009) and is therefore the basis of the screening approach to be developed in the present thesis.

This thesis is divided into two parts, (1) the identification of potential phytoeffectors and (2) the investigation of the role of a very promising target protein family in plants under abiotic stress conditions. The first part includes the establishment and verification of a new plant-based system for identifying potential phytoeffectors. The second part, based on the work with a defined group of compounds from the first part, includes the phenotypical analysis of the corresponding target protein family. The aims of both parts will be described in more detail in the following main chapters.

2 Plant-based identification of potential phytoeffectors

This part focuses on the identification of phytoeffectors, i.e. substances to enhance plant tolerance against abiotic stress, by activating resistance mechanisms at the transcriptional level. To this end, the gene encoding the light-emitting firefly luciferase (LUC) reporter was expressed under the control of the stress-responsive *RD29A* promoter.

2.1 Analysis and applications of *RD29A* and its promoter

RD29A, also known as *COR78* (*COLD-REGULATED78*) or *LTI78* (*LOW-TEMPERATURE-INDUCED78*), is a drought-, salinity-, and cold-inducible gene from Arabidopsis (Yamaguchi-Shinozaki and Shinozaki, 1993; Shinozaki *et al.*, 2003). While the function of its protein in plants is still unknown, its promoter is well studied and is frequently used for investigating plant stress tolerance (Xiao *et al.*, 2006; Quist *et al.*, 2009).

RD29A (At5g52310) and *RD29B* (At5g52300) are located in tandem on chromosome five of Arabidopsis (Yamaguchi-Shinozaki and Shinozaki, 1993). The promoters of *RD29A* and *RD29B* are 60.93% identical and contain two kinds of *cis*-acting elements: the dehydration-responsive (DRE; A/GCCGAC) and the ABA-responsive (ABRE; ACGTGG/TC) elements (Yamaguchi-Shinozaki and Shinozaki, 1994). More precisely, *RD29A* possesses one ABRE, two DREs and two DRE core motifs, while *RD29B* exhibits three ABREs and only one DRE (Seki *et al.*, 2002; Nakashima *et al.*, 2006). Therefore, the activation of the *RD29A* promoter can be conferred by two signaling pathways, ABA-dependent and ABA-independent, while the *RD29B* promoter is only activated by the ABA-dependent pathway (Jia *et al.*, 2012). The activation of *RD29A* via both signaling pathways makes it responsive to more abiotic stressors compared to *RD29B*, and for this reason *RD29A* is commonly used as a marker gene for abiotic stress (Cheong *et al.*, 2010; Yang *et al.*, 2010).

The *RD29A* gene is transcriptionally activated by ABRE-binding proteins (AREBs) and DRE-binding proteins (DREBs), which specifically bind to the

ABRE and DRE promoter elements, respectively (Jia *et al.*, 2012). An overexpression of *OsAREB1* in transgenic Arabidopsis resulted in higher expression of *RD29A* compared to wild type plants, whereas the expression of *RD22*, which lacks ABRE elements, was not altered (Jin *et al.*, 2010). Similarly, a constitutive overexpression of *DREB1A* led to a stronger expression of *RD29A* in Arabidopsis under both unstressed and stressed conditions, whereas no difference in the expression of *RD29B* was detected (Kasuga *et al.*, 1999).

The response of *RD29A* to various abiotic stress situations has been used successfully to investigate stress-response pathways in plants (Jia *et al.*, 2012). For example, Cheong *et al.* (2010) showed that an overexpression of *CBL5* (calcineurin B-like protein) changed the expression pattern of *RD29A* and demonstrated that *CBL5* may act as a positive regulator of plant responses to salt and drought stress.

The *RD29A* promoter carries both DRE and ABRE elements, which makes it an optimal tool for improving abiotic stress tolerance. Under unstressed conditions, plants transformed with stress-inducible genes under the control of a constitutive promoter (e.g. CaMV 35S) often show undesirable phenotypes, like delayed growth or reduced fresh weight, whereas the use of the *RD29A* promoter instead of the constitutive CaMV 35S promoter does not infer with normal plant development, but can improve stress tolerance (Kasuga *et al.*, 1999; Hong *et al.*, 2006). In addition, the *RD29A* promoter has been used to drive reporter genes. For example, Ishitani *et al.* (1997) used the *firefly luciferase* gene driven by the *RD29A* promoter to screen a large set of mutants with altered *RD29A-LUC* expression in response to abiotic stress and ABA and could identify many *cos* (constitutive expression of osmotically responsive genes), *los* (low expression of osmotically responsive genes), and *hos* (high expression of osmotically responsive genes) mutants.

2.2 *Luciferase* as reporter gene

Luciferase (*LUC*) is a gene naturally occurring in the firefly *Photinus pyralis* and has become a popular reporter gene for *in vitro* and *in vivo* analyses of transcriptional activity in eukaryotes (Leeuwen *et al.*, 2000). Since cloning of its cDNA by DeWet *et al.* in 1985, it has been expressed in many species, such as

tobacco and carrot plants (Ow *et al.*, 1986), mammalian cells (de Wet *et al.*, 1987) and *Drosophila* (Brandes *et al.*, 1996).

LUC catalyzes the oxidative decarboxylation of the substrate luciferin to oxyluciferin, which is accompanied by the emission of light, more precisely of one photon at 560 nm. For detecting the low levels of luminescence, a low-light-detecting charge-coupled device (CCD) camera is suitable (Southern *et al.*, 2006). LUC is very stable in the absence of luciferin, but in the presence of luciferin LUC loses activity and is only slowly regenerated. This long regeneration time together with the short half-life of LUC of about 2 to 3 hours allows the detection of both increases and decreases of the activity of the promoter of interest (Millar *et al.*, 1992; Leeuwen *et al.*, 2000). The non-invasiveness of this reporter system is a further advantage compared to the histochemical detection of expression by promoter fusions with the *β-glucoronidase* (*GUS*) gene (Jefferson *et al.*, 1987; Alvarado *et al.*, 2004).

In its native organism, firefly, LUC is located in peroxisomes. To optimize *luciferase* expression in foreign hosts, an improved version of *LUC* has been produced, *LUC+*, in which the peroxisomal translocation sequence was removed (Sherf and Wood, 1994). Furthermore, some other modifications were made, such as an improved codon usage for mammalian cells, exchange of some restriction sites without changing the amino acid sequence, removing of regulatory sites to ensure the “genetically neutral” behavior of the reporter gene, and an alteration of two consensus glycosylation sites to prevent potential occurrence of N-linked glycosylation. In Arabidopsis, *LUC+* produces 5 to 20-fold brighter luminescence compared to the native *luciferase* (Sherf and Wood, 1994; Southern *et al.*, 2006). *LUC+* is also the *luciferase* version which was used in the present study.

Because of its properties, *luciferase* can be used as reporter gene to investigate the transcriptional activity of any given gene-of-interest in plants in real time. For example, under the control of a circadian-regulated promoter, *CAB2* (*chlorophyll a/b-binding protein 2*), it was used as a reporter for transcriptional clock output (Millar *et al.*, 1995a, b; Tindall *et al.*, 2015). Under control of the *LOX2* (*lipoxygenase 2*) promoter, whose gene is involved in JA signaling, it was used to get a better understanding of the action of the phytohormone jasmonic acid (Jensen *et al.*, 2002). Finally, under control of the

abiotic stress-induced *RD29A* promoter, it was used as a reporter to investigate plant abiotic stress responses (Ishitani *et al.*, 1997).

2.3 Aims

Chemical genetics provide a promising opportunity in plant stress tolerance research. Therefore, the aim of this part was to establish an appropriate experimental setup for identifying new potential phytoeffectors.

This includes:

- construction of the *RD29A-LUC* reporter gene
- generation of Arabidopsis *RD29A-LUC* reporter plants
- development of a robust screening assay for test substances
- first experiments to verify the suitability of the screening system

2.4 Material and Methods

2.4.1 Chemicals

Table 2.1 Chemicals used in this part

Substance	Chemical formula	Company	Cat. No.
(±)-Abscisic acid	$C_{15}H_{20}O_4$	Sigma	A1049
3-Aminobenzamide	$H_2NC_6H_4CONH_2$		
Agar-Agar, Kobe I	-	Sigma	5210.2
Agarose	-	Biozym S	840004
Beef extract	-	Roth	X975.1
Dipotassium hydrogen phosphate	K_2HPO_4	Merck	
Dimethyl sulfoxide	C_2H_6OS	Duchefa	D1370.0250
D(+)-Glucose	$C_6H_{12}O_6$	Roth	HN06.2
Isonicotinamide	$C_6H_6N_2O$	SKW	-
Isopropanol	C_3H_8O	Roth	T910.1
D-Luciferin sodium salt	$C_{11}H_7N_2O_3S_2Na*H2O$	Roth	4096.2
Magnesium sulfate	$MgSO_4*7H_2O$	Sigma	63140
MS salts+vitamins	-	Duchefa	M0231
Nicotinamide	$C_6H_6N_2O$	Sigma	72340
Peptone	-	Roth	AE41.1
Phyto-Agar	-	Duchefa	P1003
PJ-34 hydrochloride hydrate	$C_{17}H_{17}N_3O_2*HCl*xH_2O$	Sigma	P4365
Polyethylene glycol6000	$(HSCH_2CH_2COOCH_2)_4C$	Duchefa	P0805
Potassium chloride	KCl	Duchefa	PO515
Potassium dihydrogen phosphate	KH_2PO_4	Fluka	60220
Silwet L-77	-	Lehle Seeds	VIS-02
Sodium chloride	NaCl	Roth	3957.1
D(+)-Sucrose	$C_{12}H_{22}O_{11}$	Roth	4621.1
Tryptone	-	Formedium	TRP02
Yeast extract	-	Formedium	YEM02

2.4.2 Enzymes

Table 2.2 Enzymes for molecular cloning

Enzyme	Source	Cat. No.
*BstB*I	New England Biolabs	R0519S
*EcoR*I-HF	New England Biolabs	R3101S
*Hind*III-HF	New England Biolabs	R3104S
Klenow Fragment	New England Biolabs	M0210S
*Nco*I-HF	New England Biolabs	R393S
Phusion Polymerase	Finnzymes	F-540S
*Pst*I	New England Biolabs	R0140S
*Sma*I	New England Biolabs	R0141S
TSAP (Thermosensitive alkaline phosphatase)	Promega	M9910
T4 DNA ligase	New England Biolabs	M0202S

2.4.3 Oligonucleotides

All oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

Table 2.3 Oligonucleotide sequences

Purpose	Name	Gene	Sequence (5´→3´)
RD29A-LUC cloning	PrRD29Afor_HindIII	PrRD29A	AAAAAAAGCTTGATATACTACCGACA TGAGTTCCA
	PrRD29Arev_EcoRI	PrRD29A	AAAAAGAATTCTCCTCTGTTTGATCC ATTTTC
colony-PCR	T7	T7	TAATACGACTCACTATAGGG
	T3	T3	AATTAACCCTCACTAAAGGG

2.4.4 Kits

Table 2.4 Kits used in this study

Kit	Source	Cat. No.
DNeasy Plant Mini Kit	Qiagen	69104
Wizard® Plus SV Minipreps DNA Purification System	Promega	A1460
Wizard® SV Gel and PCR Clean-Up System	Promega	A9282

2.4.5 Bacterial strains

Table 2.5 Bacterial strains

Strain	Species	Aim	Selection
Top 10	*E. coli*	standard cloning	Streptomycin
GV3101	*A. tumefaciens*	stable plant transformation	Rifampicin, Gentamicin

2.4.6 Cloning vectors

Table 2.6 Cloning vectors

Vector	Source	Aim	Selection
pRT100-luc+	G Reuter lab, Institute of Genetics, MLU Halle-Wittenberg	cloning *RD29A-LUC*	Ampicillin
pGreenII0229	John-Innes-Centre, Norwich, UK	cloning *RD29A-LUC*	Kanamycin, BASTA

2.4.7 Cloning of the *RD29A-LUC* construct

Genomic DNA was extracted from 5-week-old *A. thaliana* (ecotype Col-0) shoots by using the DNeasy Plant Mini Kit. The genomic sequence of At5g52310 (*RD29A*) was obtained from TAIR (http://www.arabidopsis.org/). Primer pairs used for amplifying the appropriate promoter contained *Hind*III and *EcoR*I restriction sites, respectively (Tab. 2.3). The amplification was carried out by Phusion PCR (2.4.7.1).

The vectors pRT100-luc+ and pGreenII0229 were used for constructing the *RD29A-LUC*+ plasmid. pRT100-LUC+ was double-digested with *Pst*I and *Nco*I to isolate a fragment containing *LUC*+ and a 35S terminator (2.4.7.2) and purified by using the Wizard Gel and PCR Clean-Up System Kit. Afterwards the fragment was blunted with Klenow Fragment (2.4.7.3) and purified again. pGreenII0229 was digested with *Sma*I (2.4.7.4), purified and dephosphorylated with TSAP (Thermosensitive alkaline phosphatase) (2.4.7.5) to prevent self-ligation after digestion. Ligation of pGreenII0229 and LUC+-term35S with T4 DNA ligase was carried out at 16°C overnight (2.4.7.6). *E. coli* transformation (2.4.7.7) was performed by electroporation using an Elektroporator 2510

(Eppendorf, Hamburg, Germany) with a voltage of 2.5 KV. For recovery of the bacteria, SOC medium was added and bacteria were pre-cultured on a KS 4000ic shaker (IKA, Staufen, Germany) at 37°C and 200 rpm for 1 h. Afterwards cells were plated on selective LB plates containing 50 µg/ml kanamycin and incubated overnight at 37°C. Positive colonies were identified by colony-PCR using T7 and T3 primers (Tab. 2.3) and used to inoculate liquid TB medium (2.4.7.8). Plasmid DNA was extracted from those cultures by using the Promega Wizard Plus SV Miniprep Purification system according to the manufacturer's manual. The inserted sequence was confirmed by Sanger sequencing (Sanger *et al.*, 1977) (2.4.7.9) using the ABI PRISM BigDye Terminator V1.1 Cycle Sequencing kit (Platt *et al.*, 2007). After the sequencing PCR reaction, 1 µl 125 mM EDTA (pH 0.8), 1 µl 3 M Na-acetate, and 25 µl 100% ethanol were added, and the reaction was incubated at room temperature for 15 min to precipitate the DNA, followed by centrifugation at 4°C and 1400 rpm for 45 min (5415R, Eppendorf, Germany). Afterwards, the supernatant was removed and the remaining pellet was washed with 60 µl 70% ethanol, followed by an additional centrifugation for 15 min. After removing the supernatant, the final pellet was dried at 80°C for around 10 min. Electrophoresis and fluorescence detection were performed commercially. Results were evaluated by Sequence Scanner software (Life Technologies, Darmstadt, Germany).

The *RD29A* PCR product and the new pGreen-luc+-35S vector were double-digested with *Hind*III and *EcoR*I (2.4.7.10) and purified by using the Wizard Gel Clean up and Purification System Kit. The insert was ligated into the dephosphorylated vector. Following transformation of *E. coli,* selection on selective LB plates containing 50 µg/ml kanamycin, colony-PCR, inoculation of TB medium, and sequencing reaction were performed as described above. The final construct was named *RD29A-LUC*.

2.4.7.1 Amplification of *RD29A*

H_2O	30.5 µl
5x Phusion HF Buffer	10 µl
10 mM dNTPs	1 µl
10 µM forward primer	2.5 µl
10 µM reverse primer	2.5 µl
DNA	3 µl
Phusion hot start DNA polymerase	0.5 µl
Total	50 µl

98°C for 30 s
98°C for 10 s
60°C for 30 s } 33 cycles
72°C for 15 s
72°C for 5 min

2.4.7.2 *Pst*I- and *Nco*I-digestion of pRT100-LUC+

Vector	3 µg
10x NEB Buffer 4	3 µl
10x BSA	3 µl
H_2O	x µl
20 U *Pst*I	1 µl
20 U *Nco*I	1 µl
Total	30 µl

37°C for 5 h
80°C for 20 min

2.4.7.3 Blunting of LUC+-35S terminator with Klenow Fragment

Purified fragment	25 µl
10 mM dNTPs	1 µl
10x NEB Buffer 2	5 µl
H_2O	18.8 µl
Klenow Fragment	0.2 µl
Total	50 µl

25°C for 15 min
Addition of 1 µl EDTA (0.5 M)
75°C for 20 min

2.4.7.4 *Sma*I-digestion of pGreenII0229

Vector	2 µg
10x NEB Buffer 4	3 µl
H_2O	x µl
20 U *Sma*I	1 µl
Total	30 µl

25°C for 3 h

2.4.7.5 TSAP dephosphorylation

Vector	30 µl
10x Multi-core Buffer	3.3 µl
1.5 U TSAP	2 µl
Total	35.3 µl

37°C for 20 min
74°C for 20 min

2.4.7.6 Ligation of LUC+-35S terminator and pGreenII0229

10x ligase Buffer	1 µl
100 ng vector	y µl
x ng insert*	x µl
5 U T4 ligase	0.5 µl
Total	10 µl

*: ng insert = (100 ng vector x size insert fragment (bp) / size vector fragment (bp)) x 3

2.4.7.7 *E. coli* transformation

SOC medium

Tryptone	20 g
Yeast extract	5 g
1 M NaCl	10 ml
2.5 M KCl	2.5 ml
1 M Glucose	20 ml
2 M $MgSO_4$	10 ml
H_2O	ad 1l

LB agar medium

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar-Agar, Kobe I	20 g
H_2O	ad 1l

2.4.7.8 Identification of positive *E. coli* colonies

Colony PCR

H_2O	19 µl
10x Taq Buffer	2.5 µl
10 mM dNTPs	0.5 µl
10 µM forward primer	1 µl
10 µM reverse primer	1 µl
5 U/µl Taq polymerase	1 µl
Total	25 µl

94°C for 5 min
94°C for 30 s
55°C for 30 s } 40 cycles
72°C for 2.5 min
72°C for 5 min

TB medium

Tryptone	12 g
Yeast extract	24 g
Glycerin	4 ml
TB phosphates (2.31 g KH_2PO_4, 16.43 g K_2HPO_4)	100 ml
H_2O	ad 1l

2.4.7.9 BigDye sequencing reaction

H_2O	x µl
5x Sequencing Buffer	2 µl
Plasmid	200 ng
3.2 µM primer	2 µl
BigDye	0.5 µl
Total	10 µl

96°C hot start
96°C for 1 min
96°C for 10 s
50°C for 5 s
60°C for 1min 15 s
} 15 cycles
96°C for 10 s
50°C for 5 s
60°C for 1min 30 s
} 5 cycles
96°C for 10 s
50°C for 5 s
60°C for 2 min
} 5 cycles

2.4.7.10 Digestion of purified *RD29A* PCR product and pGreen-luc+-35S

<u>*EcoR*I- and *Hind*III-digestion of purified *RD29A* PCR product</u>

Phusion PCR product	30 µl
10x NEB Buffer 2	4 µl
H_2O	4 µl
10 U *EcoR*I	1 µl
10 U *Hind*III	1 µl
Total	40 µl

37°C for 5 min
65°C for 20 min

<u>*EcoR*I- and *Hind*III-digestion of purified pGreen-LUC+-35S</u>

Vector	2 µg
10x NEB Buffer 2	4 µl
H_2O	x µl
10 U *EcoR*I	1 µl
10 U *Hind*III	1 µl
Total	30 µl

37°C for 5 min
65°C for 20 min

2.4.8 *Agrobacterium*-mediated plant transformation and screening of transformants

Transformation of *Agrobacterium tumefaciens* (Tab. 2.5; Logemann *et al.*, 2006) was performed by electroporation as described above (2.4.7). Transformed

cells were plated on selective YEB plates containing 100 mg/l rifampicin, 25 mg/l gentamicin and 50 mg/l kanamicin. Plates were incubated in darkness at 28°C for 2 to 3 days. Positive colonies were identified by colony-PCR (2.4.7.8) and plated as a lawn onto selective YEB plates. After incubation in darkness at 28°C for 2 to 3 days, densely grown bacterial lawn was collected from the plate by scraping and resuspended in 30 ml liquid YEB. This bacterial suspension was added to 120 ml sucrose solution (5%) containing 0.03% Silwet L-77.

Arabidopsis plants were transformed according to the “floral dip” method (Clough and Bent, 1999). Col-0 plants were grown on a 2:1 (v/v) mixture of soil substrate (Einheitserde ED73; Einheitserde Werkverband, Germany) and vermiculite under long-day conditions (16 h day / 8 h night, 22°C / 18°C, 65 % rh) for 5 to 6 weeks until the first flowers appeared at stalks of approximately 10 cm in length. The inflorescences of the plants were dipped for approximately 30 s and afterwards covered with a lid. The lid was removed after two days and the plants were cultivated further as above.

The seedlings of transgenic plants (i.e. the T1 generation) were selected on soil by spraying with BASTA (Bayer Crop Science). After 7 to 10 days the seedlings were sprayed with a 200 mg/l BASTA solution and the spraying was repeated after three to seven days. Resistant seedlings were grown to maturity, and their seeds were harvested (i.e. the T2 generation).

YEB medium

Beef extract	5 g
Yeast extract	1 g
Peptone	5 g
Sucrose	5 g
$MgSO_4*7H_2O$	0.49 g
Agar	20 g
H_2O	ad 1l
Adjust pH to 7.2	

2.4.9 Plant growth in the 96-well system

Seeds were surface-sterilized once with 70% ethanol and twice with 100% ethanol. Dry seeds were transferred onto a white 96-well plate (Greiner Bio-One, Germany) of which the tips of the conical wells had been removed by

grinding, described in detail in the Results section. The wells contained ½ MS-Agar supplemented with 0.5% sucrose (according to Krysan, 2004). The plate was attached to a 96-deep-well plate (Greiner Bio-One, Germany) containing liquid medium of the same composition. The construction was covered with a cover plate and plants were stratified for 2 days at 4°C. Seedlings were grown for 6 days under long-day conditions (16 h light / 8 h dark, 120 µmol m^{-2} s^{-1}, 22°C /18°C) in a growth cabinet (AR-75, Percival Scientific, USA), unless stated otherwise.

2.4.10 Measurement and analysis of *luciferase* activation

Substances were dissolved in 100% DMSO and stored frozen at -20°C in aliquots as 1 mM stock solution. Substances diluted in nutrient solution were applied to the shoots. D-luciferin was dissolved in sterile water and stored frozen in aliquots as 50 mM stock solution. Working solution of 750 µM luciferin was applied like the substances. After luciferin application seedlings were kept in the dark for 2 min. Imaging was performed for 16 h using a photon-counting CCD camera (HRPCS4, Photek, St Leonards on Sea, UK). Quantification of the acquired data was performed using the corresponding IFS32 software.

2.4.11 Statistical analysis

Statistical significances were determined by using Student's *t*-test.

2.5 Results

2.5.1 Development of a plant-based method for identifying phytoeffectors

To identify potential phytoeffectors that activate transcriptional stress responses, the *RD29A* promoter (Yamaguchi-Shinozaki and Shinozaki, 1994) and the *firefly luciferase* (*LUC*) reporter gene (Millar *et al.*, 1992) were chosen. Fig. 2.1 shows the *RD29A* promoter sequence, which was used for generating an appropriate promoter-reporter construct.

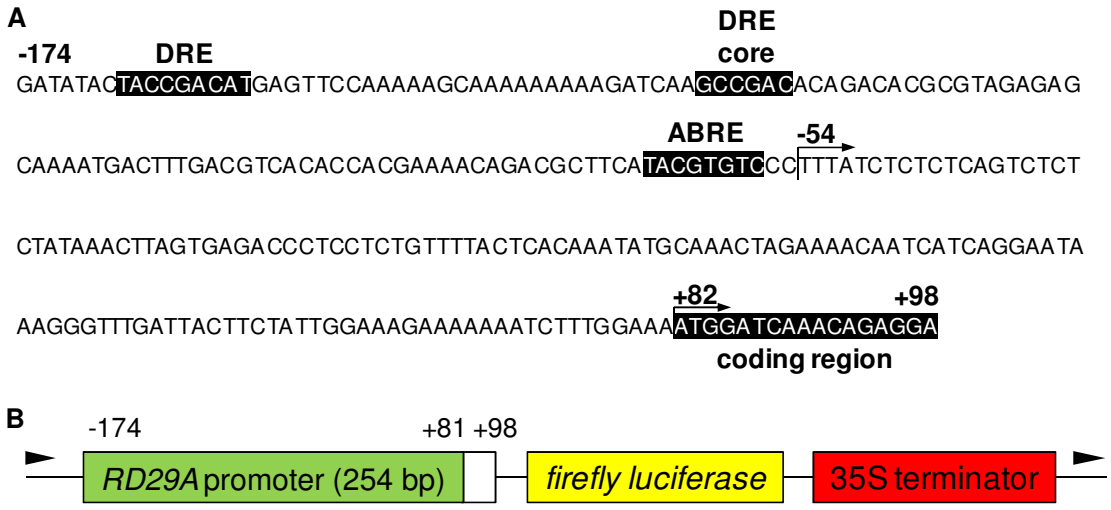

Figure 2.1 *RD29A* promoter sequence used for construction of transgenic Arabidopsis.

(A) Structure of the *RD29A* promoter including coding region (-174 to +98). **(B)** Diagrammatic representation of the *RD29A-LUC* construct used for transformation of Arabidopsis.

The used *RD29A* fragment comprises a sequence of 271 bp (Fig. 2.1A). This contains a 120 bp region including a DRE sequence (TACCGACAT), a DRE-core motif (GCCGAC) and an ABRE sequence (TACGTGTC) (-174 to -55), 134 bp of the minimal TATA sequence containing the TATA box and untranslated leader sequence (-54 to +81), and a 17 bp fragment of the coding region of *RD29A* (+82 to +98). The *RD29A* promoter sequence was fused upstream of the *LUC* reporter sequence followed by the 35S terminator from the Cauliflower Mosaic Virus (CaMV), which is responsible for terminating the transcription of the whole sequence (Fig. 2.1B). Finally, the whole *RD29A-LUC* construct was introduced in Arabidopsis.

To test a large number of substances, a method was established which was based on a system developed for high-throughput genotyping (Krysan, 2004). Fig. 2.2 shows this adapted experimental setup for the *luciferase* assay. The *RD29A-LUC* reporter plants were grown in a white 96-well plate (one plant per well) with holes in the bottoms of the wells, containing ½ MS-Agar supplemented with 0.5% sucrose (Fig. 2.2A). The plate was inserted in a 96-deep-well plate containing liquid medium of the same composition. The construction was covered with a cover plate to prevent dehydration of the agar.

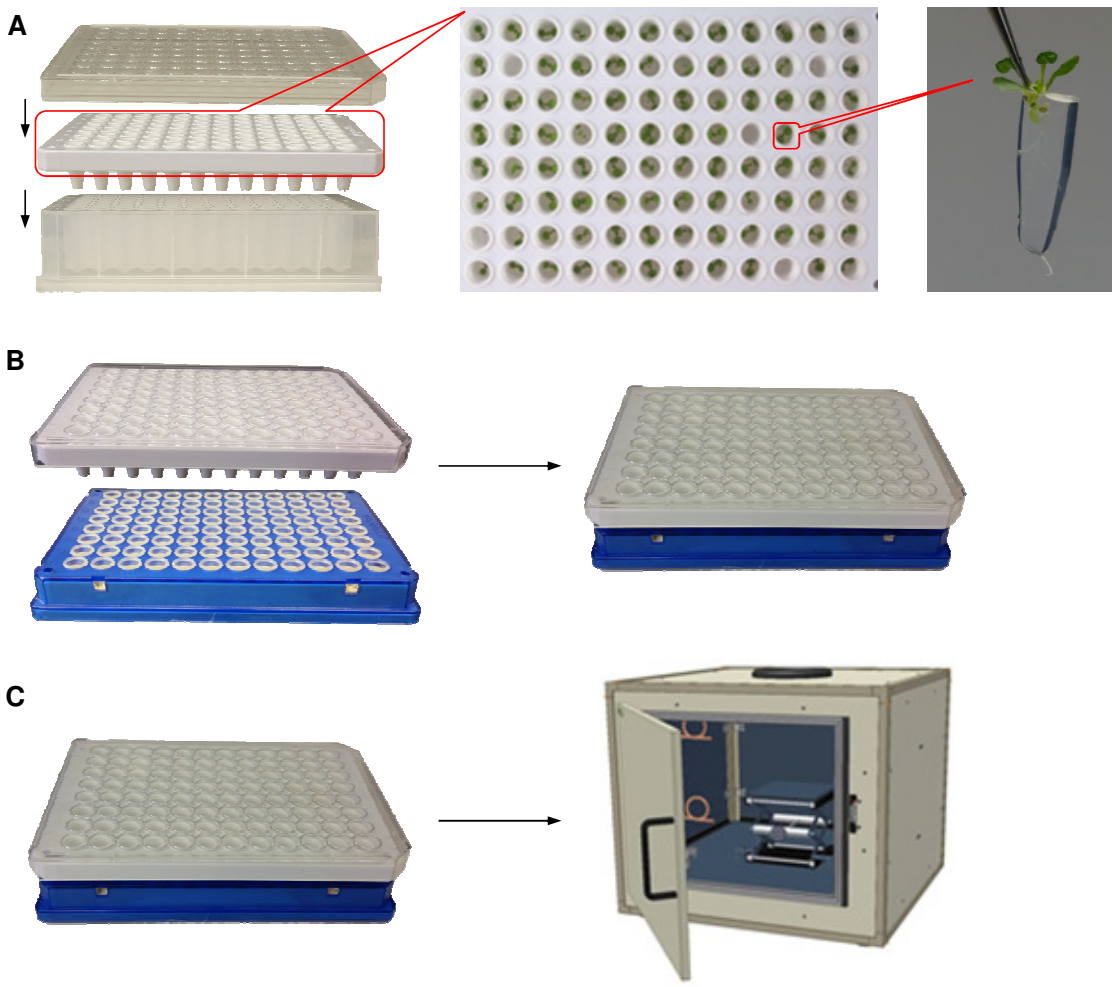

Figure 2.2 Experimental setup for the luciferase assay.

