The role of *ALDH* in drought stress

A comparative analysis in *A. thaliana* and barley

Dissertation

Zur Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Bonn, 2022

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

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Day of oral examination: 6th of July, 2022

Year of publication: 2022

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Abbreviation

°C	Celsius
µl	Microliter
aa	Amino acid
ABA	Abscisic acid
ALDH	Aldehyde dehydrogenase
APS	Ammonium persulfate
B/W	Black/White
Ba	Basal thermotolerance
bp	Base pair
cDNA	Complementary DNA
CDT	Controlled deterioration test
CO_2	Carbon dioxide
CRT	Chloroquine resistance transporter
CTAB	Cetyltrimethylammonium bromide
dH2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DRE	Dehydration response element
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EST	Expression sequence tags
EtOH	Ethanol
FLC	FLOWERING LOCUS C
g	Gram
g	gravity acceleration (9.81)
GFP	Green fluorescent protein
GUS	β-glucuronidase
HCl	hydrochloric acid
hr	Hour(s)
Kb	Kilo-base
kDa	Kilo-Dalton
KO	Knock-out
LB	Luria and Bertani medium
LiCl	Lithium chloride
M	Molar
MDA	Malonaldehyde
MgCl2	Magnesium chloride
MgSO4	Magnesium sulphate
min	Minute(s)
ml	Milliliter
mm	Millimeter
mM	Millimolar
MS	Murashige and Skoog
NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
NaOCl	Sodium Hypochlorite
mg	Milli-gram
ng	Nano-gram
nm	Nano-molar
OD	Optical density
OE	Overexpression
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	Pondus Hydrogenii
PSI	Pound per square inch
PUFAs	Polyunsaturated fatty acids
RCS	Reactive carbonyl species
redox	Reduction/oxidation
RNA	Ribonucleic acid
ROIs	Reactive oxygen intermediaries
ROS	Reactive oxygen species
RWC	Relative water content
SDS	Sodium dodecyl sulphate
SOC	Super Optimal broth with Catabolites repression
Sp.	Species
TAE	Tris base, acetic acid and EDTA.
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultra-violet
V	Volts
v/v	Volume/volume
w/v	Weight/volume
WT	Wild-type
YEP	Yeast Extract Peptone

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1 Introduction

1.1 The effect of drought stress on plants

The green-house gases resulting from man-made activities have resulted in increased number of heat-related events, such as heat waves. The increased number and intensity of such events is projected to escalate both the temperature and the drought in the Mediterranean, central Europe, the southern Amazon, and southern Africa regions. It will impact the ecosystem, followed by food security issues (Shukla *et al.*, 2019).

Drought is considered the most devastating stress that plants might encounter in their lifetime. It is defined as the lack of adequate moisture that would allow the plant to complete its life cycle (Manivannan *et al.*, 2008). It affects several aspects of plants' life as follows

1.1.1 Crop growth and yield

Cell growth is one of the most sensitive processes to water deficiency, as it reduces the cell turgor pressure. In severe water deficiency conditions, cell elongation might be inhibited in higher plants which in turn reduces cell growth. Depending on the developmental stage in which the drought stress occurs, yield reduction is a grave possibility for several crops (Taiz *et al.*, 2015). In maize, drought stress during the vegetative stages could cause yield reduction from 25 to 60%. If the drought stress happened during reproductive stages, the yield reduction could reach 92% (Atteya, 2003). In barley, if the plant experienced water-limiting conditions during the seed-filling stage, the yield could suffer 49 to 57% loss (Samarah, 2005).

1.1.2 Photosynthesis

One of the most seriously affected processes by drought stress is photosynthesis (Chaves *et al.*, 2009). The decline in the process occurs through multiple pathways. First, the stress lowers tissue water potential, which affects the ribulose bisphosphate carboxylase/oxygenase (Rubisco) enzyme activity and diminishes the activity of photosynthesis-related enzymes (Bota *et al.*, 2004). Second, drought stress activates ABA signaling, which in turn, increase stomatal closure to decrease water-loss through transpiration. The resulting closure decreases the influx of CO_2, which not only decreases the carboxylation process and declines photosynthesis, but also increases the production of reactive oxygen species (ROS) (Farooq *et al.*, 2009).

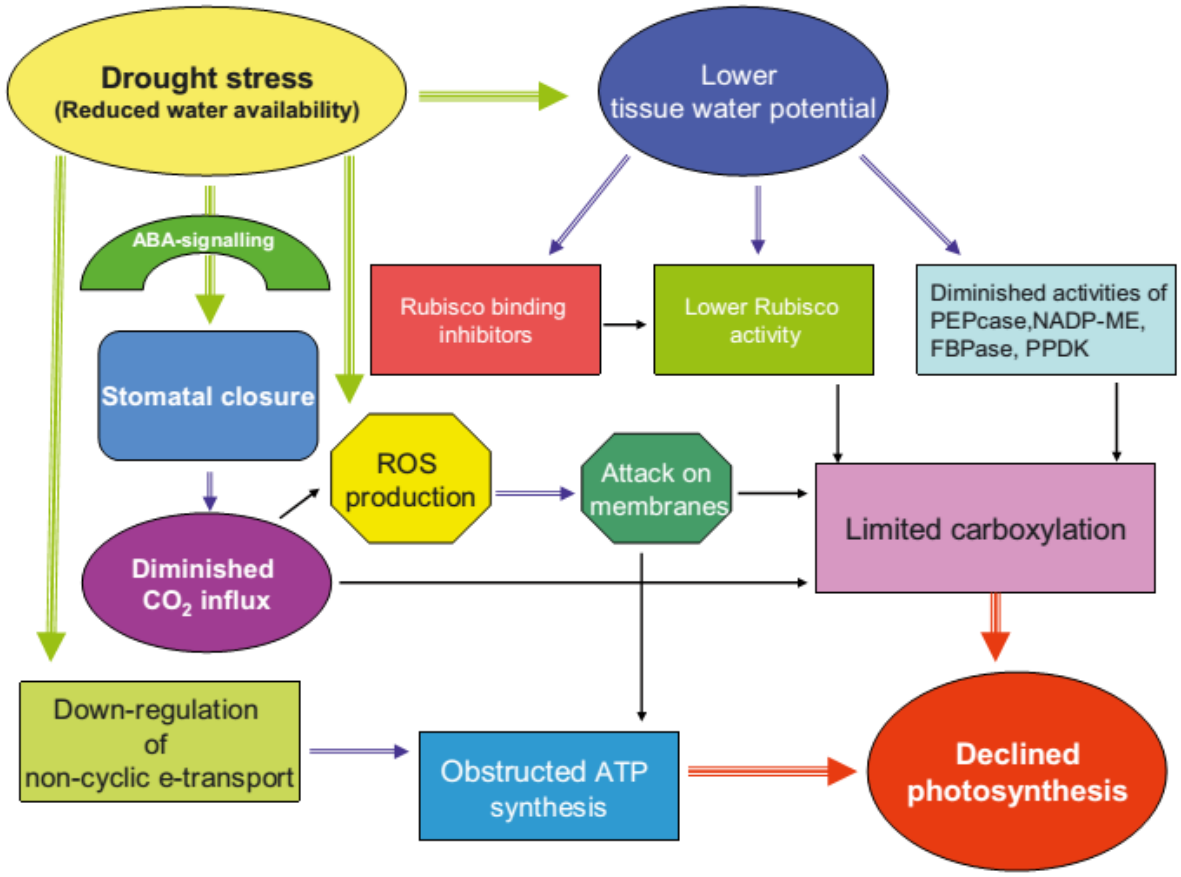


Figure 1 Schematic representation of the different ways drought stress could affect photosynthesis (Farooq *et al.*, 2009).

1.1.3 Oxidative damage

Reactive oxygen intermediates (ROIs) are unavoidable by-products of aerobic metabolism inside chloroplasts and mitochondria. They are also produced during programmed cell death and during pathogen defense (Apel & Hirt, 2004). ROIs are reduced forms of atmospheric oxygen (O_2). Upon excitation, singlet oxygen (O_2^1), super oxide radical (O_2^-), hydrogen peroxide (H_2O_2), or hydroxyl radicals (HO^-) are produced (Møller, 2001). Under non-stress conditions, ROIs are important to monitor the stress levels inside the plant. They are tightly regulated to prevent cell death (Foyer & Noctor, 2013; Mignolet-Spruyt *et al.*, 2016; Mittler, 2017). Due to their reactive status, excessive amounts of ROIs could cause several disruptive oxidative processes like membrane lipid peroxidation, protein oxidation, enzyme inhibition, and nucleic acid damage. Lipid molecules – mainly in the lipid bilayer of the cell membrane- are oxidized by ROIs through

their fatty-acid tail, especially if the tail contains polyunsaturated fatty acids (PUFAs). There are two main pathways for the PUFA to be oxidized by ROIs. First, a radical chain reaction initiated by the protonated form of superoxide radicals (HO^+, HO_2^+) taking away an H atom from PUFA resulting in a lipid radical (L^+). Adding an oxygen molecule would then produce a peroxyl radical (LOO^+). The peroxyl radical would then borrow a hydrogen atom from the neighboring lipid molecule to produce a new lipid hydroperoxide molecule (LOOH). A new L^+ radical is formed, and its oxygenation produces another LOOH and again L^+. The other pathway is initiated by an attack of a singlet oxygen ($^1O_2$) on the PUFA to form lipid endoperoxide which will be converted to LOOH. This pathway is the most common pathway for LOOH production. LOOHs are relatively unstable. They breakdown further to lipid alkoxyl radicals (LO^+) - in the presence of redox-active ions like Fe^{2+}- which are highly oxidizing. They attack neighboring lipid molecules, producing lipid radicals ($L^{\cdot}$), followed by lipoxyl radicals (LOO^+). The LO^+ radicals are further reduced by neighboring organic molecules to produce carbonyls as aldehydes and ketones (Montillet *et al.*, 2004).

1.2 Reactive- carbonyl species (RCS) detoxification mechanisms

In order to scavenge the resulted reactive carbonyl species (RCS) from the lipid peroxidation process, plants mainly produce three types of enzymes:

1- The aldo-keto reductases, and aldehyde reductase enzymes. They reduce the carbonyl groups to alcohol using NADPH as electron donor (Sengupta *et al.*, 2015; Yu *et al.*, 2020).

2- 2-alkenal reductases (AER), which reduce the C-C double bond to form saturated carbonyls, with the help of NADPH as electron donor (Jun'ichi Mano *et al.*, 2005).

3- Aldehyde dehydrogenase (ALDH) enzymes, which are the focus of this thesis.

1.2.1 Aldehyde dehydrogenases

Aldehyde dehydrogenases are a group of NAD (P)+ -dependent enzymes. They are ubiquitous and are found in almost all organisms (Brocker *et al.*, 2013b; H. H. Kirch *et al.*, 2004). They are involved in different physiological processes like flower and seed development (Xiang Li *et al.*, 2018; Shen *et al.*, 2012; Shin *et al.*, 2009), male sterility (F. Liu *et al.*, 2001; Xie *et al.*, 2020), leaf patterning (Toyokura *et al.*, 2011), glycolysis (Yang *et al.*, 2011), mannitol synthesis (Tarczynski *et al.*, 1993), amino acid *de-novo* biosynthesis (Rasheed *et al.*, 2011), redox homeostasis(T. D. Missihoun *et al.*, 2018; T. D. Missihoun & Kotchoni, 2018), and most notably biotic and abiotic stress resistance.

The ALDHs are grouped into seven groups based on their structures: A) Semialdehyde dehydrogenases. B) Non-specific aldehydes. C) Betaine dehydrogenase. D) Non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase. E) Phenylacetaldehyde dehydrogenase. F) Lactaldehyde dehydrogenase. G) ALDH-like proteins.

In the early 1990s, *ALDHs* were discovered in different organisms (Guerrero *et al.*, 1990; Ishitani *et al.*, 1995; Pereira *et al.*, 1991). By the end of the 1990s, the need for categorizing and naming these *ALDHs* became a must. So, in 1999, a nomenclature system was proposed to classify the newly discovered superfamily. According to which, the *ALDH* superfamily would be divided into families and subfamilies. Whenever the protein sequences have more than 40% similarity, they are placed in the same family. If they share more than 60% of their sequence, they are included into a subfamily (Sophos *et al.*, 2001; Ziegler & Vasiliou, 1999).

In total, *ALDHs* are organized into 24 families. Families *ALDH2*, *ALDH3*, *ALDH5*, *ALDH6*, *ALDH7*, *ALDH10*, *ALDH11*, *ALDH12*, *ALDH18*, *ALDH21*, *ALDH22*, *ALDH23*, and *ALDH24* belong to the plant ALDH superfamily. Families *ALDH2*, *ALDH3*, *ALDH5*, *ALDH6*, *ALDH7*, and *ALDH18* have mammalian orthologues, while the rest of the superfamily are plant specific (Brocker *et al.*, 2013a; H. H. Kirch *et al.*, 2004; Sophos & Vasiliou, 2003; N. Stiti *et al.*, 2021).

Due to the widespread genome sequencing efforts and annotation tools, several *ALDH* superfamilies have been studied in numerous plant species. For example, *Arabidopsis thaliana* (H. H. Kirch *et al.*, 2004), model tree species *Populus trichocarpa* (Tian *et al.*, 2015), grape (Zhang *et al.*, 2012), apple (Xiaoqin Li *et al.*, 2013), and soybean (Wang *et al.*, 2017). Several orthologues are found in every economically important crop, and they are studied to elucidate their rule during different developmental stages, and their role to resist abiotic stresses.

1.3 *Arabidopsis thaliana*

Arabidopsis thaliana is a small, dicot, flowering weed belongs to the Brassicaceae family. Although *A. thaliana* is not an economic crop, its small number of chromosomes (n=5), short life cycle (~ 6 weeks), small stature, and abundance of produced seeds -whether from selfing or cross-pollination- made it the perfect candidate to be used as a model plant for molecular biology research. It has been first nominated in 1943 by F. Laibach (Koornneef & Meinke, 2010; Sivasubramanian *et al.*, 2015). Afterwards, it has been adopted by groups all over the world. By the year 2000, the *A. thaliana* genome was completely sequenced (Marra *et al.*, 1999). It took only four years to provide the first review of the *ALDH* gene superfamily (H. H. Kirch *et al.*, 2004), with several articles exploring the expression and importance of selected members of *ALDH* in *A. thaliana* before the review (H. H. Kirch *et al.*, 2001a; Nair *et al.*, 2004; Skibbe *et al.*, 2002; Sophos & Vasiliou, 2003; Sunkar *et al.*, 2003a).

In *A. thaliana*, there are 16 *ALDH* genes which belong to 10 different families: Three genes belong to families *ALDH2*, and *ALDH3* each. Two genes belong to families *ALDH10*, and *ALDH18* each. And one gene belongs to families *ALDH5*, *ALDH6*, *ALDH7*, *ALDH11*, *ALDH11*, *ALDH12*, and *ALDH22* each.

Under the supervision of Prof. Dorothea Bartels, several *ALDH* genes have been extensively studied like *ALDH10A8*, *ALDH10A9* (T. D. Missihoun, Willèe, *et al.*, 2014; T. D. Missihoun *et al.*, 2011), *ALDH3I1* (H. H. Kirch *et al.*, 2001a; Kotchoni *et al.*, 2006; T. D. Missihoun *et al.*, 2018; N. Stiti, Adewale, *et al.*, 2011; Sunkar *et al.*, 2003a; Zhao *et al.*, 2017), *ALDH3H1* (H. H. Kirch *et al.*, 2001a; T. D. Missihoun *et al.*, 2012; N. Stiti *et al.*, 2014; N. Stiti *et al.*, 2020; N. Stiti, Adewale, *et al.*, 2011), and *ALDH7B4* (Kotchoni *et al.*, 2006; T. D. Missihoun *et al.*, 2018; T. D. Missihoun, Hou, *et al.*, 2014; Zhao *et al.*, 2017, 2018).

In this research, the focus was kept on *ALDH3H1*, *ALDH3I1*, *ALDH3F1*, and *ALDH7B4*. *ALDH3H1* was originally identified in the year 2001. The gene was discovered because it was homologous to a similar protein which was found in the desiccation tolerant plant *Craterostigma plantagineum* after a subtraction hyperdization experiment. It was called *At-ALDH4*. The open reading frame (ORF) of the gene is 1583 bp, encoding a 484 aa long protein. It has a molecular weight of 53 kDa, and an isoelectric point of 8.65. The expression pattern of *ALDH3H1* gene was constitutively low during dehydration stress and ABA-treatment in leaves (H. H. Kirch *et al.*, 2001a), where it is activated after both stresses in the roots (H. H. Kirch *et al.*, 2004). The protein of ALDH3H1 was found inside both the tonoplast and the plant cytoplasm using GFP-marking (Shimaoka *et al.*, 2004; N. Stiti, Missihoun, *et al.*, 2011). The preferred substrates for the enzyme activity are medium-to-long chain saturated aldehydes (C_6 to C_{12}), with exclusive co-enzyme dependency on NAD+. The enzyme activity is dependent on its redox state, with a 25-35% reduction of activity in its oxidized form (N. Stiti *et al.*, 2016; N. Stiti, Adewale, *et al.*, 2011). Using a yeast-2-hybrid and a library generated from seven-day-old roots, the ALDH3H1 protein was found to interact with XLG1, and XLG3 subunits. The G proteins are involved into different stress responses, but their mechanism is still unidentified (Liang *et al.*, 2017). The *ALDH3H1* RNA transcript accumulated at a lower level during recovery from high temperature stress. The protein levels increased after a three-hour heat treatment. The RNA transcript also increased after a combination of each dehydration/heat, and heat/salt stress (Zhao *et al.*, 2017).

ALDH3I1 gene was similarly discovered along with *ALDH3H1*. The open-reading frame is 1653 bp long, composing a 550 aa-long protein. The protein mass is 60.1 kDa, with a calculated isoelectric point of 8.7 (H. H. Kirch *et al.*, 2001a). The GFP-fusion protein showed that it resides inside the chloroplast (Kotchoni *et al.*, 2006). The RNA expression of the gene in the leaves was elevated in response to ABA-treatment, dehydration (H. H. Kirch *et al.*, 2001a; H. H. Kirch *et al.*, 2005), salt, heavy metals, and oxidative stress (Kotchoni *et al.*, 2006). Overexpression lines were able to better tolerate those stresses than WT lines (Kotchoni *et al.*, 2006; Sunkar *et al.*, 2003a). Similar results were shown when *ALDH3I1* was overexpressed in *Nicotiana tabacum* against WT lines, where less ROS and malonaldehyde were accumulated during salt, drought, and oxidative stress compared to native lines (Raza, 2009). T-DNA knock-out lines performed worse than WT lines when facing salt and dehydration stress (Kotchoni *et al.*, 2006). Like ALDH3H1, medium

and long-chain saturated aldehydes are the preferred substrates for the ALDH3I1 enzyme. Unlike ALDH3H1, ALDH3I1 enzyme is able to use both NAD+, and NADP+ as co-enzymes (N. Stiti, Adewale, *et al.*, 2011). Both RNA and protein expression levels were elevated after three hours of basal thermotolerance (Ba) stress in 10-day-old *A. thaliana* seedlings. Four-week-old plants showed increased RNA transcripts after one hour of heat stress, and declined transcripts after 12, and 24 hours. Both protein and RNA levels were elevated after recovery. The T-DNA lines were more susceptible to high heat stress. The protein levels were higher after dehydration/heat, and wounding/heat stress combinations (Zhao *et al.*, 2017).

ALDH3F1 was discovered in 2004 by Kirch *et al.* in an unpublished work (H. H. Kirch *et al.*, 2004). The protein resides in the cytoplasm (N. Stiti, Missihoun, *et al.*, 2011). By the time of its discovery, it was believed that unlike its closely related family members, *ALDH3F1* expression is not driven by stress (H. H. Kirch *et al.*, 2004). In 2017, the RNA transcripts of *ALDH3F1* were accumulated under Ba stress, and protein levels were elevated after three hours of heat stress. The same occurred after a combination of drought/heat, and heat/salt stresses (Zhao *et al.*, 2017). In 2020, it was discovered that *ALDH3F1* is involved in determining flowering time. When *ALDH3F1* was knocked out, it caused early flowering. Whereas an overexpression of the gene caused late flowering through an interaction with the FLC locus (Xu *et al.*, 2020).

ALDH7B4 is one of the most studied members of the *ALDH* superfamily in *A. thaliana*. *ALDH7B4* was originally found as an EST in response to high salt stress (Gong *et al.*, 2001). Afterwards, the *ALDH7B4* was recognized among a number of genes that contain a myc recognition sequence, which has been shown to be activated in dehydrated plants (Simpson *et al.*, 2003). The gene was then discovered to be the only member of the *ALDH7* family in *A. thaliana* (H. H. Kirch *et al.*, 2004). Using GFP-coupled enzyme, the ALDH7B4 enzyme was shown to reside in the cytosol (N. Stiti, Missihoun, *et al.*, 2011). Since its early discovery, it was shown that *ALDH7B4* RNA expression was high in the leaves during various abiotic stresses like drought, ABA, and salt stresses. The expression was not similarly high in the roots during the same stresses (H. H. Kirch *et al.*, 2005). Subsequently, an over-expression mutant line that is constitutively expressing the ALDH7B4 enzyme was made. It showed better adaptability and tolerance to osmotic and oxidative stress. The level of H_2O_2, and malonaldehyde (MDA) was also reduced compared to WT lines. On the other hand, the T-DNA knock-out mutant lines performed poorly compared to WT lines. It

showed the importance of the ALDH7B4 enzyme in scavenging ROS components, and decrease lipid peroxidation process (Kotchoni *et al.*, 2006). Similar results were obtained after overexpressing the gene in *Nicotiana tabacum* plants. The overexpression mutant lines performed significantly better than WT plants under salt, drought, and oxidative stress (Raza, 2009).

To further investigate the *ALDH7B4* expression during different stresses, 600 bp upstream form the translation start sequence (ATG) were analyzed and tested during different abiotic stresses to determine the *cis*-acting elements that influence the enzyme expression. It was proven that DRE/CRT, and three ACGT (ACGT1, ACGT2, ACGT3) are required for the gene induction against salt and dehydration stress. The DRE/CRT motif is important for the activation by ABA, whereas the ACGT motif is quite relevant during wounding stress. In seeds, DRE/CRT and ACGT1 particularly are important for *ALDH7B4* expression. When the promoter was attached to β-glucuronidase (GUS) reporter protein, it showed that *ALDH7B4* is highly expressed in the reproductive organs of the plants, mainly flowers, stamen, pistil, and seeds. It also showed an almost non-existent expression during non-stress conditions (T. D. Missihoun, Hou, *et al.*, 2014). In 2018, using a yeast one-hybrid technique, it was shown that ATAF1 transcription factor – which belongs to a group of the NAC transcription factors – can bind to the *ALDH7B4* promoter. The overexpression of the ATAF1 resulted in higher *ALDH7B4* expression in seeds, seedlings, and in mature plants (Zhao *et al.*, 2018). Finally, during stress combination experiment, it was revealed that the RNA expression of the *ALDH7B4* gene was elevated during all single stress treatments (drought, wounding, heat, and salt), and the expression is even higher elevated under drought/heat, heat/drought, wounding/heat, and heat/salt stress combinations. The protein expression showed a similar pattern, where the protein levels were higher during all stresses, and specifically elevated during wounding, drought/heat, and heat/salt stress combinations (Zhao *et al.*, 2017).

