Natural genetic variation in the expression regulation of chloroplast antioxidant system among *A. thaliana* accessions.

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CONTENT

LIST OF ABBREVIATIONS

A	ampere
A_{260}	absorbance at λ = 260 nm
ABA	abscisic acid
ACC	acclimated plants
Act2	Actin 2
Ago1/Ago1	Argonaute 1
Ala	alanine
ANOVA	analysis of variance
APS	ammonium persulfate
APxs/APXs	ascorbate peroxidases
AsA	ascorbate
A. thaliana	*Arabidopsis thaliana*
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
°C	Celsius degree
CAT	catalase
1CPs	1-Cys peroxiredoxins
2CPA/2CPA	2-Cys peroxiredoxin A
2CPB/2CPB	2-Cys peroxiredoxin B
2CPs/2CPs	2-Cys peroxiredoxins
CCS1/CCS1	copper chaperone for superoxide dismutases
cDNA	complementary DNA
Chl	chlorophyll
Chl a	chlorophyll a
Chl b	chlorophyll b
CBF	c-repeat binding protein
COR15A	cold-regulated 15A gene
Csd2/CSD2	Cu/Zn-superoxide dismutase
Ct	threshold cycle
Cu/Zn-SOD	copper-zinc superoxide dismutase
DAB	3,3'-diaminobenzidine
DAP1	plants on the first day after application of priming stimulus
DAP2	plants on the second day after application of priming stimulus
DAP3	plants on the third day after application of priming stimulus
DAT1	plants on the first day after application of triggering stimulus
DAT2	plants on the second day after application of triggering stimulus
DAT3	plants on the third day after application of triggering stimulus
DeA1	plants on the first day of de-acclimation phase
DeA2	plants on the second day of de-acclimation phase

DeA3	plants on the third day of de-acclimation phase
DEPC	diethylpyrocarbonate
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides
DTT	dithiothreitol
ECT	electron transport chain
EDTA	ethylenediamin-tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EXPRS	S-adenosyl-L-methionine-dependent methyltrasferase
Fe	iron
Fe-SOD	iron superoxide dismutase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPx1/GPX1	glutathione peroxidase 1
GPx7/GPX7	glutathione peroxidase 7
GPxs/GPXs	glutathione peroxidases
GR/GR	glutathione reductase
GSH	glutathione
GSSG	oxidized glutathione
G	glycine, guanine
HCl	hydrochloric acid
H_2O	water
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
HSP	heat-shock protein
KCl	potassium chloride
KCN	potassium cyanide
kDa	kilo dalton
KH_2PO_4	monopotassium phosphate
K_2HPO_4	dipotassium phosphate
LT_{50}	temperature at which 50 % freezing damage occurs
LiCl	lithium chloride
λ	wavelength
$MgCl_2$	magnesium chloride
M	molar concentration
MAPK	mitogen activated protein kinase
MDA	monodehydroascorbate
MDHAR	monodehydroascorbate reductase
Mn-SOD	manganese superoxide dismutase
mRNA	messenger RNA
miRNA	micro RNA
MOPS	3-(N-morpholino)-propanesulfonic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NA	non-acclimated plants
NBT	nitro blue tetrazolium
O_2	molecular oxygen
$^1O_2$	singlet oxygen
$O_2^{\bullet-}$	superoxide radical anion
$OH^{\bullet}$	hydroxyl radical

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDF2	protein phosphatase 2A
PetE2	plastocyanin
P_i	inorganic phosphorus
PMSF	phenylmethylsulfonyl fluoride
Primed	primed plants
pRNA	polysomal RNA
PSI	photosystem I
PSII	photosystem II
Prxs/PRXs	peroxiredoxins
PrxIIE	peroxiredoxins type IIE
PrxQ	peroxiredoxin Q
PUFA	polyunsaturated fatty acids
qRT-PCR	quantitative real-time PCR
R	arginine
r_s	Spearman's correlation coefficient
RBP	RNA-binding protein
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
ROS	reactive oxygen species
RuBisCO	ribulose-1,5-bisphosphate carboxylase oxygenase
sAPx/sAPX	stromal ascorbate peroxidase
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
SOD	superoxide dismutase
SNP	single nucleotide polymorphism
T	thymine
tAPx/tAPx	thylakoid-bound ascorbate peroxidase
TBS	Tris-buffered saline
TEMED	tetramethylethylene-diamine
TF	transcription factor
TIS	translation initiation site
TOC	translocon in the outer envelope of chloroplasts
TIC	translocon in the inner envelope of chloroplasts
Trig	triggered plants
TUA	α-tubulin
UV	ultraviolet
UTR	untranslated region
V	volt
v/v	volume per volume
w/v	weight per volume
XOD	xanthine oxidase

Chapter 1

INTRODUCTION

1.1. Reactive oxygen species (ROS) in plants – production and neutralization

About 2.7 billion years ago molecular oxygen was introduced to the environment by O_2-evolving photosynthetic organisms. Since then, the production of reactive oxygen species (ROS), being a group of free radicals, reactive molecules and ions that are derived from O_2, have been an unavoidable consequence of aerobic metabolism (Halliwell, 2006).