(A) Cultivation of the *RD29A-LUC* plants in the 96-well plate system. **(B)** For luminescence measurements the white 96-well plate including Arabidopsis plants was transferred onto an opaque 96-well plate. **(C)** Luminescence measurement was performed in a dark box with a photon-counting CCD camera mounted on top (not shown).

For the final luminescence measurements, the white 96-well plate containing 6-days-old reporter plants was transferred to an opaque 96-well plate (Fig. 2.2B). The luminescence measurement was performed in the dark box attached to a photon-counting CCD camera (Fig. 2.2C). The opaque 96-well plate was used because of signal cross talk between the wells that was observed during establishment of the luciferase assay. The approach to avoid this effect is shown in Fig. 2.3. Fig. 2.3A shows one of the initial experiments without signal cross talk prevention using a transparent 96-well base plate. Subsequently an opaque 96-well base plate was used for the treatments, which reduced the signal cross talk, albeit not completely (Fig. 2.3B). Therefore, a plaster cast was produced which could be fitted in the bottom of in the white 96-well plate containing reporter plants at the start of the luminescence measurement. The opaque 96-well plate together with the plaster cast satisfactorily prevented the signal cross talk (Fig. 2.3C).

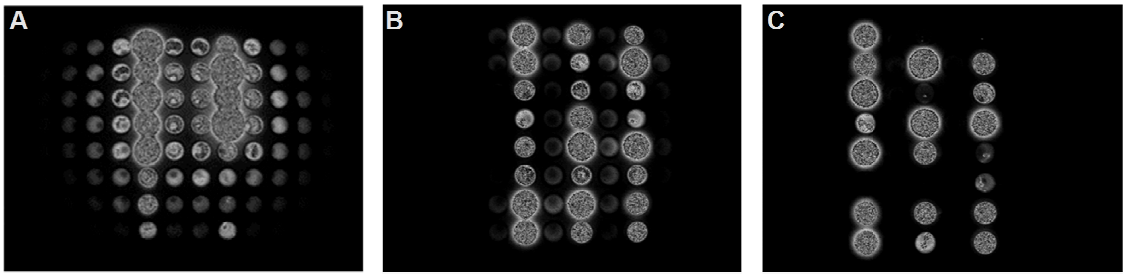

Figure 2.3 Prevention of signal cross talk in luciferase assay.

(A) Luminescence image using a transparent 96-well base plate. **(B)** Luminescence image using an opaque 96-well base plate. **(C)** Luminescence image with complete signal cross talk protection using an opaque 96-well base plate and an additional plaster cast in the bottom of the white 96-well plate.

After ensuring the prevention of signal cross talk, the general suitability of the transgenic *RD29A-LUC* plants was tested. To this end, the effect of ABA, NaCl, and PEG treatment on *LUC* expression was examined (Figs. 2.4, 2.5). Seedlings were treated with 100 µM ABA, and their luminescence was measured with the low-light CCD imaging system. As shown in Fig. 2.4D, luminescence was mainly emitted by the *RD29A-LUC* plants after ABA treatment. As expected, control plants without ABA treatment showed a much weaker luminescence.

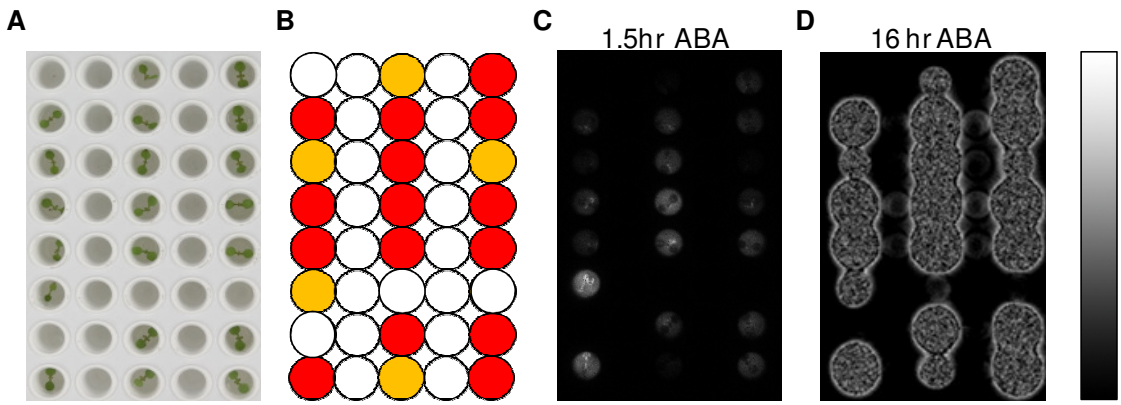

Figure 2.4 Luminescence of the *RD29A-LUC* plants in response to ABA.

(A) Photographic image of the plants. **(B)** Arrangement of the treatments in the plate. Red circles, 100 µM ABA; yellow circles, ethanol control. **(C)** Integrated luminescence image of the plants after 1.5 h of ABA treatment (100 µM). **(D)** Integrated luminescence image after 16 h of ABA treatment; the scale on the right indicates the luminescence intensity from black (lowest) to white (highest).

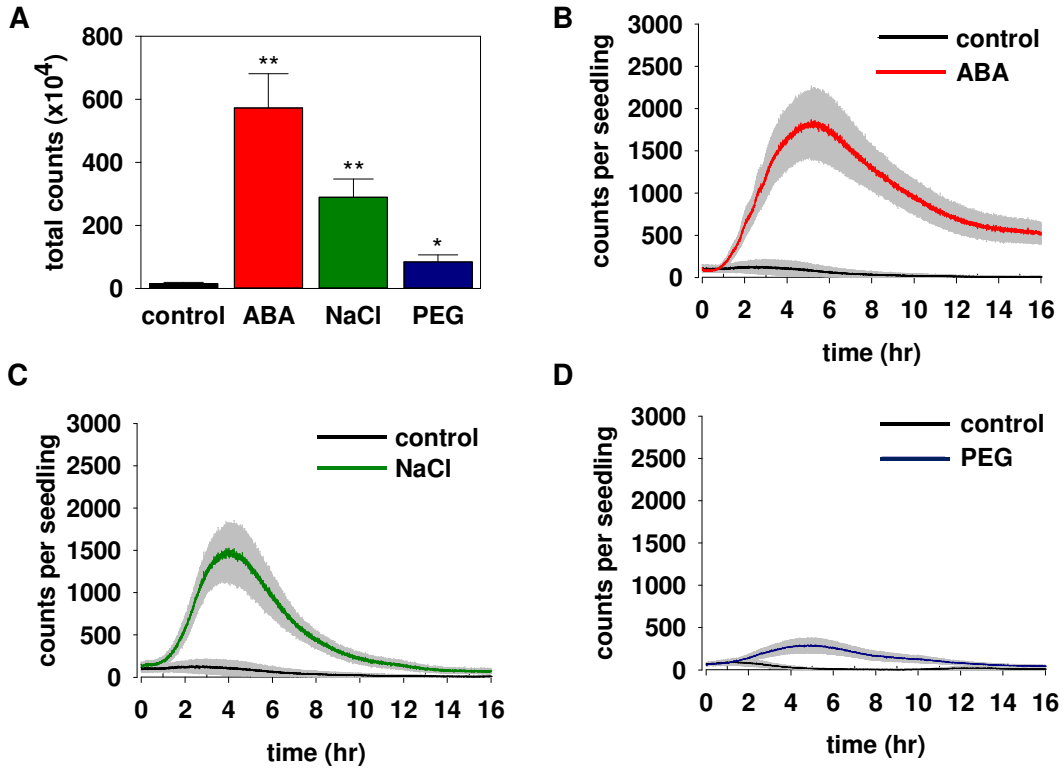

Figure 2.5 Regulation of *RD29A-LUC* activity in Arabidopsis plants by ABA, NaCl, and PEG.

(A) Total luminescence counts after treatment with ABA (1 µM), NaCl (300 mM), and PEG6000 (30%). Exposure time was 16 h. **(B)** Time course of ABA (1 µM) response. **(C)** Time course of NaCl (300 mM) response. **(D)** Time course of PEG (30%) response. Error bars represent SE of $n \geq 3$ seedlings. Significant differences to control were determined by Student's *t*-test (* $p<0.05$; ** $p<0.01$).

The typical response of *RD29A-LUC* plants to ABA, NaCl, and PEG is shown in Fig. 2.5. Both ABA and osmotic stresses, induced by NaCl or PEG, strongly induced bioluminescence in the reporter plants, whereby ABA evoked the highest and PEG the lowest response (Fig. 2.5A). Figs. 2.5B, 2.5C, and 2.5D show the time course of *RD29A-LUC* expression in response to 1 µM ABA, 300 mM NaCl or 30% PEG treatment, respectively. The response to all treatments was rapid; significant luminescence was detected after 2 h, and the expression reached peak levels after 4-5 h.

2.5.2 Analysis of potential phytoeffectors with the *luciferase* assay

After establishing an appropriate experimental setup for identifying potential phytoeffectors, first experiments to assess the suitability of this screening system were performed. Therefore, a set of 93 chemical substances with known structure provided by L. Wessjohann (IPB, Halle, Germany) was used to investigate their effect on *RD29A-LUC* expression in combination with stress treatments. On their own, none of the substances showed such an effect (data not shown). Because of intellectual property rights, the identity of the substances cannot be revealed here. In each experiment, substances were tested in one replication per plate. The experiment was repeated at least three times, and the means of the individual experiments was calculated. Fig. 2.6 shows the comparison of *RD29A-LUC* expression in response to salt stress, induced by NaCl alone (control) and in combination with each substance. The substances were sorted by descending mean values. Most of the substances were able to enhance the NaCl-induced *RD29A-LUC* expression. For example, substance 649 caused a 6-fold higher luminescence intensity, whereas substance 597 showed no difference compared to control treatment.

Fig. 2.7 shows the result of experiments to test the effect of the substances in combination with osmotic stress, induced by PEG, on *RD29A-LUC* expression. Similar to experiments performed with NaCl, over the half of the tested substances show an increased response in combination with PEG compared to the control. Some substances caused a similarly positive effect like in the NaCl experiment (e.g. 656 and 652), some of them showed a different response (e.g. 579, 611, and 640) and some of them showed no effect in both experiments (e.g. 642, 597 and 595).

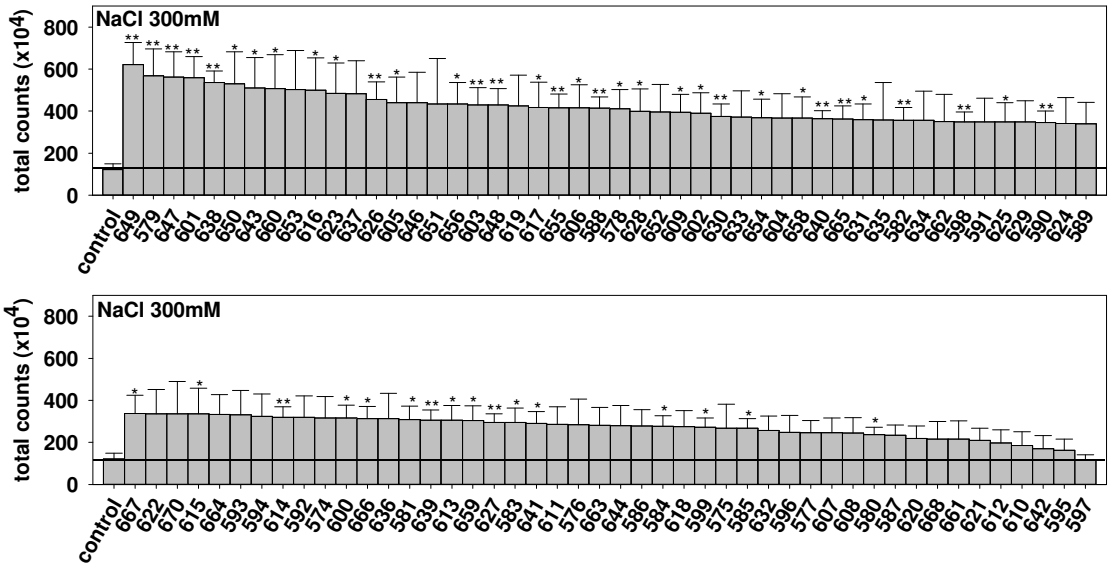

Figure 2.6 Effect of test substances on NaCl-induced *RD29A-LUC* activity in Arabidopsis plants.

Plants were treated with NaCl (300 mM) alone (control) and in combination with each of the test substances (10 µM). Exposure time was 16 h. Error bars represent SE of n ≥ 3 seedlings. Significant differences to control were determined by Student's *t*-test (* $p<0.05$; ** $p<0.01$).

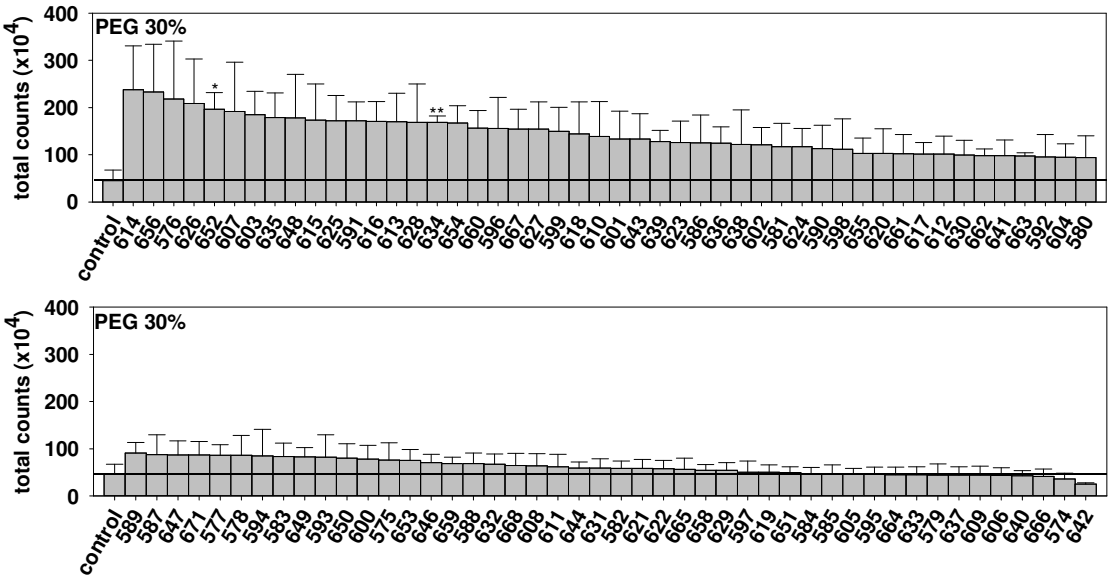

Figure 2.7 Effect of test substances on PEG-induced *RD29A-LUC* activity in Arabidopsis plants.

Plants were treated with PEG (30%) alone (control) and in combination with each of the test substances (10 µM). Exposure time was 16 h. Error bars represent SE of n ≥ 3 seedlings. Significant differences to control were determined by Student's *t*-test (* $p<0.05$; ** $p<0.01$).

The correlation of PEG- and NaCl-triggered *RD29A-LUC* activity is shown in Fig. 2.8. The data points represent the relative increase in luminescence intensity by the substances as compared to the controls. The data cloud was arbitrarily divided into four areas by using the median of luminescence intensity elevation in combination with both PEG- and NaCl-induced stress. The upper right area includes the substances, which had a positive impact on NaCl- as well as PEG-responsive *RD29A-LUC* activity. Based on this data these substances could be further analyzed regarding their potential to increase *RD29A* expression and may lead to an increased abiotic stress tolerance of plants.

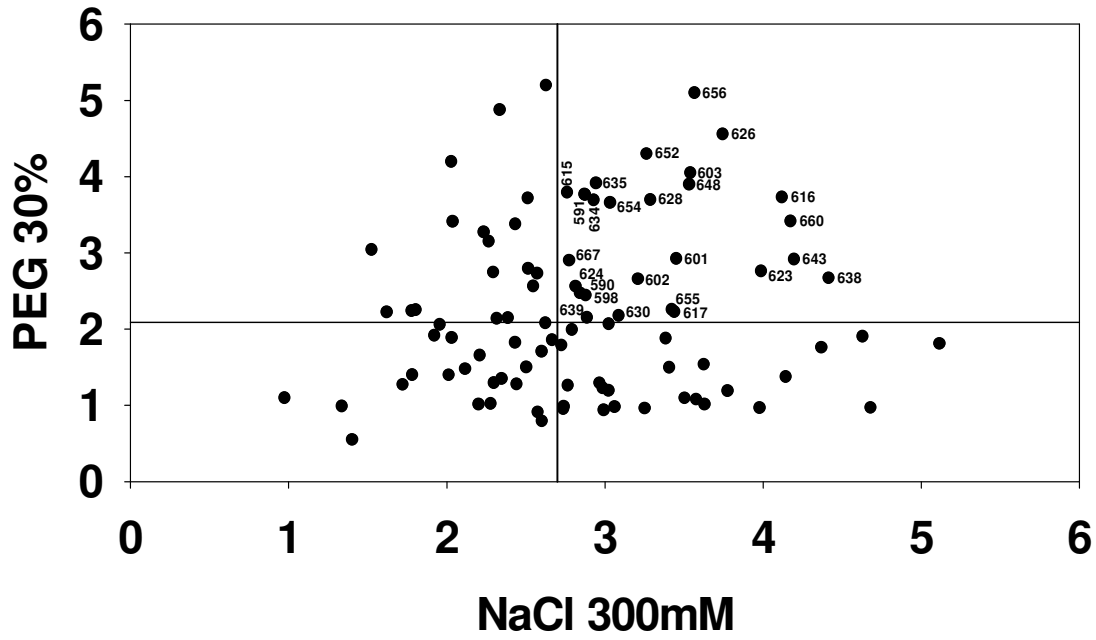

Figure 2.8 Correlation of the relative effect of substances on PEG- and NaCl-triggered *RD29A-LUC* activity in Arabidopsis.

Data was obtained from experiments shown in Figs. 2.6 and 2.7 by determining the relative luminescence intensity elevation by the substances. This was performed by dividing the total luminescence counts of each substance treatment to the control treatment.

2.6 Discussion

The aim of this part of the thesis was to develop an experimental system for the identification of potential phytoeffectors to enhance plant tolerance towards abiotic stress. For this purpose, the *firefly luciferase* (*LUC*) reporter gene (Millar *et al.*, 1992) under the control of the stress-inducible *RD29A* promoter (Yamaguchi-Shinozaki and Shinozaki, 1994) was chosen.

2.6.1 *RD29A-LUC* is a suitable reporter for the established screening assay

Many abiotic stress-inducible genes contain two kinds of *cis*-acting elements, DRE and ABRE, in their promoter region, which allow them to be transcriptionally activated via ABA-independent and ABA-dependent pathways (Narusaka *et al.*, 2003). Mishra *et al.* (2009) identified a set of 2,052 genes in Arabidopsis, whose promoter regions contain both ABRE and DRE motifs. ABA is an important regulator of plant development, growth, and stress responses. Most of the ABA-regulated genes are genes, which are involved in abiotic stress responses (Finkelstein *et al.*, 2002; Nakashima *et al.*, 2006). Therefore, the *RD29A* promoter was used as a marker for identifying possible phytoeffectors. The *RD29A* promoter is well-studied and commonly used as a marker for investigating plant stress tolerance mechanisms (Ishitani *et al.*, 1997; Kasuga *et al.*, 1999; Xiao *et al.*, 2006; Quist *et al.*, 2009). Because it contains both DRE and ABRE elements, it can be activated via the ABA-independent as well as the ABA-dependent pathway. This constellation facilitates the possibility to identify substances interfering with only one or both pathways.

The *RD29A* promoter fragment used in the present work contains one DRE element, one DRE-core motif, and one ABRE element (Fig. 2.1) and was chosen according to findings of Narusaka *et al.* (2003), who tested various deletions and base substitutions of the *RD29A* promoter using the *GUS* reporter gene in transgenic tobacco. The monomer form of the 120 bp region (-174 to -55) of the promoter showed the highest dehydration-induced *GUS* activity with a 26.7-fold increase. Deletions, base-substitutions and also the dimer form of the 120 bp region showed considerably reduced *GUS* activity (Narusaka *et al.*, 2003).

Luciferase was chosen as reporter gene for analyzing *RD29A* activity. Compared to other reporter systems, like *GUS*, its activity can be detected *in vivo* by bioluminescence using a sensitive CCD camera (Southern *et al.*, 2006). Additionally, because of its rapid turn-over in about 2 to 3 h, it is well-suited as a real-time reporter for gene expression studies (Alvarado *et al.*, 2004).

To realize a high-throughput screen for test substances, a plant growth system using 96-well plates was established by adapting a method described by Krysan (2004) (Fig. 2.2). In order to test the effect of different substances in parallel, it was necessary to grow the reporter plants separately so that the amount of *RD29A-LUC*-emitted luminescence could be determined for each individual (Krysan, 2004). In general, the handling of this growth system is comfortable and little time-consuming. It is possible to autoclave the white 96-well plate together with the 96-deep-well base plate to generate sterile growth conditions. The cover plate was sterilized by using 70% ethanol and 30 min incubation under UV light. In the present work, surface-sterilized seeds were loaded onto the agar medium by pipetting. This allowed the exact positioning of one seed in the middle of each well. Here, to save preparation time, a 96-well seed loading device may be used alternatively (Krysan, 2004). Growth of the plants in the 96-well plates proceeded homogeneously. For final luminescence measurements using a CCD camera, plants were transferred to an opaque 96-well plate (Fig. 2.3) and, additionally, a plaster cast needs to be inserted into the bottom of the white 96-well plate to satisfactorily prevent signal cross talk between the wells (Fig. 2.4). However, these plaster casts were very fragile. Here, it would be easier and time-saving to produce an opaque cast from autoclavable material that can be inserted when seeds are sown.

It is known that *RD29A* can be activated by exogenously applied ABA, salinity, and dehydration (Yamaguchi-Shinozaki and Shinozaki, 1993; Yamaguchi-Shinozaki and Shinozaki, 1994; Shinozaki *et al.*, 2003). In order to test the functionality of the *RD29A-LUC* plants, the effect of these treatments on *LUC* activity was examined. It could be shown that, compared to control treatment, both ABA and osmotic stress, induced by either NaCl or PEG, significantly enhanced the bioluminescence in the reporter plants (Fig. 2.5A). Here, 1 µM ABA led to the highest amount of luminescence, followed by 300 mM NaCl, and 30% PEG caused the lowest but also significant effect. Figures

2.5B-D show the time course of *RD29A-LUC* expression in response to 1 µM ABA, 300 mM NaCl or 30% PEG treatment, respectively. The response to all treatments was rapid; significant luminescence was detected 1-2 h after onset of the treatment. The luminescence peaked after 4-5 h of treatment. The patterns of these bioluminescence responses are similar to those reported by Ishitani *et al.* (1997). Furthermore, the endogenous *RD29A* gene shows a similar expression pattern, as previously determined by RNA gel blot analysis (Yamaguchi-Shinozaki and Shinozaki, 1993).

NaCl induced a 3.4-fold higher luminescence intensity compared to PEG, which was also observed by Ishitani *et al.* (1997). The osmolaties of 300 mM NaCl and 30% PEG are 660 and 730 mmol kg^{-1}, as determined by vapour pressure osmometry. The fact that NaCl produces a stronger signal than PEG is thus not due to a higher osmolality, but most likely due to an additive mode of action. While PEG only causes an osmotic stress, NaCl additionally causes an ionic stress (Schmöckel *et al.*, 2015).

In constrast, the difference between ABA- and NaCl-induced luminescence intensity is not in agreement with results obtained by other groups, where NaCl mostly shows a stronger effect than ABA (Ishitani *et al.*, 1997; Quist *et al.*, 2009; Xiong *et al.*, 1999). One explanation for this discrepancy may be the ABA enantiomer applied in the luciferase assays, but Quist *et al.* (2009) used the same +/- ABA product as in the present work. Ishitani *et al.* (1997) and Xiong *et al.* (1999) do not give information on the ABA entantiomer in their studies. A second explanation may be the ecotype of Arabidopsis. In the present work the Col-0 ecotype was used, whereby Ishitani *et al.* (1997), Quist *et al.* (2009), and Xiong *et al.* (1999) used C24, which is also known to respond differently to NaCl compared to Col-0 (Jha *et al.*, 2010; Schmöckel *et al.*, 2015). A third explanation may be the fragment length of the *RD29A* promoter. In this work, the *RD29A* promoter fragment was chosen according to Narusaka *et al.* (2003), whereas Ishitani *et al.* (1997) as well as Quist *et al.* (2009) and Xiong *et al.* (1999) used a longer fragment, which includes one more DRE and DRE-core element. Possibly, a higher number of DRE elements may also result in a stronger activity of the promoter in response to salt stress. However, it can be concluded that the used *RD29A-LUC* plants faithfully reflect regulation by stress

and ABA, and thus appeared to be reliable for screening potential phytoeffectors.

2.6.2 The *luciferase* assay is not applicable for high-throughput screening

To identify new potential phytoeffectors, a set of 93 chemical substances provided by L. Wessjohan (IPB, Halle, Germany) was used to investigate their effect on *RD29A-LUC* expression in combination with stress (Fig. 2.6, 2.7). Most of the substances tended to enhance the NaCl- as well as the PEG-induced *RD29A-LUC* expression. Although the identity of the substances cannot be revealed, all have been designed *in silico* against the same target, i.e. PARP proteins. It is therefore not surprising that many of the substances show a similar effect. However, the observed effect was significant for only a few substances in combination with NaCl and for even less substances in combination with PEG. It is apparent that the standard errors are quite high in both experiments. For example, in combination with NaCl the substance 653 induced a 4.2-fold higher luminescence intensity compared to control treatment, but due to the high standard error no significant effect could be determined. In combination with PEG, the variations were even more severe. These high standard errors are probably a result of the experimental setup. Theoretically, the 96-well plate growth system allows to test the time-resolved effect of 96 different substances on *RD29A* promoter activity. However, no biological repetitions within the plate are possible in this set-up. Due to the long exposure time, only one plate can be analysed in one experiment. Therefore, the experiments were repeated multiple times using individual 96-well plates. The high standard errors reflect a high experiment-to-experiment variation. In comparison, variations between biological repetitions within plates were smaller, as obvious in the values of the experiment shown in Figure 2.5A. Interestingly, the control treatment in both experiments (Fig. 2.6 and 2.7) did not show such high standard errors between plates. Therefore the variability is apparently caused by the test substances. To examine this assumption, the most promising substances, which are indicated in Fig. 2.8, should be tested again experiments should be tested again with several repetitions per plate. Generally, due to the experiment-to-experiment variation, the *luciferase* assay

seems to be only applicable as a small-scale screening procedure, but not as a high-throughput method.