1.4 Aldehyde dehydrogenase in monocots

Even though the bulk of the early research on aldehyde dehydrogenase enzymes occurred in *A. thaliana* in the early 2000s, the aldehyde dehydrogenase enzymes were originally discovered in monocots. In 1999, two *ALDH* genes were sequenced in *Zea mays*, *Oryza sativa*, and *Sorghum bicolor* each, while one gene was discovered in *Hordeum spontaneum* (Ziegler & Vasiliou, 1999). The most significant of these genes was the *ZMRF2* that was discovered in *Zea mays*. It was hypothesized and later confirmed to produce a protein involved in restoring male fertility in maize

(F. Liu *et al.*, 2001; Ziegler & Vasiliou, 1999). More *ALDH* genes were discovered in economical important monocots in the following years (Sophos *et al.*, 2001). With the advancements in genome sequencing, *ALDH* superfamilies were identified in other economical important monocots (Gao & Han, 2009; Jimenez-Lopez *et al.*, 2010).

Barley is one the most important economical crop worldwide. It is ranked fourth in both quantities produced (140 million tons), and area cultivated (55 million hectares). It is the most adaptable cereal on the planet. It can be grown on a myriad of climate zones, ranging from Mediterranean, north African, sub-Saharan climate, up to arctic and subarctic climate zones. It belongs to the Poaceae family. There are three types of barley: *Hordeum vulgare*, *Hordeum distichum*, and *Hordeum irregulare*. In my research, I focused on studying *Hordeum vulgare*, which is a six-rowed type of barley that has a spike notched on opposite sides with three spikelets on each notch (Zhou, 2009).

Barley is mainly grown as feedstock. 25% of barley grown in the US is used for beer malting, alcohol production, and malt-related food production (Zhou, 2009).

Few aldehyde dehydrogenase enzymes were discovered and studied in barley. The first *ALDH* gene was mentioned in the 1999 aldehyde dehydrogenase superfamily review, showing a betaine-aldehyde gene (Ziegler & Vasiliou, 1999). The same gene was mentioned again in the 2000 review update under the name *ALDH10A6* (Sophos *et al.*, 2001). Ever since, more research has been done on betaine-aldehyde in barley to elucidate its role (K. Bhati & K. Singh, 2011; Nakamura *et al.*, 2001).

In 2009, Guo *et al.* showed several genes that were differentially expressed between drought-tolerant and drought-sensitive barley genotypes in response to drought stress during the reproductive stage. Among the genes that were exclusively expressed in the drought-tolerant genotypes (Martin and HS41-1) was contig2924-5-at. It was annotated as aldehyde dehydrogenase without any further explanation (Guo *et al.*, 2009). In 2017, the barley genome sequence was published (Beier *et al.*, 2017; Mascher *et al.*, 2017), allowing for a more detailed exploration of the *ALDH* family in barley, and the possible role during abiotic stresses.

2 The objective of the study

This project focused on the physiological characterization of Arabidopsis *ALDHs* with specific attention to the capacity of *ALDHs* to influence redox homeostasis under dehydration stress. A new *in-vivo* method of redox visualization will be used to obtain the NAD/NADH redox measurements.

The role of *ALDHs* in seed vigor and seed germination has not been studied before. Therefore, it will also be determined using controlled deterioration and germination test respectively.

Moreover, the availability of the genome and the RNA-seq information for the differentially expressed genes during the dehydration and rehydration cycle in *Lindernia* species should allow for the identification of the ALDH members of both *L. brevidens* and *L. subracemosa*, and their expression patterns during drought stress and after rehydration.

Finally, through the presence of proper genomic tools, the elucidation of the ALDH7 expression in response to dehydration in barley has been studied, along with the identification of all possible *ALDHs* genes in barely plants, along with their expression analysis throughout the different developmental stages of the plant.

3 Materials and methods

3.1 Materials

The following lists include the name and locations of the providers, developers, and suppliers of the chemicals, equipment, databases, and programs used in this thesis. The text that follows in the later sections of the thesis will only include the manufacturer's name.

3.1.1 Chemicals

The chemicals used in this thesis were obtained from the following companies:

- Applichem GmbH (Darmstadt, Germany)
- Biomol (Hamburg, Germany)
- Bio-Rad (Munich, Germany)
- Carl Roth GmbH (Karlsruhe, Germany)
- Fermentas (St. Leon-Rot, Germany)
- GE Healthcare (Freiburg, Germany)
- Grüssing (Filsum, Germany)
- Invitrogen (Karlsruhe, Germany)
- Merck AG (Darmstadt, Germany)
- Serva Electrophoresis (Heidelberg, Germany)
- Sigma-Aldrich Chemie GmbH (Munich, Germany)
- ZVE (Bonn, Germany).

3.1.2 Equipment

- Binocular microscope: SMZ-800 (Nikon, Düsseldorf, Germany)
- Blotting chamber for proteins: "Criterion Blotter" (Biorad, Munich, Germany)
- Chemiluminescence detector: Intelligent Dark Box II (Fujifilm, Tokyo, Japan)
- Confocal Laser Scanning Microscope: ZE2000 (Nikon, Düsseldorf, Germany)
- Consumables: Pipette tips and centrifugal tubes (Sarstedt AG, Nümbrecht, Germany)
- Desalting columns: "PD–10" (GE Healthcare, Freiburg, Germany)

- Drying and heating chamber (Binder, Tuttlingen, Germany)
- Electroporation system Gene pulser II Electroporator (Bio-Rad, Hercules, USA)
- Gel electrophoresis chambers:
  – "Mini gel" (Biometra, Göttingen, Germany)
  – "Easy Cast" (Owl, Portsmouth, USA)
- Luminescent Image Analyzer LAS 1000 (Fujifilm Life Science, Stamford, USA)
- Nanodrop: Biospec – Nano (Shimadzu Biotech, Japan)
- Particle Gun: Biolistic (Bio-Rad, Hercules, USA)
- PCR–cycler: "T3 Thermocycler" (Biometra, Göttingen, Germany)
- pH–meter (SCHOTT GLAS, Mainz, Germany)
- Rotator: "neoLab–Rotator 2–1175" (neoLab, Heidelberg, Germany)
- Spectrophotometer: "SmartSpec 3000" (Biorad, Hercules, USA)
- Scanner:
  – Typhoon 9200 (Amersham, Piscataway, USA)
  – Image scanner (Amersham, Buckinghamshire, Great Britain)
  – Azure c300 gel doc system (Azure Biosystems, California, USA)
- Sonification water bath: "Sonorex Super RK102P" (Bandelin electronics, Berlin, Germany)
- T3-Thermocycler, Biometra, Göttingen, Germany
- Venticell oven (MMM Medcenter, München, Germany)
- Centrifuges:
  – Centrifuges: "5415D"; "5417R", "5810R"; Vacuum centrifuge: "Concentrator 5301" (Eppendorf, Hamburg, Germany)
  – Sorvall centrifuge: "RC50" (DuPont, Hamm–Uentrop, Germany)
  – Ultracentrifuge: "L8-70M" (Beckman Coulter, Brea, USA)

3.1.3 Computer programs and databases

3.1.3.1 Computer programs:

- APE – A Plasmid Editor v. 1.7
- Blastn – nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

- Compute pI/Mw (http://web.expasy.org/compute_pi/)
- Microsoft Office 2016 (Microsoft, Redmond, USA)
- Primer3 (http://frodo.wi.mit.edu/primer3/)
- Reverse Complement (www.bioinformatics.org)
- RNA fold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi)
- Snap Gene (GSL Biotech; available at snapgene.com)
- ImageJ application (https://imagej.nih.gov/ij/index.html)
- Graphpad prism 8 (https://www.graphpad.com/scientific-software/prism/)
- Originpro 9.1 (https://www.originlab.com/index.aspx?go=Products/Origin)

3.1.3.2 Databases:

- National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/)
- Salk Institute (http://www.salk.edu)
- T–DNA Express (http://signal.salk.edu/cgi–bin/tdnaexpress)
- UniProt (http://www.uniprot.org/)

3.1.4 Enzymes and markers

- DNA–marker (Thermo Fisher Scientific, Darmstadt, Germany)
- Phusion DNA–polymerase (Thermo Fisher Scientific, Darmstadt, Germany)
- Taq DNA–polymerase (Isolated and provided by Tobias Dieckmann, Frederik Faden)

3.1.5 Primer

Primers were designed with the help of the Primer3 website. The primers usually contained 40-60% CG content. The dimerization of the primers should not be able to self-dimerize. Both forward and reverse primers had their melting temperature at 63-65 °C. All primers in this thesis were synthesized by Eurofins Genomics (Ebersberg, Germany). All primers were stored at -20 °C at 100 mM concentrations (Table 1). A working solution of 10 mM was always used.

Table 1 List of all primers used in the thesis

Name	Sequence (5'→ 3')
cDNA synthesis primers:	
Oligo-dT adapter primer	TTTTTTTTTTTTTTTTT

Gene-specific primers:	
MLOC primer Fwd Morex	CACTACGAGCAACGAGGATT
MLOC primer Rev Morex	TGGTGCTCCTCCCTCGCGAA
Genotyping primers:	
3H1-Fwd	GAGATCGTCGCGGCTCTTCG
3H1-Rev	CCAAGCCGAGATCACTAGCACA
3I1-Fwd	TCTCTTCCGACCACACCCTTCA
3I1-Rev	TAAGATCCGCGTCCCCTGAA
3F1-Fwd	CGAGTGGGAGGACGAGGAGT
3F1-Rev	TGGAAGCTTGGACACGAGGA
7B4_new_ KO_LP	AATCCTCTTGGCATTGTTGG
7B4_new_ KO_RP	GAACAGATCGAGCCGCTAAC
FISH1	CTGGGAATGGCGAAATCAAGGCATC
dog1-2_Fwd	TTCTTTAGGCTCGTTTATGCTTTGTGTGGTT
dog1-2_Rev	CTGACTACCGAACCAAAAAATTGAATTTAGTC
rdo2-1_WT_Fwd	GGAGATTTCACCAGAGAAACTC
rdo2-1_WT_Rev	GTCACAGTTAACACATGTAACAT
rdo2-1_Fwd	GGAGATTTCACCAGAGAAACTC
rdo2-1_Rev	GTCACAGTTAACACATGTAAAGT
Peredox_FWD	CAGCTGGCTGATCACTACCA
Peredox_REV	AACTTAACCTCAGCGCGTGT
NTRA-Fwd	GCCGTCGACATGGAAACTC
NTRA-Rev	GCTCTCTGCTGCATAATCTTAG
NTRB-Fwd	GAGCGTCTAAGATTATGCAGC
NTRB-Rev	GATCTCTCTACTAAGCATGGA
Reference-gene primers:	
AthActin2fwd	ATGGCTGAGGCTGATGATATTCAAC
AthActin2rev	AAACATTTTCTGTGAACGATTCCT
ADP-370-FWD	CCCTGTGGAGGCACTACTTC
ADP-370-Rev	TTGTTGAGACATCCAGCATC

Vector-specific primers:	
pJET1.2_fwd	CGACTCACTATAGGGAGAGCGGC
pJET 1.2_rev2	GATGAGGTGGTTAGCATAGTT

3.1.6 Vectors

- pJET1.2/blunt (Fermentas)

This plasmid was used for blunt-end cloning of PCR-fragments.

- pSS02

pSS02:cyt-Peredox-mCherry_DS was a gift from Markus Schwarzländer (Institut für Nutzpflanzenwissenschaften und Ressourcenschutz (INRES), Bonn, Germany) (Addgene plasmid # 161747 ; http://n2t.net/addgene:161747 ; RRID:Addgene_161747). This plasmid was used to host the peredox sensor system, which was used to transform different *Arabidopsis thaliana* lines.

- pB10GUS

This plasmid was used during the transient expression of *ALDH7A1* promoter activity analysis in Golden promise barley genotype. The vector was provided by Aishwarya Singh.

- pSH221

This plasmid was used as a GFP vector. It was co-bombarded with GUS vector for transient expression analysis of ALDH7A1 promoter. The vector was kindly provided by Dr. Jochen Kumlehn and Dr. Stefan Hiekel. Leibniz-institut für Pflanzengenetik und Kulturpflanzenforschung IPK, Gatersleben.

- pUGAB7

The plasmid was used as an over-expression GUS vector. The vector was kindly provided by Dr. Stefan Hiekel. Leibniz-institut für Pflanzengenetik und Kulturpflanzenforschung IPK, Gatersleben

All plasmid vector maps can be found in the supplementary data.

3.1.7 Kits

- CloneJET PCR Cloning Kit, Fermentas (St. Leon–Rot, Germany)
- NucleoSpin® Gel and PCR Clean-up. Macherey Nagel (Düren, Germany)
- RevertAid First Strand cDNA Synthesis Kit. Fermentas (St. Leon–Rot, Germany)
- TRIzol Reagent. Invitrogen (Karlsruhe, Germany)

Kits were used according to manufacturer's instructions.

3.1.8 DNA-sequencing

5 µl of plasmid DNA (30-100 ng/µl), or PCR-Fragment (10-50 ng/µl), in addition to 2.5 µl of primer (10 mM), and 2.5 µl of MiliQ water was used for sequencing. Samples were sequenced at GATC Biotech AG (Cologne, Germany).

3.1.9 Quantification of DNA, and RNA

DNA and RNA quantification was done using the Biospec – Nanospectometer. 1 µl of MiliQ water was used as a blank, followed by 1 µl of the DNA or RNA of interest. Further quantification was done against the 1 Kb DNA marker band. 6 µl of the DNA marker would result in a band intensity of 60 ng, which could be quantified against the intensity of the DNA, or RNA bands.

3.2 Plant material

This study used *Arabidopsis thaliana* (Ecotype Columbia-0, and Landsberg erecta) and *Hordeum* (Species *vulgare ;*Variety Hybernum Viborg, Nutans (Rode) Alef, Species *Spontaneum*). Wild-type plants and transgenic lines were as follows:

Arabidopsis thaliana:

- *aldh3f1*
- *aldh3i1*
- ALDH3F1 OE
- ALDH7B4 OE
- *aldh7b4* (SALKseq_47949)
- *dog1*
- *rdo2*

- *hub1-ler* (*rdo4*)
- *hub1-col*

Hordeum vulgare:

- Martin (MA): obtained from (Guo *et al.*, 2009)
- Moroc (MO): obtained from (Guo *et al.*, 2009)
- HOR 4654 (Golden promise): obtained from IPK Gene Bank
- BCC 111 (Viborg): obtained from IPK Gene Bank
- HOR 18780 (Martin): obtained from IPK Gene Bank
- BCC 906 (Morex): obtained from IPK Gene Bank
- HOR 22216 (Maresi): obtained from IPK Gene Bank

Hordeum Spontaneum:

- *Hordeum spontaneum* (HS): obtained from (Guo *et al.*, 2009)

The knock-out mutant lines of ALDH3F1, ALDH3I1, along with the overexpression mutant lines of ALDH3F1, and ALDH7B4 were generated in the IMBIO lab and obtained from Prof. Dorothea Bartels. While knock-out mutant line of ALDH7B4 was obtained from the "European *Arabidopsis* Stock Centre" (NASC, Nottingham, Great Britain). Dr. Wim Soppe provided the knock-out mutant lines of DOG1, RDO2, HUB1-Ler (RDO4), and HUB1-Col.

3.2.1 Sterilization of seeds

Prior to placing on MS media for either germination or selection, seeds were surface sterilized by the following method.

Sterilization solution

7% Sodium hypochlorite (NaOCl)

0.1% Sodium dodecyl sulfate (SDS)

A. thaliana seeds were sterilized by immersing for two minutes in 70% (v/v) ethanol (EtOH) with constant shaking, followed by washing with sterilization solution for 10 minutes with occasional shaking. The seeds were washed three times with MiliQ sterilized water. The seeds were finally placed on sterilized filter paper until dry and then placed on the growth media.

3.2.2 Growth conditions

Sterilized seeds were grown on MS plates supplemented with appropriate antibiotics. Seeds to be grown on soil did not require sterilization.

3.2.2.1 Breeding on soil

Arabidopsis thaliana seeds were sown on Lizetan® (Bayer, Leverkusen, Germany)-treated soil, before being stratified for two days at 4 °C. The seeds were then placed in a short-day growth chamber for two weeks under the photoperiodic cycle of eight hours of light at 22 °C and 16 hours of darkness at 20 °C. *A. thaliana* seedlings were then transferred to a long-day growth chamber for additional three to four weeks under the photoperiodic cycle of 13 hours of light at 23 °C and 11 hours of darkness at 19 °C. Upon full growth of the plants, siliques were collected separately for each plant inside a paper bag, and further dried inside drying and heating chamber (Binder, Germany) at 30 °C for three to four days. Seeds were finally collected and placed in 1.5 ml Eppendorf tubes.

Barley plants were grown under two different conditions depending on the experiment. For seed production: Seeds were placed on wet filter-paper inside 150 mm Petri-dishes at 4 °C for vernalization. They were sown on Terrasoil (Cordel-Bau**,** Wallenborn, Germany), and kept in at long-day growth chamber for three to four months under the photoperiodic cycle of 14 hours of light at 21 °C and 10 hours of darkness at 16 °C.

For drought-stress experiments; seeds were stratified as mentioned previously, before being sown on Floragard (Floragard, Oldenburg, Germany), and kept at long-day growth chamber for three to four weeks under the photoperiodic cycle of 16 hours of light at 21 °C and eight hours of darkness at 16 °C. The stressed plants would be watered twice a week with 50 ml of -1 MPa solution of PEG-6000 to imitate the dought stress conditions for two weeks. While control plants were watered with regular water instead. After the completion of the experiments, control plants were transferred to a long-day growth chamber for one to two months under the photoperiodic cycle of 14 hours of light at 21 °C and 10 hours of darkness at 16 °C for seed production.

3.2.2.2 Breeding on MS plates

MS media (Murashige & Skoog, 1962)
4.3 g/l MS basal salt
20 g/l Sucrose

pH 5.7-5.8
8 g/l Select agar Media was autoclaved at 121 °C for 20 min

Seeds were sterilized and stratified as previously described. Followed by sowing on MS or ½ MS plates supplemented with appropriate antibiotics for selection. Arabidopsis seedlings were transferred to soil after two weeks.

3.2.2.3 Stress conditions

Before stress treatment, three to four-week-old Arabidopsis seedlings were separated individually into soil pots. For drought treatment, water was withheld from the plants for one week, and relative water content (RWC) prior to checking the peredox activity inside transformed plants.

For barley, two to three-week-old seedlings were irrigated individually with 50 ml of -1 MPa solution of PEG-8000 to mimic drought stress environment. The treatment was done for 10 days before second and third leaves were collected for RWC calculations, *ALDH7B4* expression analysis, and MDA measurements respectively.

The RWC calculation was done using the following formula:

$$\mathrm{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \times 100$$

(Pieczynski *et al.*, 2013)

Controlled deterioration test (CDT) was performed to test the seeds' vigour after stress. The seeds were placed in a defined humidity environment (75%) using an over-saturated solution of NaCl. The seeds were then placed in a dark, 37 ℃ incubator. 50-100 seeds of each line were added three days apart, then collected after 21 days inside the treatment. The seeds were left to dry for two days using silica-gel spheres at room temperature. The seeds were then placed on damped petri-dishes with two layers of filter papers. The plates were then stratified for two days inside a 4 ℃ cold room, and then moved to the long day growth chamber.

After seven days, the number of seeds able to produce radical and cotyledons were counted for all the samples. The numbers were then plotted on IBM SPSS statistics app. The experiment was repeated three times, and all the results are shown in table. 7.

3.3 Microorganisms

3.3.1 Bacterial strains

Escherichia coli DH10B (Lorow & Jessee, 1990)

Genotype: F¯mrcAΔ(mrr–hsdRMS–mcrBC)ϕ80d lacZΔ M15 Δ lacX74 endA1 recA1 deoRΔ (ara. leu) 7697 araDD139 galUgalK nup6 rpsLλ¯

This *E. coli* strain was mainly used for cloning.

Agrobacterium tumefaciens C58C1 (Deblaere R., Bytebier B., De Greve H., Deboeck F., Schell J. *et al.*, 1985)

C58 (RifR), pTiC58 cured, pGV2260 (CarbR).

This Agrobacterium strain was used to infect the Arabidopsis plants using the floral dipping method.

3.3.2 Media for growth of microorganisms

- SOC media: 2 % (w/v) Tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$.
- LB media: 1 g/l Tryptone, 10 g/l NaCl, 5 g/l yeast extract, pH 7.0
- LB agar: 15 g/l Select–Agar was added to LB-media
- YEB media: 5 g Beef extract, 5 g peptone, 5 g sucrose, 1 g yeast extract, pH 7.0. After autoclaving filter sterilized $MgCl_2$ solution (final concentration 2 mM) was added.

All media were autoclaved at 121 °C, for 21 minutes at 1.2 bar.

Media supplements:

- Ampicillin stock solution: 100 mg/ml in dH_2O. Dilution: 1:1000
- Kanamycin stock solution: 50 mg/ml in dH_2O. Dilution: 1:1000
- Spectinomycin stock solution: 50 mg/ml in dH_2O. Dilution 1:1000
- Rifampicin stock solution: 50 mg/ml in DMSO (dimethyl sulfoxide). Dilution: 1:500

3.3.3 Glycerol stocks

Glycerol stocks were made for all bacterial strains in order to keep a fresh sample upon demand. Bacterial strain would be inoculated in 5 ml of respective liquid media supplemented with appropriate antibiotics overnight. Then, 750 µl of media would be mixed with 750 µl of sterilized 100% glycerol. The mixture was inverted several times, before being dropped into liquid nitrogen, and kept in a -80 °C freezer.

3.4 Cloning methods

3.4.1 Electrophoresis of nucleic acids (Adkins & Burmeister, 1996)

50 x TAE–Buffer	**10 x Loading buffer**
2 M Tris	2.5 mg/ml Bromphenol blue
50 mM EDTA	2.5 mg/ml Xylenxyanol
pH 8.0 (Acetic acid)	30 % (v/v) Glycerol
2 % (v/v) 50 x TAE–buffer	

Nucleic acids were separated according to their molecular weight on 1% (w/v) agarose gels. 1 g of agarose powder was added to 100 ml 1x TAE-buffer. The mixture was boiled, poured inside a gel cast, and 100 µl of ethidium bromide solution (final concentration of 10 µg/ml) was added after the mixture has cooled down. A 1 kb GeneRuler was used as a molecular marker. Electrophoresis was done inside 1x TAE-buffer at 110 V and gels were analyzed under UV-light.

3.4.2 Isolation and purification of plasmid DNA (Sambrook & W Russell, 1989)

P1–buffer	**P2–buffer**	**P3–buffer**
50 mM Tris	200 mM NaOH	3 M Potassium acetate, pH 5.5 (Acetic acid)
10 mM EDTA, pH 8	1 % (w/v) SDS	

Inside a 15 ml falcon tube, seven ml of inoculated liquid media were incubated at 37 °C shaking for 16 hours. The media was centrifuged for 1 min at 16.000 g at room temperature, and the pellet was resuspended in 250 ml of P1. Additional 250 ml of fresh P2 was added. The mixture was inverted several times and incubated at room temperature for three minutes. 350 ml of P3 were added and mixed carefully. The mixture was incubated on ice for five minutes, followed by centrifugation for five minutes at 19500 *g* at 4 °C. The upper phase was added to 800 µl of phenol:chloroform (1:1) solution. The mixture was shaken and centrifuged at 19500 *g* for 5

minutes at room temperature. The upperphase was transferred to a new 1.5 ml Eppendorf tube, where 0.7 volume isopropanol was added and incubated for 10 minutes at room temperature. Finally, the mixture was centrifuged at 4 °C for 10 minutes at maximum speed. The pellet was washed with 70% (v/v) ethanol and resuspended in 40 µl MiliQ water. One µl of RNase A was added, and the sample was kept at 37 °C for 10 minutes and stored at -20 °C for further use.

3.4.3 Purification of DNA

Plasmid DNA and PCR results were purified from agarose gel using the NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel) as instructed by the manufacturer.

3.4.4 Restriction digestion and ligation

The restriction digestion and ligation were done using CloneJET PCR Cloning Kit (Fermentas) according to the manufacturer's instructions.