It has been estimated that in plant tissues about 1-2 % of O_2 consumption leads to the formation of ROS (Puntarulo *et al.* 1988) by various metabolic pathways that are localized in different cellular compartments such as chloroplasts (Asada, 2006), mitochondria (Navrot *et al.* 2007) and peroxisomes (del Río *et al.* 2006). Dependent on their concentration, ROS are well known for playing a dual role in plant biology as both, deleterious and beneficial species (Dat *et al.* 2000; Neill *et al.* 2002; Maruta *et al.* 2012). At low levels they are not toxic for the plants and act as secondary messengers in a variety of cellular processes including growth (Foreman *et al.* 2003), hormone signalling (Yan *et al.* 2007; Jannat *et al.* 2011), development (Peña-Ahumada *et al.* 2006; Bahin *et al.* 2011) and responses to abiotic and biotic stress stimuli (Mullineaux and Karpinski, 2002; Torres *et al.* 2002; Miller *et al.* 2008). However, various abiotic and biotic environmental stresses lead to the enhanced ROS production (Sharma and Dubey, 2007; Gonzáles-Pérez *et al.* 2011; Ma *et al.* 2012; Krasensky and Jonak, 2012). If insufficiently antagonized, their accumulation can pose a threat to plant cells by causing damage of cellular components, such as proteins, nucleic acids and lipids (Halliwell, 2006; Moller *et al.* 2007). However, to prevent these destructive effects of ROS, plants have evolved a highly efficient antioxidant system, consisting of antioxidant enzymes and low molecular weight antioxidants (Asada, 1999; Foyer and Noctor, 2003; del Río *et al.* 2006; Navrot *et al.* 2007).

1.1.1 Types of ROS

Molecular oxygen (O_2) in its ground state is a harmless molecule having two unpaired electrons with parallel spin. This spin restriction makes it relatively unreactive with organic molecules, unless it is activated (Cadenas, 1989). The activation of O_2 may occur in two different ways: absorption of sufficient amount of energy to reverse the spin of one of the unpaired electrons or stepwise reduction by accepted electrons (Apel and Hirt, 2004). The first mechanism leads to the formation of singlet oxygen ($^1O_2$) (Krieger-Liszkay, 2005), while in the second one O_2 is sequentially reduced to H_2O via superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) (Halliwell, 1977; Klotz, 2002; Apel and Hirt, 2004).

1.1.1.1 Singlet oxygen ($^1O_2$)

In the light, $^1O_2$ is produced via triplet chlorophyll formation in the antenna system and in the reaction centre of photosystem II. This triplet state of chlorophyll is able to transfer the excitation energy to the ground state of molecular oxygen, resulting in the formation of highly destructive singlet oxygen (Gorman and Rodgers, 1992; Krieger-Liszkay, 2005).

$^1O_2$ directly oxidizes most of the biological molecules (proteins, unsaturated fatty acids and DNA) (Wagner *et al.* 2004), causes modifications of nucleic acids through selective reaction with deoxyguanosine (Kasai, 1997) and is thought to be responsible for light-induced loss of photosystem II activity and cell death (Krieger-Liszkay *et al.* 2008). To prevent these destructive effects, plants have evolved a set of low molecular weight antioxidants (e.g. β-carotene and α-tocopherol) able to quench $^1O_2$ (Telfer, 2002; Trebst, 2002)

1.1.1.2 Superoxide radical anion ($O_2^{\bullet-}$)

Superoxide radical anion ($O_2^{\bullet-}$) is the primary reactive oxygen species formed in the stepwise reduction of molecular oxygen (Halliwell, 1977; Klotz, 2002). It is a moderately reactive ROS with short life-time (half-time of approximately 2-4 μs) and both, oxidizing and reducing properties (Halliwell, 1977; Fridovich, 1986a). It oxidizes enzymes containing [4Fe-4S] clusters (Imlay, 2003), reduces cytochrome c (Imlay, 2003) and triggers the formation of more reactive ROS, such as hydroxyl radical

(via Haber-Weiss and Fenton reactions) and singlet oxygen (Haber and Weiss, 1932; Fridovich, 1986a; Halliwell, 2006). Moreover, $O_2^{\bullet-}$ is able to accept one electron and two protons resulting in the formation of H_2O_2. The reaction takes place either spontaneously or is catalyzed by superoxide dismutase (SOD) (Fridovich, 1986a; Asada, 1999; Halliwell, 2006).

1.1.1.3 Hydrogen peroxide (H_2O_2)

The univalent reduction of $O_2^{\bullet-}$ results in production of hydrogen peroxide (H_2O_2). It is a moderately reactive ROS having a relatively long half-life (approx. 1 ms) (Halliwell, 1977; Halliwell, 2006). In plant cells the main sources of H_2O_2 are electron transport chains of chloroplasts and mitochondria, β-oxidation of fatty acids and photorespiration (Asada, 1999; Braidot *et al.* 1999; Dat *et al.* 2000; Foyer and Noctor, 2000; Wingler *et al.* 2000, Moller, 2001). Produced by them H_2O_2 has dual role depending on its concentration. Thus, at low concentrations it acts as a signal molecule involved in acclimatory signalling triggering tolerance to various biotic and abiotic stresses (Neill *et al.* 2002; Torres *et al.* 2002; Avsian-Kretchmer *et al.* 2004; Maruta et al. 2012), whereas at higher ones oxidizes sulphur-containing amino acids (cysteine and methionine), enzymes of Calvin cycle and superoxide dismutases (Cu/Zn-SODs, Fe-SOD) leading to programmed cell death (Kristensen *et al.* 2004; Groen *et al.* 2005).