2.7 Conclusions

In their natural habitats, plants are frequently exposed to abiotic stressors, such as drought and salinity, which lead to an increasing insecurity of yield stability. Hence, there is an extensive body of research for appropriate ways to promote plant performance under stress. One option, which has received only little attention so far, is the use of phytoeffectors.

This part of the present work was focused on the identification of phytoeffectors able to enhance plant tolerance against abiotic stress. For this purpose, a plant-based system was established by using the light-emitting *firefly luciferase* (*LUC*) reporter gene driven by the stress-inducible *RD29A* promoter.

Based on the luminescence in response to ABA, NaCl and the osmotic stressor PEG, it can be concluded that the *RD29A-LUC* construct is suitable for the established screening assay. Furthermore, the developed plant growth system was generally well-practicable. However, the aim of screening substances in high-throughput could not be satisfactorily achieved because of the high variations between experimental repetitions. As a consequence of this, the luciferase assay seems to be only applicable as a small-scale screening procedure.

The tested chemical compounds were *in silico*-designed to target proteins that have been associated before with abiotic stress tolerance, poly(ADP-ribose) polymerases (PARPs). For this reason, the second part of this thesis focuses on the investigation of the role of this protein family in abiotic stress responses of Arabidopsis, employing some of the methodology developed in the first part.

3 The role of Arabidopsis Poly(ADP-Ribose) Polymerases (AtPARPs) during abiotic stress

Abiotic stress induces a variety of physiological adaptation mechanisms in the plant. It is necessary to search for regulatory and functional genes that help the plant to overcome such stress situations. A protein family known as poly(ADP-ribose) polymerases (PARP) appeared to be able to meet this criteria, since the impairment of PARP activity by pharmacological or genetic approaches showed a potential to improve plant stress responses (De Block *et al.*, 2005; Vanderauwera *et al.*, 2007; Geissler and Wessjohann, 2011; Schulz *et al.*, 2012), which, however, has been debated more recently (Rissel, *et al.*, 2017b; Rissel and Peiter, 2019). The substances shown in Part 1 to promote stress-induced *RD29A* activity had been *in silico*-designed to inhibit PARP activity, thus supporting a role of PARPs during abiotic stress. This part of the thesis aimed to contribute to a better understanding of this involvement.

3.1 Poly(ADP-ribosyl)ation in humans

Poly(ADP-ribosyl)ation is a post-translational modification of proteins, in which, comparable to phosphorylation, acetylation, or ubiquitination, the function of the target protein is reversibly changed. In case of poly(ADP-ribosyl)ation, mediated by PARPs, multiple ADP-ribose subunits derived from NAD^+ are attached to target amino acid residues (Glu, Asp or Lys) of acceptor proteins, accompanied by the formation of nicotinamide. This process was first discovered in the 1960s (Lamb *et al.*, 2012; Feng *et al.*, 2015).

All eukaryotes except yeast possess PARP proteins, which are characterized by a C-terminal catalytic PARP domain (Adams-Phillips *et al.*, 2010). In humans, 17 PARPs and PARP-like proteins exist, but not all of them have PARP activity. HsPARP1, HsPARP2, and HsPARP3 have been described as DNA-dependent PARPs, which are activated upon DNA damage. The best-described member is HsPARP1, which is also the founding member of the family (Amé *et al.*, 2004; Pines *et al.*, 2013). HsPARP1 accounts for more than 90% of the catalytic PARP activity in the cell nucleus (Henning *et al.*, 2018). In response to DNA strand breaks, HsPARP1 binds to the DNA and modifies

nuclear acceptor proteins, including itself, by formation of a bond between the protein and the ADP-ribose residue using NAD^+ as a substrate (Amé *et al.*, 2004; Henning *et al.*, 2018). HsPARP2, which accounts for approximately 15% of the catalytic PARP activity, was identified as a result of the presence of residual poly(ADP-ribose) formation in mice cells lacking HsPARP1 (Amé *et al.*, 1999; Amé *et al.*, 2004). Its catalytic domain shares 69% similarity to that of PARP1 (Amé *et al.*, 1999). Together, HsPARP1 and HsPARP2 are the most important PARPs in mammalian cells, which is highlighted by the embryonic lethality of *parp1:parp2* double mutant mice (de Murcia *et al.*, 2003). Only little information is available on HsPARP3, but similar to its fellow members HsPARP1 and HsPARP2, it is also activated upon DNA damage (Gibson and Kraus, 2012).

Poly(ADP-ribosyl)ation is reversible. The modification can be reversed by hydrolyzing the ADP-ribose polymers via poly(ADP-ribose) glycohydrolases (PARGs). The function and role PARGs have not been elucidated as detailed as that of PARPs. However, it has been reported that PARG function is crucial, as deletion of the corresponding gene in mice or *Drosophila* leads to lethality (Hanai *et al.*, 2004; Koh *et al.*, 2004). By now, only one gene encodes a PARG in mammals, although alternative splicing can produce multiple isoforms (Meyer-Ficca *et al.*, 2004; Meyer *et al.*, 2007).

3.2 Poly(ADP-ribosyl)ation in Arabidopsis

3.2.1 PARPs in Arabidopsis

In Arabidopsis three *PARP* genes are present: *AtPARP1*, *AtPARP2* and *AtPARP3* (Lepiniec *et al.*, 1995; Babiychuk *et al.*, 1998; Hunt *et al.*, 2004). The nomenclature of AtPARPs has been inconsistent in the past. In this study, the loci corresponding to *AtPARP1*, *AtPARP2* and *AtPARP3* are At2g31320, At4g02390, and At5g22470, respectively (see also Tab. S1 in Rissel *et al.*, 2017b).

AtPARP1 is the biggest Arabidopsis PARP protein with a size of 114 kDa (Hunt *et al.*, 2004) and shows the highest similarities in protein structure with HsPARP1, containing a conserved catalytic domain, zinc finger motifs, and a nuclear localization motif (Doucet-Chabeaud *et al.*, 2001; see also Fig. S6 in

Rissel *et al.*, 2017b). It was found to localize primarily to the nucleus (Song *et al.*, 2015; Pham *et al.*, 2015), but also to chloroplasts and mitochondria (Pham *et al.*, 2015). Expression of *AtPARP1* was detected in roots, apices of inflorescences, the vegetative meristem, and during late stages of embryo development (Pham *et al.*, 2015).

The second member, *AtPARP2*, was the first *PARP* gene identified in plants (Lepiniec *et al.*, 1995). The cDNA of this 72 kDa protein shows 62% similarity to the catalytic domain of HsPARP1. Furthermore, a nuclear localization signal was found, but in contrast to the zinc finger domain in HsPARP1, AtPARP2 possesses a SAP domain (Lepiniec *et al.*, 1995). Like AtPARP1, cellular localization of AtPARP2 was confirmed for the nucleus (Song *et al.*, 2015; Pham *et al.*, 2015) and chloroplasts (Pham *et al.*, 2015). Expression analysis of *AtPARP2* revealed gene activity in imbibed seeds, roots, the vegetative meristem of the shoot apex, stamen of open flowers, and late stages of embryo development (Pham *et al.*, 2015).

To date, only little information is available for *AtPARP3* (encoding a 91 kDa protein; Hunt *et al.*, 2004). It was found to localize to the nucleus as well as the cytosol and its expression could be detected mainly in seeds, but also in seedlings and roots of adult plants (Hunt *et al.*, 2004; Rissel *et al.*, 2014; Feng *et al.*, 2015; Pham *et al.*, 2015).

Similar to the HsPARPs, Arabidopsis PARP proteins have been described to be involved in DNA damage responses upon oxidative stress, deciding between DNA repair or programmed cell death (Amor *et al.*, 1998). For example, AtPARP1 and AtPARP2 show higher activity upon ionizing radiation (Doucet-Chabeaud *et al.*, 2001) and, as a consequence of this, *parp1:parp2* double knockout mutants are more sensitive to DNA-damaging agents (Jia *et al.*, 2013; Boltz *et al.*, 2014).

3.2.2 PARGs in Arabidopsis

In Arabidopsis two tandemly-arrayed genes encode putative PARGs, *AtPARG1* (At2g31870) and *AtPARG2* (At2g31865). They arose from gene duplication. A third gene, At2g31860, is classified as a pseudogene, because no ESTs or cDNA have been found (Hunt *et al.*, 2004).

Both PARG proteins localize to the nucleus, but possibly also to the plasma membrane and the cytoplasm (Feng *et al.*, 2015). In contrast to AtPARPs, much less is known about the functional role of AtPARGs. However, it has been shown that PARG1 (also known as TEJ) plays a role in regulating circadian rhythms in Arabidopsis (Panda *et al.*, 2002). By now, poly(ADP-ribose) glycohydrolase activity could be validated only for AtPARG1 *in vitro* and *in vivo*, as it was able to remove poly(ADP-ribose) from auto-PARylated AtPARP2 (Feng *et al.*, 2015). In the same study, no poly(ADP-ribose) glycohydrolase activity of AtPARG2 towards auto-PARylated AtPARP1 as well as AtPARP2 could be detected. Similar to AtPARPs, AtPARGs have been linked to DNA repair mechanisms. It has been found that *parg1* mutant plants are hypersensitive to the DNA damaging agents mitomycin C, whereas *parg2* plants showed no or only weak sensitivity to DNA-damaging agents (Adams-Phillips *et al.*, 2010).

3.2.3 Poly(ADP-ribosyl)ation in the response of Arabidopsis to biotic and abiotic stress

In addition to their role in DNA repair, AtPARPs have an impact on plant responses to external stressors, biotic as well as abiotic (Lamb *et al.*, 2012). The bacterial elicitors flg22 and elf18 induce plant defense responses, such as callose or lignin deposition, by triggering cellular signaling networks (Felix *et al.*, 1999; Kunze *et al.*, 2004). In Arabidopsis these biotic stress-induced responses were abolished by the PARP inhibitor 3-AB (Adams-Phillips *et al.*, 2008; Adams-Phillips *et al.*, 2010). Furthermore, Feng *et al.* (2015) showed that *parp1:parp2* double mutants were slightly more susceptible to *Pseudomonas syringae* bacteria. However, in a recent study, a *parp* triple mutant was not affected in flg22-triggered callose deposition, which puts the involvement of PARPs in this response into question (Rissel *et al.*, 2017b).

AtPARPs have also been proposed to be involved in abiotic stress responses (De Block *et al.*, 2005; Vanderauwera *et al.*, 2007; Geissler and Wessjohann, 2011; Schulz *et al.*, 2012). For example, silencing of Arabidopsis *PARP1* and *PARP2* by RNAi knockdown led to an improved performance of the plants under drought stress (De Block *et al.*, 2005). This phenomenon was explained by a reduced stress-induced poly(ADP-ribosyl)ation and NAD^+ consumption,

preventing ATP depletion, leading to prevention of reactive oxygen species accumulation, and thereby increasing stress tolerance. A second hypothesis for the positive effect of a reduced PARP activity during stress is based on potentially increased levels of cyclic ADP-ribose (cADPR) (Vanderauwera *et al.*, 2007). Cyclic ADP-ribose is a signaling molecule known to increase the concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) in both plants and animals (Hetherington and Brownlee, 2004; Zhang and Li, 2006). The enzyme which is responsible for synthesizing cADPR from NAD^+ is ADP-ribosyl cyclase, although no proteins with homology to the known ribosyl cyclases from *Aplysia californica* or the mammalian CD38 and CD157 proteins have been identified in plants (Hunt *et al.*, 2004). However, it is assumed that *parp*-deficient plants consume less NAD^+, which may facilitate an increased synthesis of cADPR. Through Ca^{2+} signals, this increased amount of cADPR may increase the production of ABA-regulated stress response proteins, finally resulting in increased stress tolerance of the PARP-deficient plants (Vanderauwera *et al.*, 2007). However, based on phenotypical analyses of single, double, and triple *PARP* mutants, a general involvement of PARPs in abiotic stress tolerance has recently been questioned (Rissel *et al.*, 2017b).

AtPARGs were also found to be involved in plant stress signaling. *Parg1* and *parg2* mutant plants displayed an accelerated onset of symptoms upon *B. cinerea* infection, and *parg1* but not *parg2* plants showed a stronger growth inhibition in response to elf18 treatment (Adams-Phillips *et al.*, 2010). Furthermore, knocking out *PARG1* resulted in an enhanced sensitivity of the plants to drought, osmotic and oxidative stress. This behavior supports the role of PARGs as PARP antagonists, although an overexpression of *PARG1* did not lead to a different response from wild type plants to the stress treatments (Li *et al.*, 2011).

In summary, Arabidopsis PARPs and PARGs are likely to be involved in diverse biotic and abiotic stress responses, albeit this involvement is likely of a conditional nature. In this context, PARPs seem to have negative and positive effects on abiotic and biotic stress responses, respectively, whereas PARGs may act as positive regulators in both types of responses.

3.2.4 PARP-like proteins

Besides the three canonical AtPARP proteins, several other PARP-like proteins exist in Arabidopsis. The SRO (Similar to RCD One) protein family, which includes SRO1 through SRO5 and its founding member RCD1 (Radical-induced Cell Death 1) also contain a PARP catalytic domain, but seem to lack poly(ADP-ribosyl)ation activity (Jaspers *et al.*, 2010). RCD1 is involved in a wide range of stress-related and developmental processes (Katiyar-Agarwal *et al.*, 2006; Teotia and Lamb, 2009; Teotia *et al.*, 2010; Teotia and Lamb, 2011; Vainonen *et al.*, 2012). Mutation of *RCD1* resulted in the differential regulation of more than 500 genes, leading to highly pleiotropic phenotypes (Jaspers *et al.*, 2009). The *rcd1* mutant shows an increased sensitivity to apoplastic ROS (Overmyer *et al.*, 2000), but is more tolerant to chloroplastic ROS and UV-B irradiation (Ahlfors *et al.*, 2004; Fujibe *et al.*, 2004). Furthermore, *rcd1* is more tolerant to freezing (Fujibe *et al.*, 2004), but more sensitive to salt stress, which is possibly due to its interaction with SOS1, a plasma membrane Na^+/H^+ antiporter (Katiyar-Agarwal *et al.*, 2006). It also shows altered responses to hormones, such as ABA, jasmonate, salicylic acid, and ethylene (Ahlfors *et al.*, 2004; Overmyer *et al.*, 2005). Besides SOS1, RCD1 also interacts with numerous other proteins, mainly transcription factors, such as DREB2A (Jaspers *et al.*, 2009; Vainonen *et al.*, 2012). Finally, RCD1 together with its closest homolog SRO1 play redundant functions in plant development as well as in stress responses (Jaspers *et al.*, 2009; Teotia and Lamb, 2009), but SRO1 is somewhat different to RCD1, since *sro1* mutants showed resistance to both apoplastic ROS and salt stress (Teotia and Lamb, 2009).

3.3 Effects of PARP inhibitors in plants

As discussed in part 1, chemical genetics is a useful approach to overcome genetic redundancy (Cutler and McCourt, 2005; McCourt and Desveaux, 2010; Tóth and Van der Hoorn, 2010). As the PARP family is suspected to be functionally redundant, the use of pharmacological PARP inhibitors is a prevalent approach to cope with such potential redundancy, since they target the conserved enzymatically active site (De Block *et al.*, 2005; Adams-Phillips *et al.*, 2010; Briggs and Bent, 2011; Geissler and Wessjohann, 2011; Schulz *et al.*, 2012; Schulz *et al.*, 2014). However, inhibitors may lead to off-target effects that

may complicate conclusions from such studies (Rissel and Peiter, 2019). In the present study, the PARP inhibitors 3-aminobenzamide (3-AB), nicotinamide, isonicotinamide and PJ-34 were used.

The inhibitors 3-AB and nicotinamide are not only well established inhibitors of animal PARPs, they also have been demonstrated to inhibit plant PARPs (Amor *et al.*, 1998; Adams-Phillips *et al.*, 2008; Ishikawa *et al.*, 2009; Rissel *et al.*, 2017a). While nicotinamide is an inhibiting end product of the poly(ADP-ribosyl)ation reaction, the benzamide structure of the competitive inhibitor 3-AB mimics the nicotinamide moiety of NAD^+ leading to interference with the NAD^+-consuming PARP enzyme. 3-AB was shown to protect soybean and tobacco suspension cells from programmed cell death induced by oxidative stress or heat shock (Amor *et al.*, 1998; Tian *et al.*, 2000; Briggs and Bent, 2011). As a natural product of the poly(ADP-ribosyl)ation process, nicotinamide is less specific and thereby expected to interfere with any enzyme, which either has ADP-ribosylation activity or which is able to produce nicotinamide (Lamb *et al.*, 2012). Isonicotinamide is a structural analog of nicotinamide, hence it is thought to work in a similar way. It harbours a pyridine structural motif, a marker of many systemic acquired resistance (SAR) inducers (Pétriacq *et al.*, 2013) and was demonstrated to increase the defense-related phenylalanine ammonia-lyase (PAL) activity in tobacco cells (Louw and Dubery, 2000). The hydrochloride salt PJ-34 (*N*-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-*N*,*N*-dimethylacetamide) is a highly potent and specific clinical PARP inhibitor that is mainly used in cancer and stroke treatment and research (Virag and Szabo, 2002; Huang *et al.*, 2008), but its efficiency as a PARP inhibitor in plants has been less examined so far.

Preliminary results of the Plant Nutrition Laboratory (D. Rissel, MLU Halle-Wittenberg) showed that in hydroponically-grown Arabidopsis, the PARP inhibitors 3-AB and PJ-34 led to a better performance of the plants under PEG-induced osmotic stress (Fig. 3.1). Under unstressed conditions the PARP inhibitors tendentially reduced the shoot growth, whereas upon osmotic stress the inhibitor-treated plants accumulated a tendentially higher shoot dry weight (3-AB: $P = 0.07$; PJ-34: $P = 0.11$).

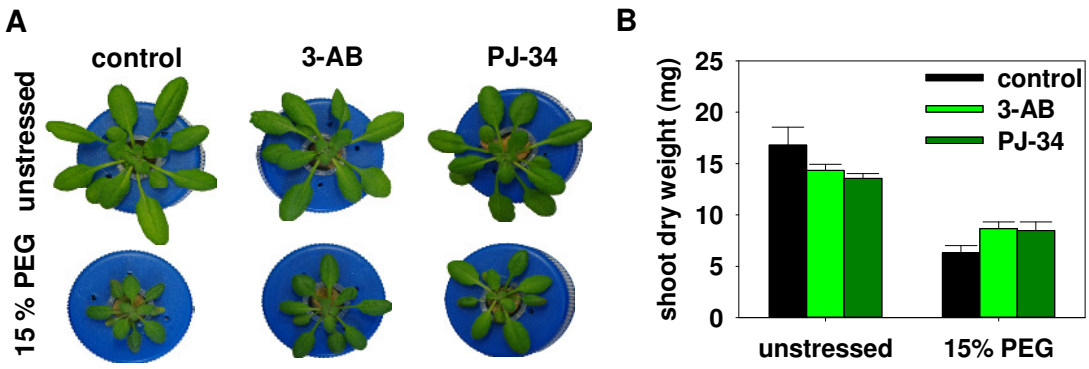

Figure 3.1 Effect of PARP inhibitors on growth of Arabidopsis under PEG-induced osmotic stress (Rissel, unpublished).

10 µM 3-AB or PJ-34 were included in the nutrient solution. **(A**) Representative pictures of 4-week-old plants grown in 50 ml nutrient solution (Arteca and Arteca, 2000) with or without 15% PEG6000. **(B)** Shoot dry weight of 4-week-old plants. Each bar represents the average from n=3 plants. Error bars represent SE.

3.4 Aims

In the first part of this thesis, chemical substances with undisclosed identity were analyzed for their ability to enhance luciferase activity under control of the stress-inducible promoter of the *RD29A* gene. Those substances had been *in silico*-designed to inhibit PARP activity. Therefore, the aim of the second part of this thesis was to investigate the role of PARPs in abiotic stress responses, making use of established PARP inhibitors, the luciferase assay, as well as of mutant plants.

This included:

- genotypical analysis of *PARP*-deficient T-DNA insertion lines
- generation of (1) *parp* double and triple knockout lines and (2) *parp* knockout lines containing the *RD29A-LUC* construct by crossing
- analysis of luciferase activation upon PARP inhibitor application and in *parp:RD29A-LUC* plants
- analysis of the effect of genetic PARP inhibition on abiotic stress tolerance and cADPR levels
- analysis of the effect of pharmacological and genetic PARP inhibition on $[Ca^{2+}]_{cyt}$ responses

3.5 Material and Methods

3.5.1 Chemicals

Table 3.1 Chemicals used in this part

Substance	Chemical formula	Company	Cat. No.
Abscisic acid	$C_{15}H_{20}O_4$	Duchefa	A0941
3-Aminobenzamide	$H_2NC_6H_4CONH_2$	SKW	-
Ammonium sulfate	$(NH_4)_2SO_4$	Fluka	09980
Boric acid	H_3BO_3	Roth	6943.1
Calcium chloride	$CaCl_2*2H_2O$	Sigma	31307
Cobalt chloride	$CoCl_2*6H_2O$	RdH	12914
Coelenterazine	$C_{26}H_{21}N_3O_3$	Roth	4094.3
Cupric sulfate	$CuSO_4*5H_2O$	RdH	31293
Dimethyl sulfoxide	C_2H_6OS	Duchefa	D1370
EDTA disodium salt dihydrate	$C_{10}H_{14}N_2NaO_3*H_2O$	Fluka	03680
Flavin mononucleotide sodium	$C_{17}H_{20}N_4NaO_9P*xH_2O$	Sigma	F2253
Iron sodium EDTA	FeNaEDTA	Duchefa	E0509
Isonicotinamide	$C_6H_6N_2O$	SKW	-
Isopropanol	C_3H_8O	Roth	T910.1
D-Luciferin sodium salt	$C_{11}H_7N_2O_3S_2Na*H2O$	Roth	4096.2
Magnesium chloride	$MgCl_2$	Merck	5833
Magnesium sulfate	$MgSO_4*7H_2O$	Sigma	63140
Manganese sulfate	$MnSO_4*H_2O$	FLuka	M7634
D-Mannitol	$C_6H_{14}O_6$	Duchefa	M0803
MS salts+vitamins	-	Duchefa	M0231
Nicotinamide	$C_6H_6N_2O$	Sigma	72340
Perchloric acid	$HClO_4$	Sigma	30755
Phyto-Agar	-	Duchefa	P1003
PJ-34 hydrochloride hydrate	$C_{17}H_{17}N_3O_2*HCl*xH_2O$	Sigma	P4365
Polyethylene glycol 6000	$(HSCH_2CH_2COOCH_2)_4C$	Roth	0158.1
Potassium dihydrogen phosphate	KH_2PO_4	Fluka	60220
Potassium nitrate	KNO_3	Sigma	P8291
Resazurin	$C_{12}H_6NNaO_4$	Sigma	R7017
Sodium chloride	NaCl	Roth	3957.1
Sodium dodecyl sulfate	$C_{12}H_{25}NaO_4S$	Roth	CN30.2
Sodium molybdate	$NaMoO_4$	Roth	0274.1
D(+)-Sucrose	$C_{12}H_{22}O_{11}$	Roth	4621.1

Substance	Chemical formula	Company	Cat. No.
1,1,2-Trichloro-trifluoroethane	$ClCF_2CCl_2F$	Fluka	91441
Tri-*n*-octyl amine	$[CH_3(CH_2)_7]_3N$	Sigma	T81000
Tris	$C_4H_{11}NO_3$	Roth	AE15.2
Zinc sulfate	$ZnSO_4*7H_2O$	RdH	31665

3.5.2 Enzymes

Table 3.2 Enzymes used in this part

Enzyme	Source	Cat. No.
ADP-ribosyl cyclase	Sigma	A9106
Alcohol dehydrogenase	Sigma	A7011
Alkaline phosphatase	Sigma	P4978
Diaphorase	Sigma	D5540
M-MLV reverse transcriptase	Promega	M1701
NADase	Sigma	N9879
Nucleotide Pyrophosphatase	Sigma	P7383
RNase A	Sigma	

3.5.3 Oligonucleotides

All oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

Table 3.3 Oligonucleotides used in this part

Purpose	Name	Gene	Sequence (5´→3´)
Genotyping PCR	GABI_pAC161-8409	GABI T-DNA	ATATTGACCATCATACTCATTGC
	SALK_LBa1	SALK T-DNA	TGGTTCACGTAGTGGGCCATCG
	PARP3.1_for	PARP3	AAAGCCTGAAACGATGACGG
	PARP3.1_rev	PARP3	AAGGCACAGTTATACAAGAGTCCAT
	PARP1.7_for	PARP1	TTGAGGCATTGACGGAGATAC
	PARP1.7_rev	PARP1	TTTCTCCCAATGCAACTTCAC
	PARP2.5_for	PARP2	AGAACACTCATGCAAAGACGC
	PARP2.5_rev	PARP2	ACGCATCTTGATTTGTTCCAC
qRT-PCR	RD29A qRT_fw	RD29A	GTTACTGATCCCACCAAAGAAGA
	RD29A qRT_rv	RD29A	GGAGACTCATCAGTCACTTCCA

3.5.4 Kits

Table 3.4 Kits used for qRT-PCR

Kit	Source	Cat. No.
Spectrum Plant Total RNA Kit	Sigma	STRN50-1KT

3.5.5 T-DNA insertion lines

T-DNA insertion lines were obtained from the Nottingham Arabidopsis Stock Center (NASC; Alonso *et al.*, 2003; Ülker *et al.*, 2008).

Table 3.5 Arabidopsis T-DNA insertion lines

Gene	AGI code	T-DNA insertion line	Mutant name	T-DNA insertion region
PARP1	At2g31320	GK-692A05	*parp1-3*	Exon
PARP2	At4g02390	GK-420G03	*parp2-1*	Exon
PARP3	At5g22470	SALK_108092	*parp3-1*	Exon

3.5.6 Genotypical analysis of T-DNA insertion lines

A piece of leaf was harvested with a 1.5 ml Eppendorf tube, and 400 µl of extraction buffer was added. The leaf material was homogenized by using a micro-pestle, and the tube was centrifuged at maximum speed for 5 min in a Minispin centrifuge (Eppendorf). 300 µl of the supernatant were transferred into a new tube. After adding of 300 µl isopropanol the tube was vortexed and centrifuged at maximum speed for 10 min. The supernatant was discarded, and the remaining pellet was washed with 300 µl of 70% ethanol. The ethanol was removed after an additional centrifugation for 10 minutes at maximum speed. The pellet was air-dried for around 45 min and dissolved in 50 µl 10 mM Tris-HCl (pH 8.5). For PCR, different primer combinations were used, containing gene-specific primers spanning the insertion site or one gene-specific plus one T-DNA-specific primer (Tab. 3.3) in order to confirm the homozygosity of the plants for the insertion. Products of the PCR reactions were loaded onto a 1% agarose gel for electrophoresis.

Extraction Buffer

200 mM Tris-HCl (pH 7.5)
250 mM NaCl
25 mM EDTA
0.5% SDS

PCR reaction

H_2O	17 µl
10x Taq Buffer	2.5 µl
10 mM dNTPs	0.5 µl
10 µM forward primer	1 µl
10 µM reverse primer	1 µl
DNA product	2 µl
5 U/µl Taq polymerase	1 µl
Total	25 µl

94°C for 5 min
94°C for 30 s
52°C for 30 s } 35 cycles
72°C for 1 min
72°C for 5 min

3.5.7 Crossing of plants

The anthers of flowers were removed using fine tweezers at a stage when the petals grew out of the sepals. All remaining older and younger flowers were removed. After one day the stigma of the carpels was pollinated.

3.5.8 Plant growth in the 96-well system

Seeds were surface-sterilized once with 70% ethanol and twice with 100% ethanol. Dry seeds were transferred onto a white 96-well plate (Greiner Bio-One, Germany) without tips containing ½ MS-Agar supplemented with 0.5% sucrose (see 2.4.9). The plate was attached to a 96-deep-well plate (Greiner Bio-One, Germany) containing liquid medium of the same composition. The construction was covered with a cover plate and plants were stratified for 2 days at 4°C. Seedlings were grown for 6 days under long-day conditions (16 h light / 8 h dark, 120 µmol $m^{-2}s^{-1}$, 22°C /18°C) in a growth cabinet (AR75, Percival-Scientific, USA), unless stated otherwise.

3.5.9 Measurement and analysis of luciferase activation

Substances were dissolved in 100% DMSO and stored frozen in aliquots as 1 mM stock solution. Substances diluted in nutrient solution were applied to the shoots. D-luciferin was dissolved in sterile water and stored frozen in aliquots as 50 mM stock solution. Working solution of 750 µM luciferin was applied like the substances. After luciferin application seedlings were kept in the dark for 2 min. Imaging was performed using a photon-counting CCD camera (HRPCS4, Photek, St Leonards on Sea, UK). Exposure time was 16 h, unless stated otherwise. Quantification of the acquired data was performed using the corresponding IFS32 software.