3.4.5 Transformation of rubidium chloride-competent *E. coli* (adapted from Hanahan, 1983)

The ligation products were used for the cloning in DH10B *E. coli* chemically-competent cells. 50 µl of DH10B chemo-competent cells were placed on ice, where three µl of the ligation product was added. The mixture was transferred to a 1.5 ml Eppendorf tube on ice. The tube was then placed on a hot plate of 42 °C for 35 seconds, then immediately 400 µl SOC solution were added. The Eppendorf tube was left on ice for five minutes, then the contents were transferred to a 15 ml falcon tube. The tube was left shaking at 37 °C for one hour. Each 200 µl of the mixture were placed on a solid LB medium plate containing Ampicillin antibiotic. The plate was incubated in the growth chamber at 37 °C overnight to allow transformed cells to grow into visible colonies. The transformed colonies were checked using vector-specific primers.

3.5 Isolation of genomic DNA

3.5.1 Quick and dirty DNA extraction method (Edwards *et al.*, 1991)

Extraction buffer
250 mM NaCl
25 mM EDTA
200 mM Tris/HCl, pH 7.5

0.5% (w/v) Sodium dodecyl-sulphate (SDS)

A single leaf was placed inside a 1.5 ml Eppendorf tube and homogenized using a plastic pestle. 500 µl of extraction buffer was added, vortexed, and incubated for five minutes at room temperature. The mixture was centrifuged at maximum speed for five minutes, and 300 µl of the upper phase was transferred to a new tube containing equal amounts of isopropanol. The mixture was well mixed, incubated for 10 minutes at room temperature, and finally centrifuged at room temperature for 10 minutes at maximum speed. The precipitate was washed with one ml of 70% (v/v) ethanol, allowed to dry, and resuspended in 50 ml MiliQ water.

3.5.2 CTAB extraction method (Rogers & Bendich, 1985)

CTAB buffer	**Precipitation solution**
30 g/l Cetrimonium bromide (CTAB)	5g/l CTAB
1.4 M NaCl	0.04 M NaCl
0.1 M Tris/HCl	
20 mM EDTA	
2% β-mercaptoethanol (freshly added)	

Inside a 15 ml falcon tube, 300 mg of pulverized plant material was added to 500 µl of CTAB buffer preheated at 65 °C. The mixture was well mixed and kept at 65 °C water bath for 30 minutes with occasional shaking to prevent lump formation. The mixture was then centrifuged at 12000 *g* for 10 minutes, and the supernatant was later added to a new tube containing 200 µl of chloroform:isoamyl alcohol (24:1) solution. The mixture was vigorously shaken and centrifuged as before. The supernatant was separated into a new tube, and two volumes of precipitation solution were added and kept at room temperature for one hour. The mixture was centrifuged at 12000 *g* for five minutes, followed by discarding the supernatant and resuspending the pellet in 350 µl of 1.2 M NaCl. Another 350 µl of chloroform was added and mixed well. The mixture was centrifuged at maximum speed for 10 minutes. 0.6 volumes of isopropanol were added to the upper phase in a new 1.5 ml Eppendorftube and centrifuged for 10 minutes at maximum speed at room temperature. The pellet was then washed twice with 70% ethanol, air-dried, and resuspended in 40 µl MiliQ water. One µl RNase-A enzyme was added and incubated at 37 °C for 10 minutes to remove excess RNA.

3.6 Amplification of DNA fragments by PCR (Mullis *et al.*, 1986)

Different DNA fragments were amplified using the PCR technique. The reaction mixture was composed as follow in table 2:

Table 2 PCR reaction mix preparation

MiliQ Water	14.3 µl
10X PCR buffer	2 µl
dNTPs (10 mM)	0.4 µl
Forward primer (10 mM)	0.4 µl
Reverse primer (10 mM)	0.4 µl
Taq polymerase	0.5 µl
DNA template (> 250 ng/µl)	2 µl
Total	20 µl

The typical PCR program consists of three distinct stages: Denaturation, annealing, and extension as follow in table 3:

Table 3 Regular PCR conditions

Initial denaturation	95 °C	5 minutes
Denaturation	95 °C	30 seconds
Annealing	Ta	45 seconds
Elongation	72 °C	30 s/500 bp
Final elongation	72 °C	10 minutes
Storage	4 °C	∞

In case of using the PhusionDNA polymerase (Thermo Fischer Scientific), both the components and the PCR program are changed to the following table 4 and 5:

Table 4 PCR reaction mix preparation for PhusionDNA polymerase

MiliQ Water	19.1 µl
5X PCR buffer	6 µl
dNTPs (10 mM)	0.6 µl
Forward primer (10 mM)	1.5 µl
Reverse primer (10 mM)	1.5 µl
Taq polymerase	0.3 µl
DNA template (> 250 ng/µl)	1 µl
Total	30 µl

Table 5 PCR conditions for PhusionDNA polymerase

Initial denaturation	98 °C	30 seconds
Denaturation	98 °C	10 seconds
Annealing	Ta + 3 °C	45 seconds
Elongation	72 °C	30 s/500 bp
Final elongation	72 °C	10 minutes
Storage	4 °C	∞

Genotyping of T-DNA insertion mutants

In order to confirm the homozygosity status of T-DNA knock-out mutant lines, PCR reactions using gene-specific and T-DNA-specific primers were carried out. Total DNA was extracted using the quick and dirty method as described, followed by a typical PCR program. The products were loaded on a 1% agarose gel. Wild-type DNA was used as a positive control.

3.6.1 Colony–PCR (Sambrook & W Russell, 1989)

One bacterial colony was inoculated in 10 µl of dH_2O. Five µl are used for a colony PCR using gene-specific and vector of interest-specific primers. The other five µl are placed on a solid LB medium supplemented with appropriate antibiotic. Positive colonies were further grown for later usage.

3.7 Extraction of RNA from plant tissue

3.7.1 RNA extraction using urea (adapted from Missihoun *et al.*, 2011)

Extraction buffer
6 M Urea
3 M LiCl
10 mM Tris-HCl, pH 8.0
20 mM EDTA, pH 8.0

Extraction buffer was autoclaved prior to use

500 µl of extraction buffer was added to 200 mg of pulverized plant material. The mixture was vortexed and additional 500 µl of phenol:chloroform:isoamylalcohol (25:24:1) solution were added to the mixture. The mixture was further vortexed and centrifuged for five minutes at 14000 *g* at 4 °C. The upper phase was transferred to a new 1.5 ml tube, where an equal volume of phenol:chloroform:isoamylalcohol solution was added and htoroughly mixed with no more vortexing. The mixture was centrifuged as before, and the upper-phase was added to an equal

volume of chloroform:isoamylalcohol (24:1) solution. The mixture was heavily mixed and centrifuged as before. The upper phase was transferred, and 0.1 volume of 3 M sodium acetate of pH 5.2 and one volume of ice-cold isopropanol was added. The mixture was well mixed and incubated on ice for 15 minutes. The mixture was centrifuged for 10 minutes at 14000 *g* at 4 °C, before washing the pellet twice using 70% (v/v) ethanol. The pellet was left to dry on ice for 10 minutes before resuspended in 10-20 µl of sterilized MiliQ water.

3.7.2 RNA extraction using Trizol reagent

RNA from barley leaves was extracted using Trizol reagent, the protocol was followed as provided by the manufacturer.

3.7.3 RNA extraction using SDS/Trizol (G. Wang *et al.*, 2012)

Extraction buffer
100 mM Tris-HCl, pH 9.0
2% (v/v) β-mercaptoethanol (freshly added)

In order to extract RNA from barley seeds, the seeds were initially crushed using pestle and mortar, and liquid nitrogen was later added to pulverize them. A volume of 400 µl of extraction buffer was added to 200 mg of powder. The sample was vortexed and incubated at room temperature for 15 minutes. Additional 20 µl of 20% (w/v) SDS was added, inverted several times, and incubated at room temperature for five minutes. The mixture was centrifuged at 12000 *g* for 10 minutes at 4 °C. The upper phase was added to two volumes of Trizol, vortexed and incubated at room temperature for 10 minutes. Additional 1/5 volume of chloroform was added, vortexed, then centrifuged at 12000 *g* for 10 minutes at 4 °C. the aqueous phase was carefully transferred to a fresh tube, and an equal amount of isopropanol was added, mixed, and incubated at -20 °C for 20 minutes. The sample was centrifuged as before, before discarding the supernatant, and resuspending the pellet in 400 µl MiliQ water. An equal amount of phenol:chloroform (1:1) was added and mixed. The mixture is centrifuged as before. The upper phase was transferred to a new tube containing 1/10 volume of 3 M sodium acetate, pH 4.8, and two volumes of ice-cooled ethanol. The mixture was inverted and incubated at -80 °C for 30 minutes. The mixture was centrifuged for 20 minutes at 12000 *g* at 4 °C. The pellet was finally washed with 70% (v/v) ethanol, air-dried, resuspended in 10-20 µl sterilized MiliQ water, and stored at -80 °C for further use.

3.8 Reverse transcription polymerase chain reaction

3.8.1 DNase treatment (adapted from Innis *et al.*, 1990)

In a first step, 500 ng of total RNA was added to 1µl RNase–free DNase I enzyme and 1µl of RNase enzyme buffer. The total volume of the mixture was brought to 10 µl. The sample was incubated at 37 °C for 10 min, and stopped by adding 1 µl of 50 mM EDTA and re-incubated at 65 °C for 10 min. The total volume was then separated into two tubes for positive and negative control.

3.8.2 Synthesis of cDNA (adapted from Innis *et al.*, 1990)

For both positive and negative samples 1 µl oligo-dT primer and 0.5 µl MiliQ was added, gently combined, and incubated at 65 °C for 5 min. The following components (Table 6) were added as indicated:

Table 6 Components of cDNA synthesis mix

Positive treatment		Negative treatment	
5x First strand buffer	2 µl	5x First strand buffer	2 µl
10 mM dNTP Mix	1 µl	10 mM dNTP Mix	1 µl
Reverse Transcriptase enzyme	0.5 µl	MiliQ Water	0.5 µl
Total Volume	10 µl	Total Volume	10 µl

Tubes were gently mixed, incubated at 42 °C for 60 min and terminated at 72 °C for 5 min. The cDNA product was diluted in 20 µl MiliQ to a total volume of 30 µl.

3.9 Extraction of proteins

3.9.1 Quick protein extraction (Laemmli, 1970)

2x sample buffer
4% (w/v) SDS
20% (v/v) glycerol
120 mM Tris, pH 6.8
0.01% (w/v) Bromophenol blue
0.2 M DTT (freshly added)

100 mg of pulverized plant material was resuspended in 250 µl of 1x sample buffer. The colloid was vortexed and heated to 95 °C for 10 minutes. The upper phase was then transferred and kept on -20 °C for further use.

3.10 Quantification of nucleic acids and proteins

The quantification of nucleic acids was done using a spectrophotometer (Nanodrop: Biospec – Nano). The concentration (c) could be calculated with the optical density at 260 nm (OD_{260}), in combination with the dilution factor (V) and a DNA/RNA– specific multiplication as follows:

Double-stranded DNA: c [µg/ml] = OD260 x V x 50

RNA: c [µg/ml] = OD_{260} x V x 40

The OD_{260}/OD_{280} quotient describes the purity of the solution. A value between 1.8 and 2.0 indicates pure nucleic acids without too much protein contamination.

3.11 Electrophoresis of proteins

3.11.1 SDS-PAGE (adapted from Laemmli, 1970)

	Separating gel (12%)	**Stacking gel (4%)**
dH_2O	2.88 ml	2.16 ml
1.5 M Tris-HCl, pH 8.8	2.34 ml	
1 M Tris-HCl, pH 6.8		375 µl
Rotiphorese gel 30	3.60 ml	410 µl
10% (v/v) SDS	90 µl	30 µl
10% (w/v) APS	90 µl	30 µl
TEMED	3.6 µl	3 µl

A cast of two glass pieces was attached together using a holder and plastic clips and surrounded with an elastic band. The separating gel was placed inside the cast, leaving a space of three centimeters on top of the cast. Three ml of water were placed on top of the gel to ensure a smooth surface. The stacking gel was prepared and poured instead of the water. The comb was placed to create wells, in which 10 µl of proteins solutions (total protein extraction dissolved in sample buffer, heated to 95 °C for 10 minutes) were loaded. The electrophoresis was performed in 1x running buffer at 20 mA for two hours.

3.11.2 Staining of polyacrylamide gels

3.11.2.1 Coomassie staining (adapted from Zehr, Savin and Hall, 1989)

Fixation solution	Staining stock solution	Staining solution
10 % (v/v) Acetic acid	10 % (w/v) Ammonium sulfate	80 % (v/v) Staining stock solution
40 % (v/v) Methanol	1 % (v/v) Phosphoric acid	20 % (v/v) Methanol
0.1 % (w/v) Coomassie G250		

The polyacrylamide gels were taken out of the glass cast, removing the stacking gel, and immersing the separating gel in 50 ml of fixation solution for one hour. The gel is washed several times with distilled water and incubated in 50 ml of staining solution overnight on a shaker of 50 rpm. The gel was then washed several times with distilled water and scanned. The Coomassie staining is sensitive to 10-50 ng protein per band.

3.12 Protein blot (adapted from Towbin, Staehelin and Gordon, 1979)

The proteins were transferred from the polyacrylamide gels onto nitrocellulose membranes using transfer buffer and electric current. The membranes were used for protein immunodetection.

Towbin–buffer	TBS	TBST	Ponceau red solution	Blocking solution
25 mM Tris	20 mM Tris, pH 7.5	0.1 % (v/v) Tween–20 in TBS	0.2 % (w/v) Ponceau S	2 g of low-fat milk powder
0.2 M Glycine	0.15 M NaCl		3 % (w/v) TCA	50 ml TBST
20 % (v/v) Methanol				

The polyacrylamide gels were placed inside western blotting device filled with pre-chilled Towbin buffer and ice packet. Proteins were transferred onto nitrocellulose membranes at 70 V for 1-1.5 hours. The blots were immersed in 50 ml ponceau red solution for 15 minutes to monitor the protein transfer. The blots were then washed with TBST solution and placed overnight inside 50 ml of blocking solution on a shaking surface at 4 °C to decrease the unspecific binding of antibodies when applied. The blocking solution was discarded, and the membrane was washed once with TBST. The first antibody was introduced to the membrane for one hour at room

temperature on a shaking surface. Then, the antibody was collected, and the membrane was washed three times (1x fast wash, 1x 15 minutes, 3x five minutes) with TBST solution. The second antibody (1:5000 inside the solution) was then introduced to the membrane on the shaking surface and incubated for 45 minutes. The washing steps were repeated, and the membrane was left in the TBST solution for protein detection.

The membrane was placed on a Whatman paper and dried with another piece on top. The bioluminescent was detected using "ECL Western Blotting Detection Reagent" (GE Healthcare) according to the manufacturer`s instructions. The secondary antibody coupled with horseradish peroxidase was used to form a complex with the first antibody. The membrane was then placed inside Azure c300 gel doc system (Azure Biosystems), where the ECL solution was excited to show the attached protein bands.

3.13 Stable plant transformation

3.13.1 *A. tumefaciens*-mediated stable transformation of *A. thaliana* (adapted from Clough and Bent, 1998)

A positive clone of *Agrobacterium tumefaciens* bearing the peredox sensor vector was inoculated in10 ml of YEP medium supplemented with two different antibiotics for selection (rifampicin, and kanamycin, both at 50 µg/ml) for the further selection process and extensive growth for 24 hours at 28 °C. Afterward, the whole medium is added to 250 ml of YEP medium supplemented with selection markers and incubated for several hours until reaching an optical density of 0.6 - 0.8 which is optimum for a floral dip. *Arabidopsis thaliana* plants were prepared by removing the flowers siliques, brown and dead leaves. Afterward, 125 µl of silwet gold was added to 250 ml of YEP medium containing the transformed Agrobacterium to increase the attachment of Agrobacterium to the plant. The plants were dipped into the solution for 20-25 seconds and subsequently covered with perforated plastic bags for 24 hrs to keep a high level of humidity and prevent the Agrobacterium transfer to other plants. After one week of the first floral dip, the process was repeated to increase the possibility of transformation. The plants were grown for two to three more weeks to produce seeds. The putatively transformed seeds were collected and sown on Murashige-Skoog Medium supplemented with hygromycin as selection marker.

3.13.2 Hygromycin selection of peredox-transformed seeds (adapted from Harrison *et al.*, 2006)

The transformed seeds were placed on MS agar plates supplemented with 20 µg/ml hygromycin. The plates were first placed at the 4 °C chamber in the dark to induce stratification in the seeds. Then, the plates were placed in direct light for six to eight hours at 22 °C to jump-start the germination process. The plates were then kept in the dark for 48 hours at 22 °C to allow elongation of the roots for the transformed seedlings. Finally, the plates are kept at constant light for another 48 hours at 22 °C. Transformed seedlings are characterized by longer roots and green cotyledons. The transformed seedlings are then transferred to pots filled with soil to allow them to complete their life cycles, and they could produce seeds to be further tested. T3 plants are the ones to be used for further experiments as they are genetically stable.

3.14 Transient transformation

3.14.1 Particle co-bombardment of barley leaves

3.14.1.1 Plasmid extraction

Plasmid extraction was done using Machery and Nagel midi-kit. The manual protocol was followed.

3.14.1.2 Micro-carrier preparation (Sanford *et al.*, 1993)

Gold particles (1.6 µm in diameter) were used as a microcarrier for particle bombardment. 3.33 µl of gold particles (50 mg/ml stock) were transferred to 1.5 ml Eppendorf tube. They were washed with one ml of 70% ethanol. The Eppendorf tube was vortexed for three to five min, followed by 15 min of soaking for the particles. Once done, the particles were centrifuged for 5 sec to form a pellet. Three rounds of water washing followed: 1) one ml of sterile water was added. 2) the Eppendorf tube was vortexed for one min. 3) The particles were allowed to settle. 4) Centrifuge the Eppendorf tube shortly. 5) Discard the supernatant and repeat the process. After washing, the gold particles were resuspended in 4.665 µl water.

3.14.1.3 Microcarrier coating

In order to ensure an equal coating of the gold particles, the Eppendorf tube was vortexed for five minutes. While vortexing, 2 µl of plasmid DNA (pSH221 GFP plasmid, and pUGAB7 as positive

control/ pB10GUS including *ALDH7A1* promoter) (1 mg/µl), 0.65 µl spermidine, and 1.65 µl $CaCl_2$ were added to the 1.5 ml Eppendorf. After vortexing, the microparticles were allowed to settle for two mins. Another washing step using 70% (v/v) ethanol was performed. The particles were resuspended by tapping. The liquid part was once again removed, and 4 µl 100% ethanol were added.

3.14.1.4 Carrier loading

The carrier loading was done inside a sterile bench. The microcarrier metal support holder was first dipped in 70% ethanol, followed by quick burn using Bunsen burner. The microcarrier holder was placed in the metal support disc and pressed using a corex tube. The 4 µl of 100% ethanol containing the coated gold particles were loaded on the middle of the holder and left for 30 minutes to completely dry out. 650 PSI rupture discs were placed in the dish together with the stopping discs.

3.14.1.5 Leaf preparation

The leaves were excised from three to four-week-old barley seedlings. The leaves were placed in the middle of an MS agar plate. The leaves should overlap to reduce any empty area which would not be affected by the gold particles.

3.14.1.6 Particle bombardment

The machine was used according to the manufacturer's recommendation and to remarks from Dr. Martin Becker from LIMES (Becker, 2019). The leaves were kept on the MS plates overnight. They were checked the following day for GFP spots.

3.14.1.7 Stress treatment

After GFP counting, the leaves were subjected to different stresses to evaluate the role of the *ALDH7A1* gene during such stresses. For salt stress, the leaves were placed onto MS agar plates supplemented with 100 mM NaCl. For drought stress, the MS agar plates were supplemented with 20% PEG-4000. For heat stress, the leaves were kept inside a 37 °C chamber. Finally for the cold stress, the leaves were placed inside the 4 °C chamber. All stresses were applied for 48 hours.

3.14.1.8 Determination of GUS activity in bombarded leaves

Glucuronidase tissue staining solution	$NaPO_4$ buffer (PH=7)	Staining solution
10 mM EDTA 10 % Triton x-100 100 mM $NaPO_4$ - pH 7	10 mM EDTA 10 % (w/v) Triton x-100 100 mM NaH_2PO4 100 mM Na_2HPO4 0.5 mM potassium ferrocyanide (KaFe). In 200 ml of dH2O	25 mg of X-Gluc in 0.5 ml of DMSO 50 ml of $NaPO_4$ buffer 25 µl of H_2O_2 (0.05 % H_2O_2) for 50 ml of the solution Vacuum the leaves for 5 min Incubate overnight at 37 °C.

3.14.1.9 De-staining process

Remove the staining solutions and add 1.5 ml of 70% (v/v) ethanol. Incubate the samples at 80 °C for one or two hours until the green color is completely washed out. Remove the ethanol and keep the leaves in 50 ml tubes for further inspection under confocal microscope.

4 Results

4.1 The effect of *ALDH* on redox homeostasis

We have previously shown that overexpression and knock-out of several *ALDH* genes have an effect on the *A. thaliana* seedlings during biotic and abiotic stresses (Kotchoni *et al.*, 2006; Martens, 2009; Sunkar *et al.*, 2003a; Zhao *et al.*, 2017). One of the areas that we did not investigate was the effect of *ALDHs* on redox homeostasis. We used several ALDH overexpression and knock-out mutant lines to research redox homeostasis as will be shown in the results.

4.1.1 Transformation of *ALDH* overexpression and knock-out mutant lines with vector carrying the peredox reporter

The peredox sensor vector was presented to our lab by Dr. Markus Schwarzländer (Steinbeck *et al.*, 2020). It was originally designed by Hung *et al.*, 2011. The biosensor consists of a circularly permuted GFP T-sapphire, and a bacterial NADH-binding protein. Combining both elements allowed for sensing the cytosolic NADH-NAD^+ redox state of the cell. The biosensor also included a red m-Cherry gene to normalize the signal for protein expression (Hung *et al.*, 2011; Steinbeck *et al.*, 2020). We received both transformed WT Arabidopsis plants which will be referred to as

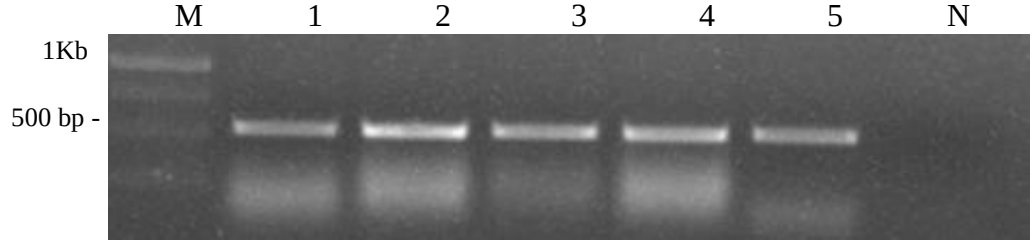


Figure 2 Colony PCR results for peredox-transformed Agrobacterium cells grown on selective kanamycin YEP media. The first lane (**M**) shows molecular-weight size marker. Lanes marked 1-5 show different colonies used in the PCR. The last lane (**N**) is a negative control.

peredox plants, along with a plate of transformed Agrobacterium cells on kanamycin YEP plates. Colony PCR was first performed using peredox-specific primers to assure their transformation status. The PCR bands were expected at 548 bp. All colonies that were tested turned out to be positive and showed a band in the right position (Fig. 2).

The positive colonies were further inoculated in a 250 ml YEP liquid media for floral dipping. Several five-week-old seedlings from WT, *aldh7b4*, ALDH7B4 OE, ALDH3F1 OE, and *aldh3i1* with closed siliques were chosen for transformation. All seeds were collected and plated on MS

hygromycin plates. Plates were stratified at 4 ℃ for four days, then moved to a long day growth chamber with 16 hr light & eight hr darkness. Transformed plants were picked, genotyped, and allowed to grow until generating seeds. The cycle was repeated with the new seeds (Fig. 3), and T3 seeds were used to ensure stable transformation of the peredox vector in plants.