1.1.1.4 Hydroxyl radical ($OH^{\bullet}$)

Both, $O_2^{\bullet-}$ and H_2O_2 are only moderately reactive. However, in the presence of transitional metals (especially Fe) they can be converted to more reactive hydroxyl radical ($OH^{\bullet}$) (Haber and Weiss, 1932; Rigo *et al.* 1977; Mori and Schroeder, 2004).
$OH^{\bullet}$ is the most reactive among all ROS, having a single unpaired electron. It interacts with all biological molecules and causes subsequent cellular damages, such as lipid peroxidation (Schopfer, 2001; Bailly, 2004). In plant cells there is no enzymatic mechanism for the elimination of this highly reactive ROS, therefore excess production of $OH^{\bullet}$ ultimately leads to cell death (Pinto *et al.* 2003).

1.1.2 Sources of ROS

Because of the aerobic metabolism and presence of polyunsaturated fatty acids (PUFA) in the chloroplast membrane, photosynthetic plants are especially at risk of oxidative damage. In their cells, ROS are continuously produced under both, stressful and non-stressful conditions at several locations in mitochondria, chloroplasts, plasma membrane, peroxisomes, apoplast, endoplasmic reticulum and cell walls (Asada, 2006; del Río *et al.* 2006; Navrot *et al.* 2007; Gill and Tuteja, 2010). In the light, chloroplasts and peroxisomes are the main source of ROS (Foyer and Noctor, 2003; Asada, 2006), while in the darkness the most of ROS is produced in mitochondria (Moller, 2001).

1.1.2.1 Chloroplasts

Photosynthesis, which takes place in chloroplasts, is the predominant source of ROS in plants in light (Asada, 1994; Foyer and Noctor, 2003). It is estimated that approximately 30 % of the electrons transferred through the photosynthetic electron transport chain can flow into ROS metabolism (Miyake *et al.* 1991).

The main sites of ROS production in chloroplasts are photosystems I (PSI) and II (PSII) of the chloroplast electron transport chain (ETC) (Asada, 1994; Navari-Izzo *et al.* 1999). Production of ROS by these sources is enhanced by conditions limiting CO_2 fixation, such as drought, salt, heat and high light stresses. Under such stressful conditions, electrons in the chloroplast ETC are not transferred, as under non-stressful conditions, from the excited photosystems to $NADP^+$ leading to its reduction to NADPH, but there is a leakage of electrons from ETC to O_2, resulting in reduction of O_2 to $O_2^{\bullet-}$ (Mehler, 1951). There are several sites in the chloroplast ETC, from which such leakage of the electrons may occur, namely ferredoxin, Q_A and Q_B at the acceptor sites of PSII and [4Fe-4S] clusters in PSI (Takahashi and Asada, 1988; Cleland and Grace, 1999). Moreover, limited electron transport under high excitation pressure, supports also the formation of singlet oxygen from triplet chlorophyll and pheophytin (Anderson *et al.* 1992; Krieger-Liszkay, 2005).

The reduction of O_2 to $O_2^{\bullet-}$ in chloroplast is a rate limiting step in ROS formation. However, once formed $O_2^{\bullet-}$ can be used for generation of more reactive ROS, such as H_2O_2 and $OH^{\bullet}$. H_2O_2 is produced from $O_2^{\bullet-}$ spontaneously or in enzymatic reactions catalyzed by chloroplast superoxide dismutases (Asada, 1999; Foyer and Shigeoka,

2011), while the transformation of $O_2^{\bullet-}$ to $OH^{\bullet}$ through Fenton reaction is Fe^{2+}-dependent and takes place at [4Fe-4S] clusters of PSI (Haber and Weiss, 1932; Asada, 1994).

1.1.2.2 Mitochondria

In plant mitochondria $O_2^{\bullet-}$ is generated at several sites of respiratory electron transport chain, including the flavoprotein region at the matrix site of NADH dehydrogenase (Complex I) and the ubiquinone-cytochrome region of cytochrome bc_1 (Complex III) (Turrens, 1997; Murphy, 2009; Moller, 2001). Moreover, $O_2^{\bullet-}$ can be also produced by several enzymes present in the mitochondrial matrix, such as aconitase and 1-galactono-γ-lactone dehydrogenase (Moller, 2001; Andreyev *et al.* 2005; Rasmusson *et al.* 2008). Generated by these sources $O_2^{\bullet-}$ is the primary ROS, which is further converted by Mn-SOD to hydrogen peroxide or in Fenton reaction to extremely active hydroxyl radical (Haber and Weiss, 1932; Scandalios, 1993; Rhoads *et al.* 2006).

1.1.2.3 Peroxisomes

As a result of their essential oxidative metabolism, peroxisomes are the major sites of intracellular H_2O_2 production (del Río *et al.* 2006). ROS are generated in these organelles mainly during photorespiration and β-oxidation of fatty acids. The main enzymes involved in these processes are: localized in the peroxisome matrix xanthine oxidase (XOD) and two enzymes of the small electron transport chain located in the peroxisomal membrane, flavoprotein NADH and cytochrome c (Sandalio and del Río, 1988; del Río *et al.* 1998; del Río *et al.* 2006).

1.2.2.4 Other sources of ROS generation in plants

Other important sources of ROS production in plants that have received less attention are plasma membranes, apoplast and cell walls. In plasma membranes, $O_2^{\bullet-}$ generation is attributed to the action of at least two enzymes, an NADPH oxidase and, in the presence of menadione, a quinone reductase (Heyno *et al.* 2011). In the apoplast, the cell-wall-associated oxalate oxidase and amine oxidase-like enzymes have been proposed as a source of H_2O_2 (Wojtaszek, 1997; Lane, 2002). Both of these apoplastic enzymes may contribute to defence responses occurring in the apoplast following biotic stress, mainly through H_2O_2 production (Cona *et al.* 2006). Also cell-wall-peroxidases are regarded as

active sites of H_2O_2 generation (Gross, 1977; Kim *et al.* 2010). Among them, horseradish peroxidase catalyzes the formation of H_2O_2 in the presence of NADH and diamine oxidases produce ROS using diamine or polyamines to reduce quinone that autooxidizes to form peroxides (Elstner and Heupel, 1976).