3.5.10 Stress experiments on agar plates

Col-0 and *parp* mutant plants were pre-cultured for one week on ½ MS agar (pH 5.8) supplemented with or without 0.5% sucrose under long-day conditions (16 h light / 8 h dark, 120 µmol $m^{-2}s^{-1}$, 22°C /18°C) in a growth cabinet (AR75, Percival-Scientific, USA). For stress experiments plants were transferred to agar plates containing either 50 or 100 mM NaCl, or 100 or 200 mM mannitol and grown further for one week. Afterwards root growth was measured, and plants were transferred to recovery plates (½ MS agar, ±0.5% sucrose, pH 5.8) for at least one week. The fresh weight was measured at the day of harvest.

3.5.11 $[Ca^{2+}]_{cyt}$ measurements

Col-0 and *pap2-3* plants expressing the *APOAEQUORIN* gene under control of the CaMV 35S promoter were used for $[Ca^{2+}]_{cyt}$ analyses (Knight *et al.*, 1991). Seeds were surface-sterilized once with 70% ethanol and twice with 100% ethanol. Plants were grown in liquid MS medium (½ MS, 0.5% sucrose, pH 5.8) in 96-well plates (Greiner Bio-One, Germany) for 6 days under long-day conditions (16 h light / 8 h dark, 120 µmol m^{-2} s^{-1}, 22°C /18°C) in a growth cabinet (Percival-Scientific, USA). Aequorin was reconstituted with 10 µM coelenterazine overnight in the dark. Luminescence was measured using a microplate reader (Berthold Technologies, Wildbad, Germany). After 10 s of recording, the treatment was applied by addition of 75 µl of a twofold concentrated solution via an automatic dispenser, and measurements were continued for the indicated time. Controls were performed by addition of an

equal volume of water. Remaining aequorin was discharged by automatic injection of one volume of a solution containing 2 M $CaCl_2$ and 20% ethanol. $[Ca^{2+}]_{cyt}$ concentrations were calculated according to Rentel and Knight (2004):

$$pCa = 0.332588(-\log k) + 5.5593$$

$$k = \frac{\text{luminescence counts per sec}}{\text{total luminescence counts remaining}}$$

3.5.12 cADPR assay

Twenty-day-old plants, cultivated as described above (3.5.10), were transferred to liquid medium of the same composition and cultivated for additional 3 days. Afterwards plants were treated with 300 mM NaCl, collected and rapidly frozen in liquid N_2 (Sanchez *et al.*, 2004). cADPR was extracted from 0.2 g of powdered plant tissue (Dodd *et al.*, 2007). To this end, 1000 µl of perchloric acid (600 mM) were added, and samples were homogenized by mixing. Afterwards samples were centrifuged (10,000 *g*) at 4°C for 10 min, and 1 ml of supernatant was recovered in 15 ml Falcon tubes (Greiner Bio-One, Germany). For removing the perchloric acid 4 ml of 1,1,2-trichloro-trifluoroethane and tri-*n*-octyl-amine mixture (3:1) were added and briefly vortexed. Samples were centrifuged again (1,000 *g*) at 4°C for 10 min, and 500 µl of neutral sample from the upper aequous phase were recovered. The pH was adjusted to 8 by adding 160 µl of 20 mM Tris base. To remove NAD and NADH from the samples, 140 µl of a hydrolytic enzyme mixture was added and incubated at 37°C overnight. Next day hydrolytic enzymes were removed by ultrafiltration (4,000 *g* for 30 min) using Amicon-3 filters (Merck, Germany). Finally, the cADPR was measured using a fluorescence-based cycling assay (Graeff and Lee, 2002; 2003). First, 50 µl of final sample were added into a black 96-well plate (Greiner Bio-One, Germany) followed by addition of 25 µl of Reagent 1 and incubation at room temperature for 30 min. Finally, 50 µl of Reagent 2 were added, and the cADPR-dependent increase in fluorescence (540 nm excitation, 600 nm emission) was measured with a microplate reader (Berthold Technologies, Germany) over a 2 hour period with a cycle time of 10 min.

Hydrolytic enzyme mixture

6.25 mg NADase

0.44 U/ml nucleotide pyrophosphatase

12.5 U/ml alkaline phosphatase

2.5 mM $MgCl_2$

Reagent 1

30 mM nicotinamide

0.3 U/ml *Aplysia* cyclase

Reagent 2

100 mM sodium phosphate, pH 8 (95 mM Na_2HPO_4 and 5 mM NaH_2PO_4)

2% ethanol

10 mM nicotinamide

0.1 mM FMN

0.1 mg/ml alcohol dehydrogenase

0.01 mg/ml diaphorase (purified with 2% charcoal in 20 mM $Na_xH_yPO_4$, pH7)

0.01 mM resazurin

3.5.13 Statistical analyses

The relative standard errors (SE_{rel}) were calculated via the relative standard deviation (SD_{rel}) according to Applied Biosystems (2001) using the following formula:

$$SD_{rel} = \left(\sqrt{\left(\frac{SD_1}{MV_1}\right)^2 + \left(\frac{SD_2}{MV_2}\right)^2} \right) \times MV_{rel} \qquad MV = \text{mean value}$$

$$SE_{rel} = \frac{SD_{rel}}{\sqrt{n}}$$

The statistical significances were determined by using Student's *t*-test.

3.6 Results

3.6.1 Pharmacological PARP inhibition promotes *RD29A* promoter activity

During the establishment of the luciferase assay for identifying potential phytoeffectors (part 1), known PARP inhibitors were used to investigate their effect on stress-induced *RD29A-LUC* expression. To determine the effect of the PARP inhibitors on *RD29A-LUC* activity under unstressed conditions, three concentrations (1, 10, 100 µM) of 3-AB, PJ-34, nicotinamide, and isonicotinamide were tested (Fig. 3.2). No significant differences to the control were observed at any of the concentrations.

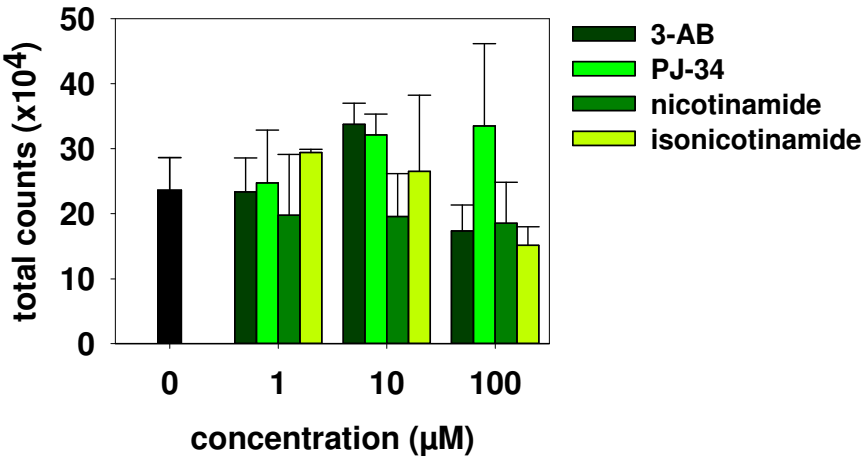

Figure 3.2 Effect of PARP inhibitors on *RD29A* activity in Arabidopsis.

Plants were treated with water or with 3-AB, PJ-34, nicotinamide, or isonicotinamide (1-100 µM). Exposure time was 16 h. Each bar represents the average luminescence from n ≥ 3 seedlings. Error bars represent SE.

Figure 3.3 shows the effect of the PARP inhibitors on NaCl-induced *RD29A-LUC* expression. Total counts of PARP inhibitor-treated plants are displayed relative to the total counts of the control plants. Under NaCl stress, all inhibitors provoked a dose-dependent effect. For PJ-34, nicotinamide, and isonicotinamide, the inhibitor concentration was positively correlated with the NaCl-induced RD29A promoter activity. Nicotinamide and isonicotinamide showed the highest effect at 100 µM, whereas for PJ-34 and 3-AB, the

maximum enhancement of *RD29A-LUC* expression was already observed at 10 µM. Only for 3-AB, the activating activity declined again at 100 µM.

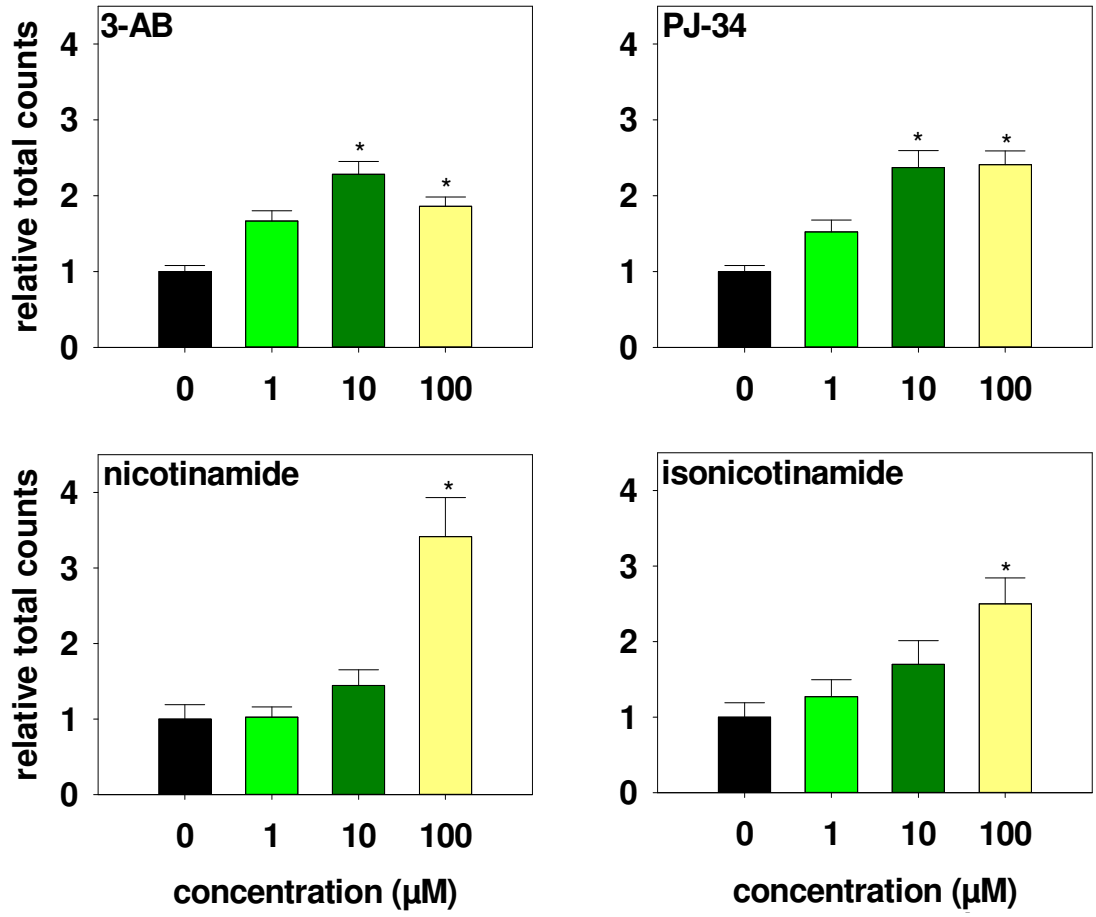

Figure 3.3 Effect of PARP inhibitors on NaCl-induced *RD29A* activity in Arabidopsis.

Plants were treated with 300 mM NaCl alone (0 µM) or in combination with 3-AB, PJ-34, nicotinamide, or isonicotinamide (1-100 µM). Exposure time was 16 h. Data show the total counts of PARP inhibitor-treated plants relative to the total counts of the non-inhibitor-treated plants. Each bar represents the relative average luminescence from n ≥ 5 seedlings. Error bars represent relative SE. Significance was determined by Student's *t*-test (* $p<0.05$).

The effect of the PARP inhibitors on *RD29A-LUC* expression in combination with osmotic stress induced by PEG was similar to that in combination with NaCl (Fig. 3.4). Comparable to the NaCl experiment, nicotinamide and isonicotinamide showed the maximum effect at 100 µM, while 10 µM were the optimum concentration for 3-AB and PJ-34. The enhancement of the stress-induced *RD29A-LUC* expression by 3-AB, and also PJ-34 was absent again at 100 µM.

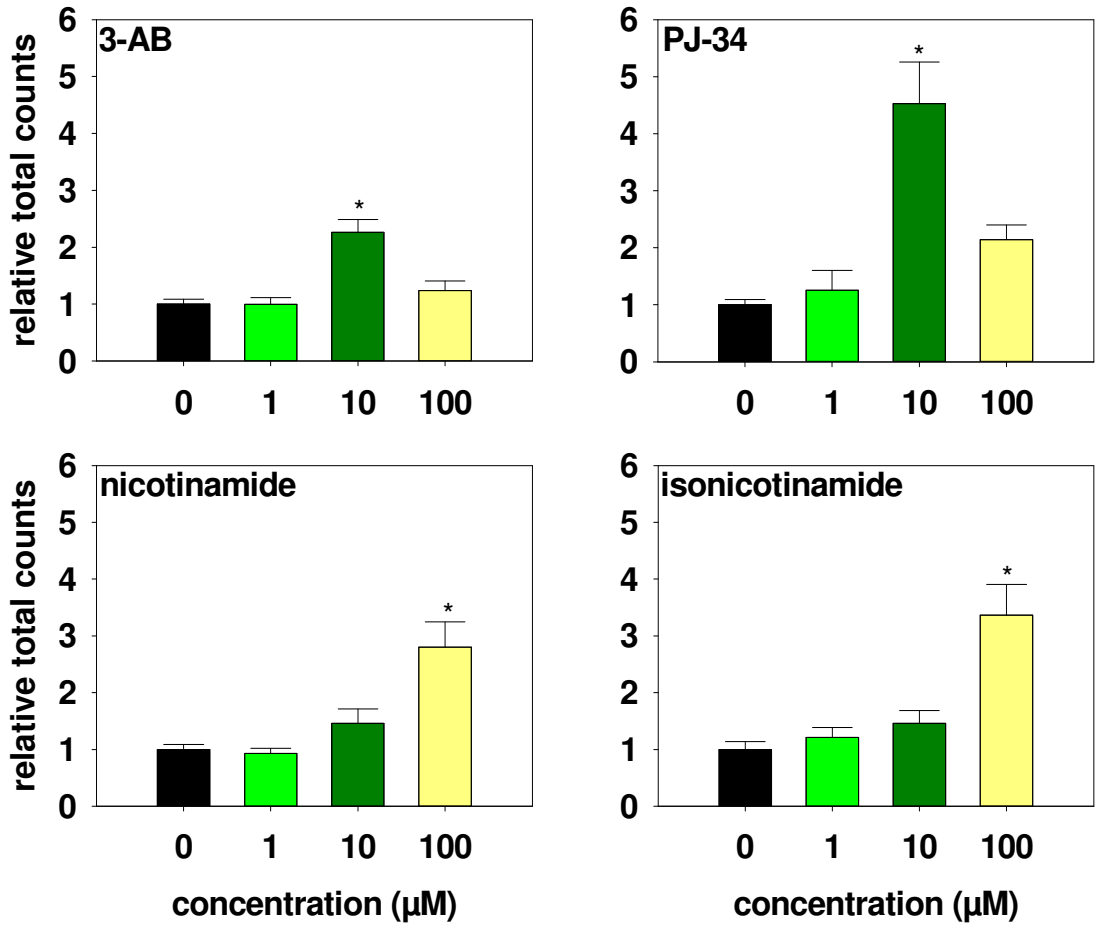

Figure 3.4 Effect of PARP inhibitors on PEG-induced *RD29A-LUC* activity in Arabidopsis.

Plants were treated with 30% PEG alone (0 µM) or in combination with 3-AB, PJ-34, nicotinamide or isonicotinamide (1-100 µM). Exposure time was 16 h. Data show the total counts of PARP inhibitor-treated plants relative to the total counts of the non-inhibitor-treated plants. Each bar represents the relative average luminescence from n ≥ 5 seedlings. Error bars represent relative SE. Significance was determined by Student's *t*-test (* $p<0.05$).

Based on these results, it can be concluded that a pharmacological inhibition of PARP activity leads to a higher *RD29A* promoter activity under abiotic stress, but has no such effect under non-stressed conditions.

3.6.2 *RD29A* promoter activity responds differently to salt stress in *parp* mutants

Based on the above findings, the effect of a genetic inhibition of PARPs on *RD29A* activity was investigated. To this end, the *parp* loss-of-function (knockout) mutants *parp1-3*, *parp2-1*, and *parp3-1* were crossed with the *RD29A-LUC* reporter line and tested for the response of *RD29A-LUC* expression to abiotic stress using the luciferase assay (Fig 3.5). Luminescence

of all mutant lines was comparable to the wild type under control conditions, allowing an interpretation of the *RD29A-LUC* expression induced by PEG and NaCl. PEG caused an increase in luminescence, as shown before in part 1 (Fig. 2.5D). There was no difference between wild type and mutant plants in this response. NaCl provoked a stronger response than PEG, again confirming the findings in part 1 (Fig. 2.5C). While *parp1-3* responded similarly to the wild type, the *parp3-1* mutant showed a strongly alleviated *RD29A-LUC* expression. In contrast, the response was significantly enhanced in the *parp2-1* mutant compared to the wild type.

Although the *parp* knockout mutants responded in different ways in this experiment, it can be concluded that a genetic PARP inhibition alters the NaCl-stimulated expression of *RD29A*.

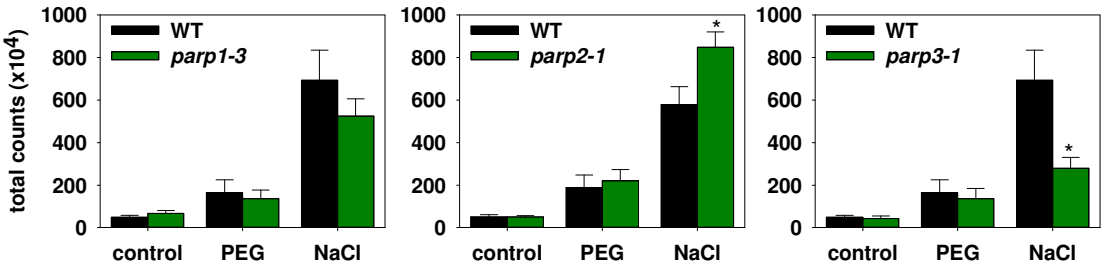

Figure 3.5 PEG- and NaCl-responsive *RD29A* activity in Arabidopsis wild type, *parp1-3*, *parp2-1*, and *parp3-1* mutant plants.

Plants were treated either with water (control), PEG (30%) or NaCl (300 mM). Exposure time was 16 h. Each bar represents the average luminescence from n ≥ 3 seedlings. Error bars represent SE. Significance was determined by Student's *t*-test (* $p<0.05$).

3.6.3 *parp* mutant plants recover differently from abiotic stress

In literature PARPs are proposed to be involved in plant abiotic stress tolerance (De Block *et al.*, 2005; Vanderauwera *et al.*, 2007; Geissler and Wessjohann, 2011; Schulz *et al.*, 2012), which is supported by preliminary data from our laboratory (Fig. 3.1), but debated by other studies (Rissel *et al.*, 2017b; Rissel and Peiter, 2019). Because of the altered *RD29A-LUC* expression in *parp* knockout mutants (Fig. 3.5), the effect of a genetic PARP inhibition on abiotic stress tolerance of Arabidopsis was further investigated using knockout mutants for each *PARP* gene, as well as *parp* double and triple mutants. Different to

previous studies, the ability of the plants to recover from the stress was also examined.

3.6.3.1 Recovery of *parp* mutants from salt stress

First, salt stress experiments on wild type and *parp* mutant plants grown on agar plates were performed. The plants were grown for one week under non-stressed standard growth conditions. For stress induction, plants were transferred to agar plates containing 50 or 100 mM NaCl for one week. Afterwards plants were transferred to recovery plates without NaCl and grown for another week.

Figure 3.6 shows the impact of salt stress on Arabidopsis wild type and *parp1-3* mutant plants. After one week of stress no visible differences in growth between wild type and mutant plants could be observed. While 50 mM of NaCl had only marginal effects on plant growth, 100 mM of NaCl led to a comparable growth inhibition in both genotypes (Fig. 3.6A), which was also manifested in primary root elongation (Fig. 3.6B). After one week of recovery, there are still no growth differences between wild type and mutant plants (Fig. 3.6A), which is also reflected in plant fresh weight (Fig 3.6C).

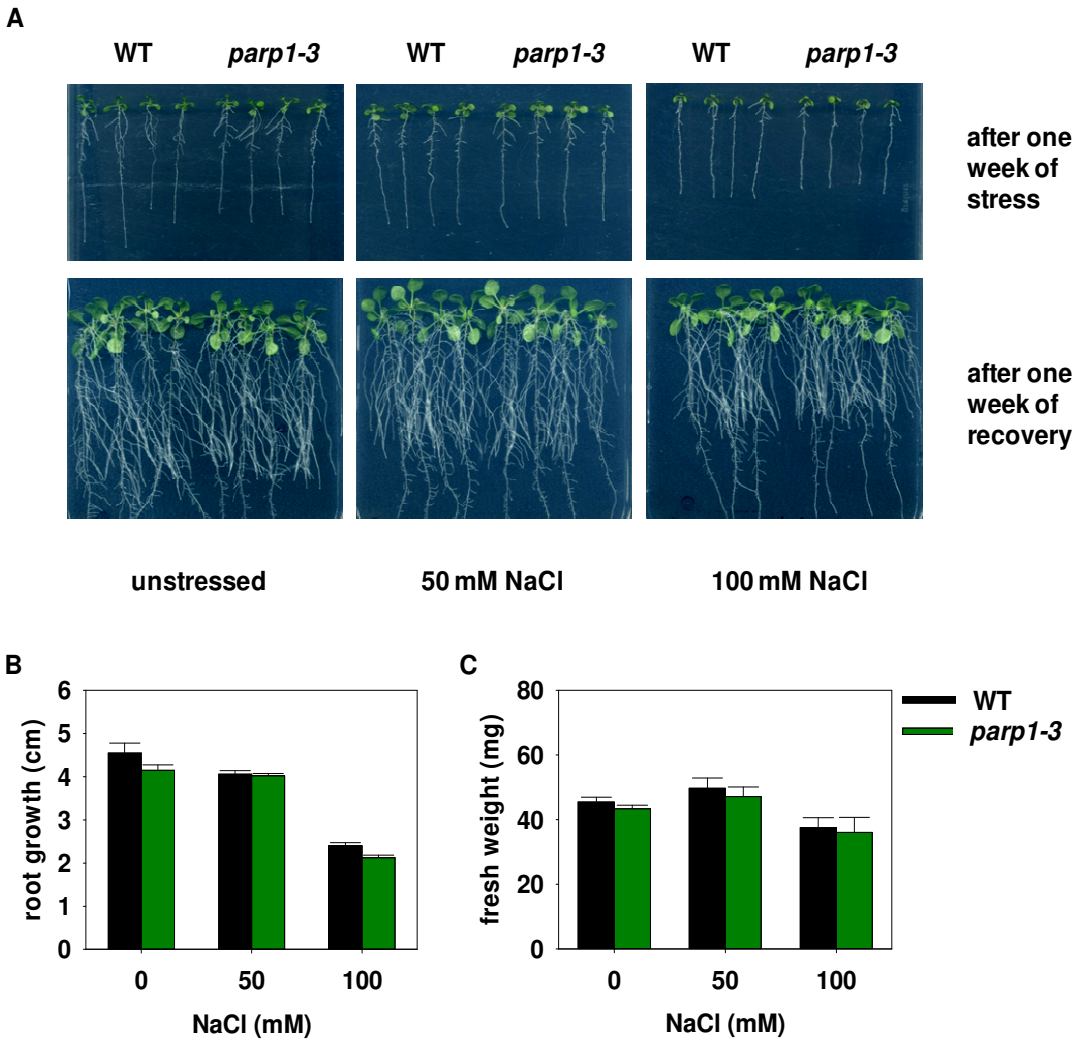

Figure 3.6 Effects of salt stress on wild type and *parp1-3* mutant plants.

(A) Representative pictures of plants after salt stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. The experiment was performed twice with similar results.

In contrast to *parp1-3*, the *parp2-1* mutant showed a different response to NaCl than the wild type (Fig. 3.7). After one week of stress no significant differences were observable between wild type and mutant plants under control conditions as well as upon 50 mM NaCl treatment (Fig. 3.7B). However, when treated with 100 mM NaCl the mutant plants showed smaller shoots and significantly shorter roots (Fig. 3.7B). Moreover, the mutant plants showed strongly reduced potential to recover from both 50 and 100 mM NaCl. This severe hypersensitivity of the *parp2-1* plants is reflected in smaller shoots, a

lower lateral root density (Fig. 3.7A) and a significantly lower fresh weight (Fig. 3.7C).

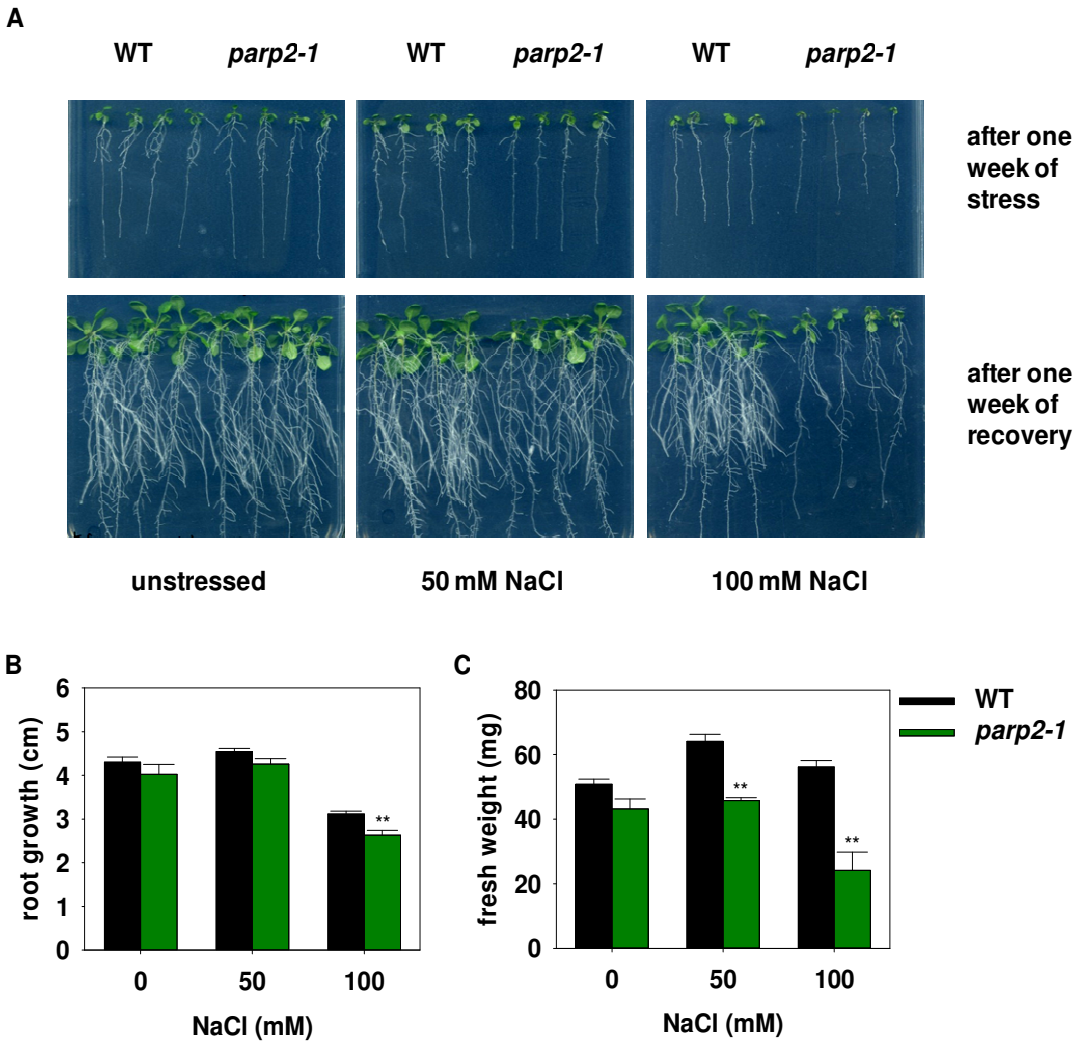

Figure 3.7 Effects of salt stress on wild type and *parp2-1* mutant plants.