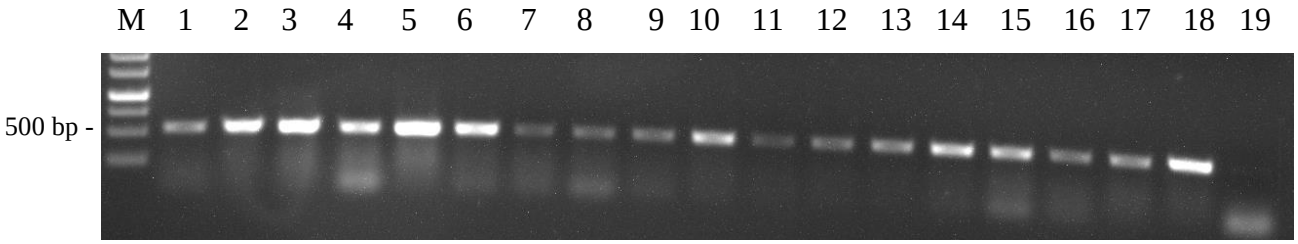


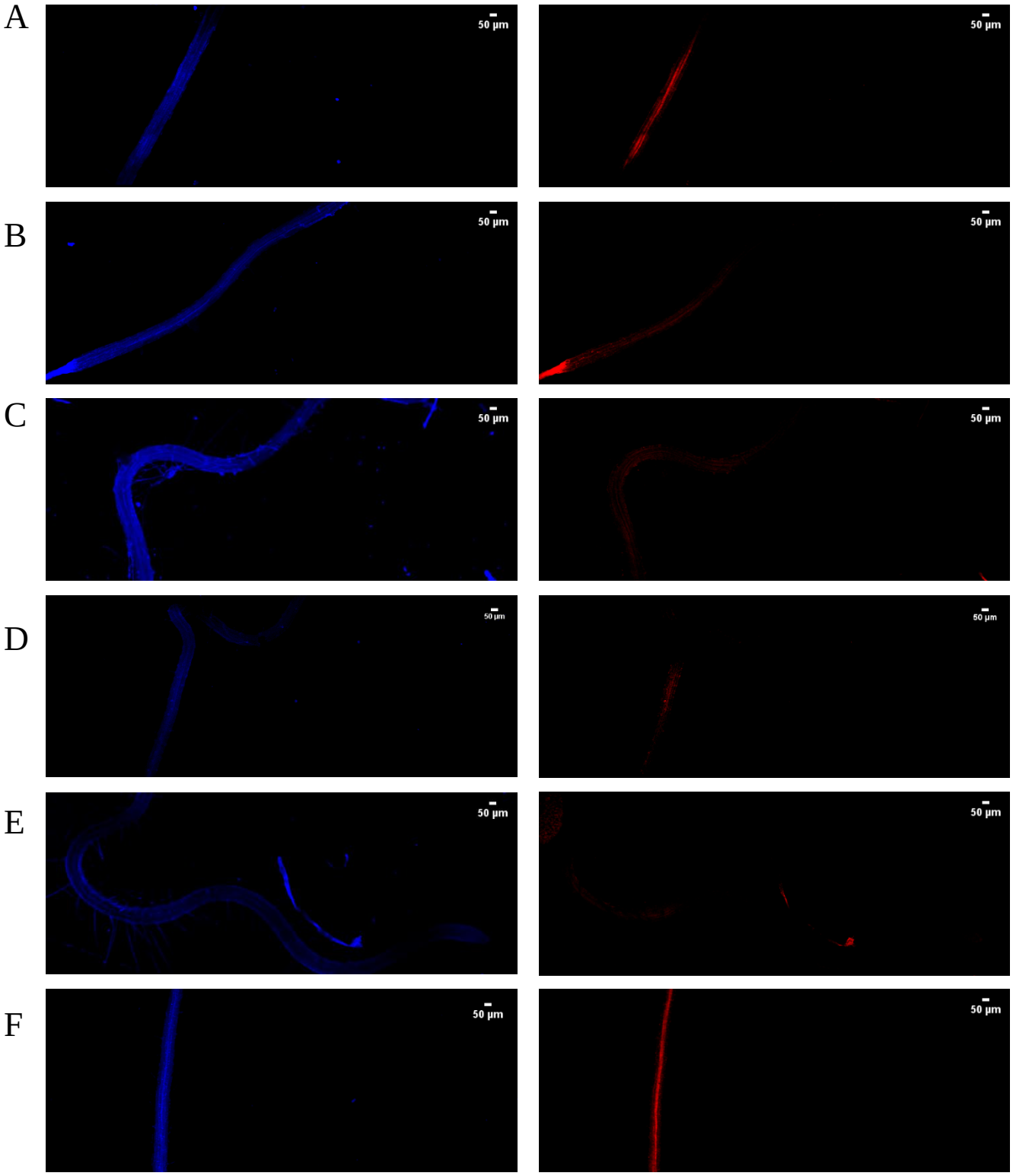
Figure 3 Genotyping PCR results for three-week-old peredox-transformed plants. DNA was extracted using quick method. Peredox fwd and rev primers were used to ensure stable transformation with 548 bp band expected. The first lane (**M**) shows molecular-weight size marker. Lanes marked 1-3 shows WT plants. Lanes 4-7 shows *aldh7b4* plants. Lanes 8-11 shows ALDH7B4 OE plants. Lanes 12-15 ALDH3F1 OE plants. Lanes 16-19 shows *aldh3i1* plants.

4.1.2 Evaluation of Cellular NADH levels

T3 seeds were selected using the hygromycin selection method (**3.13.2**). Half of the transformed seeds were then moved to ½ MS plates with 300 mM sorbitol for two days to generate osmotic stress conditions. While the other half was moved to ½ MS plates with no antibiotic as a control. At least four seedlings per genotype were selected (Fig. 4), and they were observed under the confocal laser scanning microscope.

Figure 4 Samples of five seedlings which are chosen to be observed under the confocal laser microscope. The main focus is on the roots of the seedling as the leaves and stem have chloroplasts which interfere with the?fluorescence detected.

The seedling roots were analyzed under the confocal microscope. Three or more pictures (according to the length of the root) were taken of the root (Fig. 5). Two different lasers with excitation at 405 and 543 nm were used to excite both the T-sapphire green and c-Peredox m-Cherry respectively. The third channel was B/W channel to ensure that the cells were in-focus (not included).


A
B
C
D
E
F
50 µm

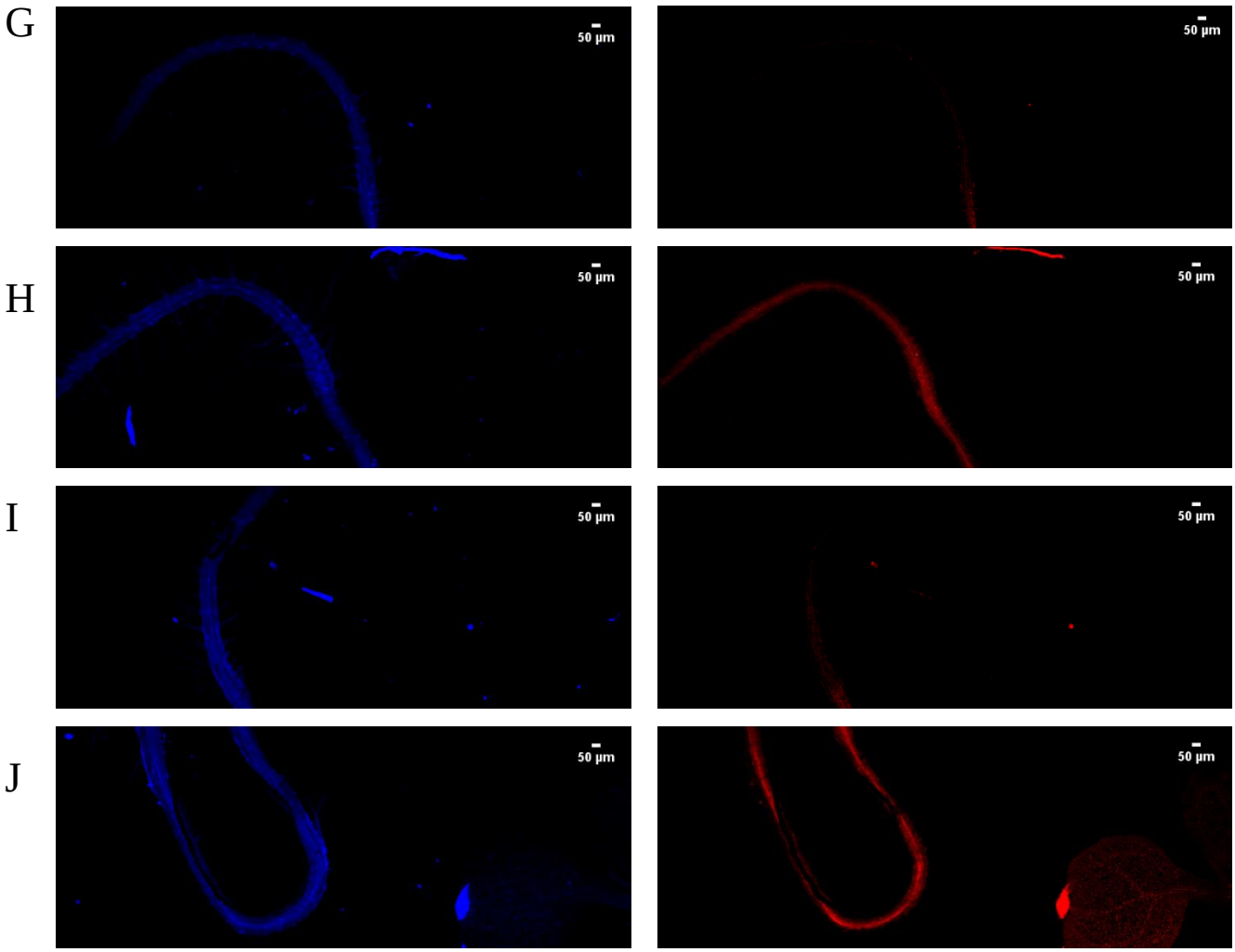


Figure 5 Sample of pictures taken of different ALDH overexpression and knock-out mutant lines under control and osmotic stress. **A-E** shows WT, *aldh7b4*, ALDH7B4 OE, ALDH3F1 OE, and *aldh3i1* roots under control conditions. **F-J** shows WT, *aldh7b4*, ALDH7B4 OE, ALDH3F1 OE, and *aldh3i1* roots under osmotic stress conditions. All the pictures on the left represent the T-sapphire green fluorescenc. They were taken at 405 nm excitation peak. The pictures on the right represents the c-peredox mcherry red fluorescence. They were taken at 543 nm excitation peak.

The pictures were analysed using imageJ application (**https://petebankhead.gitbooks.io/imagej-intro/content/**). All the fluorescence means were used to calculate the average mean of fluorescence of the lines under control conditions and osmotic stress conditions (Fig. 6). Under control conditions, the values of the ALDH7B4 OE line were 35% higher than WT. while other lines were insignificantly different compared to the WT. On the other hand, all mean values under osmotic stress were higher compared to mean values under control conditions, except for ALDH7B4 OE values, which were almost identical in both cases. The mean values were 77%, 32%, 38%, 41% higher in WT, *aldh7b4*, ALDH3F1 OE, and *aldh3i1* respectively.

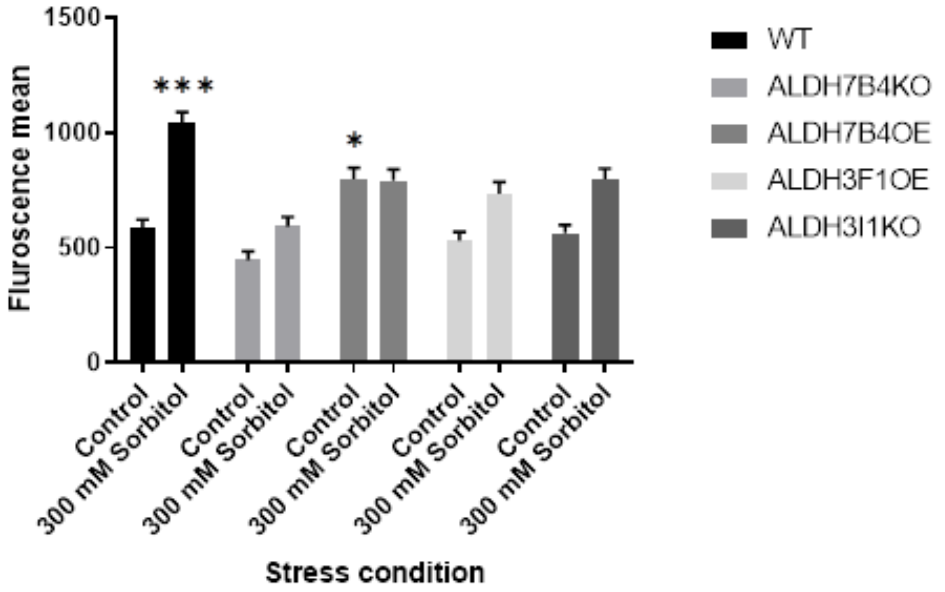


Figure 6 Bar chart shows the mean fluorescence value of each A. thaliana line under control and osmotic stress. The means were calculated using the imageJ application. The data reported are means ± SE (n = 9). The star indicates the levels of significance in comparison to the WT control sample (two-way ANOVA, Tukey method): * $p < .05$; ** $p < 0.01$; *** $p < 0.001$.

4.2 The effect of ALDH enzymes on seed germination and longevity

4.2.1 The expression of ALDH7B4 enzyme in *dog1*, *rdo2-4* lines

The *DOG1*, *RDO2*, and *RDO4* genes have been previously shown to play a major rule during seed germination, and to affect seed longevity as well (Y. Liu, Geyer, Zanten, *et al.*, 2011; Nakabayashi *et al.*, 2015; Nguyen *et al.*, 2012; Xiang *et al.*, 2014). Seed germination and longevity have not been studied of the different *ALDHs* overexpression and knock-out mutant lines.

As previously investigated, the effect of knocking-out the *ALDH7B4* gene was immense, as the knock-out line performed poorly against abiotic stress (Kotchoni *et al.*, 2006) compared to WT and other mutant lines. The main idea here was to monitor the effect it would have on germination and longevity. First, the expression of the *ALDH7B4* gene was checked on both RNA and protein levels.

Seeds of WT, *rdo2*, and *rdo4* were used for RNA extraction (Fig. 7). cDNA was synthesized (**3.8.2**), and PCR reactions were done using actin primers to ensure equal amount of cDNA from all samples (Fig. 7). *ALDH7B4* gene specific primers were then used to check for the RNA

expression level in all samples (Fig. 7). The results show that RNA expression levels are higher in the WT sample compared to both *rdo2* (Col), and *rdo4* (Ler). This demonstrates that *rdo2* (Col), and *rdo4* (Ler). influence expression of *ALDH7B4* genes.

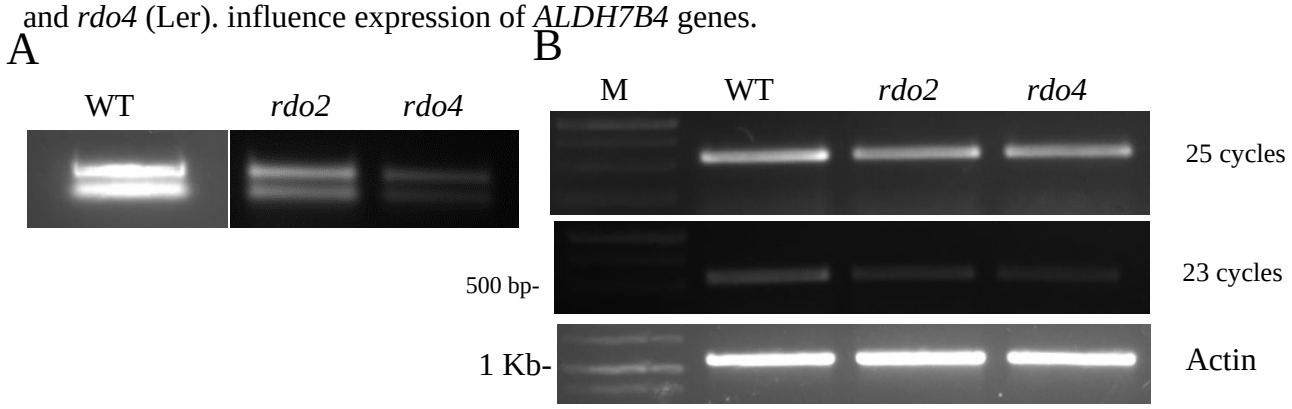


Figure 7 RNA expression analysis of the *ALDH7B4* gene in *rdo2* and *rdo4* lines. **A**: shows 2 µl of RNA extract on 1% agarose gel. **B**: shows the amplification of *ALDH7B4* from the cDNA samples of WT, *rdo2*, and *rdo4*. The actin PCR was first done to ensure equal amount of cDNA used from all samples. Same amounts were used to determine the expression of the *ALDH7B4* gene. PCRs were set to perform 23, and 25 cycles.

Protein samples were extracted from both seeds and leaves (3.9.1). More lines were added to have a more comprehensive idea about the expression of the *ALDH7B4* gene in germination-mutant lines. Protein samples were separated on a PAGE gel and proteins were transferred to a nitrocellulose membrane. Then, ponceau red staining was done to ensure equal amounts of proteins in all samples. Finally, ALDH7B4 antibody was added to the membrane, and the ALDH7B4 protein expression was detected under azure biosystem. The protein level in WT was inversely proportional to all other samples tested (Fig. 8). It was higher in seed samples compared to all others. In leaf samples, the ALDH7B4 protein was almost non-existent in WT- as it was previously shown in our research (T. D. Missihoun, Hou, *et al.*, 2014), while it showed higher amounts in *rdo2*, *rdo4* (Col) (Ler). ALDH7B4 protein level in *dog1* line were high in both seeds and leaves (Fig. 9).

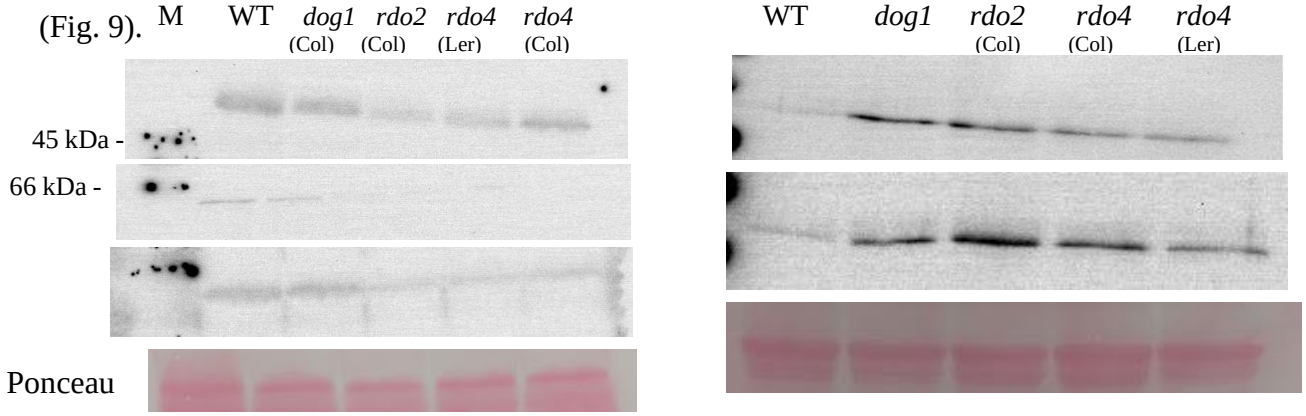


Figure 8 ALDH7B4 protein level in different germination-compromised mutant lines. **M:** Unstained protein markers. On the left are the protein samples extracted from seeds. On the right, the protein samples extracted from leaves. Ponceau red stains were used on the membrane to ensure equal protein amounts for all samples.

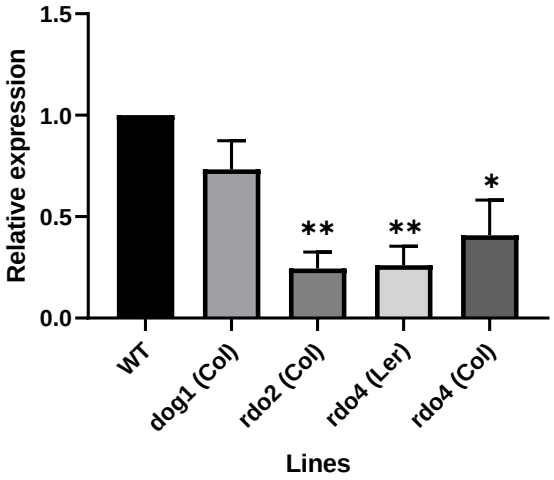


Figure 9 ALDH7B4 relative protein expression in WT and a variety of samples. The WT sample was used as the reference for all other samples. The gel readings were calculated using imageJ application. The reading came from the seed samples, as the ALDH7B4 protein is barely visible in WT leaf samples. The protein shows significant reduction in rdo2, and rdo4 samples. The data reported are means ± SE (n = 3). The star indicates the levels of significance in comparison to the WT control sample (one-way ANOVA, Tukey method): * p < .05; ** p < 0.01; ***

4.2.2 The effect of *ALDH* on seed germination

The different ALDH knock-out and overexpression lines were tested along with wild-type, and *dog1* line for seed germination. Two filter papers were damped and placed inside a 150 mm petri dish. The outside of the petri dish was divided into eight sections, and more than 30 seeds were placed inside each section to represent each line (Fig. 10). After seven days, seeds that were able to form roots and cotyledons were counted. We could not find a significant difference of seed germination percentage between WT and *ALDH* overexpression and knock-out mutant lines. The germination rate of all those lines falls between 86 – 92 %. The *dog1* line germination rate was significantly lower compared to the other rates and it dropped by almost 15% compared to the other lines.

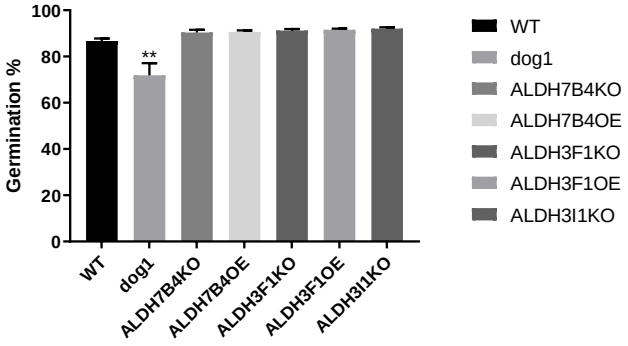


Figure 10 Germination rate percentages of several lines. The data reported are means ± SE (n > 100). The star indicates the levels of significance in comparison to the WT control sample (one-way ANOVA, Tukey method): * p < .05; ** p < 0.01

4.2.3 The effect of *ALDH* on seed longevity

The seed longevity of *ALDH* overexpression and knock-out mutant lines has not been tested before. The controlled deterioration test (CDT) has been recommended for such task. As the name suggests, the CDT is designed to rapidly deteriorate the seed vigor and ability to germinate through increasing its moisture content, followed by heat treatment (Matthews, 1980; a. a. Powell & Matthews, 2005). The test has been accepted as a reproducible way for testing seed storability and longevity. It has been used for several plant families to determine the seed longevity of their different seed lots (Demir *et al.*, 2005; Duczmal & Minicka, 1987; Larsen *et al.*, 1998; A. A. Powell *et al.*, 1984).