1.1.3 Role of ROS in redox signalling

The participation of ROS in the redox signalling requires equilibrium between ROS production and scavenging (Noctor and Foyer; 1998; Foyer and Noctor, 2003; Mittler *et al.* 2004, Gill and Tuteja, 2010). The homeostasis is controlled by highly efficient antioxidant system, which, by detoxification of ROS excess, maintains the ROS concentration at low levels (Asada, 1999; Mittler *et al.* 2004; del Río *et al.* 2006, Moller, 2007). When accumulated in low amounts, ROS function as secondary messengers in intracellular signalling cascades that mediate several responses of plant cells, such as stomatal closure (Yan *et al.* 2007; Zhang *et al.* 2011; Puli and Raghavendra; 2012), programmed cell death (Bethke and Jones, 2001; Kadono *et al.* 2010; Vannini *et al.* 2012), gravitropism (Joo *et al.* 2001; Clore *et al.* 2008) and acquisition of tolerance to both, biotic and abiotic stresses (Torres *et al.* 2002; Miller *et al.* 2008; Alboresi *et al.* 2011). The role of ROS in the generation of stress tolerance was shown for tomato in response to wounding (Orozco-Cárdenas *et al.* 2001; Chang *et al.* 2004) and in *Arabidopsis thaliana* subjected to osmotic, cold or drought stress (Xiong *et al.* 2002; Gémes *et al.* 2011; Suzuki *et al.* 2012). Wounding led to the ROS-dependent activation of defence genes in mesophyll cells (Orozco-Cárdenas *et al.* 2001), osmotic stress resulted in the up-regulation of DREB2A transcription factor (Desikan *et al.* 2001) and the combination of both, osmotic and cold stress, induced mitogen-activated-protein kinase 6 (MAPK6) (Yuasa *et al.* 2001).

1.1.4 Overproduction of ROS under stressful conditions and oxidative damage to molecules

Under normal, non-stressful growth conditions ROS are generated in plants only in low amounts as by-products of several metabolic processes including photosynthesis, photorespiration and respiration (Asada, 2006; del Río *et al.* 2006; Navrot *et al.* 2007). However, in response to various environmental stresses (e.g. drought, cold, heat, high light, UV-B stress) the production of ROS drastically increases (Sharma and Dubey,

2007; Gonzáles-Pérez *et al.* 2011; Ma *et al.* 2012; Krasensky and Jonak, 2012). If insufficiently antagonized, they can cause damage of cellular components, such as proteins, nucleic acids and lipids (Moller *et al.* 2007).

1.1.4.1 Proteins

In plant cells there are many proteins which undergo oxidation and subsequent inactivation by ROS (Moller *et al.* 2007). Among them, the most susceptible are complexes I (NADH dehydrogenase) and V (ATP synthase) of the mitochondrial electron transport chain (Sweetlove *et al.* 2002; Taylor *et al.* 2004), RuBisCO (Luo *et al.* 2002; Nakano *et al.* 2006), mitochondrial Mn-SOD (Moller and Kristensen, 2006) and chloroplast ascorbate peroxidases (sAPX and tAPX) (Shigeoka *et al.* 2002; Miyake *et al.* 2006). The attack of ROS on these proteins causes many modifications in their structure and physical properties, including site-specific amino acids modifications (e.g. nitrosylation, carbonylation, disulphide bond formation), fragmentation of the peptide chain, alternation of electric charge and increased susceptibility to proteolysis (Moller *et al.* 2007).

The most susceptible sites for damage by ROS in proteins are [4Fe-4S] clusters and sulphur-containing amino acids (Moller *et al.* 2007). The oxidation of iron-sulphur centres by $O_2^{\bullet-}$ is irreversible and leads to the enzyme inactivation, while the oxidation of methionine to methionine sulfoxide and cysteine to sulfenic ion, disulfide or sulfonic ion can be reversed using thioredoxin or glutathione as electron donors (Ghezzi *et al.* 2003).

1.1.4.2 Lipids

Besides proteins, also lipids are susceptible for oxidation by ROS. The reaction takes place in both, cellular and organellar membranes and results in the production of lipid peroxides (Moller *et al.* 2007).

The most sensitive for attack by ROS in membrane phospholipids are the unsaturated (double) bond between two carbon atoms and the ester linkage between glycerol and the fatty acid (Moller *et al.* 2007; Gill and Tuteja, 2010). The products generated during peroxidation of these phospholipids, especially malondialdehyde (MDA), are highly reactive, bind to the membrane proteins leading to their degradation and subsequent

damage of membranes (Eyidogan and Öz; 2007; Pan *et al.* 2006; Agarwal, 2007; Moller *et al.* 2007).

1.1.4.3 DNA

Also nuclear, mitochondrial and chloroplastic DNAs can be modified by ROS in many different ways, including deoxyribose oxidation, strand breakage, removal of nucleotides, changes in the organic bases of nucleotides and formation of DNA-protein crosslinks (Imlay and Linn, 1988; Oleinick *et al.* 1987; Tuteja *et al.* 2001; Gill and Tuteja, 2010). Since DNA is cell's genetic material such destructive changes can result in malfunction or complete inactivation of the encoded by this DNA protein (Halliwell, 2006). Damages of DNA by ROS, especially by $OH^{\bullet}$ are generally random and have been observed for many genes, however no gene has been identified as being particularly susceptible to ROS damage (Moller *et al.* 2007).