(A) Representative pictures of plants after salt stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. Significance was determined by Student's *t*-test (** $p<0.01$). The experiment was performed three times with similar results.

Figure 3.8 shows the impact of salt stress on wild type and the *parp3-1* mutant plants. Comparable with the experiment with *parp1-3*, there are no significant differences between wild type and mutant plants, neither after one week of salt stress nor after recovery. In this experiment, and less pronounced in the previous ones, wild type plants had a tendentially higher fresh weight after recovery from 50 mM NaCl compared to control conditions (Fig. 3.8C).

Taken together, amongst the canonical *PARP* genes, only *PARP2* appears to play a role in salt tolerance and recovery under the given experimental conditions, with its knockout leading to a hypersensitivity of the plants.

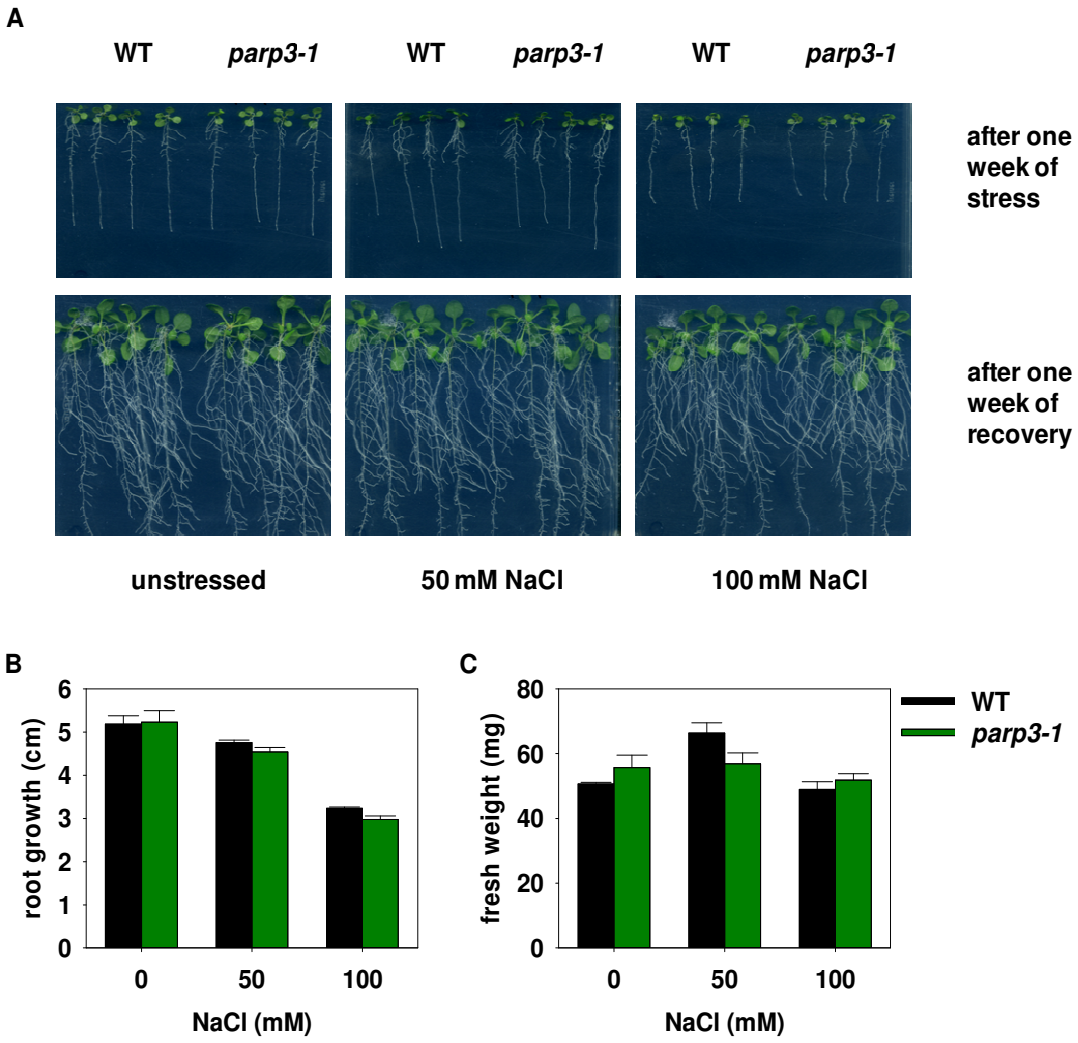

Figure 3.8 Effects of salt stress on wild type and *parp3-1* mutant plants.

(A) Representative pictures of plants after salt stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. The experiment was performed once.

To test for redundancy effects, two *parp* double knockout lines were tested for their performance under salt stress. Figure 3.9 shows the effects of salt stress on wild type and *parp1-3:parp2-1* mutant plants. There were no visible differences or differences in primary root length between wild type and mutant plants, neither after one week of 50 mM NaCl nor after recovery from this stress

(Fig. 3.9A,B). Interestingly, after one week of recovery from 100 mM NaCl, it is clearly visible that the *parp1-3:parp2-1* mutant plants showed bigger shoots and higher lateral root density compared to the wild type plants (Fig. 3.9A), which is also reflected in a significantly higher plant fresh weight (Fig. 3.9C).

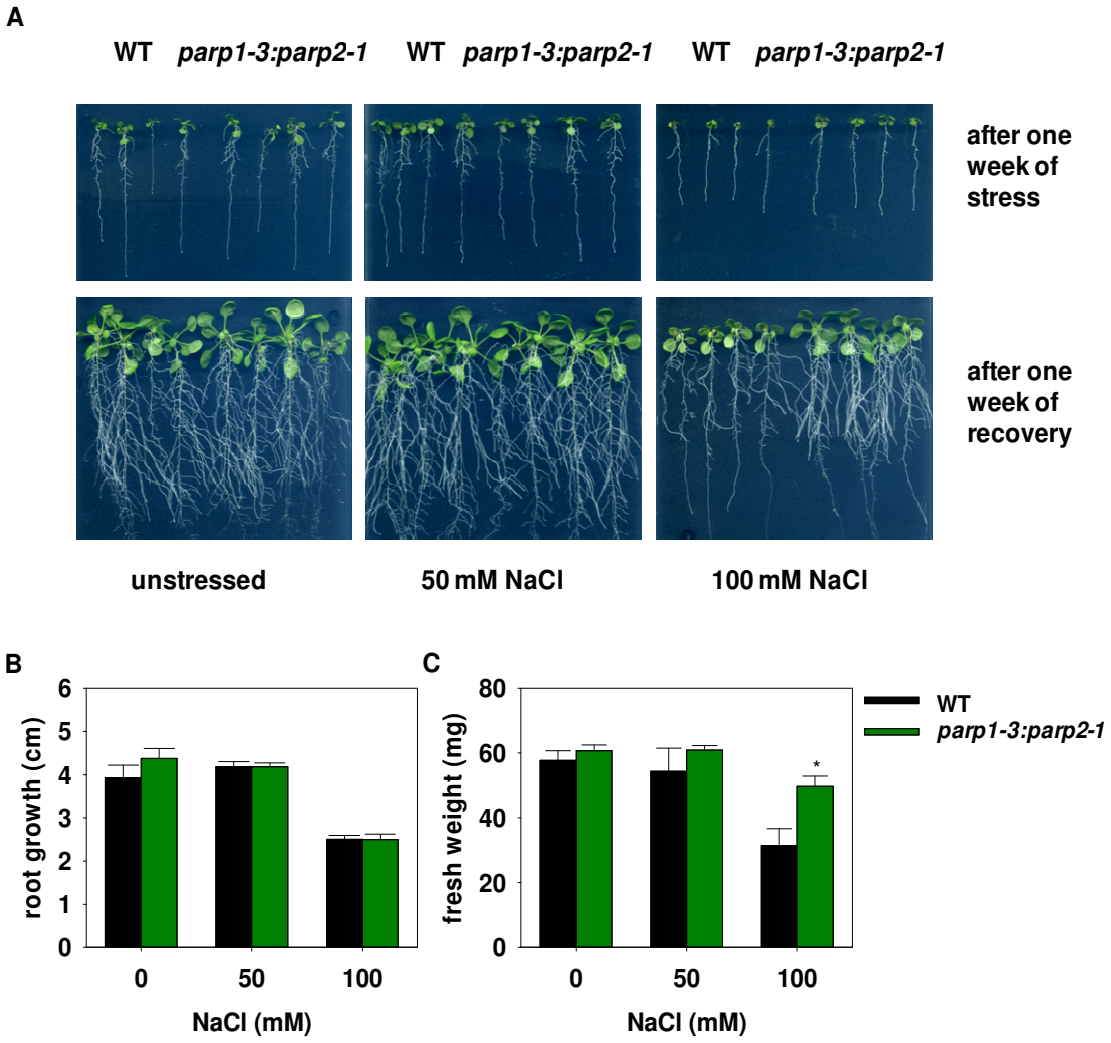

Figure 3.9 Effects of salt stress on wild type and *parp1-3:parp2-1* mutant plants.

(A) Representative pictures of plants after salt stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. Significance was determined by Student's *t*-test (* $p<0.05$). The experiment was performed three times with similar results.

Figure 3.10 shows the impact of the knockout of both *PARP2* and *PARP3* on Arabidopsis salt tolerance. After one week of stress no visible differences in growth between wild type and *parp2-1:parp3-1* mutant plants were observed. In

both genotypes, 100 mM NaCl led to a comparable growth inhibition (Fig. 3.10A), which was also apparent in the primary root lengths (Fig. 3.10B). Also, after one week of recovery from both 50 and 100 mM NaCl, no differences in growth between wild type and mutant plants were observed, which is also reflected in the plant fresh weight (Fig 3.10C).

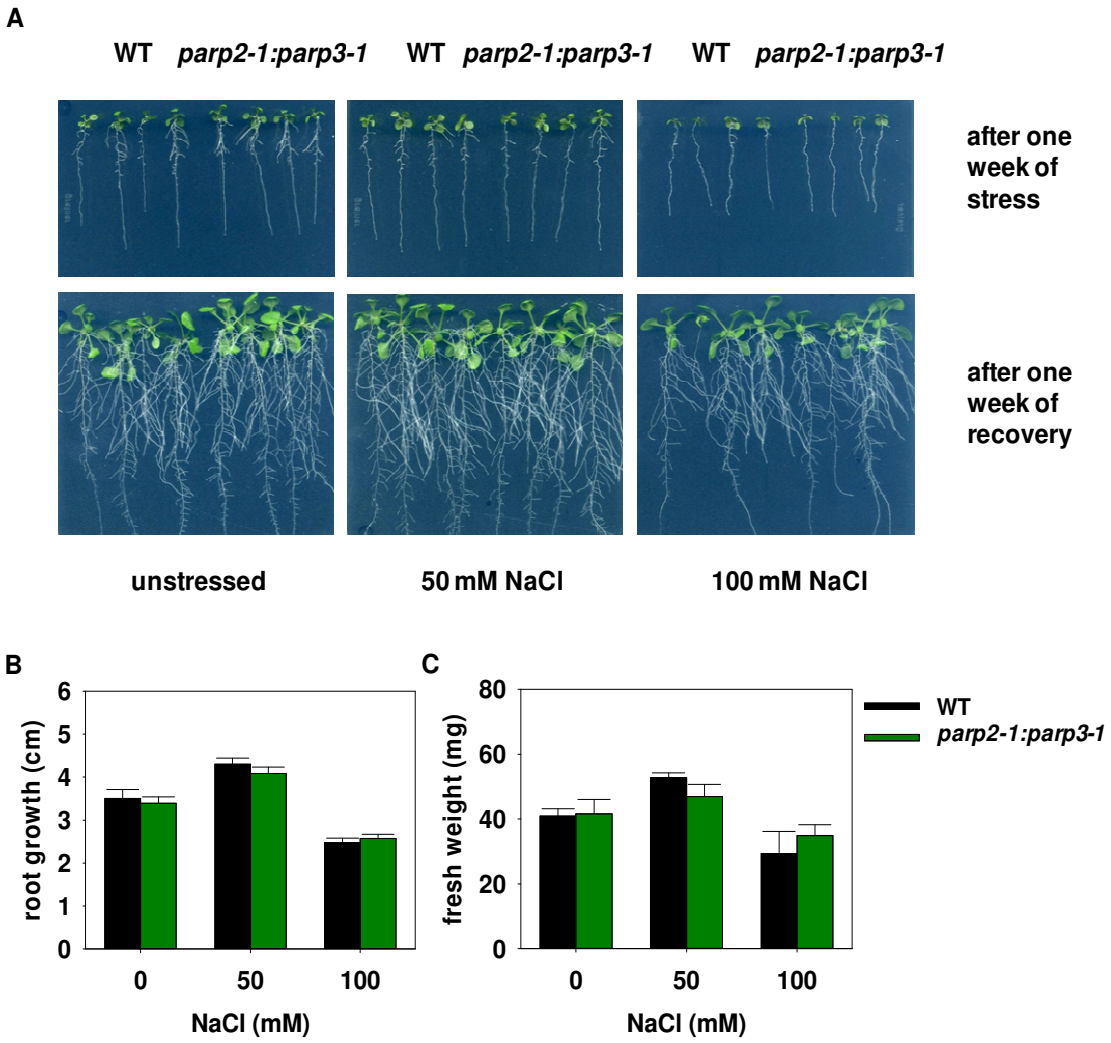

Figure 3.10 Effects of salt stress on wild type and *parp2-1:parp3-1* mutant plants.

(A) Representative pictures of plants after salt stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. The experiment was performed once.

Conclusively, while a knockout of *PARP2* alone has a negative impact on growth, an additional deletion of *PARP1* leads to an improved ability to recover

from salt stress. Interestingly, a knockout of both *PARP2* and *PARP3* has no effect.

Finally, a triple knockout of all three *PARP* genes was analyzed (Fig. 3.11). After one week of salt stress no differences between wild type and *parp1-3:parp2-1:parp3-1* mutant plants were observed, neither on 50 nor on 100 mM NaCl (Fig.3.11A,B). There were also no differences between both genotypes after one week of recovery (Fig 3.11A,C).

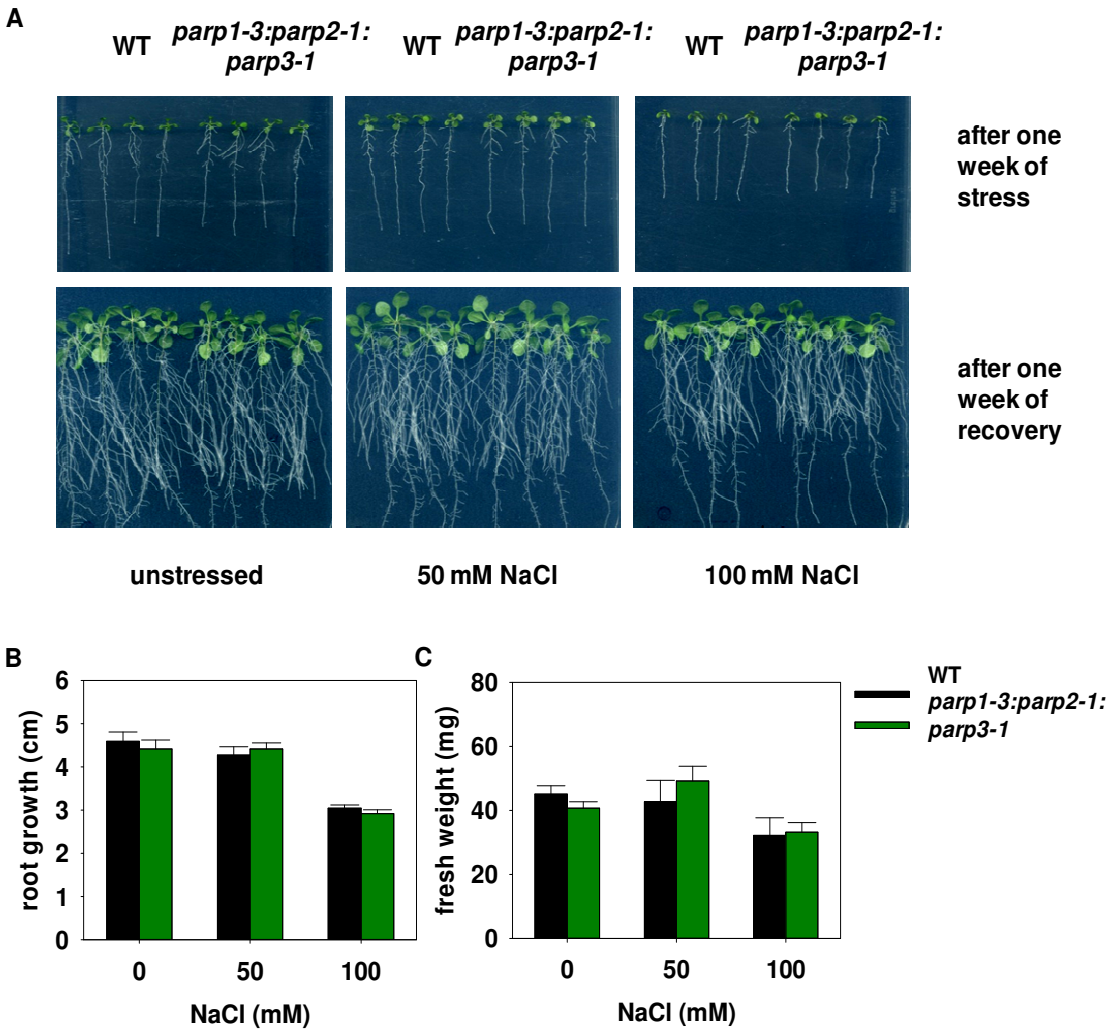

Figure 3.11 Effects of salt stress on wild type and *parp1-3:parp2-1:parp3-1* mutant plants.

(A) Representative pictures of plants after salt stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. The experiment was performed three times with similar results.

Taken together, a knockout of *PARP2* leads to a hypersensitivity of Arabidopsis to salt stress, whereas a knockout of both *PARP1* and *PARP2* has the opposite effect, leading to an improved ability to recover from salt stress. Further complicating this situation, a knockout of all three *PARP* genes has no impact on Arabidopsis salt stress tolerance.

3.6.3.2 Recovery of *parp* mutants from osmotic stress

The effect of mannitol-induced osmotic stress to mimick drought stress was investigated. Again, the plants were grown for one week under standard growth conditions. For stress induction, plants were transferred to agar plates containing either 100 or 200 mM mannitol for one week. 100 and 200 mM of mannitol were chosen to impose the same osmotic stress as 50 and 100 mM NaCl, respectively. Afterwards plants were retransferred to recovery plates without mannitol and grown further for another week.

Figure 3.12 shows the impact of mannitol-induced osmotic stress on Arabidopsis wildtype and *parp1-3* mutant plants. After one week of stress no differences in growth between wild type and mutant plants could be observed. Similarly, after one week of recovery, there are no differences between both genotypes in visual appearance and plant fresh weight (Fig 3.12B, C).

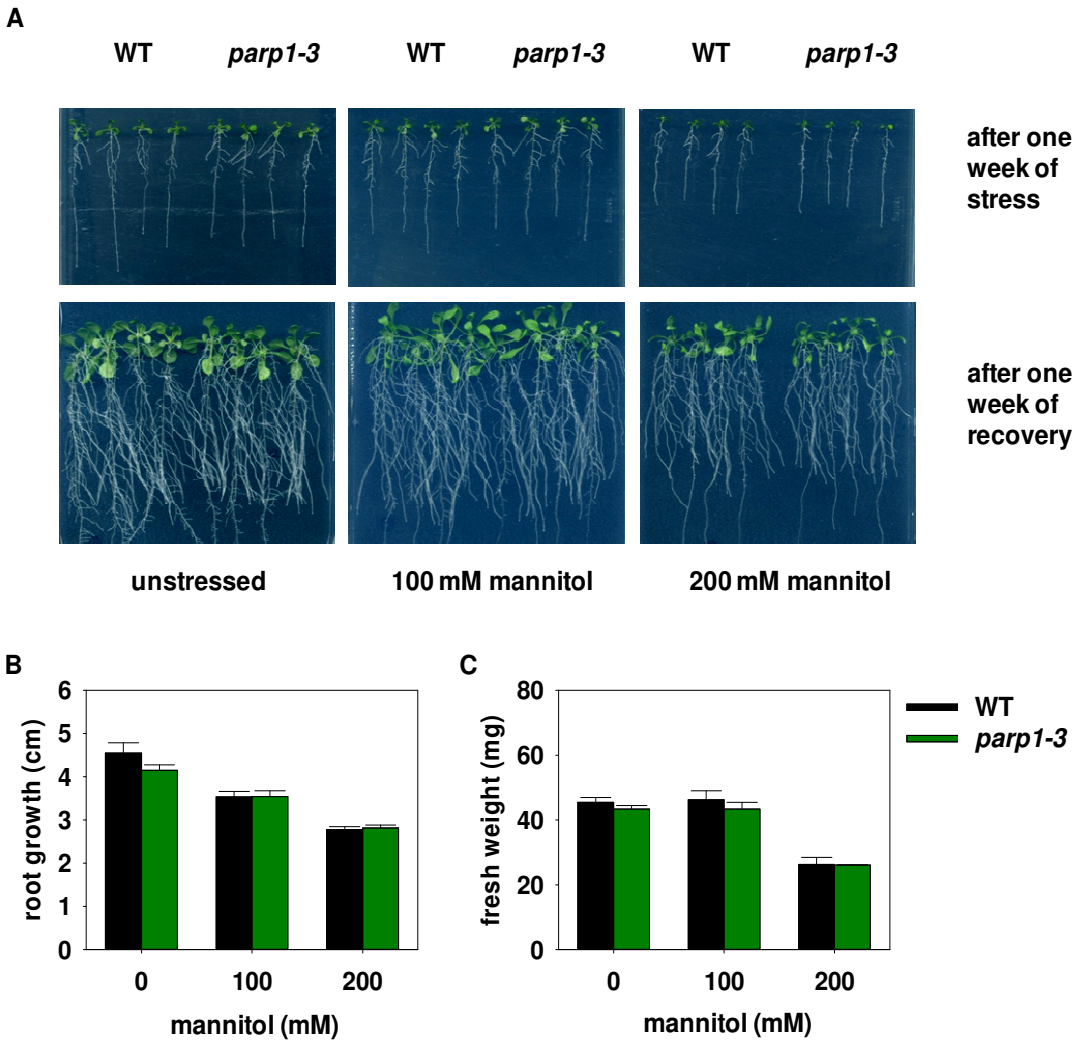

Figure 3.12 Effects of mannitol-induced osmotic stress on wild type and *parp1-3* mutant plants.

(A) Representative pictures of plants after osmotic stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. The experiment was performed twice with similar results.

In contast to *parp1-3, parp2-1* mutant plants responded differently to mannitol as compared to the wild type (Fig. 3.13). There were no significant differences observable between wild type and mutant plants after one week of stress, neither under control conditions nor upon 100 or 200 mM mannitol treatment, and, in contrast to the corresponding salt stress experiment (Fig. 3.7B), mannitol-stressed *parp2-1* mutants showed the same primary root length as wild type plants (Fig. 3.13B). However, the potential of *parp2-1* mutant plants to recover from both 100 and 200 mM mannitol was lower than that of the wild

type. This hypersensitivity of the *parp2-1* plants is reflected by smaller shoots, a lower lateral root density (Fig. 3.13A), and a significantly lower plant fresh weight (Fig. 3.13C).

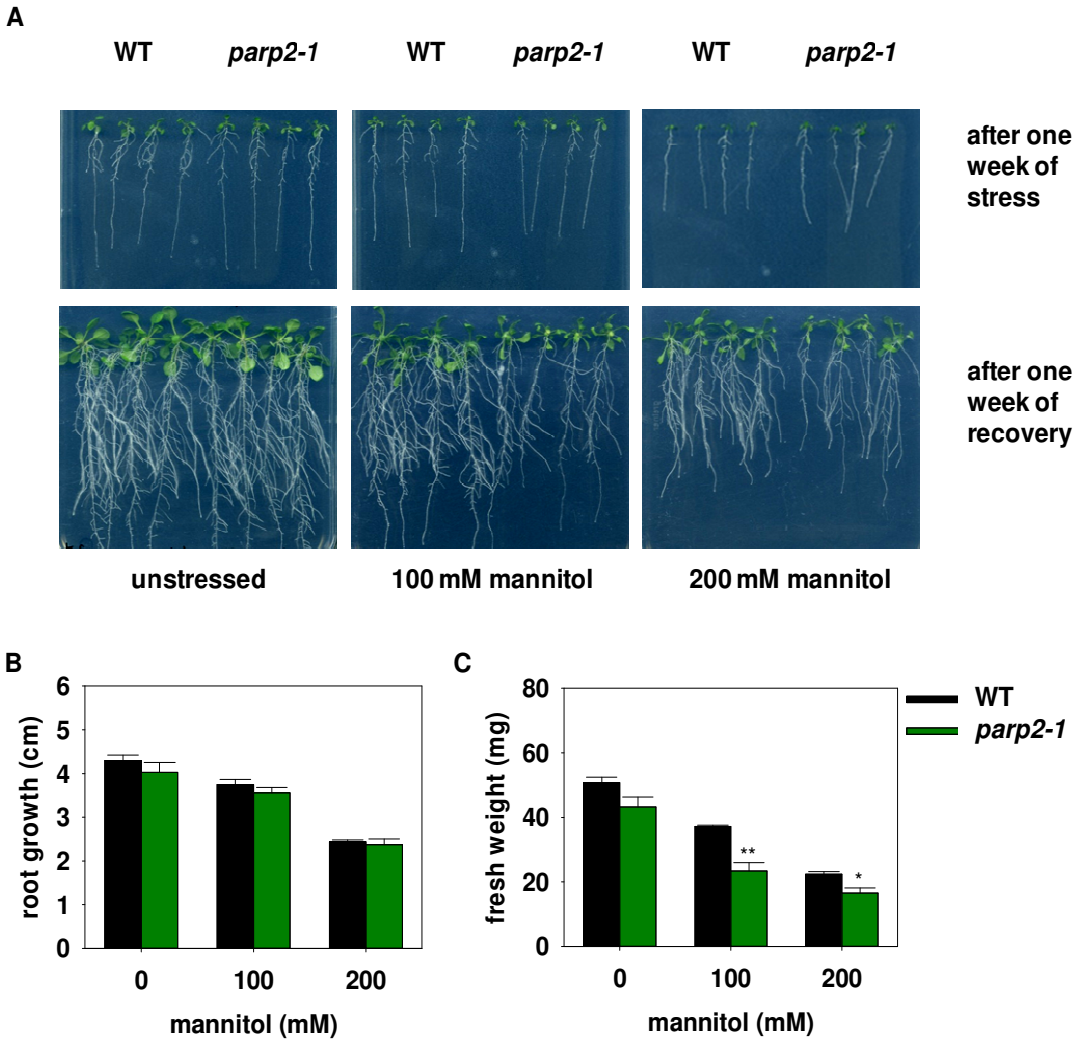

Figure 3.13 Effects of mannitol-induced osmotic stress on wild type and *parp2-1* mutant plants.

(A) Representative pictures of plants after osmotic stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. Significance was determined by Student's *t*-test (* $p<0.05$; ** $p<0.01$). The experiment was performed three times with similar results.

Figure 3.14 shows the impact of mannitol-induced osmotic stress on Arabidopsis wild type and the *parp3-1* mutant plants. Like in the salt stress

experiments (Fig. 3.8), no differences between both genotypes were observed, neither after one week of osmotic stress nor after recovery from stress.