At day 0, all seeds achieved a germination rate between 83% and 96%. After three days of stress, the germination rate did not change significantly for any of the lines, with most of the lines showing a slight decrease in germination apart from *dog1*, and ALDH3F1 OE lines which showed a slight increase in the germination rate. By the 6th day of stress, five lines showed a slight decrease in the germination rate. The *dog1* line showed a steep decrease from 91% to 59%, while *aldh3i1* line kept its germination rate. After three additional days, WT and *aldh3i1* germination rate slightly increased, while the germination rate for all the other lines decreased, with the *dog1* line losing 23% of its former germination capacity. At 12 days of stress, the *dog1* germination rate continued to descend reaching 35%. While *aldh3f1*, and *aldh3i1* lines' rate decreased to 83% and 76% respectively. The rest of the lines showed a slight increase in germination rate. WT seeds lost 40% of the germination rate after 15 days of stress, followed by a further decrease to 32% by day 18. It had a sudden increase to reach 66% by 21 days of stress. *dog1* seeds' germination rate increased to 57% by day 15, to be later decreased reaching 5% by day 18. The germination rate was then increased once more at 21 days reaching 27%. *aldh7b4*, ALDH7B4 OE, and ALDH3F1OE lines showed a slight decrease when reaching day 15 of stress. *aldh7b4* and ALDH3F1 OE reached a 90% germination rate by the 18th day of the treatment. The ALDH7B4 OE line continued its decrease reaching 80% germination rate. The *aldh3f1* line arrived at a 90% germination rate, before declining to 77% at day 18. The *aldh3i1* line showed a slight increase to 80%, before losing 27% rate at 18 days of stress. At day 21, the lines displayed various changes and the overexpression lines showed a big decrease in their germination rates. While the WT, *dog1*, and *aldh3i1* lines

showed a sudden increase in their gemination rates. The *aldh7b4*, and *aldh3f1* lines suffered a slight decrease in their germination rates.

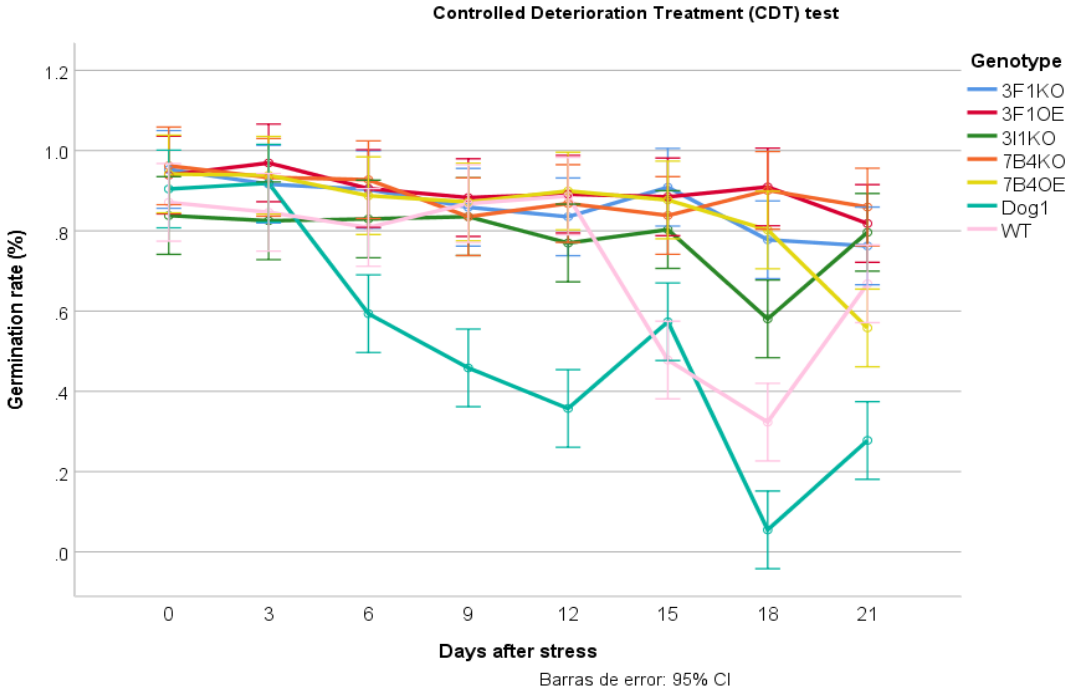


Figure 11 Schematic representation of the germination rate means for different seeds from various lines following a controlled deterioration test. The germination rates of all the lines showed a downward trendline throughout the test. The test was repeated for three biological replicates. The data reported are means ± SE (n > 100). The means were analysed using one-way ANOVA, Tukey method.

Table 7 Germination rates of the different *ALDH* and *dog1* mutant lines.

Line/treatment day	0	3	6	9	12	15	18	21
WT	87	84	80	86	88	47	32	66
dog1	90	91	59	45	35	57	5	27
aldh7b4	96	93	92	83	86	83	90	85
ALDH7B4 OE	94	93	88	87	89	87	80	55
ALDH3F1 OE	93	96	90	88	89	88	90	81
Aldh3f1	95	91	90	85	83	90	77	76
Aldh3i1	83	82	82	83	76	80	58	79

4.3 Aldehyde dehydrogenase family in Lindernia family

The drought-tolerant *L. brevidens*, and the closely related drought-susceptible *L. subracemosa* plants have been providing us with an insight of the expression of different genes under drought stress (Phillips *et al.*, 2008). We have analysed a number of desiccation-related genes, late embryogenesis abundant (LEA) proteins, and drought-related element motifs (Giarola *et al.*, 2018; Phillips *et al.*, 2008; Van Buren *et al.*, 2018). Using RNA-seq data that have been generated using dehydrated and rehydrated *Lindernia brevidens* and *subracemosa* RNA samples (Van Buren *et al.*, 2018), a comparative *ALDH* map was generated for both species. The *ALDH* DNA, and protein sequences from *Arabidopsis thaliana* were used as reference. The Lindernia species contain 19 *ALDH* genes (Fig 12). 10 genes belong to the *ALDH2* family. Three genes belong to *ALDH10* family, two genes belong to *ALDH11* family, and one gene belongs to *ALDH5*, *ALDH6*, *ALDH7*, and *ALDH22* family each. The genes are grouped into four main phylogenic classes. The RNA-seq expression data shows that class four has the lowest activity during dehydration and rehydration in both species, except for an ascending expression of Lsu_021525 gene in *Lindernia subracemosa*. In class two and three, *ALDH* genes are expressed at a moderate level during both dehydration and rehydration in both species. Lbr_028666 - which belongs to the *ALDH5* family in class one - gene expression is significantly elevated during dehydration, starting from day seven. The expression then declines once rehydration occurs. The corresponding gene expression in the *L. subracemosa* species does not seem to elevate from its basic expression during non-stress conditions. In conclusion, only a limited number of *ALDH* genes seems to be involved in the drought stress response in the Lindernia family. Most of the involved genes belong to class one in both Lindernia species, and in class three for *L. subracemosa*.

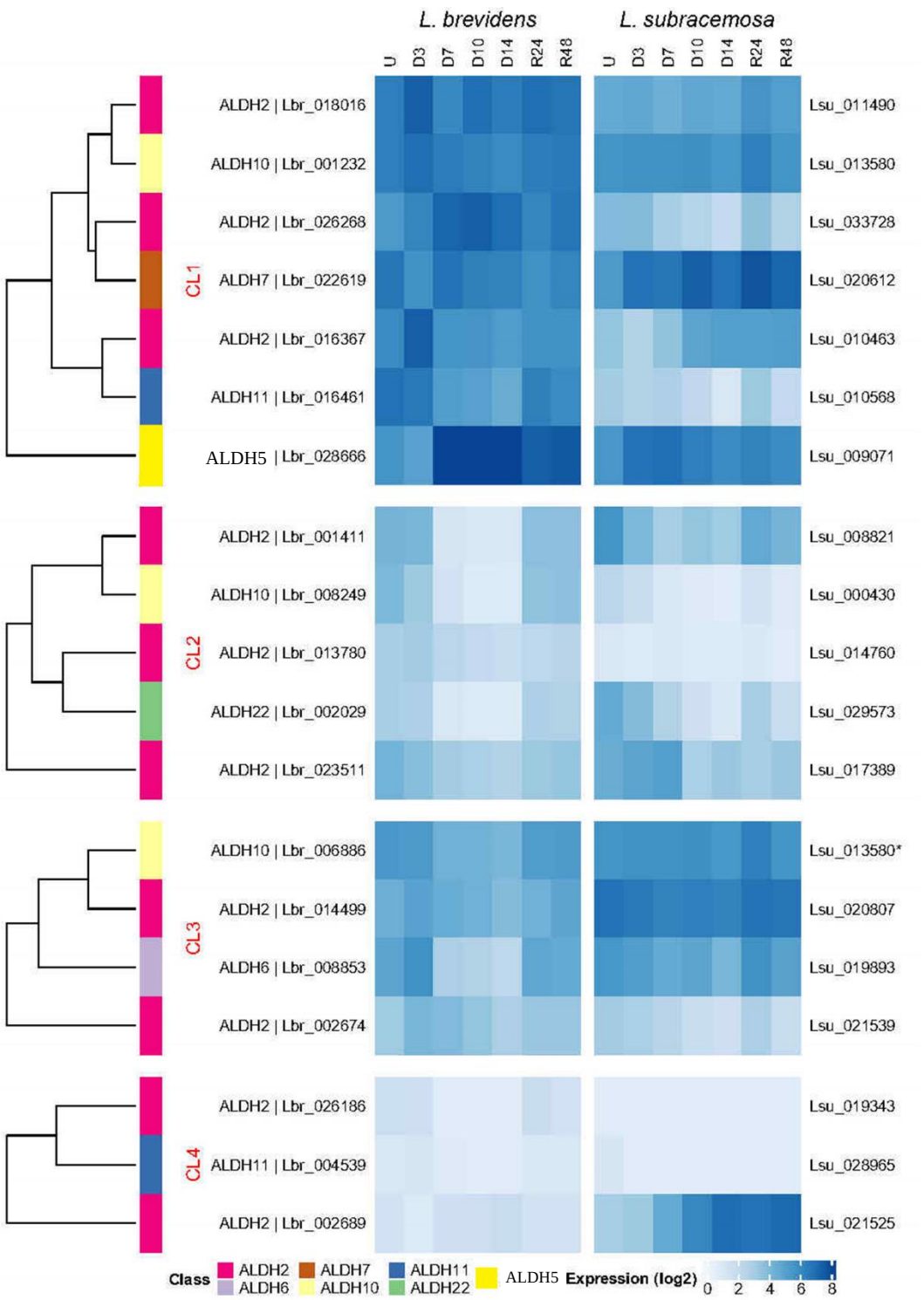


Figure 12 Aldehyde dehydrogenase expression heat map in *Lindernia brevidens* and *subracemosa* species. The genes' expression was extracted from RNA-seq data generated from control, four dehydration, and two rehydration stages. The map was created using the R program, using ComplexHeatMap package. **U**: untreated samples. **D3**: 3 days after dehydration. **D7**: 7 days after dehydration. **D10**: 10 days after dehydration. **D14**: 14 days after dehydration. **R24**: 24 hours after rehydration. **R48**: 48 hours after rehydration.

4.4 The role of the aldehyde dehydrogenase enzyme family in monocots during drought stress

The aldehyde dehydrogenase enzyme family has not been extensively studied in monocots. The main focus was studying the enzyme families in the economical crops such as *Oryza sativa* (rice), *Sorghum bicolor* (sorghum), and *Zea mays* (maize) (Brocker *et al.*, 2013a). In our study, we wanted to add *Hordeum vulgare* as another economic crop to the list.

4.4.1 Bioinformatic searches for genes encoding aldehyde dehydrogenase enzymes in *Hordeum vulgare*

The ALDH enzyme family has not been extensively studied in *Hordeum vulgare*. The recent barley genome sequencing (Beier *et al.*, 2017; Mascher *et al.*, 2017) allowed for a more intense analysis of the *ALDH* family with a possible insight of its role during drought stress. The protein, and mRNA sequences from *Arabidopsis thaliana* and *Zea mays* aldehyde dehydrogenase members have been used to search for *ALDH* sequences in barley. The BARLEX website was used for BLAST along with extracting expression analysis of the *ALDH* genes from several developmental stages. The barley genome contains 26 *ALDH* genes that belong to eight *ALDH* families (Fig 13). Eight genes belong to the *ALDH2* family. Eleven genes belong to the *ALDH3* family, two genes belong to the *ALDH6* family. One gene belongs to *ALDH5*, *ALDH7*, *ALDH10*, *ALDH11*, and *ALDH22* family each. The *ALDH* genes can be grouped into four classes. Classes two, three, and four are not highly expressed during the different stages of barley development. Class one contains most of the genes highly expressed throughout the different developmental stages. As drought tolerance genes are our focus, we concentrated on the genes that were able to increase their expression rate during grain development, inflorescence, and in early stages of development, as well as those having a lower expression level during non-stress vegetative stages. Two genes that fit the profile, namely the *HORVU6HG0519800-1*, which belongs to *ALDH2* family, and *HORVU5HG0398150* belonging to the *ALDH7* family were selected for further studies. The expression of *HORVU6HG0519800-1* is particularly high during grain development (CAR5, CAR15), four-day-old embryos (EMB), developing tiller (NOD), two inflorescence parts (palea (PAL), and rachis (RAC)), roots (ROO2), and senescing leaves (SEN). *HORVU6HG0519800-1* shows less activity in the other tissues of the plant during vegetative growth. The activity of the

gene *HORVU5HG0398150* is noticeable during the late stage of grain development (CAR15), palea part of inflorescence (PAL), and in roots (ROO2). The activity was less noticeable in four-day-old embryos (EMB), developing tillers (NOD), early grain development (CAR5), and in both lemma (LEM) and lodicule (LOD) parts of the inflorescence.

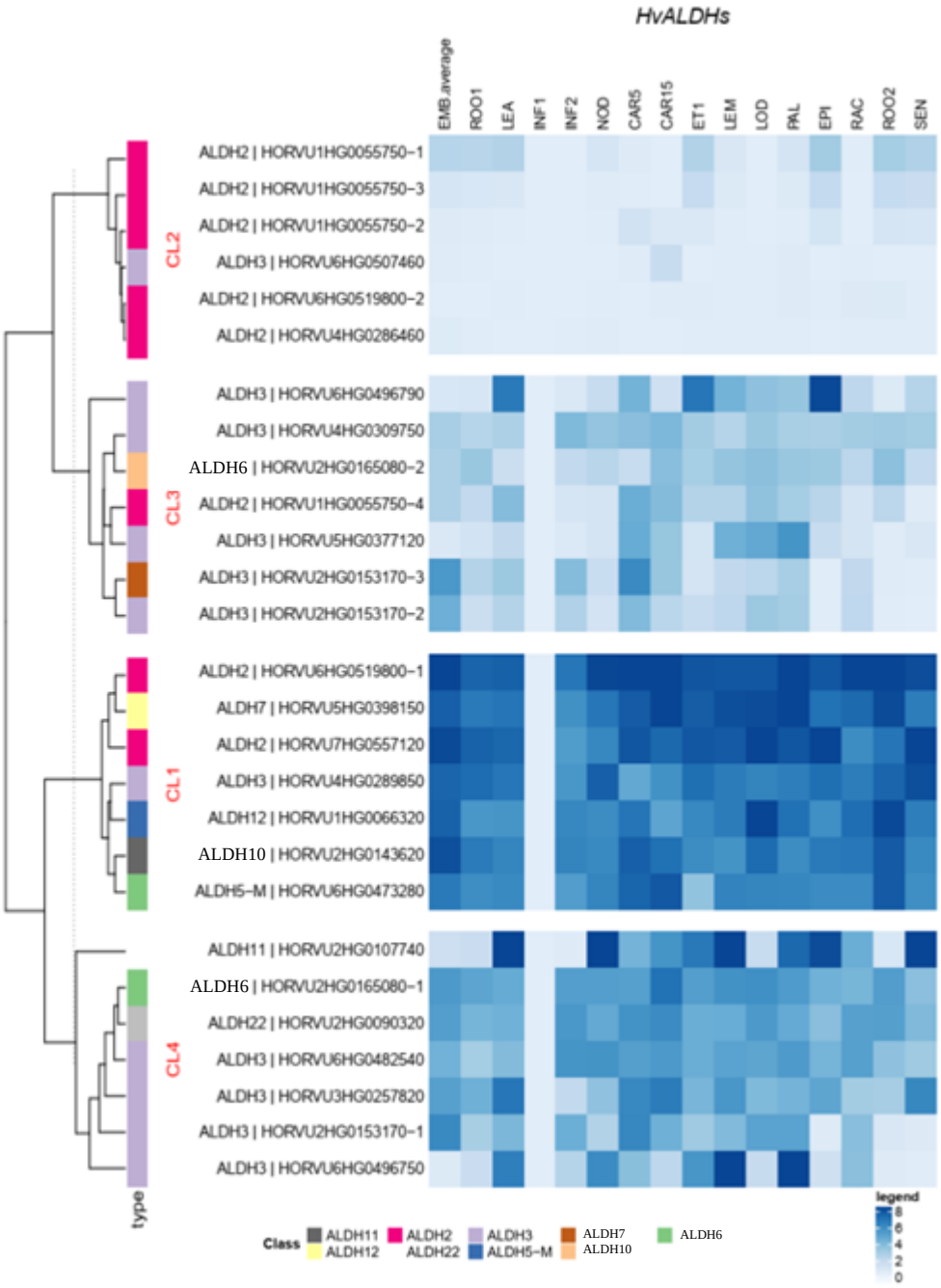


Figure 13 Aldehyde dehydrogenase expression heat map in *Hordeum vulgare*. The Morex line was used for genome extraction and compiling. The genes' expression was extracted from BARLEX website, along with the expression information during the different barley developmental stages. The map was created using the R program, using ComplexHeatMap package. after rehydration. **EMB**: Four-day Embryo. **ROO1**: Roots from seedlings. **LEA**: Shoots from seedlings. **INF1**: Young developing inflorescence. **INF2**: Developing inflorescence. **NOD**: Developing tillers, 3rd internode. **CAR5**: Developing grain. **CAR15**: Developing grain. **ETI1**: Etiolated seedling. **LEM**: Inflorescences, lemma. **LOD**: Inflorescences, lodicule. **PAL**: Dissected inflorescence, palea. **EPI**: Epidermal strips. **RAC**: Inflorescences, rachis. **ROO2**: Roots. **SEN**: Senescing leaves.

4.4.2 The expression analysis of *ALDH7A1* in different barley spices during drought stress

The *ALDH7A1* gene was first discovered to be differentially expressed during drought stress between drought-tolerant and drought-sensitive barley genotypes in 2009. It was identified as Contig2924 (Guo *et al.*, 2009). It was then BLAST aligned against the newly generated barley genome in BARLEX. The *ALDH7A1* gene was identified as the gene of interest and the three genotypes (Martin, Moroc, and HS41-1) used in aforementioned paper were obtained from Dr. Michael Baum from ICARDA in Lebanon. The seeds were grown in the green house (**3.2.2.1**). The T2 seeds were used for the expression analysis of the *ALDH7A* gene. The seeds were grown in a long day growth chamber. After reaching the third tiller (three weeks after sawing), plants were watered using 50 ml of -1 MPa PEG-6000 for 14 days. The third tiller was collected from three replicates, and RNAs were extracted (Fig 14).

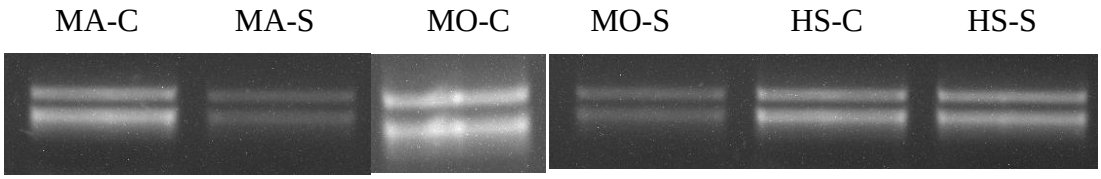


Figure 14 RNA extraction of three Hordeum vulgare genotypes. **MA-C**: Martin genotype under control conditions. **MA-S**: Martin genotype under drought stress conditions. **MO-C**: Moroc genotype under control conditions. **MO-S**: Moroc genotype under drought stress conditions. **HS-C**: Hordeum spontaneum genotype under control conditions. **HS-S**: Hordeum spontaneum genotype under drought stress conditions.

ADP-ribosylation factor 1-like protein (ADP) was used as a house-keeping gene for the barley genotypes under drought stress (Ferdous *et al.*, 2015). Primers for gene-specific expression analysis were designed to produce a 371 bp amplicon from cDNA, which was generated from previously extracted RNA (**3.8.2**). The cDNA was used as the starting material for two types of PCR reactions. First, the house-keeping PCR was done using ADP primers. Both control and drought stress samples produced equal amounts of product ensuring equal amounts of cDNA added to the reaction (Fig 15).

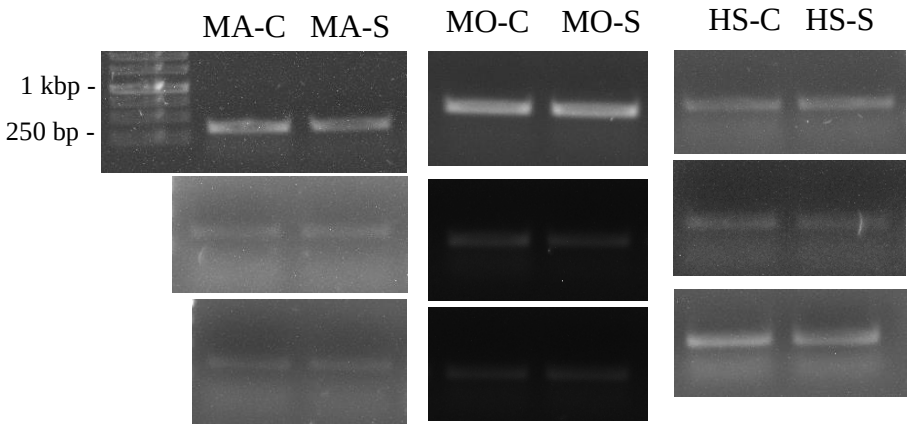


Figure 15 ADP PCR results. The results were reproduced in triplets to insure equal amounts of cDNA were used in the reaction. **MA-C**: Martin genotype under control conditions. **MA-S**: Martin genotype under drought stress conditions. **MO-C**: Moroc genotype under control conditions. **MO-S**: Moroc genotype under drought stress conditions. **HS-C**: *Hordeum spontaneum* genotype under control conditions. **HS-S**: *Hordeum spontaneum* genotype under drought stress conditions.

The same reaction constituents were used, except for using *ALDH7A1*-specific primers to analyse *ALDH7A1* RNA expression. The expression was elevated in all genotypes under drought stress conditions (Fig 16). The RNA expression was significantly higher by two-fold in Martin genotype, and three-fold in *H. spontaneum* genotype compared to control conditions. The expression increased by 1.3-fold in case of the Moroc genotype. In control conditions, the expression of *ALDH7A1* was similar in Moroc and Martin genotypes, but the relative expression was lower in case of *H. spontaneum*.