1.1.5 Antioxidant system – ROS scavenging

To protect from damage of cellular components caused by ROS, plants evolved highly efficient antioxidant system comprising of antioxidant enzymes and low molecular weight antioxidants. Such systems are found in different cellular compartments, namely cytosol, chloroplasts, mitochondria and peroxisomes (Noctor and Foyer, 1998; Asada, 1999; del Río *et al.* 2006; Navrot *et al.* 2007; Gill and Tuteja, 2010). All of them are well coordinated and play a role in maintaining equilibrium between ROS production and scavenging under both, non-stressful and stressful conditions (Noctor and Foyer, 1998; Mittler *et al.* 2004; Almeselmani *et al.* 2006; Ahmad *et al.* 2010).

1.1.5.1 Enzymatic components of antioxidant system

The enzymatic antioxidative defence system includes several antioxidant enzymes, namely superoxide dismutases (SOD), catalases (CAT), glutathione peroxidases (GPXs), peroxiredoxins (PRXs), ascorbate peroxidases (APXs), monodehydroascorbate reductases (MDHAR), dehydroascorbate reductases (DHAR) and glutathione reductases (GR). The enzymes are localized in different cellular compartments, where they neutralize ROS produced in these sites (Mittler *et al.* 2004; Asada, 1999; del Río *et al.* 2006; Navrot *et al.* 2007).

Superoxide dismutase (SOD)

Superoxide dismutases (SOD, EC 1.15.1.1) are the enzymes constituting the first line of defence against many environmental stimuli leading to the oxidative stress (Scandalios, 1993). Their activity has been reported to increase in plants exposed to various environmental stresses, such as salt stress (Gapinska *et al.* 2008; Nounjan *et al.* 2012), metal toxicity (Drazkiewicz *et al.* 2004; Hasan *et al.* 2008; Shah and Nahakpam, 2012), drought stress (Sharma and Dubey, 2005; Zlatev *et al.* 2006; Regier *et al.* 2009) and UV-B stress (Agarwal, 2007). Since the increased activity of SODs in these plants was often correlated with higher tolerance against environmental stresses, it is proposed that SODs might be essential for the survival of plants subjected to various stressful stimuli (Drazkiewicz *et al.* 2004; Sharma and Dubey, 2005; Agarwal, 2007).

SODs belong to the group of metalloenzymes and catalyze the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 (Figure 1-1) (Fridovich, 1986b). Based on the metal cofactors they are classified into three types: copper-zinc SOD (Cu/Zn-SOD), manganese SOD (Mn-SOD) and iron SOD (Fe-SOD) (Bowler, 1992). All of these isoenzymes are nuclear encoded and post-translationally targeted to their respective subcellular compartments (Bowler, 1992; Kliebenstein *et al.* 1998).

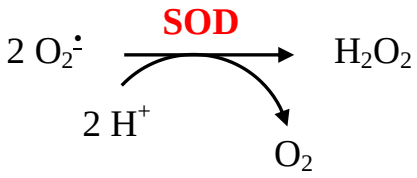

Figure 1-1. Reaction of superoxide dismutation catalyzed by superoxide dismutases (SODs).

Genome of *Arabidopsis thaliana* encodes several isoforms of SODs: three Cu/Zn-SODs (CSD1, CSD2 and CSD3), three Fe-SOD (FSD1, FSD2 and FSD3) and one Mn-SOD (MSD1) (Kliebenstein *et al.* 1998). They are localized in the cytosol (CSD1), plastids (CSD2, Fe-SODs), mitochondria (Mn-SOD) and peroxisomes (CSD3), where they form dimeric (Cu/Zn-SODs and Fe-SODs) or tetrameric (Mn-SOD) complexes (Bowler, 1992; Kliebenstein *et al.* 1998).

Chloroplast Cu/Zn-SOD (CSD2) in *A. thaliana* is a nuclear encoded homodimer composed of two identical 16-kDa-subunits, each containing one atom of Cu and Zn (Kitagawa *et al.* 1991). The immmunoblot analyses revealed that the most (70 %) of

this localized in the chloroplast stroma soluble protein is attached to the thylakoid membranes close to the PSI, being the major site of $O_2^{\bullet-}$ generation (Ogawa *et al.* 1995).

Catalase (CAT)

Catalase (CAT, EC 1.11.1.6) is an ubiquitous tetrameric haem-containing enzyme with potential to directly reduce H_2O_2 to H_2O and O_2 without consumption of cellular reducing equivalents (Figure 1-2) (McClung, 1997) .

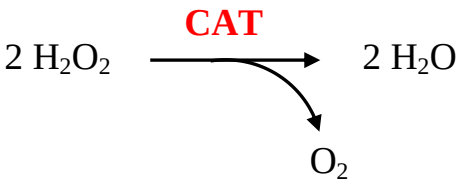

Figure 1-2. Reaction of hydrogen peroxide (H_2O_2) catalyzed by catalase (CAT).

Among all H_2O_2-reducing enzymes, it has one of the highest turnover rates (one molecule of CAT can reduce approx. six million of H_2O_2 per minute) and relatively high specificity for H_2O_2, but weak against organic peroxides (e.g. lipid peroxides) (Mhamdi *et al.* 2012). Moreover, it was shown that environmental stresses cause either enhancement or depletion of its activity, dependent on the type, intensity and duration of the stress. Thus, decreased activity of CAT was observed under cadmium stress in *A. thaliana* (Cho and Seo, 2005) and *Glycine max* (Balestrasse *et al.* 2001), while in response to drought stress in wheat the activity of this enzyme increased (Simova-Stoilova *et al.* 2010).