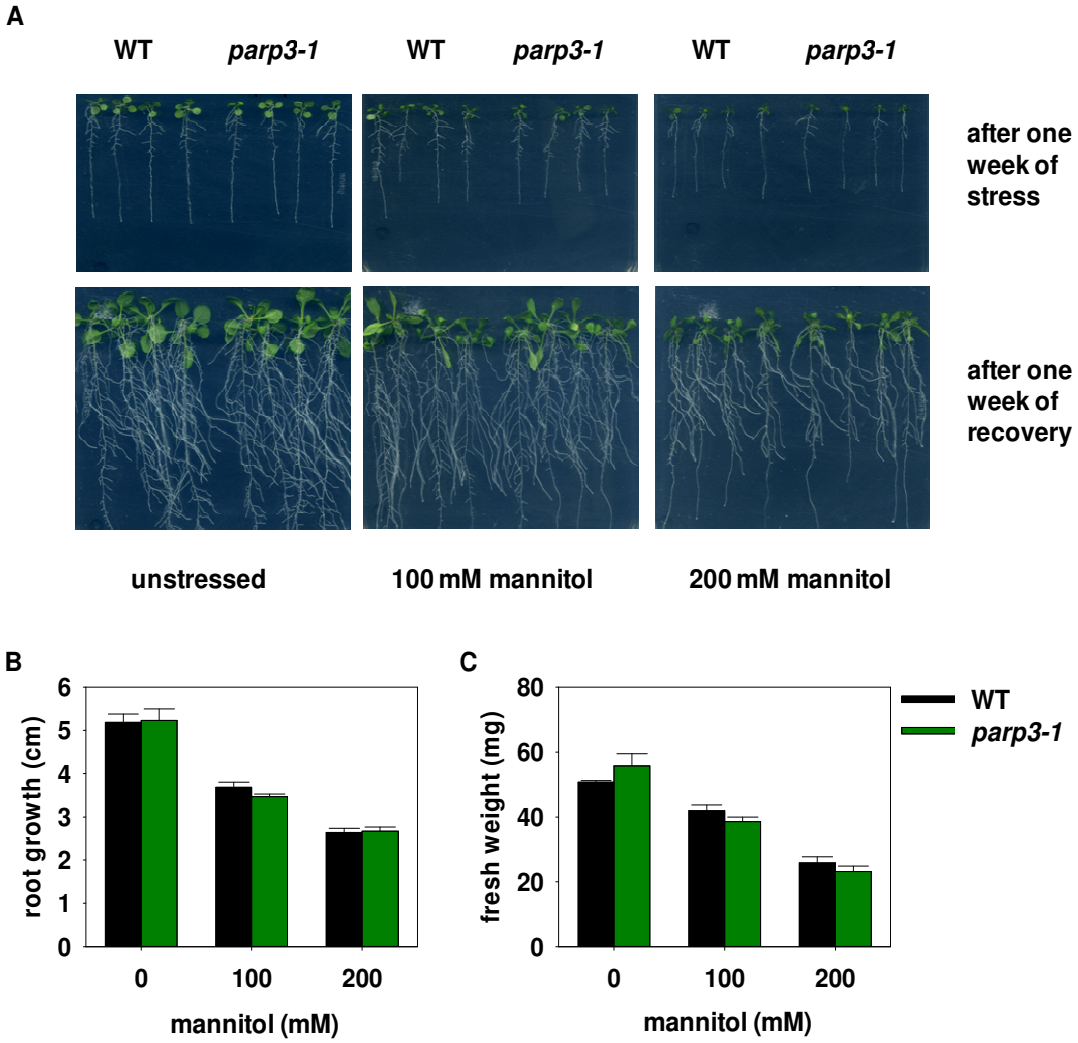

Figure 3.14 Effects of mannitol-induced osmotic stress on wild type and *parp3-1* mutant plants.

(A) Representative pictures of plants after osmotic stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. The experiment was performed once.

Taken together, the *PARP2* gene, but not *PARP1* or *PARP3*, appears to play a role in the tolerance of Arabidopsis to salt as well as osmotic stress, because its knockout leads to a hypersensitivity of the plants to both stresses.

To again test for genetic redundancy, the *parp1-3:parp2-1* and *parp2-1:parp3-1* double knockout lines were tested for their performance under

osmotic stress conditions. Figure 3.15 shows the effects of osmotic stress on wild type and *parp1-3:parp2-1* mutant plants. There were no differences between wild type and mutant plants after one week of osmotic stress (Fig. 3.15A,B). However, in line with the salt stress experiment (Fig. 3.9), the *parp1-3:parp2-1* mutant plants showed a better potential to recover from 100 and 200 mM mannitol, which is manifested by bigger shoots and a higher lateral root density (Fig. 3.15A), as well as a higher plant fresh weight compared to the wildtype plants (Fig. 3.15C).

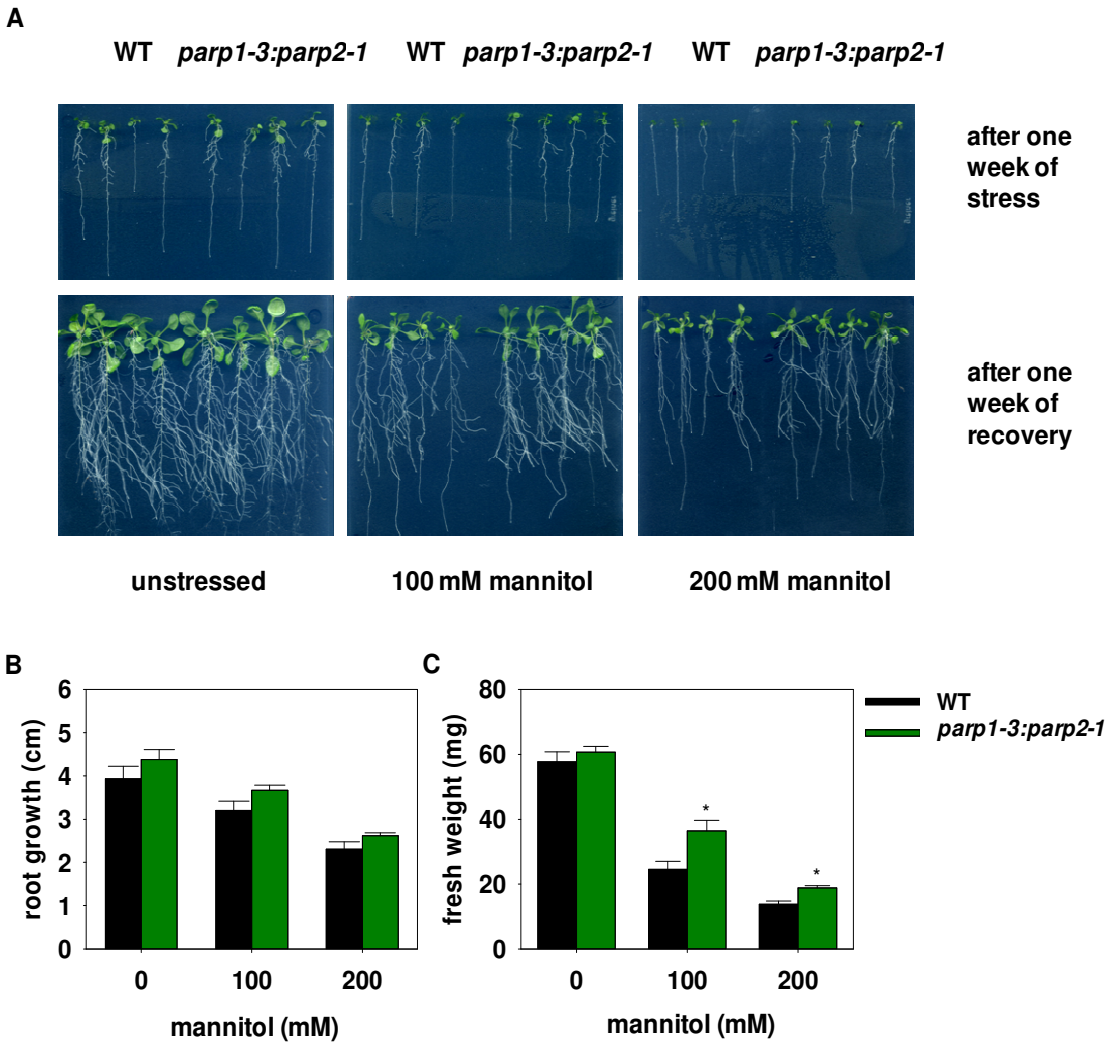

Figure 3.15 Effects of mannitol-induced osmotic stress on wild type and *parp1-3:parp2-1* mutant plants.

(A) Representative pictures of plants after osmotic stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. Significance was determined by Student's *t*-test (* $p<0.05$). The experiment was performed three times with similar results.

Comparable to the corresponding salt stress experiment (Fig. 3.10), no differences in growth between *parp2-1:parp3-1* mutant and wild type plants under mannitol stress were observed, neither after one week of stress nor after recovery (Fig. 3.16).

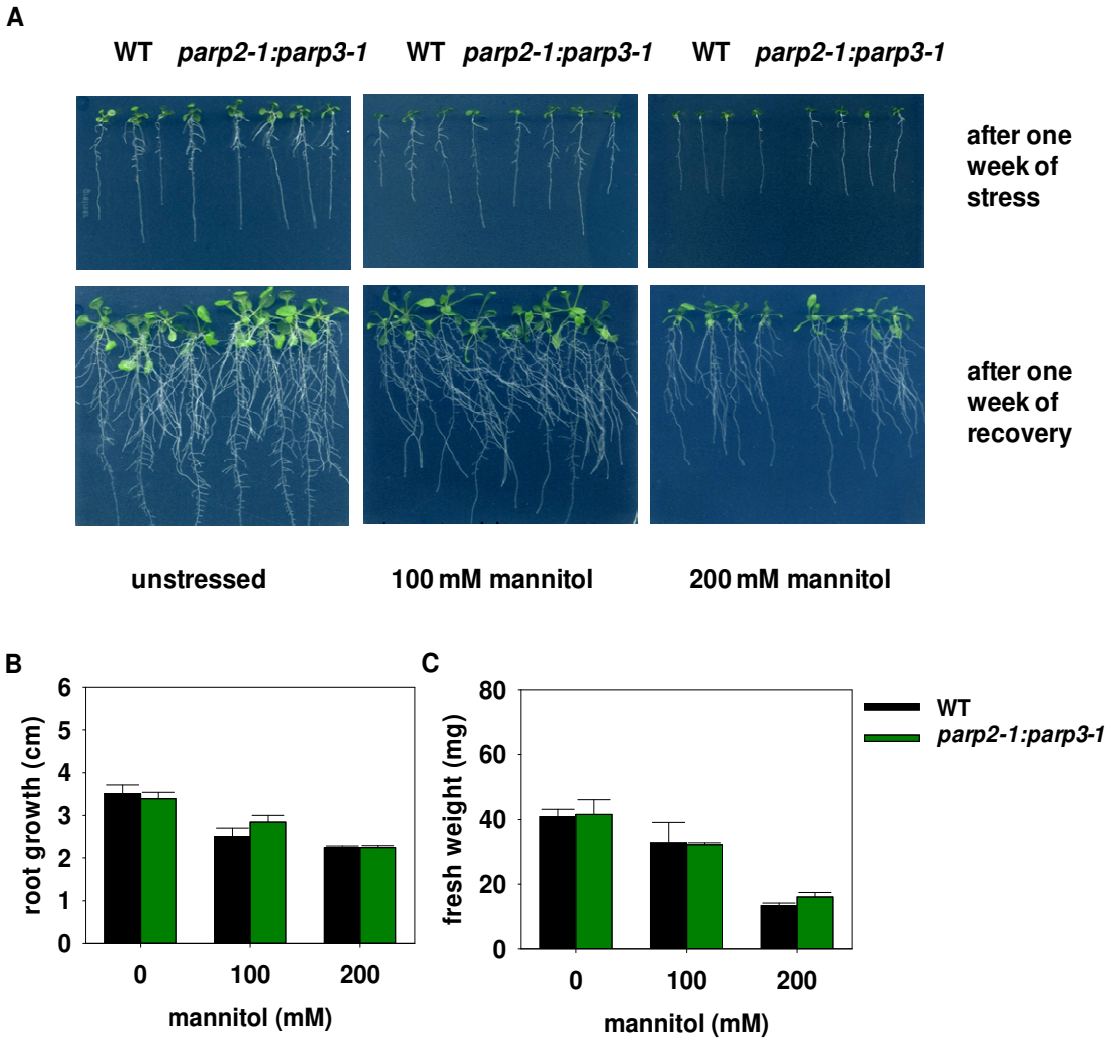

Figure 3.16 Effects of mannitol-induced osmotic stress on wild type and *parp2-1:parp3-1* mutant plants.

(A) Representative pictures of plants after osmotic stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. The experiment was performed once.

Finally, a triple knockout mutant for all three *PARP* genes was analyzed (Fig. 3.17). Comparable to the salt stress experiment (Fig. 3.11), no differences

between mutant and wild type plants were observed after one week of osmotic stress as well as after recovery.

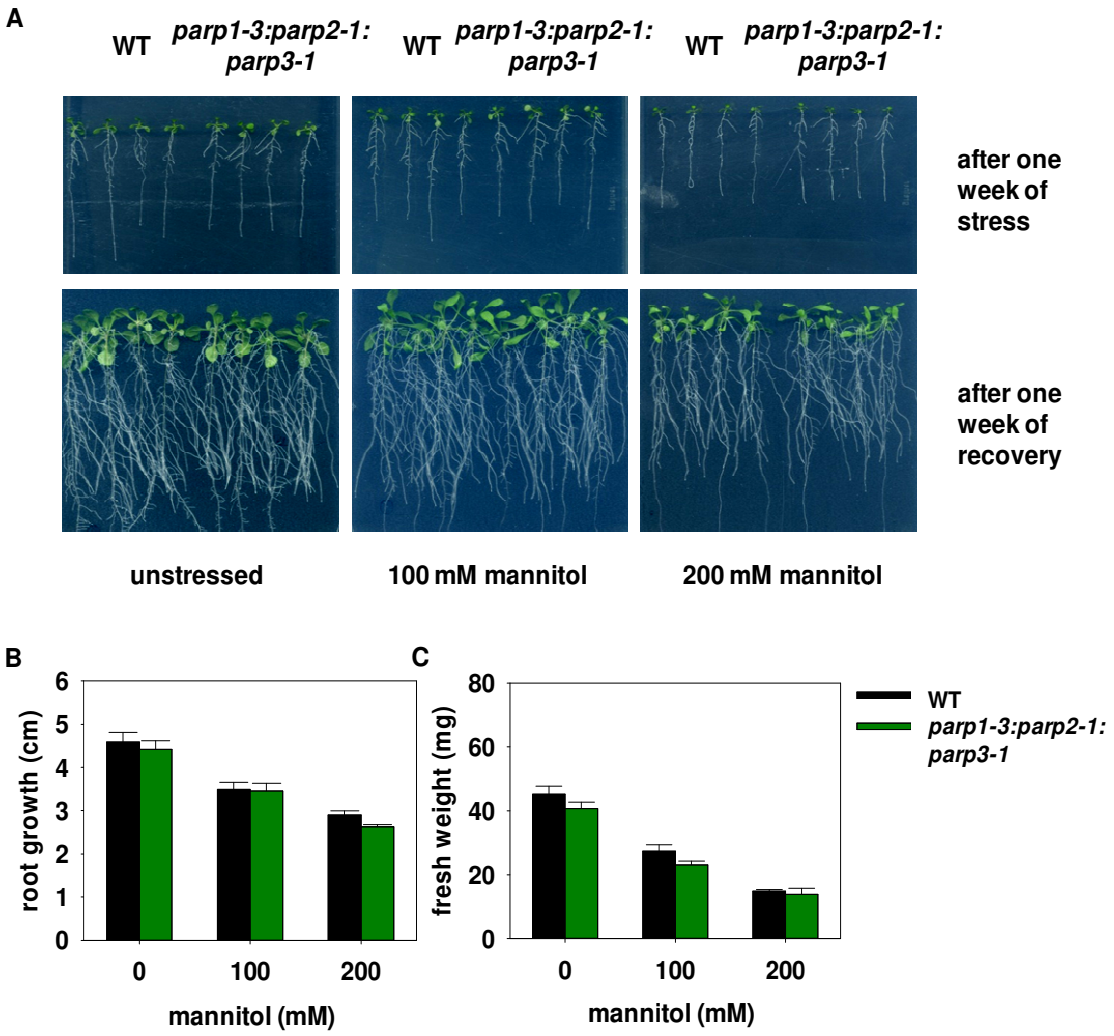

Figure 3.17 Effects of mannitol-induced osmotic stress on wild type and *parp1-3:parp2-1:parp3-1* mutant plants.

(A) Representative pictures of plants after osmotic stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. The experiment was performed three with similar results.

Conclusively, while a knockout of *PARP2* leads to a hypersensitivity of Arabidopsis to salt stress and osmotic stress, a knockout of both *PARP1* and *PARP2* surprisingly leads to an improved ability to recover from both stresses. Again, a triple knockout of all three *PARP* genes has no impact on tolerance

towards those stresses. The mannitol experiments thus confirmed the unexpected interactions of the *PARP* genes observed under salt stress.

3.6.3.3 The effect of sucrose supplementation on recovery from stress

The addition of sucrose to the growth medium is a common practice in the cultivation of plants under sterile conditions to facilitate a uniform development, but it may have unexpected physiological effects (Kwaaitaal *et al.*, 2011; Ranf *et al.*, 2012; Tracy *et al.*, 2008). In a recent study performed by Rissel *et al.* (2017b), in which growth of *parp* mutants under stress was assessed, no sucrose was added to the growth medium. In that study, mutation of *PARP*s had no effect on osmotic and salt stress tolerance, albeit the recovery was not examined. To test for a possible effect of sucrose on the observed phenotypes of *parp2-1* and *parp1-3:parp2-1*, the impact of salt and osmotic stress was again investigated in the absence of sucrose.

Figures 3.18 and 3.19 show the impact of sucrose supplementation on the *parp2-1* mutant under salt and osmotic stress conditions, respectively. The plants, wild type as well as mutant, show similar growth after one week of stress compared to the experiments including sucrose (Figs. 3.7, 3.13). The roots of both genotypes were generally a bit shorter under unstressed conditions as well as upon stress treatments. The hypersensitivity of *parp2-1* root growth to 50 mM NaCl (Fig. 3.7B) was not observed if sucrose was omitted (Fig. 3.18B). In contrast, the sensitivity of *parp2-1* to 200 mM mannitol was higher than that of the wild type on the sucrose-free plates (Fig. 3.19B), what was not observed before (Fig. 3.13B). After recovery, the plants were generally somewhat smaller compared to the experiments, which were performed with sucrose in the medium. Intriguingly, on the sucrose-free plates, the strong *parp2-1* phenotypes that were observed before (Figs. 3.8C, 3.13C) were absent upon recovery from salt stress (Fig. 3.18C) and mild mannitol stress (3.19C).

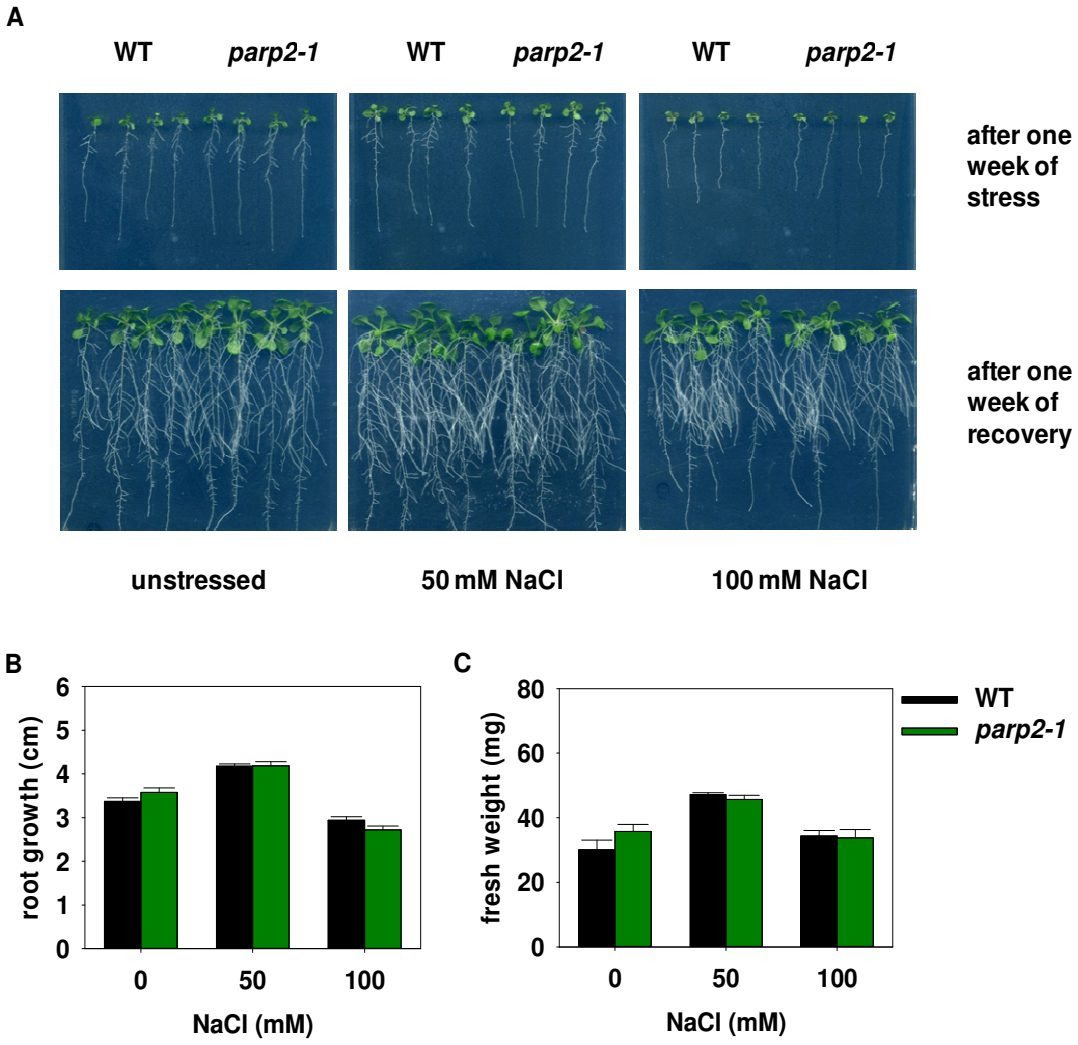

Figure 3.18 Effects of salt stress on wild type and *parp2-1* mutant plants in the absence of sucrose.

Plants were grown on agar plates without sucrose supplementation. **(A)** Representative pictures of plants after salt stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. The experiment was performed once.

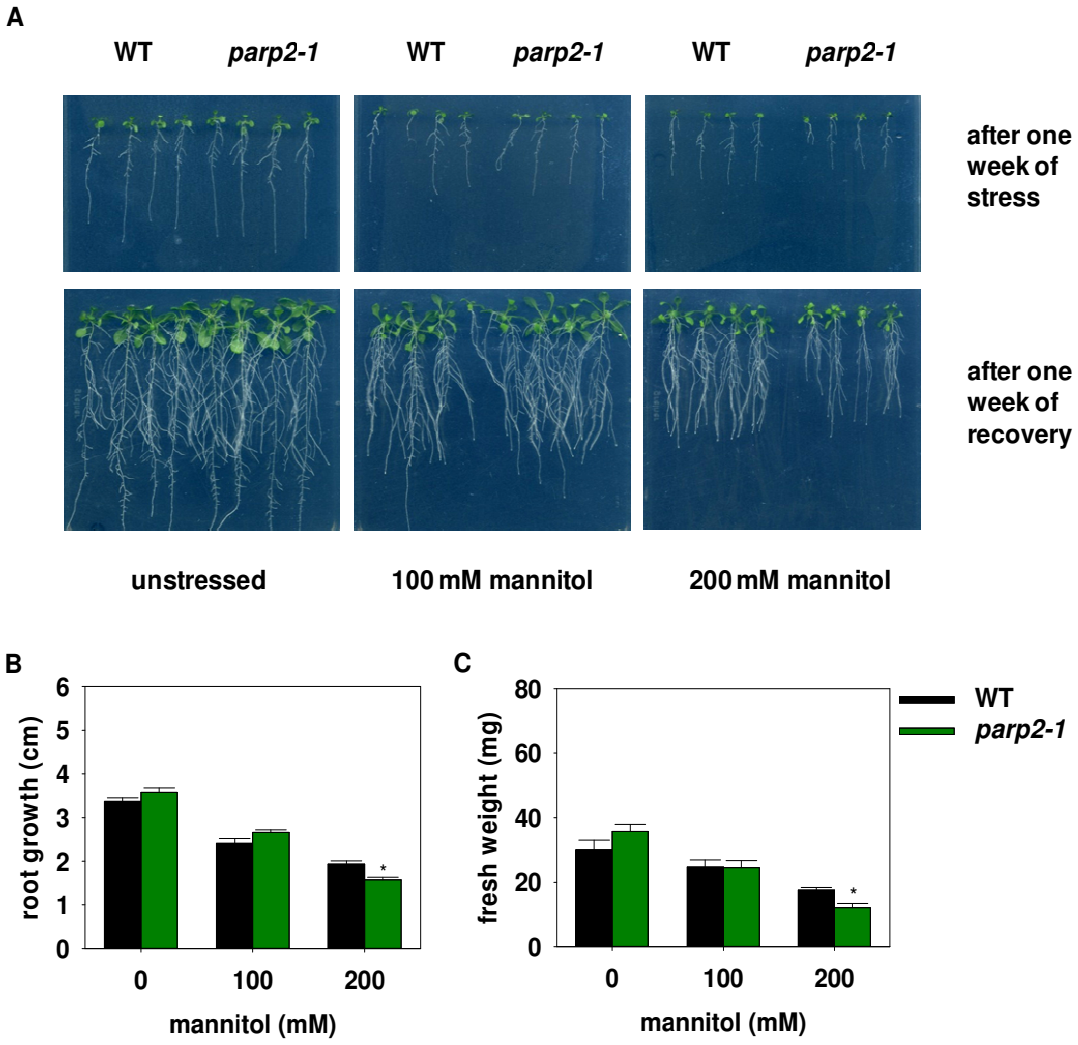

Figure 3.19 Effects of mannitol-induced osmotic stress on wild type and *parp2-1* mutant plants in the absence of sucrose.

Plants were grown on agar plates without sucrose supplementation. **(A)** Representative pictures of plants after osmotic stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. Significance was determined by Student's *t*-test (* $p<0.05$). The experiment was performed once.

A similar phenomenon was observed when the *parp1-3:parp2-1* double mutant was stressed with NaCl or mannitol in the absence of sucrose (Figs. 3.20, 3.21). If sucrose was supplemented, the *parp1-3:parp2-1* mutant showed an improved ability to recover from both stresses (Figs. 3.9, 3.15). However, in the absence of sucrose, this phenotype was not apparent after recovery from salt stress and high mannitol stress (Figs. 3.20C, 3.21C), and it was alleviated after recovery from mild mannitol stress (Fig. 3.21C).

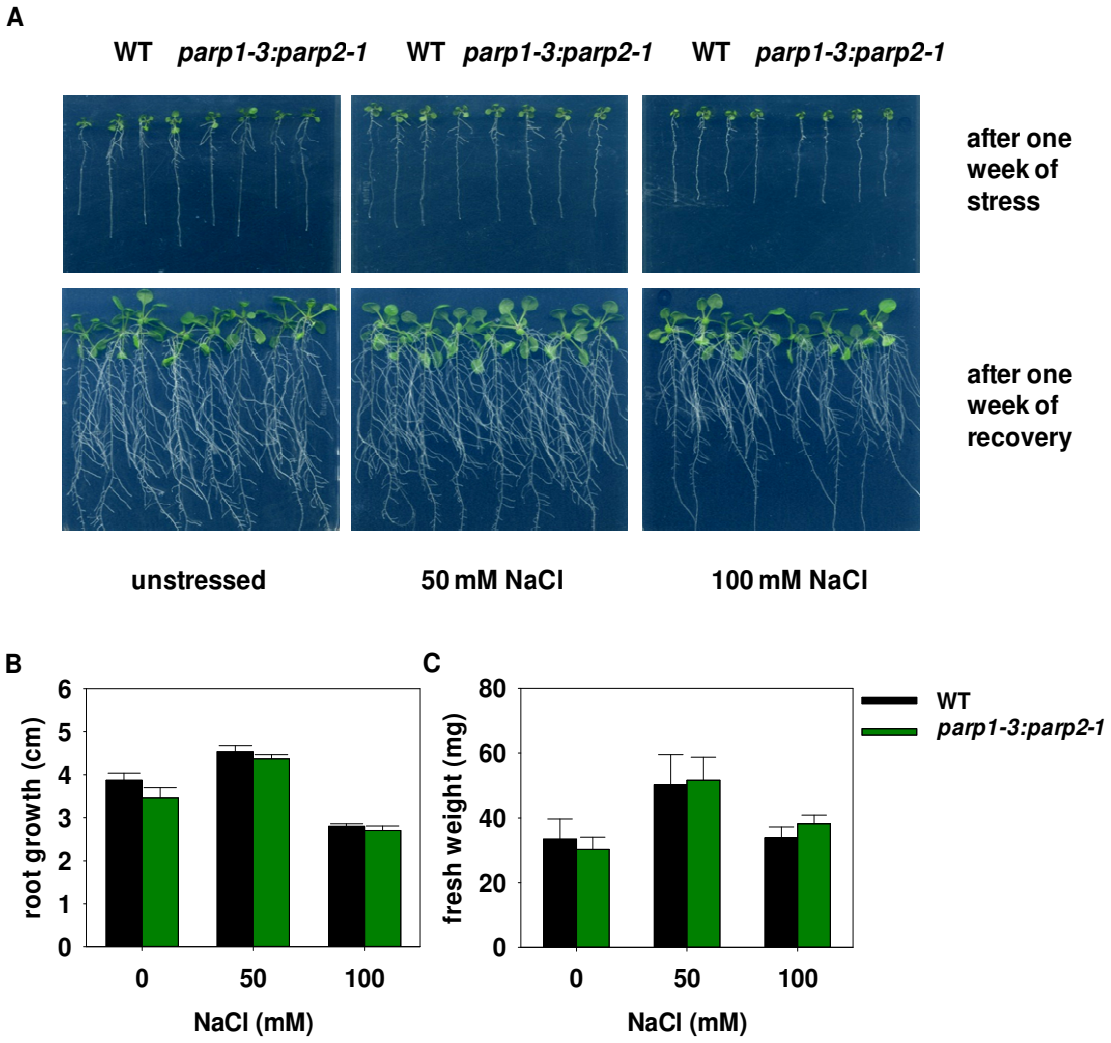

Figure 3.20 Effects of salt stress on wild type and *parp1-3:parp2-1* mutant plants in the absence of sucrose.