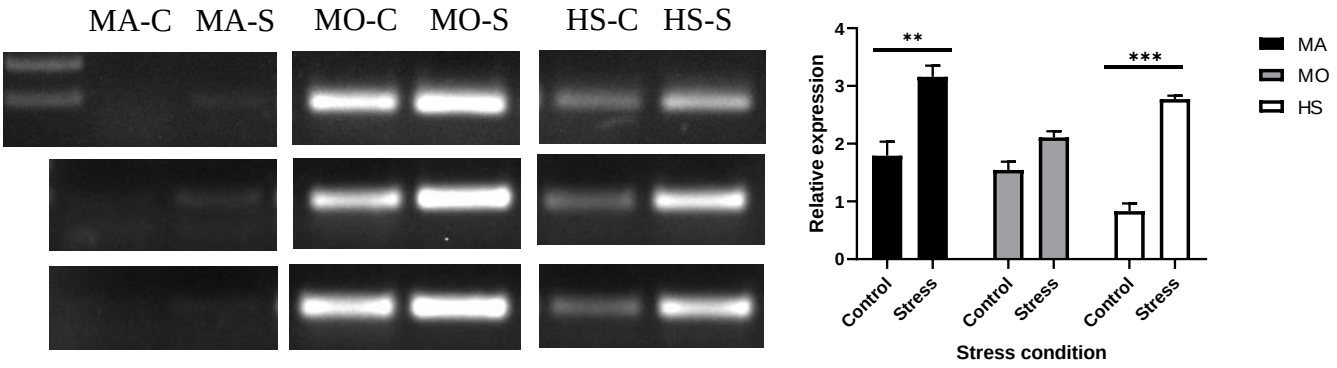


Figure 16 *ALDH7A1* PCR results. The results were reproduced in triplets to insure equal amounts of cDNA were used in the reaction. The graph shows the expression of *ALDH7A1* gene in relation to the expression of ADP gene expression in each genotype. The data reported are means ± SE (n=6). The star indicates the levels of significance in comparison to the control sample (two-way ANOVA, Tukey method): * $p < .05$; ** $p < 0.01$; *** $p < 0.001$ **MA-C**: Martin genotype under control conditions. **MA-S**: Martin genotype under drought stress conditions. **MO-C**: Moroc genotype under control conditions. **MO-S**: Moroc genotype under drought stress conditions. **HS-C**: *Hordeum spontaneum* genotype under control conditions. **HS-S**: *Hordeum spontaneum* genotype under drought stress conditions.

In order to check if the increase of the *ALDH7A1* gene expression responds to improved drought tolerance, two main drought stress signals have been calculated in all genotypes. Relative water content (RWC) in leaves is an important indicator of how well the plants perform during drought stress (Bornare *et al.*, 2012; Mullan & Pietragalla, 2012). After applying the drought stress for two weeks, the leaves were collected and weighed. They were then completely dried in an oven for two hours and weighed again, before being placed inside a water-filled 50 ml Eppendorf tube to reach the turgor weight and weighed. RWC was calculated (**3.2.2.3**). The three genotypes did not experience a significant loss of water from their leaves (Fig. 17). The Martin genotype lost 14% of its leaf water content, while the other genotypes lost less than 10% of their RWC.

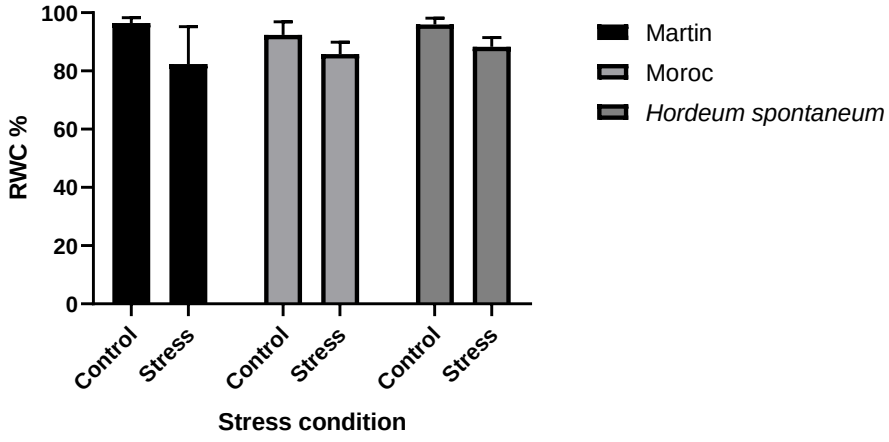


Figure 17 Diagram showing the difference in leaf relative water content (RWC) for Martin, Moroc, and *Hordeum spontaneum* genotypes under control and drought stress conditions. The data reported are means ± SE (n=6) (two-way ANOVA, Tukey method)

The other parameter that was determined for the three genotypes under normal and drought stress conditions was the malondialdehyde (MDA) content (Kotchoni *et al.*, 2006; Morales & Munné-Bosch, 2019; Srivastava *et al.*, 2006). The MDA was measured using the thiobarbituric acid (TBA) test following the Sunkar, Bartels and Kirch, 2003 paper. The MDA levels were higher in all genotypes after drought stress conditions compared to control conditions. The increase in the MDA levels was similar between Martin and *H. spontaneum* genotypes with 2.3 and 2.6-fold respectively, while the level in the Moroc genotype increased only by 1.4-fold between the stress level and control level (Fig. 18).

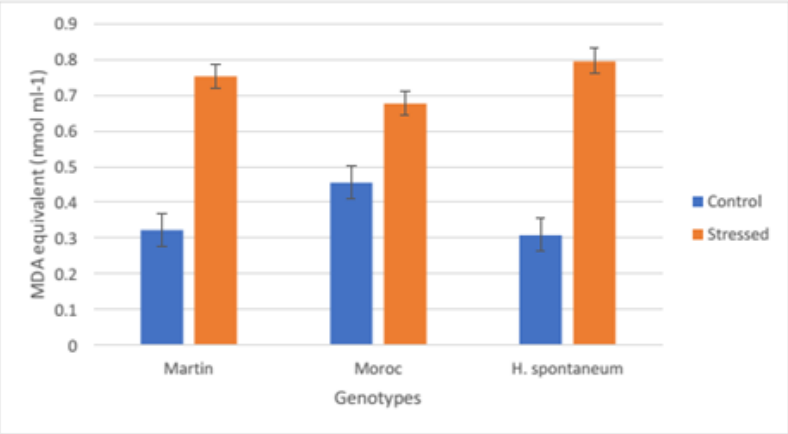


Figure 18 Diagram shows the difference in malonaldehyde (MDA) content for Martin, Moroc, and Hordeum spontaneum genotypes under control and drought stress conditions. The data reported are means ± SE (n=3)

During the germination of three genotypes, two other genotypes were grown for seed production. These two lines (111, and 4654) were used to check for the ALDH7A1 gene expression during three main developmental stages. The line 4654 is the line Golden Promise, which was used for the genome sequencing project (Beier *et al.*, 2017), and on which the bioinformatic results are based. While the line 111 (Viborg) was used as a control to check if the results were the same in other lines. Tillers, seeds inside kernels, and seeds were collected. RNA was extracted from the first two samples, with seeds proven to not allow clear RNA extraction (Fig. 19). cDNA was synthesized from extracted RNA, and usable cDNA was only obtained from tillers and seeds inside kernels. PCR reactions using ADP primers as house-keeping gene were used to ensure equal amounts of cDNA used in r reactions between tillers and seeds inside kernels.

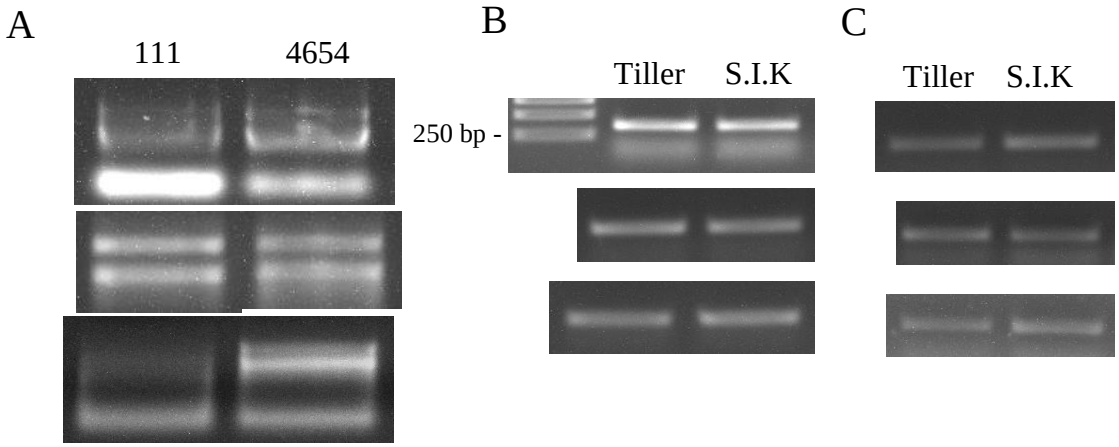


Figure 19 Several RNA extractions, and PCR results using *ADP* primers from 111, and 4654 genotypes. **A**: RNA extraction from tillers, seeds inside kernels, and developed seeds samples from both 111, and 4654 genotypes. **B**: PCR results using ADP primers as house-keeping gene in both tillers and seeds inside kernels from 111 genotype. **C**: PCR results using ADP primers as house-keeping gene in both tillers and seeds inside kernels from 4654 genotype.

ALDH7A1 gene expression was measured using PCR reactions with specific primers. The reactions were repeated three times. The relative expression of the gene of interest was higher in seeds inside kernels compared to the relative expression inside tillers in both genotypes (Fig. 20).

The expression of *ALDH7A1* gene in the line Golden Promise showed 70% increase between tillers and seeds, while only increasing by 33% in the case of line Viborg. The results came in accordance with the previous bioinformatic results which showed that *ALDH7A1* is highly expressed during seed maturation stages.

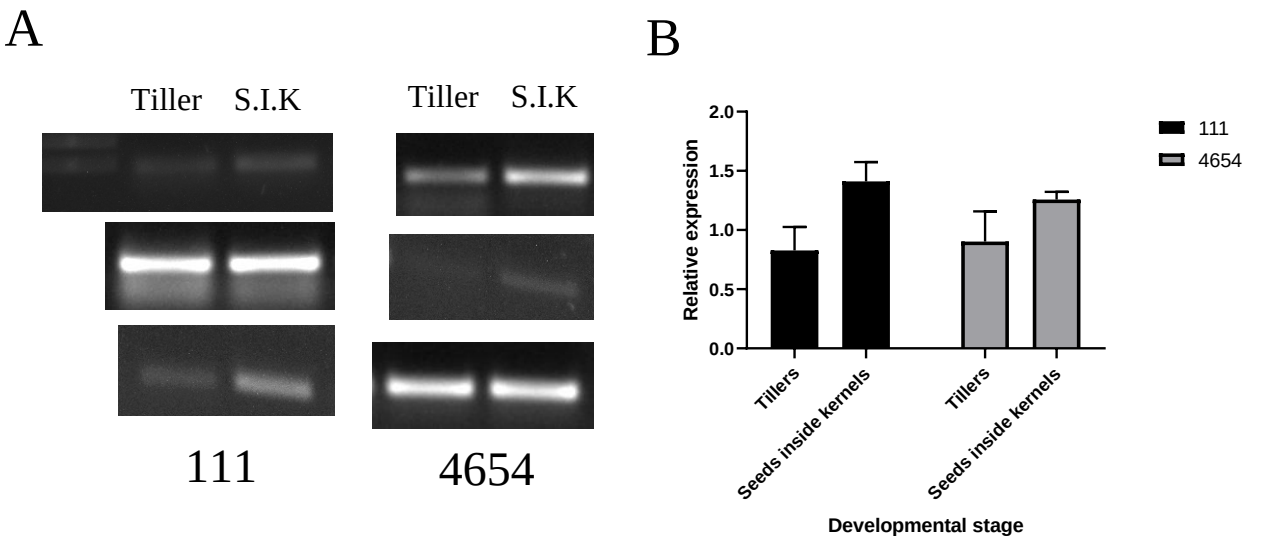


Figure 20 Expression analysis of *ALDH7A1* gene in tillers and seeds inside kernels. **A**: PCR results using *ALDH7A1* specific primers in tillers and seeds inside kernels in both 111, and 4654 genotypes. **B**: Relative expression of *ALDH7A1* in tillers and S.I.K in both genotypes compared to *ADP* expression. The data reported are means ± SE (n=6) (two-way ANOVA, Tukey method)

4.4.3 Analysis of the *ALDH7A1* promoter activity in the genotype Golden Promise

The final resort to understand the different expression levels of the *ALDH7A1* gene between the *H. spontaneum* genotype, and the other genotypes was to transiently transform a barley genotype with the *H. spontaneum ALDH7A1* promoter, and further analyze the GUS activity under different stress conditions.

First, the promoter region was amplified using two specific primers. The primers were generated based on the genome sequence of barley (Beier *et al.*, 2017; Mascher *et al.*, 2017). The PCR result from the amplification process was an amplicon of 904 bp. It was only possible to amplify the

promoter from the *H. spontaneum* genotype (Fig 21), while it was not possible to amplify it from Martin and Moroc genotypes were unable to replicate it.

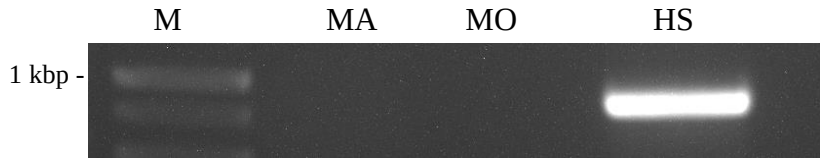


Figure 21 PCR result using promoter-specific primers. Only H. spontaneum was able to amplify the promoter of interest with 904 bp. **M**: 1kb DNA marker. **MA**: Martin genotype. **HS**: H. spontaneum genotype. **MO**: Moroc genotype.

The PCR band result was excised, eluted from the agarose gel, and ligated into the pJET vector using blunt end ligation. Colony PCR was performed using pJET-fwd and rev primers to ensure colony transformation at 1046 bp. Positive colonies were chosen (Fig 22) and inoculated into LB media overnight. The bacteria were pelleted, and the plasmids were extracted and sent for sequencing.

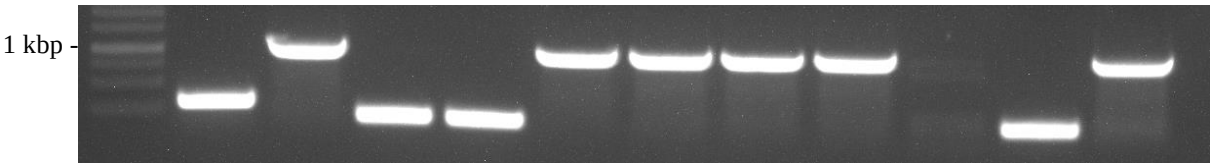


Figure 22 Colony PCR results using pJETfwd and rev primers. Two positive colonies were selected for further germination and vector extraction.

After sequencing, the promoter sequence was aligned with the published sequence to fill in the empty gaps. The cloned promoter fragment was then excised from the vector using XhoI and NcoI restriction enzymes (Fig 23) and ligated into the pBT10GUS vector (**3.1.6**) for further analysis.

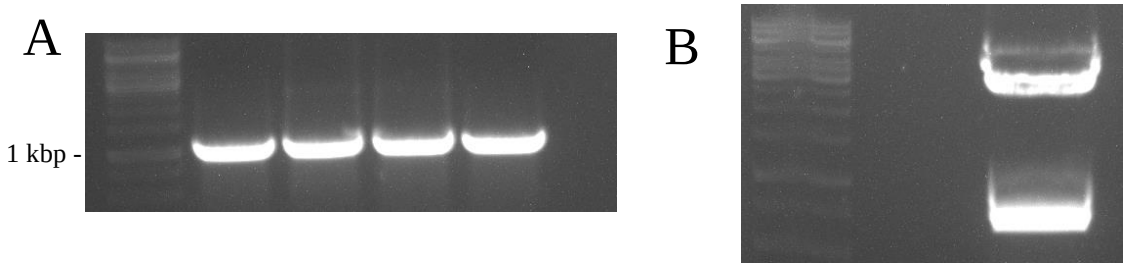
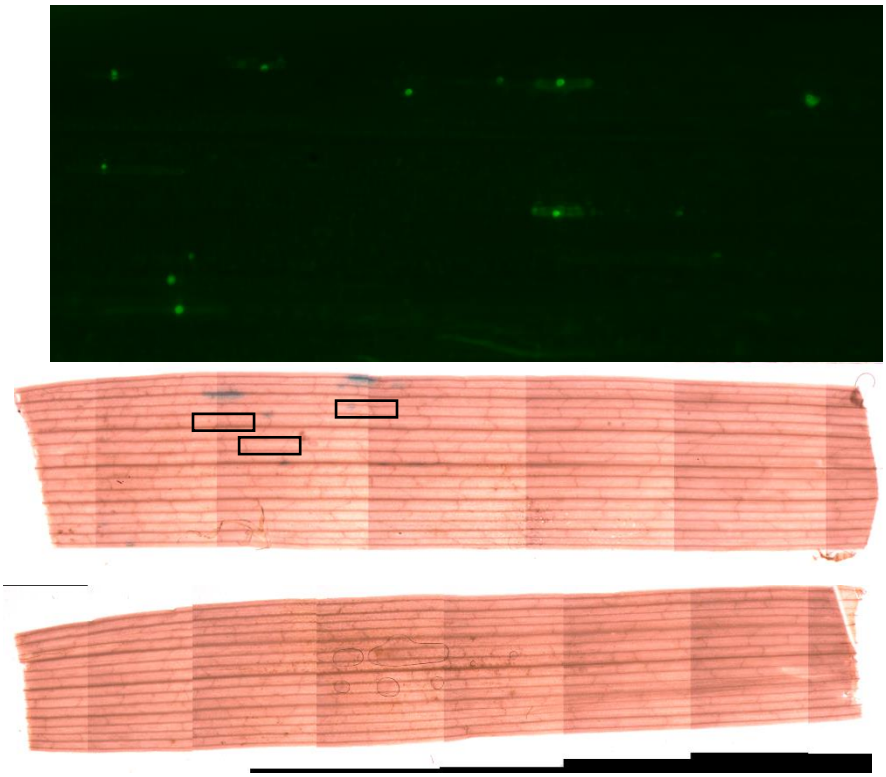


Figure 23 A: Confirmation PCR using pJETfwd and rev primers. **B**: Restriction digestion using XhoI and NcoI enzymes. The lower band conformed with the promoter sequence, and the higher band represented the rest of the vector.

In order to analysis the promoter activity during different stress conditions, the GUS vector along with the pSH221-GFP vector were co-bombarded using particle bombardment system (**3.14.1**) into three-week-old leaves of Golden Promise (**3.14.1.5**). The pUGAB7 vector - where GUS reporter gene is influenced by home-keeping Ubi-p promoter was used as a control vector for control samples. The leaves were placed on MS-media. The GFP fluorescence was first checked to ensure a successful bombardment. The GFP spots were counted (Fig. 24). Afterwards, the plates containing the leaves were transferred, and stressed under several conditions. For salt stress, the media was supplemented with 100 mM NaCl. For drought stress, the media was supplemented with 20% (/w/v) PEG-4000. For heat stress, the leaves were left inside a growth chamber at 37 ℃ for two days. Finally, for the cold treatment. the leaves were left at the 4 ℃ fridge. After 48 hours of stress treatment, the leaves were stained using glucuronidase tissue-staining technique (**3.14.1.8**). The leaves were kept at 37 ℃ overnight. The de-staining technique (**3.14.1.9**) was performed the next day until the leaves were completely white. The goal was to count all the blue spots resulting from the hydrolysis of the GUS enzyme to the X-gluc substrate to an intermediate product that dimerizes to an insoluble blue dye known as dichloro-dibromo-indigo. In the control samples, I was able to distinguish a small number of blue spots (Fig 24). Unfortunately, we were unable to identify any blue spots in any of the leaves that were under stress conditions.



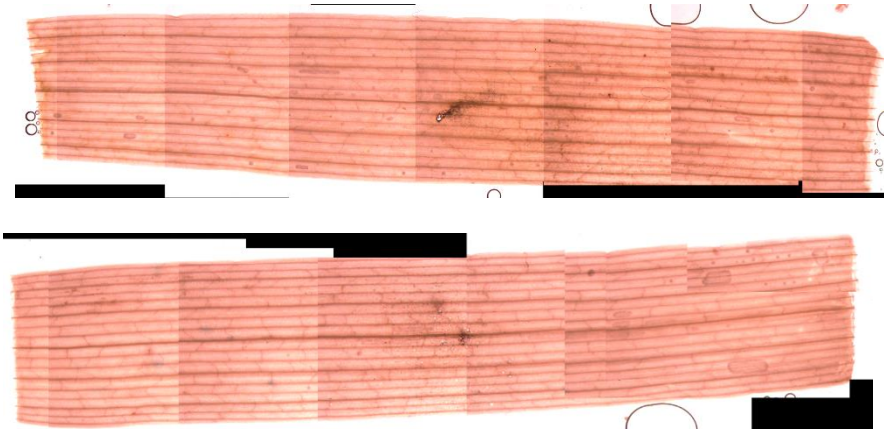


Figure 24 The results of the co-bombardment of *ALDH7A1* promoter-GUS vector, and GFP vector transiently in golden promise barley genotype leaves. The pUGAB7 vector replaced the promoter-GUS vector in control samples. The first picture show and example of GFP spots that were spotted following the co-bombardment process. The GFP spots were present in all samples. The second picture show the control sample, with the blue dots framed by the black rectangles. The third to the fifth picture represent the rest of the leaves that were placed under different stresses. No blue spots were noticed in any of the leaves irrespective of the stress.

4.4.4 Bioinformatic analysis of the *ALDH7A1* promoter

Since the promoter of *ALDH7A1* is newly defined and sequenced, a bioinformatic analysis was done to identify the different transcription factors that might influence the expression of the gene. All the motifs from *A. thaliana*, *H. vulgare*, and *Z. mays* were collected from the MEME suite website database. The motifs were then combined and used to compare with the promoter sequence to identify all the motifs. There were 27 motifs that were identified. All the motifs were then searched in available data bases to identify. 24 motifs and are shown in the figure below (Fig 25), seven motifs that are related with stress were placed in relation to the ATG sequence at the beginning of the gene. These motifs are as follow: TGA7 responsible for genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in *Arabidopsis thaliana*. BIM1 which is a new class of transcription factors mediates brassinosteroid-regulated gene expression in *Arabidopsis thaliana*. NAC029 is a gibberellin-mediated Della-Nac signaling cascade regulates cellulose synthesis in rice Nac29/31 directly regulates Myb61, which in turn activates CESA expression. DREB2D is dehydration response element binding factors (DREBs). It is one of the principal plant transcription factor subfamilies that regulate the expression of many abiotic stress-inducible genes. MYB63 is a transcriptional activator of the lignin biosynthetic pathway during secondary cell wall formation in *Arabidopsis thaliana*. MYB61

improves water-use efficiency in *Arabidopsis thaliana* by stomata regulation by tissue-specific expression of the *Citrus sinensis*. Finally, SPL1 was identified which confers plant thermotolerance at the reproductive stage.

Figure 25 Schematic representation of the *ALDH7A1* promoter found in Morex and *H. spontaneum* genotype. The different transcription factors are shown in relation with their distance from ATG sequence at the beginning of the gene sequence.

5 Discussion

The research of this thesis had several objectives. First, we wanted to investigate the effect of ALDH enzyme knock-out and overexpression mutants on the redox homeostasis of the cell during osmotic stress in *Arabidopsis thaliana*. Secondly, we wanted to examine the effect of ALDH7B4 enzyme knock-out and overexpression on seed germination and longevity. Finally, we wanted to check the effect of aldehyde dehydrogenase enzymes in monocots during drought stress, with the main focus on *Hordeum vulgare* species.

5.1 Objectives of *ALDH* analysis in *Arabidopsis thaliana*

5.1.1 Redox homeostasis status during osmotic stress

Plants are sessile organisms, which makes them more prune to environmental stressors. Drought is one of the biggest stresses threatening plants on earth (Rao *et al.*, 2006).