In *Arabidopsis thaliana* the catalase multi-gene family includes three genes encoding individual subunits which associate to form at least six isozymes. All of these isoenzymes are localized to peroxisomes, where they scavenge H_2O_2 generated during photorespiratory oxidation, β-oxidation of fatty acids and purine metabolism (McClung, 1997).

Enzymes of ascorbate-glutathione cycle (AsA-GSH cycle)

In plant cells the ascorbate-glutathione cycle (Figure 1-3), also referred to as Halliwell-Foyer-Asada cycle, is present in the cytosol (Ishikawa *et al.* 1996), chloroplasts

(Asada, 1999; Madhusudhan *et al.* 2003), mitochondria (Jimenez *et al.* 1997; Teixeira *et al.* 2006) and peroxisomes (Jimenez *et al.* 1997). It involves successive reduction of H_2O_2, as well as oxidation and subsequent reduction of AsA, GSH and NADPH. The reduction of H_2O_2 to H_2O and O_2 is catalyzed by ascorbate peroxidases (APXs), using ascorbate (AsA) as an electron donor. Generated in this reaction oxidized form of ascorbate, monodehydroascorbate radical (MDHA), spontaneously disproportionates to ascorbate and dehydroascorbate (DHA) or is reduced to ascorbate by the NAD(P)H-dependent monodehydroascorbate reductase (MDHAR). DHA is then reduced to AsA by dehydroascorbate reductase (DHAR) using glutathione (GSH) as an electron donor. The reaction results in the GSSG formation, which is then regenerated to GSH by NAD(P)H-dependent glutathione reductase (GR) (Jimenez *et al.* 1997; Asada, 1999; Foyer and Shigeoka, 2011).

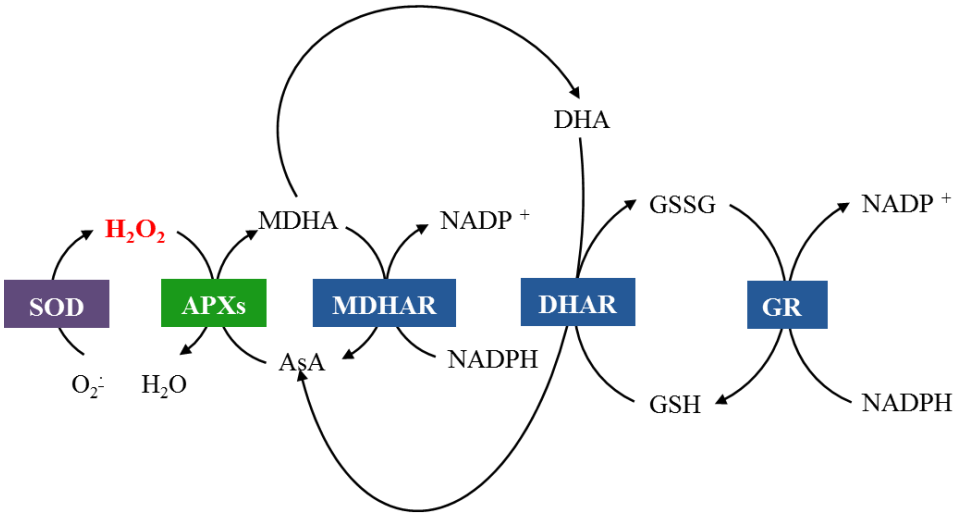

Figure 1-3. Ascorbate-glutathione cycle (Halliwell-Foyer-Asada cycle) in plant cells. Ascorbate peroxidases (APXs) catalyze the reduction of H_2O_2 to H_2O and O_2 at the expense of ascorbate (AsA), oxidizing it to monodehydroascorbate radical (MDHA) and dehydroascorbate (DHA). AsA is regenerated from MDHA and DHA in the reaction catalyzed by NADPH-dependent monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), respectively. DHAR reduces DHA to AsA at the expense of glutathione (GSH), oxidizing it to GSSG. GSH is regenerated from GSSG by NADPH-dependent glutathione reductase (GR). The scheme was drawn according to Asada (1999).

Ascorbate peroxidases (APXs, EC 1.1.11.1) are the central components of AsA-GSH cycle and play an essential role in the control of intracellular ROS levels. They are haem-binding enzymes that use two molecules of AsA to reduce H_2O_2 to water with concomitant generation of two MDHA molecules (Welinder *et al.* 1992; Asada, 1999).

In *Arabidopsis thaliana* APX family consist of five different isoforms, including cytosolic APX1, APX2 and APX6 (Santos *et al.* 1996; Panchuk *et al.* 2002), microsomal APX3, APX4 and APX5 (Jespersen *et al.* 1997; Panchuk *et al.* 2002; Narendra *et al.* 2006) and chloroplast sAPX (soluble stromal APX) and tAPX (thylakoid-bound APX) (Jespersen *et al.* 1997; Kieselbach *et al.* 2000). The chloroplast sAPX and tAPX are respectively 33 kDa and 38 kDa-proteins, each encoded by single nuclear gene (*sAPx* and *tAPx*) and post-translationally targeted to chloroplasts (Jespersen *et al.* 1997; Asada, 1999; Kieselbach *et al.* 2000). The activity of both, sAPX and tAPX was shown to be responsive to environmental stresses, such as drought stress (Sharma and Dubey, 2005; Secenji *et al.* 2010) and photooxidative stress (Yabuta *et al.* 2002; Maruta *et al.* 2010). Moreover, the overexpression of gene encoding tAPX (*tAPx*) in *A. thaliana* or tobacco resulted in enhanced tolerance to oxidative stress (Yabuta *et al.* 2002; Murgia *et al.* 2004). However, inconsistent with the role of chloroplast APXs in photooxidative stress tolerance, they are known to be inactivated by H_2O_2 if ascorbate is present at low levels (Shigeoka *et al.* 2002; Miyake *et al.* 2006).