Plants were grown on agar plates without sucrose supplementation. **(A)** Representative pictures of plants after salt stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. The experiment was performed once.

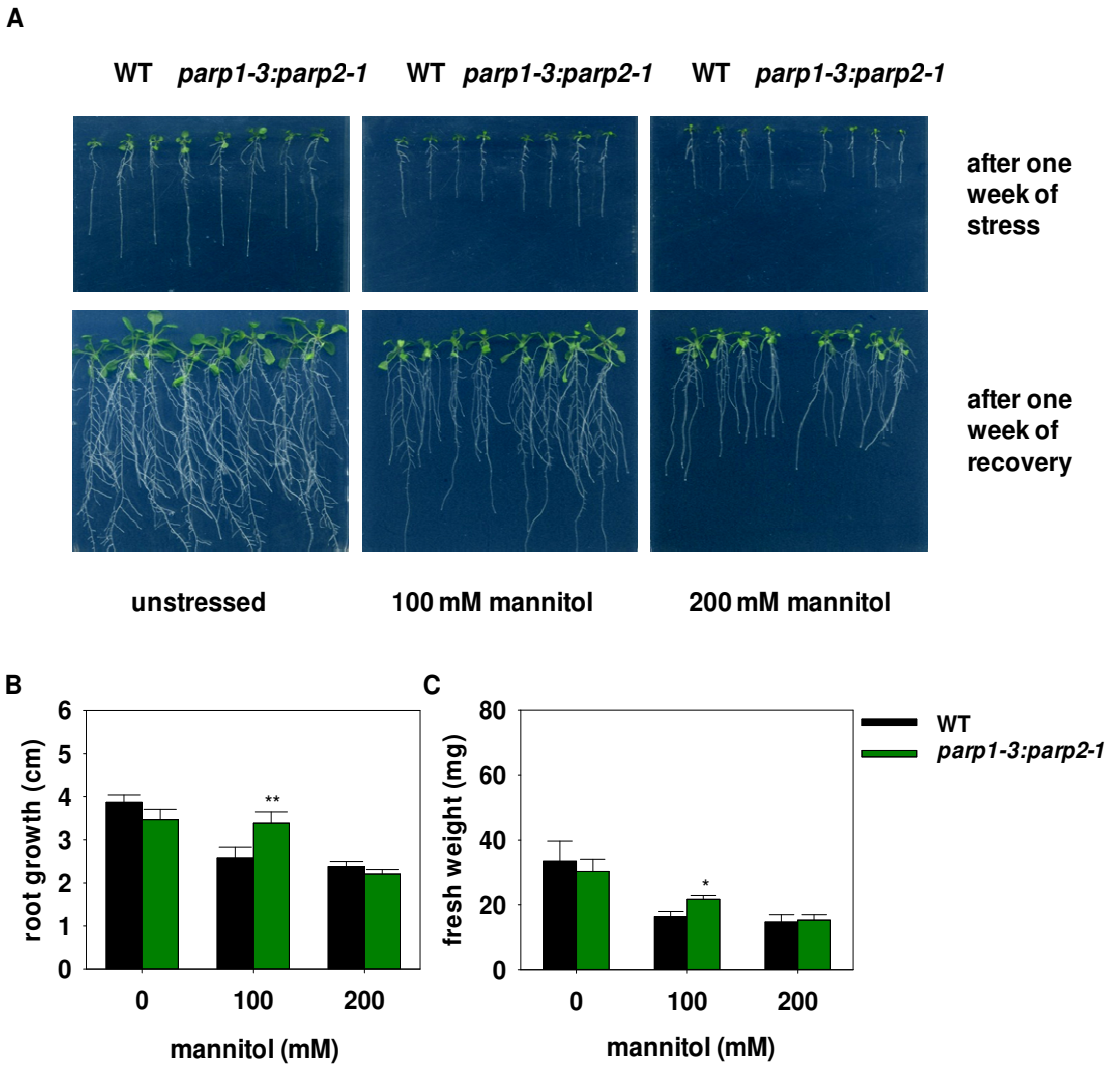

Figure 3.21 Effects of mannitol-induced osmotic stress on wild type and *parp1-3:parp2-1* mutant plants in the absence of sucrose.

Plants were grown on agar plates without sucrose supplementation. **(A)** Representative pictures of plants after osmotic stress and recovery. **(B)** Root growth after one week of stress. Each bar represents the average from 12 seedlings. Error bars represent SE. **(C)** Plant fresh weight after one week of recovery. Each bar represents the average from 3 plates, each containing 4 seedlings. Error bars represent SE. Significance was determined by Student's *t*-test (* $p<0.05$; ** $p<0.01$).The experiment was performed once.

Based on these findings, two conclusions can be drawn: First, based on the generally better growth of all plants in the presence of 0.5% sucrose, the availability of sucrose in the medium can have a positive impact on plant growth in general. Second, based on the attenuated phenotypes of both mutant lines in the absence of sucrose, the availability of sucrose determines to what extent PARPs interfere with the tolerance of the plant to and its recovery from abiotic stress.

3.6.4 Double knockout of *PARP1* and *PARP2* alters cADPR levels upon salt stress

It has been speculated that a reduced PARP activity during stress leads to increased levels of cyclic nucleotide ADP-ribose (cADPR) (Vanderauwera *et al.*, 2007), which again may impact stress resistance. Based on the better performance of the *parp1-3:parp2-1* double mutant in the stress experiments (Figs. 3.9, 3.15), it was hypothesized that the mutant may also accumulate higher cADPR levels upon NaCl treatment. Therefore, the double mutant and its wild type were analyzed for cADPR concentrations during salt stress.

Figure 3.22 shows the cADPR concentration in both genotypes upon treatment with 300 mM NaCl. Concentration and kinetics of cADPR differ strongly between both lines. Already under non-stressed conditions at the start of the experiment, the *parp* double mutant showed a significantly higher cADPR concentration than the wild type. This concentration nearly tripled within 1 h of salt stress, followed by a steady decline. In contrast, in the wild type, the cADPR level was only increased after 3 h of stress, albeit to a level exceeding that of the mutant.

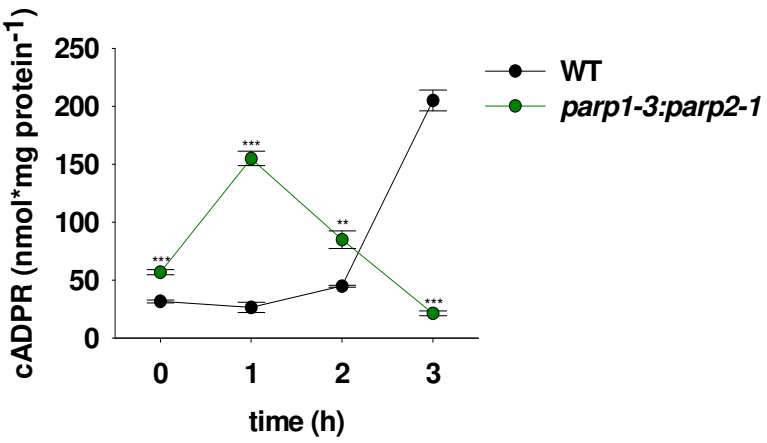

Figure 3.22 Effect of 300 mM NaCl on cADPR concentrations in wild type and *parp1-3:parp2-1* mutant plants.

Plants were treated with 300 mM NaCl at 0 h, and cADPR was extracted every hour post treatment. Data represent means ± SE from three technical replicates. Mean values were normalized to protein concentration. Significance was determined by Student's *t*-test (** $p<0.01$; *** $p<0.001$).

Conclusively, the knockout of *PARP1* and *PARP2* leads to an altered cADPR response upon salt stress, which is designated by a faster increase of the cADPR level.

3.6.5 Neither pharmacological nor genetic PARP inhibition affect early stress-induced changes in $[Ca^{2+}]_{cyt}$

It has been assumed that in PARP-deficient plants a higher level of cADPR causes an increased production of ABA-induced stress response proteins due to the Ca^{2+}-mobilizing activity of this second messenger, hence triggering Ca^{2+} signaling cascades (Vanderauwera *et al*., 2007). To investigate the effects of PARP activity on the generation of Ca^{2+} signals upon salt and osmotic stress, $[Ca^{2+}]_{cyt}$ of *APOAEQUORIN*-transformed wild type plants pre-treated overnight with the PARP inhibitors 3-AB, PJ-34, nicotinamide, or isonicotinamide, was determined using the aequorin bioluminescence reporter system (Fig. 3.23). As expected, injection of H_2O elicited a weak instantaneous touch response, whereby NaCl and mannitol triggered a fast response of greater magnitude. Upon H_2O injection, no difference in $[Ca^{2+}]_{cyt}$ between untreated and PARP inhibitor pre-treated plants was observed (Fig. 3.23 A-D left graphs). When plants were challenged with 300 mM NaCl or 500 mM mannitol, an immediate increase in $[Ca^{2+}]_{cyt}$ for both untreated and PARP inhibitor pre-treated plants was observed. In both cases $[Ca^{2+}]_{cyt}$ rapidly declined within a few minutes to the base level. For both stress treatments, no differences in the $[Ca^{2+}]_{cyt}$ response between untreated and PARP inhibitor pre-treated plants was apparent.

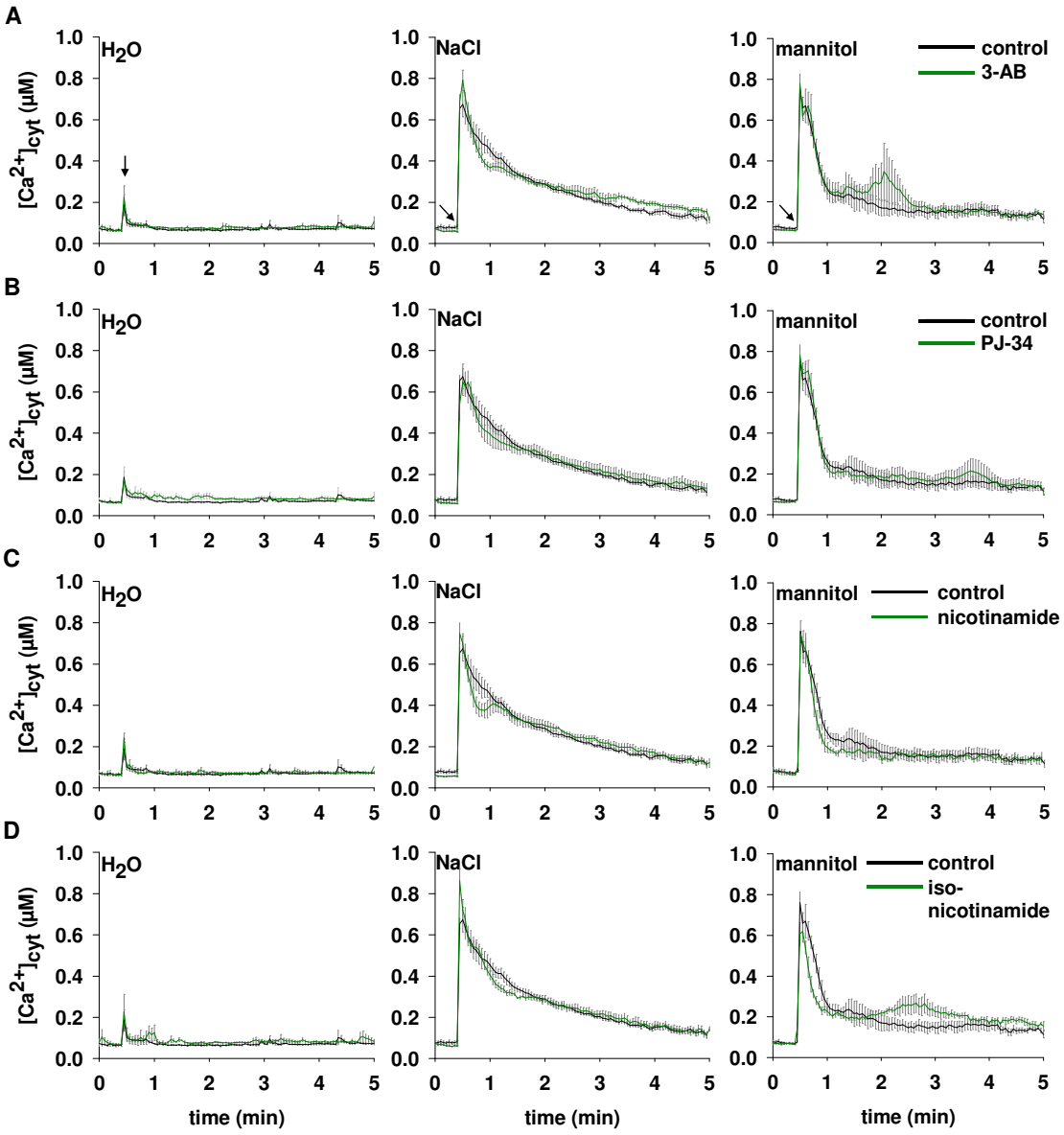

Figure 3.23 Response of cytosolic free Ca^{2+} concentration to NaCl and mannitol in PARP inhibitor pre-treated *APOAEQUORIN*-expressing wild type plants.

Plants were pre-treated with 10 µM 3-AB **(A)**, 10 µM PJ-34 **(B)**, 100 µM nicotinamide **(C)** or 100 µM isonicotinamide **(D)** overnight and treated either with H_2O (left), 300 mM NaCl (middle) or 500 mM mannitol (right). Arrows mark time of treatment injection. Data were recorded every 3 sec. Data represent means ± SE, $n \geq 4$.

To examine the initiation of the fast $[Ca^{2+}]_{cyt}$ elevation induced by NaCl and mannitol in more detail, the response was recorded with higher temporal resolution (Fig. 3.24). Again, upon injection of H_2O as well as of NaCl or mannitol, no difference in $[Ca^{2+}]_{cyt}$ was observed between untreated and PARP inhibitor pre-treated plants.

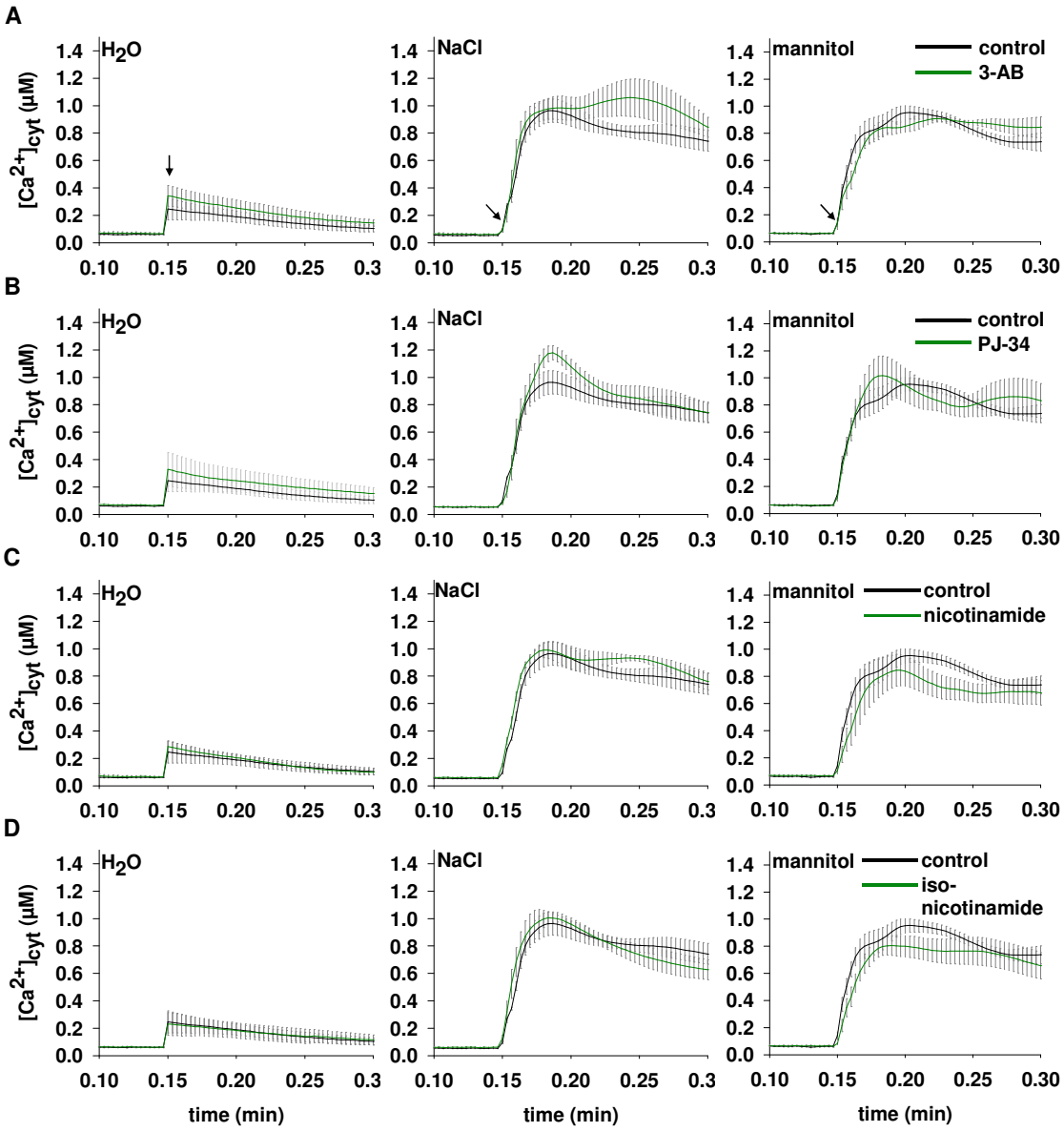

Figure 3.24 Response of cytosolic free Ca^{2+} concentration to NaCl and mannitol in PARP inhibitor pre-treated *APOAEQUORIN*-expressing wild type plants (high time resolution).

Plants were pre-treated with 10 µM 3-AB **(A)**, 10 µM PJ-34 **(B)**, 100 µM nicotinamide **(C)** or 100 µM isonicotinamide **(D)** overnight and treated either with H_2O (left), 300 mM NaCl (middle) or 500 mM mannitol (right). Arrows mark time of treatment injection. Data were recorded every 0.2 sec. Data represent means ± SE, $n \geq 3$.

To investigate the effect of a genetic interference with PARP activity on stress-induced Ca^{2+} signals, the *parp2-1* mutant line, which before showed a phenotype under osmotic and salt stress (Figs. 3.7, 3.13), was crossed with an *APOAEQUORIN*-transformed reporter line. A homozygous offspring line was compared to the wild type in its $[Ca^{2+}]_{cyt}$ responses to abiotic stress (Fig 3.25). The response of the *parp2-1* mutant was not different to that of the wild type, neither after H_2O injection nor upon treatment with NaCl or mannitol.

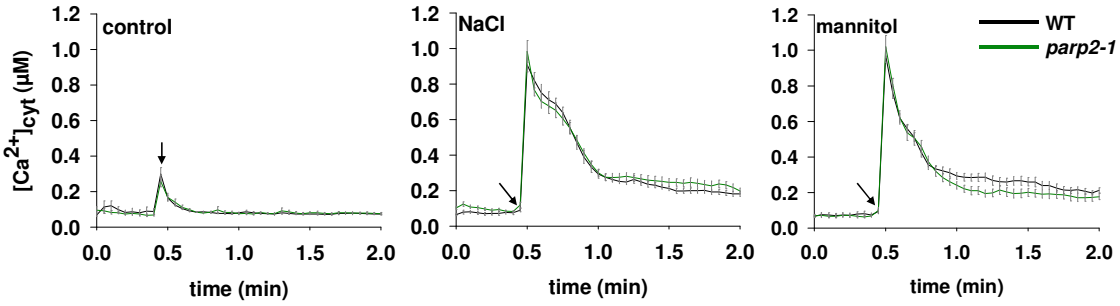

Figure 3.25 Response of cytosolic free Ca^{2+} concentration to NaCl and mannitol in *APOAEQUORIN*-expressing wild type and *parp2-1* mutant plants.

Plants were treated either with H_2O (left), 300 mM NaCl (middle) or 500 mM mannitol (right). Arrows mark time of treatment injection. Data were recorded every 3 sec. Data represent means ± SE, $n \geq 4$.

In conclusion, neither a pharmacological inhibition of PARP activity nor a genetic abolition of *PARP2* has an effect on fast $[Ca^{2+}]_{cyt}$ responses of Arabidopsis to salt and osmotic stress.

3.7 Discussion

Under abiotic stress conditions poly(ADP-ribosyl)ation is suspected to be counterproductive, which does mean that an impairment of PARP activity should have a positive impact on protecting the plant against negative effects of environmental stress (De Block *et al.*, 2005; Vanderauwera *et al.*, 2007; Geissler and Wessjohann, 2011; Schulz *et al.*, 2012). However, based on mutational analyses, this view has recently been debated (Rissel *et al.*, 2017b; Rissel and Peiter, 2019). The aim of this part was therefore to further examine the role of PARPs in plant abiotic stress responses. To this end, the effect of pharmacological and genetic PARP inhibition on abiotic stress response approaches was investigated.

3.7.1 Pharmacological PARP inhibition promotes transcriptional stress responses

In the first part of this thesis, the *RD29A* promoter, which is responsive to abiotic stress (Ishitani *et al.*, 1997; Kasuga *et al.*, 1999; Xiao *et al.*, 2006; Quist *et al.*, 2009), was chosen as a marker for identifying potential phytoeffectors by using the luciferase assay. The use of chemicals to enhance abiotic stress tolerance has been successfully applied in recent years, for example by applying hormones such as ABA, brassinosteroids, ethylene or even molecules with an unknown mode of action (Hwang and VanToai, 1991; Clarke *et al.*, 2004; Jakab *et al.*, 2005; Divi *et al.*, 2010). PARP inhibitors have also been used experimentally for such purposes (De Block *et al.*, 2005; Schulz *et al.*, 2012). In this work, this system was employed to specifically investigate the effect of the known PARP inhibitors 3-AB, PJ-34, nicotinamide, and isonicotinamide on *RD29A-LUC* activity during salt and osmotic stress (Fig. 3.3 and 3.4). It could be shown that all PARP inhibitors caused a higher *RD29A* activity upon both stressors. This result supports a positive role for pharmacological PARP inhibition during abiotic stress, at least at the transcriptional level.

There are several hypotheses regarding the positive effect of PARP inhibition under abiotic stress conditions. One of them has been put forward by Vanderauwera *et al.* (2007): A down-regulation of PARP activity during stress leads to increased levels of cADPR, which thereby induces the expression of

many ABA-responsive genes (Sanchez *et al.*, 2004). A link of PARP activity and ABA signalling is supported by the study of Vanderauwera *et al.* (2007), who found that *RD29A* expression during high-light stress was higher in *hpAtPARP2* RNAi than in wild type plants. The involvement of cADPR in ABA-dependent gene expression pathways was shown before by Wu *et al.* (1997), who microinjected the *GUS* reporter gene fused to the *RD29A* promoter into plant cells. *GUS* expression was enhanced upon injection of either cADPR or the *Aplysia* ADPR cyclase. This gene expression was blocked by the antagonistic cADPR analogue 8-NH_2-cADPR (Walseth and Lee, 1993; Wu *et al.*, 1997).

3.7.2 PARP inhibitors may have additional targets

PARPs are suspected to be functionally redundant, although it is not clear to what extent (Rissel *et al.*, 2017b). The application of PARP inhibitors, that caused an increased *RD29A-LUC* activity, likely targeted all PARP proteins. To get a deeper insight how an inhibition of each single *PARP* gene may affect the transcriptional stress response, we further analyzed the response of the *parp* knockout mutants *parp1-3*, *parp2-1*, and *parp3-1* harboring the *RD29A-LUC* construct to abiotic stress (Fig 3.5). Only the *parp2-1* mutant showed a higher *RD29A-LUC* activity upon NaCl treatment, whereas, surprisingly, the response of the *parp3-1* mutant was lower, and *parp1-3* showed no difference to the wild type. The response to osmotic stress by PEG was not altered in any of the mutants. These findings indicate that pharmacological PARP inhibition seems to be more effective than genetic knockout, what may be explained by the suspected redundancy of PARPs, but also by the existence of other or additional proteins targeted by PARP inhibitors. In line with this is a recent published transcriptomics study, which revealed that, under normal growth conditions, a treatment with the PARP inhibitors 3-AB and 3-MB alters the expression of 228 and 3935 genes, respectively (Briggs *et al.*, 2017). Additionally, the PARP inhibitor nicotinamide has a wide range of other roles in plants, and may inhibit several other enzyme activities (Hunt *et al.*, 2004). It is employed, for example, to inhibit cADPR formation in plants by the elusive ADPR cyclase (Dodd *et al.*, 2007). However, a parallel inhibition of PARPs and ADPR cyclase with different inhibitory constants would lead to complex results,

since inhibition of both enzymes is suggested to increase and decrease cADPR levels, respectively.

Possible further targets may be the previously described members of the SRO protein family, which not only contain a presumed catalytic PARP domain but are also proposed to be key regulators 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). Especially RCD1 and SRO1, the best-described members of this protein family, contain in addition to the catalytic PARP domain an N-terminal WWE domain and a C-terminal RST domain, which are known to mediate protein-protein interactions (Rissel *et al.*, 2017b). RCD1 undergoes interactions with numerous proteins, predominantly transcription factors, such as those of the DREB2 type (Belles-Boix *et al.*, 2000; Katiyar-Agarwal *et al.*, 2006; Jaspers *et al.*, 2009). Especially DREB2A, one of the main regulators of drought and heat responses, is regulated through protein stability (Sakuma *et al.*, 2006), and there is strong evidence that binding of RCD1 to DREB2A leads the protein to degradation (Vainonen *et al.*, 2012). Therefore, RCD1 acts as a negative regulator of DREB2A. Since SRO1 is partially redundant to RCD1, a similar role could also be assumed for this protein (Jaspers *et al.*, 2009). This would mean that a pharmacological inhibition of the PARP domain of RCD1 and perhaps of SRO1 may lead to a greater stability of DREB2A by prohibiting the interaction between these proteins, followed by an increased DREB2A activity and finally leading to a greater stress resistance. Since DREB2A is one of the transcription factors which activate the *RD29A* gene during abiotic stress (Jia *et al.*, 2012), this would be a possible explanation for the increased *RD29A-LUC* activity in the PARP inhibitor experiments (Fig. 3.3 and 3.4). It should, however, be noted that in addition to altered stress responses, *rcd1* loss-of-function mutants show severe developmental defects (Fujibe *et al.*, 2004; Jaspers *et al.*, 2009; Teotia and Lamb, 2009; Hiltscher *et al.*, 2014), and there is no literature which shows a similar effect by PARP inhibitors (De Block *et al.*, 2005; Adams-Phillips *et al.*, 2010; Geissler and Wessjohann, 2011; Schulz *et al.*, 2012). However, in contrast to a genetic knockout, a pharmacological inhibition of *RCD1* may affect the catalytic PARP domain only, with the RST domain still being active and possibly able to interact with transcription factors involved in plant development (Rissel *et al.*, 2017b).