As a result of drought stress, stomatal closure results in decreasing the carbon dioxide concentration in chloroplasts. It leads to increased levels of photorespiration, followed by elevated ROS production inside chloroplasts and other cellular compartments (Smirnoff, 1993). Under non-stress conditions, ROS acts as a secondary messenger that modulates developmental processes inside the plant (Mittler *et al.*, 2004). Upon prolonged stress conditions, ROS starts to interact with enzymes and causes lipid peroxidation. One of the main products of the reaction between ROS and polyunsaturated fatty acids are reactive carbonyl species (RCS) (Alché, 2019; Jun'ichi Mano, Biswas, *et al.*, 2019; Jun'ichi Mano, Kanameda, *et al.*, 2019). Reactive aldehydes are part of RCS. They are small, mobile, and highly reactive compounds that target proteins, and genetic material causing damage and leading to cell death (H. H. Kirch *et al.*, 2001b; Sunkar *et al.*, 2003b).

In previous research, mutant lines overexpressing *ALDH7B4* showed better performance under abiotic stress compared to other overexpression mutant lines and wild-type plants. While knock-out mutant lines of the same gene were not able to survive or germinate under the same conditions (Kotchoni *et al.*, 2006). So far, no clear reason was proposed why the *ALDH7B4* overexpression mutant line performed better than the other lines. In an effort to better understand the effect, several *ALDH* overexpression and knock-out mutant lines were transformed using a peredox sensor

system. The peredox system allows for *in vivo* detection of NADH, and thus reports on the cytosolic NADH:NAD^+ ratio in the cytoplasm (Hung *et al.*, 2011; Steinbeck *et al.*, 2020).

After floral dipping, a faster selection process adapted from Harrison *et al.*, 2006 was used to determine the successfully transformed seedlings. The hygromycin selection media along with alternating light and dark periods showed a definitive way to select for positive seedlings. The long incubation in the dark at 22 ℃ allowed seedlings to solely depend on the media, with non-transformed seedlings not able to resist the hygromycin antibiotic and later death. The transformed seedlings were further grown on the selective media until true leaves were visible to ensure their ability to survive on that medium. They were then transferred to potting soil and allowed to produce seeds. The seeds were collected and further tested to ensure the homozygosity of the lines using peredox specific primers. All the positive lines showing only one band were allowed to continue growing on soil, and seeds of the T3 generation were used in this experiment.

After several alterations of the proper age and concentration of sorbitol for osmotic stress, nine-day-old seedlings which had been stressed for two days showed more consistent results than younger seedlings not being able to withstand the stress. 300 mM sorbitol was the concentration used to provide sufficient osmotic shock to the seedling (Claeys *et al.*, 2014). We were able to define which aldehyde enzyme is better suited to allow seedlings to tolerate osmotic stress during such young age and stress intensity. The roots of the seedlings were chosen to be inspected to avoid chlorophyll fluorescence under the confocal microscope.

The results in this research showed elevated levels of NADH under osmotic stress in all samples with the exception of the *ALDH7B4* OE mutant line. It showed that other lines were not able to cope with the demand for neutralizing the produced reactive aldehyde. It also showed that the overexpression of any ALDH enzyme does not always rescue the seedlings from osmotic stress. The *ALDH3F1* OE mutant line struggled to meet the needs to detoxify the cytoplasm. The *ALDH7B4* OE mutant line showed that the seedling was already producing enough enzyme, that when the stress occurred, it was able to detoxify the aldehydes, and keep the integrity of the cell intact. From these observations, we concluded that from the proposed line of ALDH enzymes, the ALDH7B4 is the most effective in scavenging the reactive aldehydes from the cytoplasm and allowing the plant to continue its development under osmotic stress.

5.1.2 The effect of ALDH enzymes on seed germination and longevity

Despite the long interest in understanding the effects of ALDH enzymes on the plant cell (H. H. Kirch *et al.*, 2001b; H. H. Kirch *et al.*, 2004; Sophos & Vasiliou, 2003), there has been little to no interest in understanding the effect they have on seed germination and longevity. In previous studies, the *ALDH7B4* RNA expression has been shown to be mainly located in the reproductive organs of the *A. thaliana* plants, namely flowers, stamens, pistils, and seeds, with no expression in leaves (T. D. Missihoun, Hou, *et al.*, 2014). In this study, we shed a light on the effect ALDH7B4 enzyme has using several knock-out mutants affecting germination and longevity.

The *DOG1*, *RDO2*, and *RDO4* genes have been studied and shown to be detrimental in the germination and longevity of *A. thaliana* seeds (Bentsink *et al.*, 2006; Y. Liu, Geyer, Zanten, *et al.*, 2011; Nakabayashi *et al.*, 2015; Nguyen *et al.*, 2012; Xiang *et al.*, 2014). *Delay of germination* (DOG) genes have been first discovered in 2003. They were described as a quantitative trait locus (QTL), which is essential for induction of seed dormancy (Alonso-Blanco *et al.*, 2003). The generation of the *dog1* mutant line showed it still requires light and gibberellic acid (GA) for germination. The *DOG1* gene expression is seed-specific (Bentsink *et al.*, 2006). The *RDO2* gene was discovered in 1996. The gene was discovered within a search for reduced dormancy mutants among freshly harvested M2 seeds. The *rdo2* mutant line showed normal levels of abscisic acid, and reduced sensitivity to GA biosynthesis inhibitors. The *RDO2* gene is believed to be part of the dormancy induction machinery in seeds (Léon-Kloosterziel *et al.*, 1996). The *RDO4* gene – also known as *Histone Monoubiquitination 1* (HUB1) was detected as a homolog for the E3 yeast enzyme responsible for H2B monoubiquitination (Fleury *et al.*, 2007). Loss of function mutants showed early flowering by upregulating the expression of the *Flowering Locus C* (FLC) gene (Cao *et al.*, 2008; Y. Liu *et al.*, 2007). The *rdo4* mutant line showed reduction in seed dormancy. But unlike the *rdo2* mutant line, the *rdo4* mutant line does not require GA for germination (Peeters *et al.*, 2002). In the first step, we wanted to check if there is a change in the expression level of the *ALDH7B4* enzyme in any of these lines. Both RNA and protein levels were analysed in both seeds and leaves (Fig.). In both cases, the expression level of the *ALDH7B4* enzyme was inversely disproportionate between WT and *rdo2*, and *rdo4* lines, with the *dog1* line having similar protein expression in both stages. The protein levels in leaves were unique, as there was relatively little expression of the gene of interest in WT as expected from previous studies. The expression of the

gene of interest was visible in *dog1*, and both *rdo4* genotypes. In *rod2*, the expression was relatively higher than in all other samples. In seeds, of the expression of *ALDH7B4* in WT seeds was three-fold higher than in *rdo2* (Col), and *rdo4* (Ler) mutant lines. It was 1.5 times higher than in *rdo4* (Col) mutant line, and only 0.3 times higher than in the *dog1* mutant line. These findings demanded both germination and longevity tests.

The germination test results were analogous to previous studies (Sunkar *et al.*, 2003a). Under normal conditions, the germination rates of all the lines were almost identical apart from the *dog1* mutant line reaching a germination rate of around 70%. It was a significantly lower germination rate compared with previous results (Graeber *et al.*, 2014). This showed that the overexpression or knock-out of the proposed ALDH enzymes do not affect germination rates under normal conditions.

In order to test the seed longevity, a CD test was carried out using all the ALDH mutant lines along with WT and *dog1* mutant lines as controls. The *dog1* mutant line performed as expected (Bentsink *et al.*, 2006, 2010; Finch-Savage *et al.*, 2007). It was unable to retain its seed viability after being exposed to a deterioration treatment for three days. Starting from day six, it showed a lower germination rate than the rest of the samples. It continued its downward trajectory until reaching a 27% germination rate after 21 days of treatment. The WT line was able to keep consistent germination rates until 12 days of treatment. Afterwards, the germination rate suddenly dropped to 47% at day 15, and germination rate of 32% at day 18, before suddenly rising to 66% at 21 days of treatment. The rest of the samples showed fluctuations in the germination rate. The samples showed a steady decrease until day 12. At day 15, *aldh3f1* and *aldh3i1 mutant lines* showed an increased germination rate by 7% and 4% respectively. At day 18, both *aldh7b4* and *ALDH3F1* mutant lines had an increase to 90%, before decreasing at the next point. The sudden increase that occurred in the germination rates of these lines might be due to using different seed batches for each repetition. Those batches might have different initial germination potential, which means they would not be affected in the same way by the same stresses that they face in this experiment.

The minor increases that were spotted in the first days of the deterioration treatment could be explained by taking into consideration that the number of seeds was not always the same between the different treatments. So even though the fitness of the line may decrease, if the seed number is higher, it might end up scoring higher germination rates. In previous studies, the CD test was

performed with a maximum of 10 days (Bueso *et al.*, 2014; Gordin *et al.*, 2015; Leão-Araújo *et al.*, 2017; Nguyen *et al.*, 2015; Xiang *et al.*, 2014; Zinsmeister *et al.*, 2016). It seems that using much longer deterioration treatments leads to the bigger fluctuations in germination rates. From the results in our research, we would suggest that this experiment should not be performed for more than 12 days.

Considering the results up to 12 days, the overexpression of *ALDH7B4* and *ALDH3F1* helped the mutant lines to maintain up to 89% of seed vigor. While using the knock-out mutant line of *aldh7b4*, *aldh3f1*, and *aldh3i1* reduced the longevity of the seeds by 17%, 15%, and 17% respectively. The WT line came third affected by the deterioration conditions, while the *dog1* mutant line lost 65% of its seed viability in the process. These results agree with previous reports. The *OsALDH7* protein has shown to be essential for seed maturation, longevity by detoxifying MDA inside dehydrated seeds (Shin *et al.*, 2009). *ALDH3F1* has been shown to be expressed during oxidative stress, with overexpression lines showing less H_2O_2 and MDA content upon salt stress (N. Stiti, Missihoun, *et al.*, 2011).

The expression of the *ALDH7B4* protein in *dog1*, *rdo2*, and *rdo4* mutant lines was unique. The enzyme was found in both leaf and root samples of the *dog1* line, suggesting no direct interaction between both genes. In the case of *rdo2*, the expression of the *ALDH7B4* protein was significantly higher than in WT under normal conditions in leaf samples. It might be due to the fact that the *RDO2* gene encodes a TFIIS transcription elongation factor (Y. Liu, Geyer, van Zanten, *et al.*, 2011). This elongation factor has been proposed in helping to reduce oxidative and transcription stress. Transcription stress occurs due to several environmental conditions (ultraviolet light), along with reactive oxygen species (ROS), and the resulting hydroxyl species can cause DNA lesions (Hoeijmakers, 2001; Lindahl, 1993). Those lesions can affect the DNA transcription process. It can physically impede the progress of RNA polymerase II (W. Wang *et al.*, 2018). This stress can produce mutant transcripts, decrease the abundance of vital mRNAs, and increase genome instability (Lans *et al.*, 2019) . In the *rdo2* mutation. the need for stress-related enzymes like ALDH7B4 is high, since the main product of *RDO2* gene is missing, which has been shown to be involved in resisting biotic stress. The *RDO4* (**HUB1**) gene is involved in H2B (histone 2B) monoubiquitination. This H2B is involved in several plant processes like flowering and defense against biotic and abiotic stress (Chen *et al.*, 2020; Dhawan *et al.*, 2009;

Patel *et al.*, 2015). The *hub1* mutant line was described with less biomass, pale leaves, and modified leaf shape, which resulted from defects in cell cycle processes (Fleury *et al.*, 2007). So, it was not surprising that a stress-related enzyme would also be expressed in the *rdo4* mutant line. Finally, it has previously been reported that several "stress-related" genes are down-regulated in mature seeds in both *rdo2*, and *hub1* mutant lines (Y. Liu, Geyer, Zanten, *et al.*, 2011). Upon reviewing the supplementary material including a list of differentially expressed genes in both *hub1-2* and *rdo2-1* mutant lines, we were not able to confirm the presence of the *ALDH7B4* sequence. *ALDH7B4* is one of the stress-related genes that are not present in mature seeds of both lines. It might be a direct target for either of them, or theses lines were not able to produce enough of the protein during seed maturation to allow it to be detected. The lines have also been described with reduced dormancy due to the down-regulation of the *DOG1* gene.

5.1.3 The aldehyde dehydrogenase family in *L. brevidens* and *L. subracemosa*

Lindernia brevidens was established as a desiccation tolerant plant in 2008. It has been shown that late embryogenesis abundant (LEA) proteins were abundantly expressed in desiccated leaves of *L. brevidens* (Phillips *et al.*, 2008). In a more detailed approach to understand the origins of this desiccation tolerance ability, a comparative genome and RNA-seq study was established between the desiccation tolerant *L. brevidens* against its desiccation sensitive relative *L. subracemosa*. The paper describes the ability of *L. brevidens* plants to upregulate a cluster of genes related to ABA-responsive and seed-specific elements that enabled it to withstand harsh conditions without dying, and giving it the ability to flourish in 48 hours upon rehydration (Van Buren *et al.*, 2018). The genome sequence information and the RNA-seq data have been used to identify the aldehyde dehydrogenase family in both plants. A heat map was established with all candidates describing their expression during dehydration and rehydration. We were mainly interested in the ALDH genes that were upregulated during dehydration, and downregulated once the rehydration started. Lbr_028666 was the only gene that fulfilled these conditions. It was differentially expressed in *L. brevidens* and not in *L. subracemosa*. After comparing the protein sequence with the *A. thaliana* and *Zea mays* protein databases, it shared over 50% of its sequence with *ALDH5F1*. *ALDH5F1* encodes a succinic semialdehyde dehydrogenase (SSADH) enzyme. The enzyme resides in the mitochondria (Busch & Fromm, 1999). The SSADH enzyme is part of the ɣ-aminobutyrate (GABA) pathway, which was shown to be elevated during abiotic stresses. Ssadh mutant lines

showed leaf necrosis under UV light stress and heat stress. Along with accumulating higher H_2O_2 under the same conditions (Bouche *et al.*, 2003). It seems that the homolog gene in *L. brevidens* is involved as well in helping the plant against abiotic stresses such as drought.

5.2 Aldehyde dehydrogenase in monocots

5.2.1 The Aldehyde dehydrogenase family in *Hordeum vulgare*

In 2009, Guo *et al.* were able to identify a number of differentially expressed genes in two drought tolerant genotypes of barley (Martin and *Hordeum spontaneum*), and the drought sensitive genotype (Moroc) (Guo *et al.*, 2009). One of these genes was identified as Contig2924_s_at and defined as an aldehyde dehydrogenase. Upon further bioinformatic search, we found out that it the gene encodes the ALDH7A1 enzyme. As a result, we decided to examine the expression level of the gene during drought stress, and to analyse the promoter of the gene to identify any transcription factor binding sites related to drought stress. The three genotypes (Martin, Moroc, and HS41-1) were donated by Dr. Michael Baum (International Center for Agricultural Research in the Dry Areas (ICARDA), Rabat, Morocco), and they were sown to produce new seeds for our experiments. The T2 seeds were grown until the third leaf was visible, and then the seedlings were drought stressed for 10 days. The third leaf was separated, and RNA extraction was performed. The *ADP* gene was used as the house-keeping gene as recommended by Ferdous *et al.*, 2015 (2.6.1). The relative expression of ALDH*7A1* was significantly higher under drought stress in both drought tolerant genotypes, and it did not increase significantly in the Moroc genotype which is drought sensitive. Therefore the expression level was in agreement with the results from Guo *et al.*, 2009 and it showed a positive correlation between the level of *ALDH7A* expression and drought tolerance. The *ALDH7A1* gene is upregulated in drought tolerant genotypes, and not upregulated in drought sensitive genotypes.

In order to check if the increase in the *ALDH7A1* expression is translated into better drought tolerance inside the genotypes, both RWC and MDA levels were determined in all genotypes under both conditions. The changes in RWC were insignificant under normal and stress conditions for all the genotypes. On the MDA front, the MDA levels in both drought tolerant genotypes were similar. In the Moroc genotype, the MDA level was unique. Under non-stress conditions, the MDA level was higher than in the other genotypes. But once under drought stress, MDA levels were

lower than in the other genotypes. This could be explained by the fact that in general, barley plants are one of the most abiotic stress tolerant crops (Jamshidi & Javanmard, 2018). The drought conditions that have been applied could have not been severe enough to get a proper response from the drought resistant genotypes. The other reason could be that even, though the *ALDH7A1* expression was higher in the drought tolerant genotypes. The ALDH7 family members are not as involved in drought tolerance in barley as they are involved in *A. thaliana*.

While these experiments were running, two different barley genotypes were used to detect the relative expression of the *ALDH7A* gene in three different developmental stages in non-stress conditions. RNA extraction was successful in both tillers and undeveloped seeds (seeds inside kernels). It was not possible to extract usable RNA from fully developed seeds using Trizol reagent. Therefore, we proceeded with the first two stages. The relative expression of ALDH*7A* was higher in the undeveloped seeds than in the tillers. The results were analogous to the bioinformatic results obtained from the Barlex website (https://apex.ipk-gatersleben.de/apex/f?p=284:10). The gene expression profile showed an increase in the gene expression during grain development (IPK Gatersleben, 2020).

We were able to identify several motifs in the promoter region of the *ALDH7A* gene. The promoter region of the barley gene was compared to motif databases from both *A. thaliana* and *Z. mays*. DREB2D and MYB61 motifs were identified in the *ALDH7A* promoter region. The DREB2D belongs to a family of dehydration response element binding factors (Morad-Talab & Hajiboland, 2016). The MYB61 motif was found to affect the stomatal regulation to increase water-use efficiency (Meraj *et al.*, 2020; Morad-Talab & Hajiboland, 2016). Both motifs alongside others can increase the possibility that *ALDH7A* is involved in tolerance of several abiotic stresses.

The final approach used to explain the difference in the expression level of *ALDH7A* genes between the different barley genotypes was a promoter analysis. Based on the published barley genome (Beier *et al.*, 2017; Mascher *et al.*, 2017), promoter-specific primers were designed. The primers were able to amplify the promoter region only from the *Hordeum spontaneum* genotype. It suggests that the difference in the expression of the *ALDH7A* gene between the different genotypes might be due to the lack of some transcription binding sites or motifs in the promoter region of Moroc or Martin genotypes due to DNA insertions or deletions**.** The promoter sequence was cloned inside a pBT10GUS vector, and co-bombarded with pSH221-GFP vector inside

excised leaves from 10-day-old Golden Promise seedlings. In control samples, pUGAB7 vector including a Ubi-p promoter driven GUS was used instead of the promoter vector. After a day's rest on MS media, the GFP spots were visible in all samples. That allowed to conclude that the co-bombardment was successful. The leaves were then exposed to different stress conditions (drought, salt, heat, and cold). In the control samples, we were able to identify a small number of blue spots. In all the other samples from stress-treated leaves, we were not able to identify any blue spots. The lack of blue spots in any of the stress samples poses a contradiction to our previous results showing that the *ALDH7A* gene is upregulated in the *Hordeum spontaneum* genotype during drought stress. It could be explained by a number of reasons. First, that the stress conditions on the MS media were not severe enough to get a response from the promoter sequence. Second, that there were several stress-related transcription factors that were lacking in the Golden Promise genotype. Therefore, although the transformation was successful, the promoter was not able to properly function in the leaf. Third, it could be hypothesized that a longer period of stress would be able to have a noticeable effect on the leaves.

Finally, a more comprehensive bioinformatic search for all aldehyde dehydrogenase included in *Hordeum vulgare* Morox sp. was performed. All the aldehyde dehydrogenase genes from *A. thaliana* and *Z. mays* were aligned against the published barley genome using the BLAST function in the BALREX website. All the identified genes were grouped in several classes. The expression profiles for each gene were collected and displayed in a heatmap. The expression was displayed for several developmental stages as shown in figure 13. In our case, we were interested in genes that would be differentially upregulated in barley reproductive organs, and during grain development as it represents the natural dehydration stages inside the plant life cycle. Only two genes fitted our criteria. *HORVU6HG0519800-1*, and *HORVU5HG0165080-1* belonging to the *ALDH2*, and *ALDH7* families respectively. The former matches to the same family in which the *L. brevidens ALDH* genes were also upregulated during drought stress. The latter belonged to the *ALDH7* family which has been reported to affect seed maturity and germination (Shin *et al.*, 2009).

The *ALDH2* gene is a good candidate for further analysis during drought stress. It would be a good approach to analyze the promoter region to further motifs related to tolerance against abiotic stress.

6 Future perspectives

The extensive characterization of aldehyde dehydrogenase in this thesis provides insights on the role of them during osmotic stress, and how they help *Arabidopsis thaliana* plants tolerate the production rise of ROS and RCS that cause lipid peroxidation and could lead to cell death. Additional investigation during other types of stresses like salt or dehydration stress could show which aldehyde gene is more efficient during those stresses, and if the overexpression of said gene would help the plant face that stress.

In order to further understand the role of the aldehydes during seed germination and storage, the aldehyde dehydrogenase mutant lines were grown and stressed to determine how would they fare under harsh conditions. The expression analysis of *ALDH7B4* gene in *dog1*, *rdo2*, and *rdo4* mutant lines showed a significant expression decrease in *rdo2*, and *rdo4* mutant lines. The next step is to investigate through protein-protein interaction approaches if there is a physical interaction between those proteins, and if the knock-out of the *ALDH7B4* gene could cause a different expression level of both genes.

The newly constructed *Lindernia brevidens* and *Lindernia subracemosa* ALDHs heat maps was able to show the different aldehyde expressions during dehydration and rehydration cycle. More extensive research regarding the differentially expressed *ALDH* genes during dehydration might shed a light on the role of these gene during dehydration stress.

Finally, more physiological and biochemical analysis is needed for the ALDH gene family in barley, with more stresses needed to elucidate the role of ALDH genes during different stresses and throughout the life cycle of the plant.

7 Summary

Drought stress is one of the most severe abiotic stresses that negatively affects plant germination and crop production worldwide. It affects several biochemical processes like photosynthesis, and increase ROS production and cell death. If drought stress occurs during reproductive stages, it can cause up to 90% yield loss in maize and 56% in barley.

In order to withstand this abiotic stress, plants use a combined strategy of mechanical and biochemical processes to increase water retention and to reduce negative effects of ROS on cell metabolism. In our lab, we have been focusing on studying ALDH enzymes and their effect on improving abiotic stress tolerance in plants. This thesis focuses on new aspects of ALDH in *Arabidopsis thaliana* and barley. Our main focus was studying the effect of overexpression of ALDH enzymes and knock-out on the redox homeostasis during oxidative stress, as well as the role of *ALDHs* during the germination, and the longevity of *A. thaliana* seeds. We also wanted to compare the *ALDH* gene family in both drought resistant *L. brevidens* and its closely related drought sensitive counterpart *L. subracemosa*. Finally, we wanted to unveil the *ALDH* family in *Hordeum vulgare*, and its possible roles under drought conditions.

First, the WT, *aldh7b4*, ALDH7B4 OE, ALDH3F1 OE, and *aldh3i1* mutant lines were transformed with Agrobacterium containing a peredox sensor vector via floral dipping. The resulting mutant lines were grown on hygromycin media for selection, followed by growing them again to obtain T3 seeds. The seeds were placed on ½ MS plates supplemented with sorbitol to initiate oxidative stress. The roots of the collected seedlings were placed under the confocal microscope for observation. Only the roots of ALDH7B4 OE mutant line showed a significant increase in fluorescence compared to WT roots under normal conditions. This showed that the ALDH7B4 enzyme in the mutant line is more active in neutralizing the reactive aldehydes even under non-stress conditions. When oxidative stress was applied, the ALDH7B4 OE mutant line was the only line able to match the increase in ROS and reactive aldehyde production, whereas all the other lines were not able to keep up with the increase. This allows to conclude that the ALDH7B4 enzyme is the most effective of the enzymes tested in neutralize the reactive aldehydes and to protect the plant facing drought and oxidative stress.