In the reaction catalyzed by ascorbate peroxidases AsA is oxidized to monodehydroascorbate radical (MDHA). AsA is regenerated from MDHA by monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), which is a flavin adenin dinucleotide enzyme (Miyake *et al.* 1998) using NAD(P)H as an electron donor (Hossain and Asada, 1984; Asada, 1999). The activity of MDHAR is widespread in plants. The MDHAR isoenzymes are present in several cellular compartments, such as cytosol (Dalton *et al.* 1993), chloroplasts (Hossain and Asada, 1984; Asada, 1999), mitochondria (Jimenez *et al.* 1997) and peroxisomes (Jimenez *et al.* 1997; Leterrier *et al.* 2005). The chloroplastic one is a soluble, stromal 55-kDa-protein, which, similar to CSD2, localizes near to the thylakoid membranes (Hossain and Asada, 1984; Asada, 1999). It is proposed that this isoenzyme might have two physiological functions: the regeneration of AsA from MDHA and mediation of O_2 photoreduction to $O_2^{\bullet-}$, when the substrate MDHA is absent (Miyake *et al.* 1998).

Beside reduction to AsA catalyzed by MDHAR, MDHA can spontaneously disproportionate to dehydroascorbate. AsA is regenerated from DHA by dehydroascorbate reductase (DHAR, EC 1.8.5.1) using glutathione (GSH) as an electron donor (Hossain and Asada, 1984; Asada, 1999). DHAR is a monomeric 23 kDa-thiol enzyme abundantly found in dry seeds, roots as well as in etiolated and

green shoots (Hossain and Asada, 1984; Trümper *et al.* 1994). So far, it has been purified from chloroplasts of spinach leaves (Hossain and Asada, 1984; Shimaoka *et al.* 2000) and potato tubers (Dipierro and Borraccino, 1991). The activity of this enzyme was shown to be enhanced in response to several environmental stresses, namely drought (Sharma and Dubey, 2005), metal toxicity (Sharma and Dubey, 2007; Pandey *et al.* 2009) and chilling (Fryer *et al.* 1998). Moreover, the overexpression of *A. thaliana* gene encoding DHAR in *N. tabacum* revealed that this enzyme is able to enhance plant tolerance against various environmental stimuli, such as drought, high salinity and ozone (Ushimaru *et al.* 2006; Eltayeb *et al.* 2007).

Generated in the reaction catalyzed by DHAR oxidized glutathione (GSSG) is reduced to glutathione (GSH) by glutathione reductase (GR, EC 1.6.4.2) (Asada, 1999; Foyer and Shigeoka, 2011). GR is an NAD(P)H-dependent enzyme belonging to the group of flavoenzymes and containing a disulfide group, which is essential in GSH reduction (Ghisla and Massey, 1989; Stevens *et al.* 1997). In plant cells it is localized to chloroplast, cytosol, mitochondria and peroxisomes, however approximately 80 % of GR activity in photosynthetic tissues is accounted for chloroplastic isoform (Edwards *et al.* 1990). The enzyme plays a crucial role in determining the tolerance of plants to many environmental stresses, such as drought (Sharma and Dubey, 2005) and metal toxicity (Pandey *et al.* 2009). Since, the resistance of plants to oxidative stress generated by these environmental stimuli was well correlated with increase in the GR activity, it is suggested that H_2O_2 stimulates GR *de novo* synthesis, probably at the level of translation (Pastori and Trippi, 1992).

Glutathione peroxidases (GPXs)

Glutathione peroxidases (GPXs, EC 1.11.1.9) belong to another important group of ROS scavenging enzymes. They catalyze the reduction of H_2O_2 and lipid hydroperoxide to water and alcohol, respectively, using thioredoxin as the electron donor (Navrot *et al.* 2006).

In *Arabidopsis thaliana* GPXs are encoded by a gene family consisting of eight members (*GPx1 – GPx8*). The sequence analyses and transit peptide comparison revealed that encoded by these genes proteins are localized to the cytosol (GPX2, GPX4, GPX6 and GPX8), chloroplasts (GPX1 and GPX7), mitochondria (GPX3) and endoplasmic reticulum (GPX5) (Rodriguez Milla *et al.* 2003; Chang *et al.* 2009).

The chloroplast glutathione peroxidases, GPX1 and GPX7, are small proteins with a molecular weight of approximately 25 kDa, sharing 82 % sequence similarity (Rodriguez Milla *et al.* 2003). GPX1 resides on the thylakoid membrane (Ferro *et al.* 2003) or in the chloroplast stroma (Zybailov *et al.* 2008), while the precise location of GPX7 is unclear (Meyer *et al.* 2005). Both of them were shown to have an important regulatory and protective role during acclimation to photooxidative and salt stress conditions, metal toxicity, wounding and hormone treatments, as well as in limiting programmed cell death in response to infection by virulent and avirulent biotrophic pathogens (Rodriguez Milla *et al.* 2003; Chang *et al.* 2009).