3.7.3 PARPs genetically interact during abiotic stress in a complex way

Besides the presence of alternative or additional inhibitor targets, the different effects of pharmacological PARP inhibition (Figs. 3.3, 3.4) and genetic knockout of individual *PARP* genes (Fig. 3.5) on stress-induced *RD29A-LUC* activation may be a consequence of functional redundancy of the *PARP* genes (Rissel *et al.*, 2017b), because in contrast to a pharmacological inhibition that targets all PARP proteins, only one gene is affected in the single knockout plant. However, mutation of *PARP2* and *PARP3* showed opposite effects on *RD29A-LUC* activity, and *PARP1* mutation was without effect, which give rise to doubts regarding a simple functional redundancy. Alternatively, *PARPs* may be candidates for an unequal redundancy, whereby the knockout of one gene has a visible phenotype and the knockout of another gene has no distinguishable phenotype, but exacerbates the knockout phenotype of the first gene (Briggs *et al.*, 2006). Supporting a genetic interaction of *PARP* genes, Boltz *et al.* (2014) and Rissel *et al.* (2017b) showed that *PARPs* regulate each other, with *PARP1* and *PARP2* expression being increased in *parp2* and *parp1* mutants, respectively. Interestingly, *PARP3,* normally expressed only in seeds (Rissel *et al.*, 2014), showed dramatically increased transcript levels in both *parp1* and *parp2* plants (Boltz *et al.,* 2014). Therefore, the observed *RD29A-LUC* activity in the *parp* single mutants may not be the result of the elimination of each single *PARP* gene alone, but also affected by the increased transcription of the remaining *PARP* genes, which may functionally differ to the eliminated one. To follow up this idea, the transcript levels of the remaining *PARP* genes in stressed single mutant plants should be examined, and the *RD29A-LUC* reporter should be introduced into *parp* double and triple mutants.

The altered *RD29A* activity in some *parp* single mutants led to the assumption of a role for *PARPs* in transcriptional abiotic stress responses in Arabidopsis. Therefore, the effect of a genetic PARP inhibition on osmotic and salt stress tolerance was investigated using the *parp* single mutants, as well as double and triple mutants (Fig. 3.6-17). With the exception of *parp2-1* on 100 mM NaCl, the mutant lines showed no differences to wild type plants after one week of stress. This observation is in agreement with findings by Rissel *et al.* (2017b) that questioned a general importance of PARPs in plant performance under abiotic stress. However, in the present work the stress

treatment was followed by a recovery phase, in which a phenotype for some of the *parp* mutants could be clearly detected. Among the single *parp* mutants, only *parp2-1* showed a difference to the wild type. In fact, these plants recovered worse from both salt and osmotic stress. Intriguingly, this is opposite to the promotion of *RD29A-LUC* activity in this mutant (Fig. 3.5) and also in contrast to the generally presumed positive effect of *parp* knockout on stress tolerance. Knockout of *parp1-3* did not cause a recovery phenotype, which is in accordance with the absence of a difference in the luciferase experiment. However, the *parp3-1* mutant, which showed a significant lower *RD29A-LUC* activity during salt stress, did also not differ from the wild type in its recovery potential. This discrepancy may be explained by the different experimental conditions and stress intensities. The stress experiments were performed on plate-grown plants exposed to 50 or 100 mM NaCl, whereas in the luciferase assay 96-well-grown plants were treated with 300 mM NaCl. Alternatively, *RD29A-LUC* activity may not be a suitable proxy for stress recovery potential. As discussed in part 1, the *RD29A* promoter integrates different ABA-dependent and -independent stress response pathways, which renders this possibility unlikely. Hence, *RD29A* activity needs to be determined under the conditions of the stress and recovery experiments, which is currently technically challenging.

Regarding multiple *parp* knockout mutants, only *parp1-3:parp2-1* showed a phenotype in that is was better able to recover from abiotic stress than the wild type. Albeit this result is in line with the suggestion from the literature that an impairment of PARP activity has a positive impact on abiotic stress tolerance (De Block *et al.*, 2005; Vanderauwera *et al.*, 2007; Geissler and Wessjohann, 2011; Schulz *et al.*, 2012), it is very surprising, as the *parp2-1* phenotype is reversed if *PARP1* is deleted simultaneously, albeit knockout of *PARP1* alone has no effect. This complexity is further increased by the fact that deletion of *PARP3* in the triple mutant nullifies both the negative effect of *parp2* single knockout, as well as the positive effect of *parp1-3:parp2-1* double knockout.

These opposing results further suggest that PARPs are not simply functionally redundant, but may be unequally redundant and function in dependence of each other: the loss of function of *PARP2* has a strong phenotype and the loss of function of *PARP1* has no distinguishable phenotype, but in the *parp1-3:parp2-1* double mutant a novel phenotype appeared, which

both may again be altered in the *parp3-1* mutant. The mechanistic causes of these genetic interactions remain to be determined.

3.7.4 The role of PARPs during abiotic stress is context-dependent

The PARP proteins, particularly AtPARP1 and AtPARP2, have been suggested to be counterproductive during abiotic stress in plants (De Block *et al.*, 2005). For example, a reduced PARP activity caused by the expression of *PARP* hairpin constructs led to an increased tolerance of Arabidopsis plants to drought and oxidative stress (De Block *et al.*, 2005). In contrast, Rissel *et al.* (2017b) showed that Arabidopsis wild type and *parp* T-DNA insertional single, double and triple knockout mutants responded similarly to drought, salt, osmotic, and oxidative stress. In the present study, the *parp1-3:parp2-1* double mutant exhibited a better performance upon salt and osmotic stress compared to wild type plants, but only after recovery (Fig. 3.9 and 3.15). These opposing results lead to the conclusion that the role of PARPs during abiotic stress is context-dependent, meaning that they have an effect only under certain conditions. The plant genotype might be a decisive factor. For instance, De Block *et al.* (2005) used the Arabidopsis ecotype C24 in their experiments, whereas in the present work the Col-0 ecotype was used as a background for mutants. It is not unlikely that there are differences in osmotic and salt stress tolerance between these genotypes, since C24 has been found to be more susceptible to cold stress and UV-B irradiation as compared to Col-0 (Klotke *et al.*, 2004; Kalbina and Strid, 2006), and is also known to respond differently to NaCl treatment (Jha *et al.*, 2010; Schmöckel *et al.*, 2015). Growth conditions are also likely to determine the effects of genetic variations. In particular, Rissel *et al.* (2017b) did not add sucrose to the growth medium in their stress experiments, which is commonly used in the cultivation of plants under sterile conditions to promote uniform plant growth (Kwaaitaal *et al.*, 2011; Ranf *et al.*, 2012; Tracy *et al.*, 2008). To assess whether the presence of sucrose is essential for the observed phenotypes, in the present study, the *parp2-1* and *parp1-3:parp2-1* mutants were also analyzed for their performance under salt and osmotic stress in the absence of sucrose (Fig. 3.18-21). Intriguingly, the phenotype of both mutants after salt and osmotic stress was fully abolished and strongly weakened, respectively. These findings further support the idea of context-dependence or conditionality of *parp* mutant

phenotypes (Rissel and Peiter, 2019). Conditional phenotypes are not infrequent in loss-of-function mutants. For example, Lloyd and Meinke (2012) have found 522 conditional phenotypes among 2400 analyzed loss-of-function mutants. The availability of sucrose has been shown before to be responsible for the conditionality of a phenotype. For instance, Hauser *et al.* (1995) demonstrated that the conditional root expansion mutant *quill* exhibits comparable root length to the wild type on 0.5% sucrose, but shows dramatically shorter roots on 4.5% sucrose medium.

With respect to the hypothezised interrelationship of PARPs, cADPR, and $[Ca^{2+}]_{cyt}$ mentioned before and further discussed below, it is interesting to note that sucrose abolishes circadian $[Ca^{2+}]_{cyt}$ oscillations that are driven by cADPR (Johnson *et al.*, 1995; Dodd *et al.*, 2007). It remains to be determined if this effect of sucrose is mechanistically linked to the effect of sucrose on stress-related phenotypes of *parp* mutants.

In conclusion, there are several clues which suggest that phenotypes derived from *parp* loss-of-function mutants are context-dependent.

3.7.5 PARPs determine cADPR homeostasis

It has been suggested by Vanderauwera *et al.* (2007) that a reduction of PARP activity during stress leads to increased levels of cADPR. This cyclic nucleotide is a signaling molecule that can evoke increases in the concentration of $[Ca^{2+}]_{cyt}$ in both plants and animals (Hetherington and Brownlee, 2004; Zhang and Li, 2006) and was first described in sea urchin eggs (Lee *et al.*, 1989). It is assumed that *parp*-deficient plants consume less NAD^+ (Vanderauwera *et al.*, 2007), which is thereby available for synthesizing cADPR by an ADP-ribosyl cyclase (Lee, 2001). A further indirect hint that cADPR levels are affected by PARylation reactions is provided by the poly(ADP-ribose) glycohydrolase mutant *tej*, which shows a general defect in the circadian clock (Panda *et al.*, 2002) that, as discussed above, is regulated by cADPR (Dodd *et al.*, 2007).

Therefore, owing to its better performance in the stress experiments, the *parp1-3:parp2-1* double mutant was analyzed for its cADPR concentration during salt stress (Fig. 3.22). It was shown that the mutant plants not only exhibited a faster increase of the cADPR level in response to NaCl compared to wild type plants, but already had a higher basal cADPR concentration in non-

stressed conditions. Presumably, this may be the consequence of the assumed higher amount of NAD^+ in *parp*-deficient plants (Vanderauwera *et al.*, 2007), which remains to be determined. By now, there is no experimental information in the literature on the role of PARPs in cADPR homeostasis, but there is evidence for increased NAD^+ levels upon PARP inhibition. For example, Ishikawa *et al.* (2009) and Schulz *et al.* (2012) showed that Arabidopsis wild type plants treated with the PARP inhibitors 3-AB and 3-MB contained higher concentrations of NAD^+ than untreated plants, respectively. Furthermore, Pham *et al.* (2015) observed higher levels of NAD^+ in *parp3* plants under normal growth conditions. Hence, it should further be investigated whether *parp1-3:parp2-1* mutant plants also contain altered amounts of NAD^+.

It should however be noted that higher levels of NAD^+ may not necessarily result in higher amounts of cADPR because NAD^+ is a ubiquitous coenzyme in plants, which is required for numerous anabolic and catabolic pathways, and is therefore generally essential for plant growth and development (Hunt *et al.*, 2004; Pétriacq *et al.*, 2013). For example, it is also consumed for histone deacetylation through sirtuins (Hunt *et al.*, 2004). However, the present data indicate that a loss of function of both *PARP1* and *PARP2* leads to an altered cADPR status in Arabidopsis.

3.7.6 PARPs do not play a role in the generation of Ca^{2+} signals induced by osmotic and salt stress

Ca^{2+} acts as a second messenger involved in many different plant processes, and alterations in $[Ca^{2+}]_{cyt}$ represent one of the first steps in plant stress signaling (Batistič and Kudla, 2012; Schmöckel *et al.*, 2015). This also includes abiotic stimuli like salt and drought stress. Using the aequorin-based bioluminescence assay, Knight et al. (1997) already showed an increase in $[Ca^{2+}]_{cyt}$ in intact seedlings of Arabidopsis upon NaCl and mannitol treatment. In such situations, the influx of Ca^{2+} into the cytosol can be evoked by specific Ca^{2+} channels from both the apoplast and intracellular stores (Kudla *et al.*, 2010). Thereby, intracellular Ca^{2+} release is mediated by several Ca^{2+}-releasing second messengers, including cADPR (Sanders *et al.*, 2002). The first demonstration of cADPR-sensitive Ca^{2+}-channels in plants was reported by

Allen *et al.* (1995) using a Ca^{2+}-release assay, as well as the patch-clamp technique.

In the present work it could be shown that a knockout of *PARP* genes can to an altered cADPR homeostasis in Arabidopsis. According to Vanderauwera *et al.* (2007), such changed levels of cADPR may be causative for an increased production of ABA-regulated stress response proteins via Ca^{2+}-mediated signaling cascades. Therefore, it was investigated whether PARP inhibition also affects the initial $[Ca^{2+}]_{cyt}$ response upon salt or mannitol-induced osmotic stress (Fig. 3.23-25). Interestingly, neither a pharmacological PARP inhibition nor the loss of function of *PARP2* altered the magnitude or kinetics of the Ca^{2+} signal. These results suggest that PARPs do not play a role in the early salt and osmotic stress-induced Ca^{2+} signaling network.

So far, there are no comparable investigations on the role of PARPs in Ca^{2+} signaling in the literature, but in a different context Tracy *et al.* (2008) used nicotinamide to inhibit cADPR-induced Ca^{2+} release from internal stores, finding that the height of the initial NaCl-induced $[Ca^{2+}]_{cyt}$ peak was not altered, supporting the present findings. Furthermore, studies with the Ca^{2+} chelator EGTA, which captures the Ca^{2+} from the external medium, have shown that the main, but not the only, source of Ca^{2+} in the generation of osmotic and salt-induced Ca^{2+} signals is the apoplast (Knight *et al.*, 1997).

However, an involvement of PARP activity in the generation of Ca^{2+} signals, possibly through altered cADPR homeostasis, cannot be excluded yet based on the present experiments. First, cellular Ca^{2+} signatures or systemically propagating Ca^{2+} signals may be altered that both go unnoticed in whole-plant measurements using aequorin (Choi *et al.*, 2014; Thor and Peiter, 2014; Kiep *et al.*, 2015). Second, owing to the time needed for cADPR synthesis, additional Ca^{2+} signals may be generated a later time points. Those possible scenarios call for further elucidation of the role of poly(ADP) ribosylation in Ca^{2+} signaling.

3.8 Conclusions

In times of climate change, more frequently occurring situations of abiotic stress, such as drought and salinity, lead to an increasing insecurity of yield stability. To satisfy food supply it is needed to make crops more tolerant against those kinds of stress. The inhibition of PARPs, pharmacologically or genetically, has in the past been suggested as an approach to stabilize crop yields, since PARP inhibition has been shown to improve plant stress responses.

In the present study, the role of PARPs as negative regulators of abiotic stress responses could be confirmed only partially. In contrast to previous findings by some other authors, the genetically generated loss-of-function of *PARPs* led to a better performance under abiotic stress only by the double knockout of *PARP1* and *PARP2* and was mainly evident in the recovery phase. Moreover, the knockout of *parp2* alone was even deterimental to stress recovery. Additionally, those phenotypes were context-dependent. This not only questions the suitability of PARPs as stable regulators of plant abiotic stress responses, but there is also a need for further analyzing the relevant developmental stages and conditions in which stress responses are modified by PARPs.

The present study further revealed that pharmacological PARP inhibition increases the transcriptional stress response, implicating PARP inhibitors as direct or indirect positive regulators of the stress-responsive transcriptional pathway. Albeit this was confirmed by the transcriptional response of a *parp2* mutant, it was opposite to that of a *parp3* mutant. Moreover, PARP inhibitors have been shown to affect plant abiotic stress responses more consistently than *parp* loss-of-function mutants. Hence, there are strong indications that PARP inhibitors have other targets than PARPs, which remain to be identified.

Conclusively, the use of PARP inhibition to stabilize crop yield and sustain food safety should be treated with caution, since the role of PARPs in plant stress responses is still far from being well understood.

4 Summary

Global warming threatens the worldwide agriculture, since abiotic stress factors are increasing presently and in the future. Thus, crop productivity and therefore food supply are in danger, and crops that are better adapted to abiotic stress are needed. Since the use of genetically modified crops is still poorly accepted in some parts of the world, the application of phytoeffectors offers a promising alternative to support the plant's own defense mechanisms. This thesis thus aimed (1) to establish a plant-based method for the identification of phytoeffectors, which could be able to enhance the plant's tolerance against abiotic stress, and (2) to elucidate the role of poly(ADP-ribose) polymerases (PARPs), which are targeted by PARP inhibitors as potential phytoeffectors, in abiotic stress responses.

To allow the identification of potential phytoeffectors, an assay based on the activation of resistance mechanisms at the transcriptional level was established. To this end, the light-emitting *firefly luciferase* (*LUC*) reporter gene expressed under control of the stress-responsive *RD29A* promoter was used. Arabidopsis plants expressing this construct were treated with abiotic stressors in combination with test substances potentially able to increase the transcriptional stress response. Those substances had been *in silico*-designed to target PARPs. Although this assay could not serve the purpose of a high-throughput procedure, some substances could be identified as positive regulators of the transcriptional stress response.

Poly(ADP-ribosyl)ation is a post-translational modification of proteins that was first discovered in mammalian cells. In response to DNA strand breaks, HsPARP1 uses NAD^+ as a substrate for transferring ADP-ribose residues to acceptor proteins and itself. In plants, PARPs have also been described to be involved in DNA damage responses. Additionally, they have been shown to have an impact on plant responses to external stressors, biotic as well as abiotic. In abiotic stress responses, PARPs have been suggested to act as negative regulators, since an impairment of PARP activity, conferred by pharmacological or genetic approaches, has been shown to improve plant performance under abiotic stress. However, such a role has recently been

debated. To further clarify the role of PARPs under abiotic stress, T-DNA knockout mutants for *AtPARP1*, *AtPARP2* and *AtPARP3* as well as two double and a triple knockout mutant were subjected to salt and osmotic stress. Surprisingly, *parp2-1* mutant plants were shown to recover worse from both salt and osmotic stress compared to the wild type, whereas the *parp1-3:parp2-1* mutant was able to recover better. The remaining *parp* mutants did not differ from the wild type in these experiments. These results indicate that PARPs function in dependence of each other. In addition, the *parp1-3:parp2-1* mutant was analyzed for its cADPR concentration during salt stress, since cADPR has been predicted to increase during abiotic stress in PARP-deficient plants. Indeed, the double mutant showed an altered cADPR status compared to the wild type. The observed phenotypes of *parp2-1* and *parp1-3:parp2-1* were strongly affected by the availability of sucrose, revealing these phenotypes as being context-dependent, which is supported by previous studies. In contrast to the unstable phenotypes achieved by *parp* loss-of-function mutants in this and other studies, pharmacological PARP inhibition has been shown to affect plant abiotic stress responses more robustly. When comparing the *RD29A-LUC* activity as proxy for the transcriptional stress response of pharmacologically PARP-inhibited plants and *parp* knockout mutant plants under abiotic stress, the pharmacological inhibition led to a more consistent result, indicating the existence of unequal functional redundancy and/or additional target proteins of PARP inhibitors.

In summary, phytoeffectors may offer a promising opportunity for plants to cope with situations of abiotic stress, and the screening approach developed in this thesis may contribute to their identification. Due to the context-dependence of PARP-related stress phenotypes and the uncertainty which proteins are potentially further targeted by PARP inhibitors, it cannot be stated whether targeting PARPs by phytoeffectors provides a suitable tool to stabilize plant performance under abiotic stress.

5 Zusammenfassung

Die globale Erwärmung stellt für die weltweite Landwirtschaft eine ansteigende Bedrohung dar, da abiotische Stressfaktoren sowohl aktuell als auch zukünftig immer mehr zunehmen werden. Die daraus resultierende Gefährdung der landwirtschaftlichen Produktivität und somit auch der Nahrungsmittelversorgung bedingt, dass besser an abiotischen Stress angepasste Nutzpflanzen von Nöten sind. Da der Einsatz genetisch modifizierter Pflanzen in einigen Regionen auf der Welt nur wenig Akzeptanz erfährt, bietet die Verwendung von Phytoeffektoren eine vielversprechende Alternative, um die pflanzeneigenen Abwehrmechanismen zu unterstützen. Die vorliegende Arbeit zielt daher darauf ab, (1) ein pflanzenbasiertes System für die Identifizierung von Phytoffektoren, welche die pflanzliche Stresstoleranz gegenüber abiotische Faktoren erhöhen könnten, zu entwickeln und (2) die Rolle von Poly (ADP-Ribose) -Polymerasen (PARPs), auf die PARP-Inhibitoren als potenzielle Phytoeffektoren abzielen, bei abiotischen Stressreaktionen genauer zu untersuchen.

Für die Identifizierung potentieller Phytoffektoren wurde ein Assay basierend auf der Aktivierung von Resistenzmechanismen auf transkriptioneller Ebene entwickelt. Zu diesem Zweck wurde das Licht-emittierende *firefly luciferase* (*LUC*) Reportergen unter Kontrolle des durch Stress aktivierten *RD29A* Promotors genutzt. Arabidopsis-Pflanzen, die dieses Konstrukt exprimieren, wurden abiotischen Stimuli in Kombination mit Testsubstanzen ausgesetzt, welche die transkriptionelle Stressantwort potentiell erhöhen könnten. Diese Substanzen wurden *in silico* designt und zielen auf PARPs ab. Obwohl das Assay nicht dem angestrebten Ansatz eines Hochdurchsatzverfahrens entsprach, konnten einige Substanzen als positive Regulatoren der transkriptionellen Stressantwort identifiziert werden.

Poly(ADP-ribosyl)ierung ist eine posttranslationale Proteinmodifikation, die zuerst in Säugetierzellen beschrieben wurde. Aktiviert durch DNA Strangbrüche überträgt HsPARP1 ADP-Ribose-Einheiten auf sich selbst und auf andere Zielproteine, wobei NAD^+ als Substrat fungiert. In Pflanzen wurde PARP-Proteinen ebenfalls eine Beteiligung bei DNA-Reparaturprozessen

zugeschrieben. Darüber hinaus wurden sie mit der pflanzlichen Antwort auf verschiedene, sowohl biotische als auch abiotische, Stressoren in Verbindung gebracht. Bei der Reaktion auf abiotische Stressfaktoren wird angenommen, dass PARPs als negative Regulatoren agieren, da festgestellt werden konnte, dass eine durch pharmakologische oder genetische Ansätze verursachte Beeinträchtigung der PARP-Aktivität die Pflanzenleistung unter abiotischem Stress verbessert. Eine solche Rolle wurde jedoch erst kürzlich diskutiert. Um die Funktion von PARPs bei der Reaktion auf abiotischen Stress weiter aufzuklären, wurden T-DNA-Insertionsmutanten für *AtPARP1*, *AtPARP2* und *AtPARP3* sowie zwei *parp*-Doppel- und eine *parp*-Dreifachmutante osmotischem und Salzstress ausgesetzt. Überraschenderweise erholte sich die *parp2-1* Mutante gegenüber dem Wildtyp schlechter von beiden Stressstimuli, während sich die *parp1-3:parp2-1* Mutante vergleichsweise besser erholte. Die verbleibenden *parp*-Mutanten zeigten in diesen Experimenten keinen Unterschied zum Wildtyp. Dieses Ergebnis legt nahe, dass PARPs in Abhängigkeit voneinander arbeiten. Zusätzlich wurde die *parp1-3:parp2-1* Mutante hinsichtlich ihrer cADPR-Konzentration unter Salzstress untersucht, da gezeigt werden konnte, dass das cADPR-Level in PARP-defekten Pflanzen bei abiotischem Stress ansteigt. In der Tat konnte in der *parp*-Doppelmutante ein veränderter cADPR-Status gegenüber dem Wildtyp detektiert werden. Weiterhin konnte festgestellt werden, dass die erzielten Phänotypen von *pap2-1* und *parp1-3:parp2-1* stark abhängig von der Verfügbarkeit von Saccharose waren. Dies spricht, wie auch in anderen Studien postuliert wird, für einen kontextabhängigen Phänotyp. Im Gegensatz zu den unsteten Phänotypen, die durch die Verwendung von *parp*-Mutanten in dieser und anderen Arbeiten erzielt wurden, konnte die pharmakologische PARP-Inhibierung die pflanzliche Reaktion auf abiotischen Stress konsistenter beeinflussen. Beim Vergleich der *RD29A-LUC* Aktivität als Indikator für die transkriptionelle Stressantwort von pharmakologisch erzeugten PARP-beeinträchtigten Pflanzen und *parp*-Mutanten unter abiotischem Stress, zeigte die pharmakologische Inhibierung ein konsistenteres Ergebnis. Dies spricht für eine ungleiche funktionale Redundanz und/oder zusätzliche Zielproteine von PARP-Inhibitoren.

Zusammenfassend kann gesagt werden, dass Phytoeffektoren eine vielversprechende Möglichkeit für Pflanzen darstellen, um mit abiotischen

Stresssituationen umzugehen und der in dieser Arbeit entwickelte Screening-Ansatz zu deren Identifizierung beitragen kann. Aufgrund der Kontextabhängigkeit der durch PARP beeinflussten Stressphänotypen und der Unsicherheit, auf welche Proteine PARP-Inhibitoren möglicherweise darüber hinaus abzielen könnten, kann nicht klar gesagt werden, ob PARPs als Ziel für Phytoeffektoren ein geeignetes Instrument zur Stabilisierung der Pflanzenleistung unter abiotischem Stress darstellen.

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Eidesstattliche Erklärung / *Declaration under Oath*

Hiermit versichere ich, 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 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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Publications and contributions to meetings

Publications

Kiep, V., Vadassery, J., Lattke, J., Maaß, J-P., Boland, W., Peiter, E., Mithöfer, A. (2015) Systemic cytosolic Ca^{2+} elevation is activated upon wounding and herbivory in Arabidopsis. *New Phytologist* 207: 996-1004

Talks

Kiep, V., Peiter, E.
Molecular and pharmacological approaches to enhance abiotic stress tolerance in plants
18. Statusseminar des Agrochemischen Institutes Piesteritz e.V.,
Piesteritz 2015

Kiep, V., Peiter, E.
From beetle to phytoeffectors
17. Statusseminar des Agrochemischen Institutes Piesteritz e.V.,
Piesteritz 2014

Kiep, V., Peiter, E.
Boosting the transcriptional stress response by phytoeffectors
16. Statusseminar des Agrochemischen Institutes Piesteritz e.V.,
Piesteritz 2014

Kiep, V., Neugebauer, K., Peiter-Volk, T., Peiter, E.
Ein pflanzenbasiertes System zur Identifizierung von Wirkstoffen zur Verbesserung der Stresstoleranz von Nutzpflanzen
7. Agrosnet-Doktorandentag, Halle 2014

Kiep, V., Peiter, E.
Plant based identification of phytoeffectors
15. Statusseminar des Agrochemischen Institutes Piesteritz e.V.,
Piesteritz 2013

Kiep, V., Peiter, E.
Plant based identification of phytoeffectors
14. Statusseminar des Agrochemischen Institutes Piesteritz e.V.,
Piesteritz 2013

Poster presentations

Kiep, V., Rissel, D., Peiter-Volk, T., Janssen, A., Peiter, E.
Phytoeffectors: (From) Molecules to the Field
International Conference of the German Botanical Society, München 2015

Kiep, V., Rissel, D., Peiter-Volk, T., Janssen, A., Peiter, E.
Phytoeffectors - the rescue from abiotic stress conditions?
Meeting of the German Society of Plant Nutrition, Göttingen 2015

Kiep, V., Rissel, D., Peiter-Volk, T., Peiter, E.
Boosting the transcriptional stress response: A plant-based system for identifying phytoeffectors, International Conference of the German Society of Plant Nutrition, Halle 2014

Gong, X., **Kiep, V.**, Janssen, A., Peiter, E., Thor, K.
Characterization of Arabidopsis homologues to a leguminous Ca^{2+} signal modulator.
International Conference of the German Society of Plant Nutrition, Bonn 2012

Thor, K., Gong, X., **Kiep, V.**, Janssen, A., Peiter, E.
From nodules to stress response – towards the function of Arabidopsis homologues to a leguminous Ca^{2+} signal modulator
International Symposium "Communication in Plants and their Responses to the Environment", Halle 2011

Thor, K., Gong, X., **Kiep, V.**, Janssen, A., Peiter, E.

More than nodules? Investigating Arabidopsis homologues to a leguminous Ca^{2+} signal modulator

International Symposium “Plant Calcium Signaling”, Münster 2010

Thor, K., Gong, X., **Kiep, V.**, Janssen, A., Peiter, E.

More than nodules? Investigating Arabidopsis homologues to a leguminous Ca^{2+} signal modulator

Meeting of the German Society of Plant Nutrition, Hannover 2010