Secondly, the RNA and protein expression of the ALDH7B4 gene was measured in mutant lines which affect germination: *dog1* (Col), *rdo2* (Col), *rdo4* (Ler), and *rdo4* (Col). The relative protein expression of the gene was significantly lower in *rdo2* and *rdo4* lines compared to WT. Under non-stress conditions, the germination rates of all the lines were similar except for the *dog1* mutant line which showed -as predicted- significantly lower germination rates. That shows that the over-expression or knocking out of ALDH genes does not affect the germination rate under non-stress conditions. In order to measure the effect of the *ALDH* genes on seed longevity, the over-expression and knockout mutant lines were subjected to a controlled deterioration test (CDT), in which, the seeds are subjected to accelerated deterioration conditions of high moisture content, followed by heat treatment. The Seeds were collected after 3 days periodically, and after 21 days the seeds were germinated. After 12 days of stress, both over-expression mutant lines were performing the best out of all the tested lines with 89% germination rate. This was followed by the WT line, followed by the knockout mutant lines, and finally the *dog1* mutant line as expected. It shows that the over-expression of *ALDH* genes in *A. thaliana* can help to increase seed longevity.

Afterwards, we explored the *ALDH* gene family in the Linderniaceae family. An RNA-seq map of dehydration and rehydration cycles of the desiccation tolerant *L. brevidens* against its desiccation sensitive relative *L. subracemosa* was used to look for *ALDH* genes in both plant species. Nineteen *ALDH* genes were uncovered. They belong to four different classes, with class one ALDH being the most active during the dehydration rehydration cycle. Specifically, the ALDH5 Lbr_028666 gene was the only gene to increase during the dehydration period, and decrease after rehydration. This points to its importance during the drought stress.

Then, we embarked to explore the role of the *ALDH* gene family in barley. In 2009, an *ALDH7A1* homologue gene was found to be differentially expressed during drought stress in two drought tolerant genotypes of barley (Martin and *Hordeum spontaneum*), and not in a drought sensitive genotype (Moroc). First, a bioinformatic search was done on the promoter region of the *ALDH7A1* gene to identify all possible motifs which might indicate its involvement in the resistance of other abiotic stresses. DREB2D and MYB61 motifs were identified. They have been previously found to be involved in dehydration responses, and to affect the stomatal regulation to increase water-use efficiency. Those and other motifs point to the involvement of the gene in abiotic stress tolerance. The relative expression of the gene was measured in the three genotypes after drought

stress for confirmation. The expression analysis was in line with the previous research, in which the relative *ALDH7A1* gene expression was significantly higher in the drought resistant genotypes after drought stress than in drought sensitive genotype. We also measured the relative expression of the *ALDH7A1* gene in two developmental stages in the plant (vegetative, reproductive). In both genotypes, the relative expression of the gene was higher in seeds-inside-kernels than in tillers. It was in agreement with previous studies showing that the *ALDH7B4* gene had the highest expression level in reproductive organs and seeds.

In order to check if the increase in the relative *ALDH7A1* gene expression is related to better drought resistance, two measurements (RWC, MDA) were taken for the three genotypes (Martin, Moroc, *Hordeum spontaneum*) under non-stress and drought conditions. Unfortunately, the readings were not conclusive of the direct participation of the gene-of-interest in drought resistance.

Next, the ALDH7A1 promoter region was co-bombarded into the barley var. Golden Promise genotype to investigate the promoter reaction to several abiotic stresses. The transformed plants were subjected to drought, heat, cold, and salt stress. While the GFP inserts were active after the transformation confirming the validity of the transformation technique, the promoter region was not activated after any of the stresses. It might have been because the stresses were not severe enough for the promoter region to be activated.

Finally, an extensive bioinformatic heatmap was constructed for all potential *ALDH* gene family members. The data was collected from BARLEX website. Twenty-six potential genes belonging to eight classes were found and analyzed. The focus was on the genes that showed an increase in later developmental stages and seed formation. Both *HORVU6HG0519800-1*, and *HORVU5HG0165080-1* genes fit that description. They belong to ALDH2 and ALDH7 families respectively. More research is needed on the gene belonging to the ALDH2 family, as it showed higher expression during developing 3rd tiller, developing grains, four-day-old embryo, and was less expressed otherwise.

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9 Supplementary material

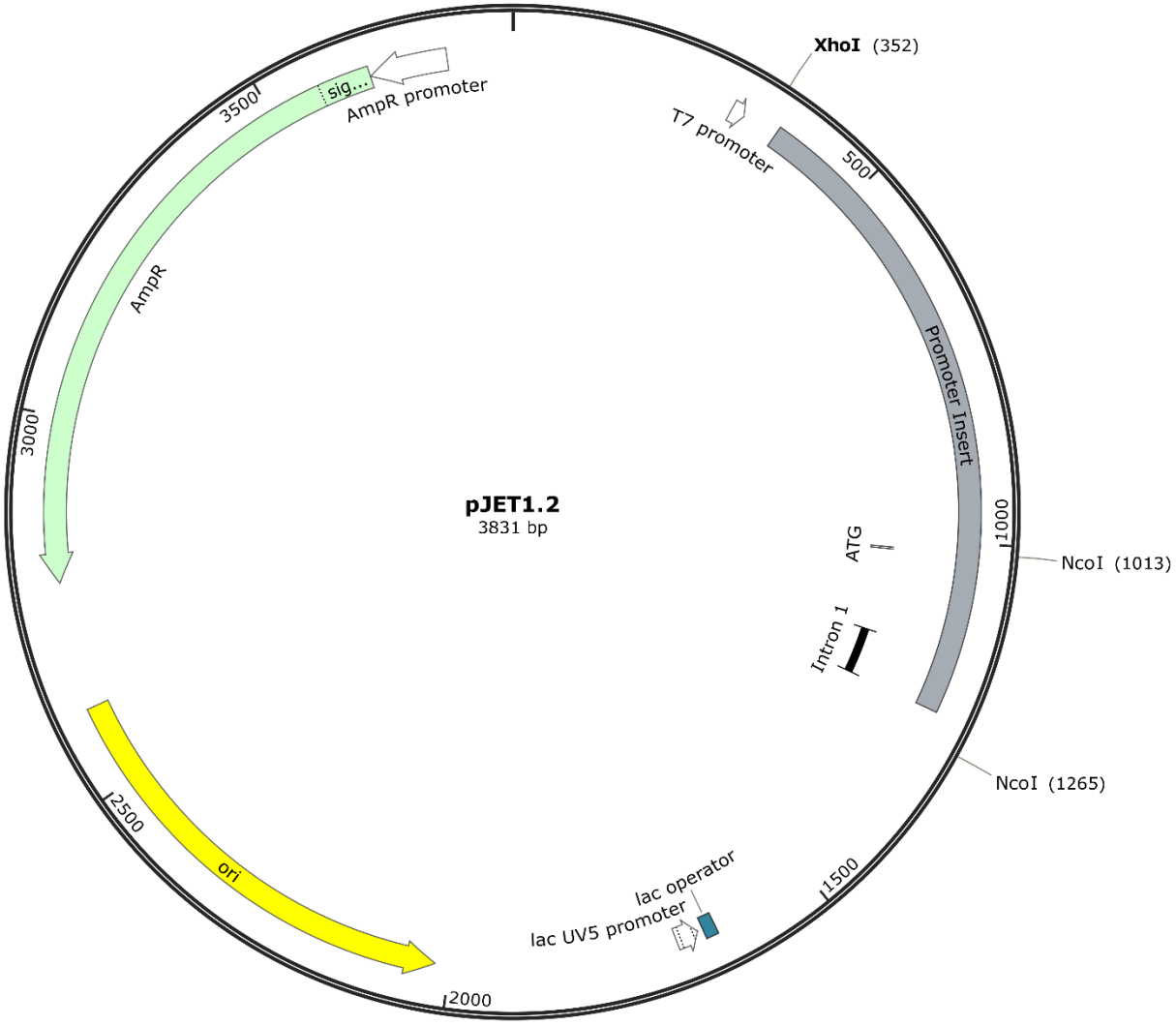

Figure 26 pJET1.2 with ALDH7A1 promoter insert map

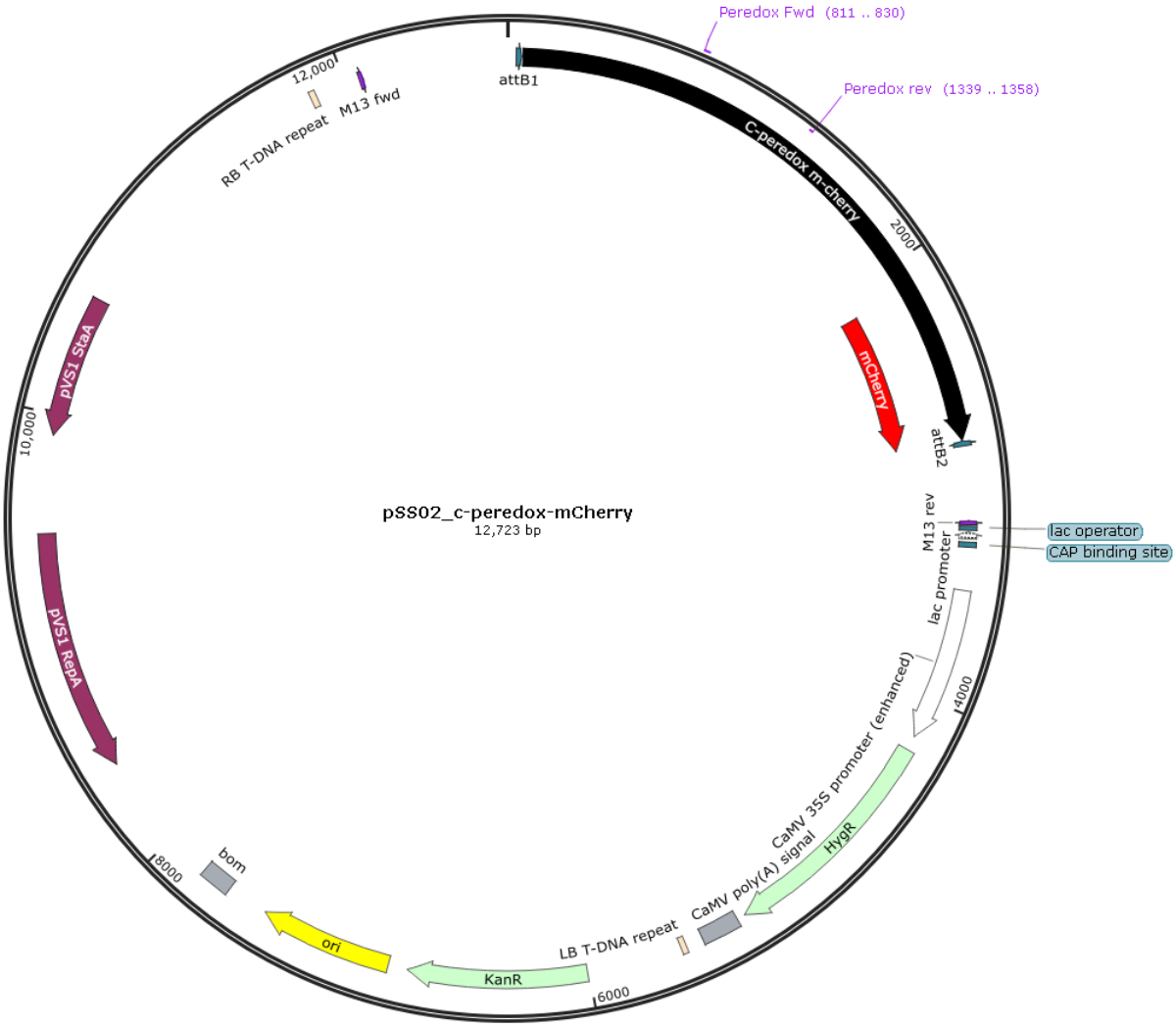

Figure 27 pSS02_c-peredox-mCherry map

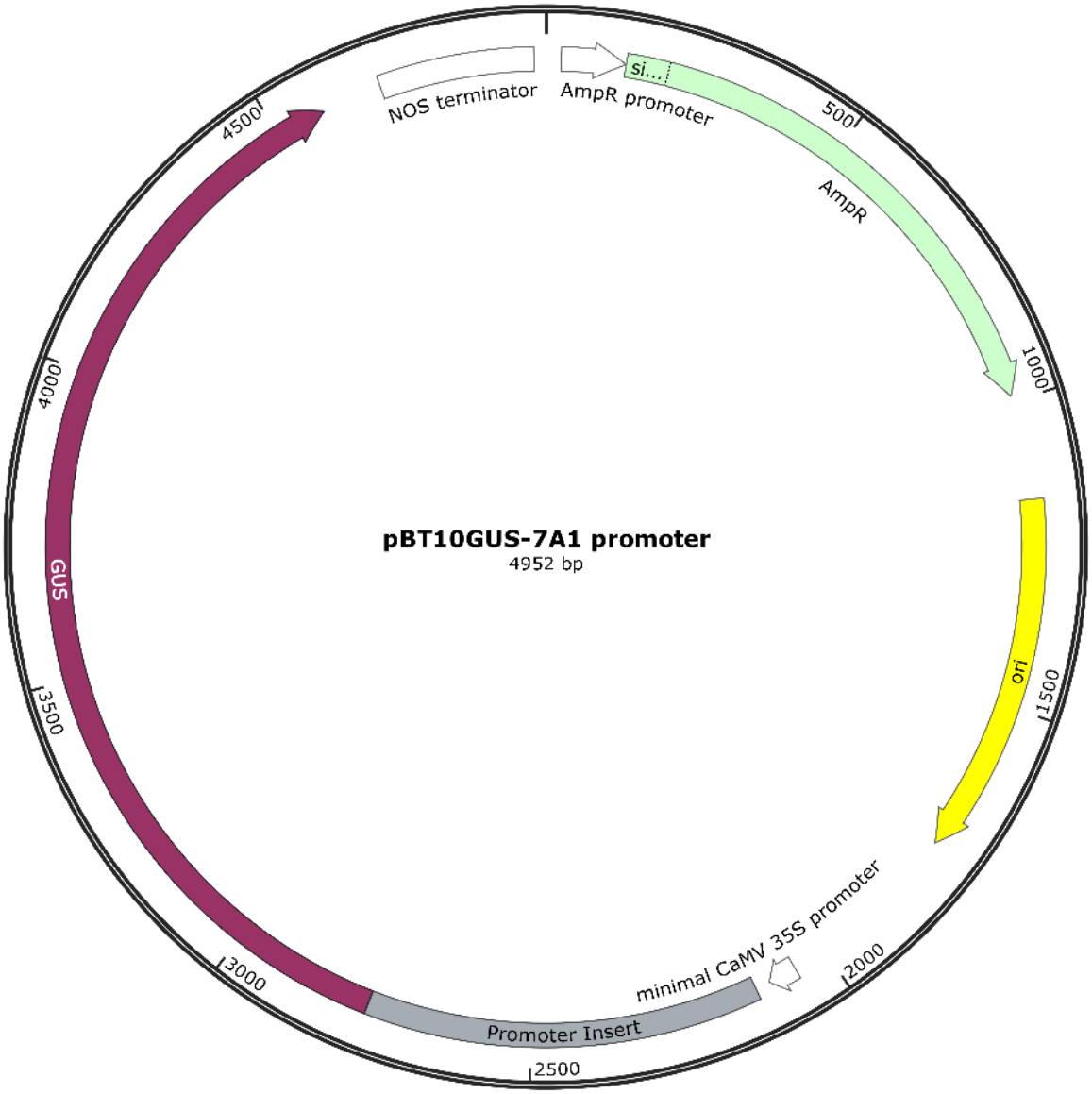

Figure 28 pB10GUS vector including *ALDH7A1* promoter

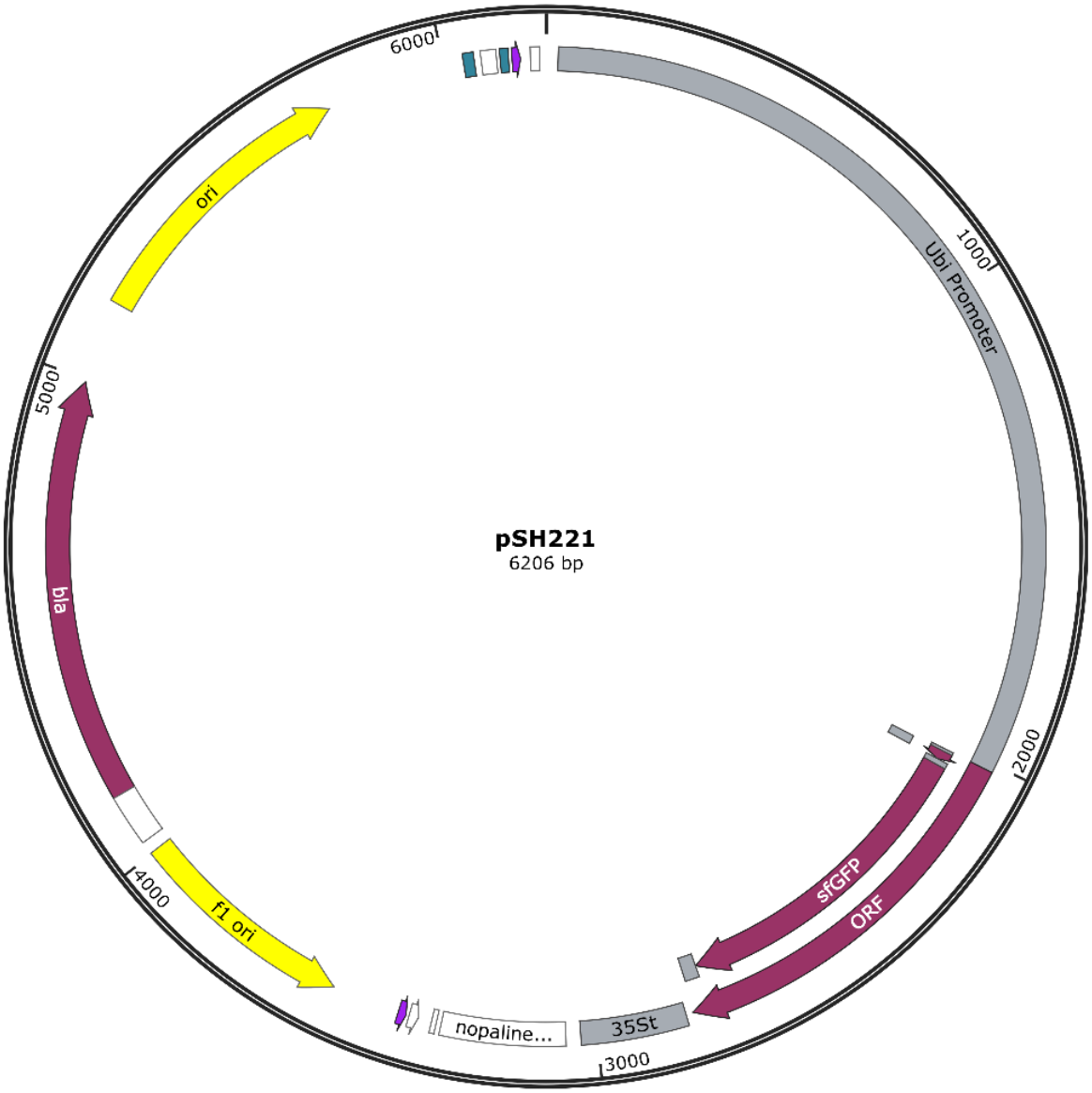

Figure 29 pSH221 GFP vector

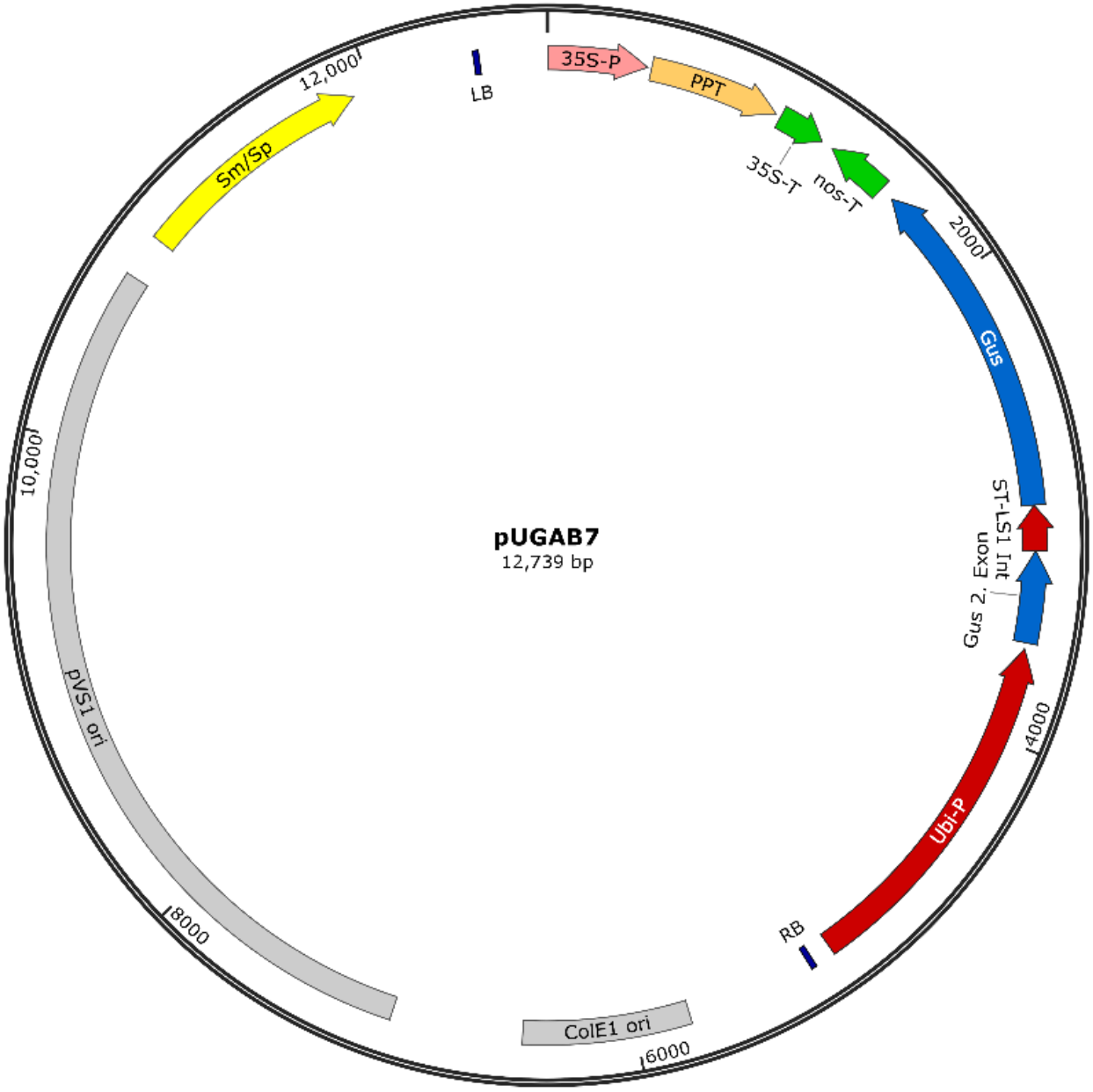

Figure 30 pUGAB7 GUS vector

10 Acknowledgements

I would like to give a special thanks to my supervisor Prof. Dorothea Bartels. She has believed in me since day one in my master's degree. Her understanding, guiding and patience has made all the difference throughout my PhD and the COVID time as well. I would also like to thank the DFG-supported Graduierentenkolleg for the funding of my thesis.

I would like to thank all my Graduiertenkolleg 2064 colleagues for all their help, advice and time working with new plants and different techniques. I would like to extend my gratitude with all the people working in Dr. Jochen Kumlehn in Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) institute for all their help and hospitality during my work with the barley plants.

I want to thank all my lab mates and students who have been essential for the results of this thesis. I want to thank Guido, Verena, Sarah, Jennifer, Aish, Selva, Niklas, and all my labmates from labs one and two.

And finally, I would like to thank my family for their continuous support, and my friends for their time and compassion in facilitating my life in Germany and helping me whenever possible.