Peroxiredoxins (PRXs)

Peroxiredoxins (PRXs, EC 1.11.1.15) are ubiquitous thiol peroxidases with broad substrate specificity. In addition to the reduction of H_2O_2, they also detoxify alkyl hydroperoxides and peroxynitrite (Dietz *et al.* 2002; König *et al.* 2003; Laxa *et al.* 2007). Their catalytic activity is based on the conserved cysteine residue in the active site of the enzyme. During the reaction cycle, the cysteine residue is oxidized to sulfenic acid, while hydrogen peroxide, alkyl hydroperoxides and peroxynitrite are reduced to water, corresponding alcohol or nitrite, respectively. The oxidized cysteine residue is then regenerated via intra- or inter-molecular disulphide formation and electron transfer from donors such as thioredoxin, glutaredoxin or cyclophilins (König *et al.* 2003).

All PRXs have a similar basic protein structure with a thioredoxin fold. Their molecular masses range between 17 and 22 kDa. Based on sequence similarities and catalytic mechanisms, they are divided in four types, namely 1-Cys PRXs (1CPs), 2-Cys PRXs (2CPs), type II PRXs (PrxII) and peroxiredoxin (PrxQ) (Dietz *et al.* 2002).

Genome of *Arabidopsis thaliana* encodes nine PRXs, including one 1-Cys PRX (1CP), two 2-Cys PRXs (2CPA and 2CPB), five type II PRXs (PrxIIA-E) and one peroxiredoxin Q (PrxQ) (Dietz *et al.* 2002). Among them, 1CP with only one catalytic cysteine residue in the active centre of the enzyme is localized to the cytosol and nucleus (Haslekas *et al.* 1998), 2CPs (2CPA and 2CPB) and PrxQ with two catalytic cysteine residues are localized to the chloroplasts (Baier and Dietz, 1997; Petersson *et al.* 2006), while also having two catalytic cysteine residues in the active site PrxIIs (PrxIIE) or mitochondria (PrxIIF) (Bréhélin *et al.* 2003; Horling *et al.* 2003; Finkemeier *et al.* 2005).

Chloroplast PRXs, namely 2CPA, 2CPB, PrxQ and PrxIIE, are highly abundant enzymes involved in many cellular processes (Dietz *et al.* 2002, Dietz, 2007). Beside their participation in the antioxidative defence (Baier and Dietz, 1996; Baier and Dietz, 1997; Horling *et al.* 2003; Muthuramalingam *et al.* 2009), all of them are also involved in the H_2O_2 signalling (Baier *et al.* 2000; Baier *et al.* 2004), 2CPs function as molecular chaperones (Aran *et al.* 2008; Kim *et al* 2009) and PrxQ is linked to the pathogen defence (Kiba *et al.* 2005).

1.1.5.2 Non-enzymatic components of antioxidant system

Non-enzymatic components of antioxidant system include the major cellular redox buffers ascorbate (AsA) and glutathione (GSH), as well as tocopherol, carotenoids and phenolic compounds (Noctor and Foyer, 1998; Maeda and DellaPenna, 2007; Agati *et al.* 2012).

Ascorbate (AsA)

Ascorbate (AsA) is the most abundant (10-100 mM) low molecular weight antioxidant in plants (Noctor and Foyer, 1998). It plays a key role in the defence against oxidative stress caused by enhanced levels of ROS (Conklin *et al.* 1996; Huang *et al.* 2005; Gao and Zhang, 2008). Under these stressful conditions, AsA directly scavenges ROS ($O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$), acts as an electron donor for ascorbate peroxidases in AsA-GSH cycle and regenerates another low molecular weight antioxidant α-tocopherol (Thomas *et al.* 1992). AsA is also linked to cell growth, division and metabolism (Liso *et al.* 1988; Barba-Espin *et al.* 2010), as well as acts as an electron donor for violaxanthin de-epoxidase (Eskling and Akerlund, 1998).

In plants ascorbate is synthesized in mitochondria by L-galactono-γ-lactone dehydrogenase and then transported to other cell compartments through a proton-electrochemical gradient (Wheeler *et al.* 1998). It is detected in the majority of plant cells, organelles and apoplast and is particularly abundant in the photosynthetic tissues (Noctor and Foyer, 1998).

Glutathione (GSH)

Glutathione (GSH) is a major low molecular weight thiol-based antioxidant in plants (Noctor and Foyer, 1998). It plays a key role in the defence against oxidative stress and is involved in diverse biological processes, including cell growth, regulation of sulphate transport, signal transduction, conjugation of metabolites, synthesis of proteins and nucleic acids, detoxification of xenobiotics and synthesis of chelating transition metals phytochelatins (Wingate *et al.* 1988; Foyer *et al.* 1997; Noctor and Foyer, 1998; Noctor *et al.* 2002; Ball *et al.* 2004). Under oxidative stress conditions GSH functions as an antioxidant in many ways, such as direct scavenging of ROS ($O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$), protection of macromolecules (e.g. proteins, lipids, DNA) from oxidative damage by their glutathiolation and regeneration of AsA in the AsA-GSH cycle (Noctor and Foyer, 1998; Ball *et al.* 2004).

In plant cells glutathione is synthesized only in the cytosol and chloroplasts by the compartment-specific isoforms of γ-glutamyl-cysteinyl synthase (γ-ECS) and glutathione synthase (GS), but afterwards is transported to almost all other cellular compartments such as endoplasmic reticulum, vacuoles and mitochondria (Noctor and Foyer, 1998). GSH in these compartments occurs predominantly in the reduced form (GSH) and its concentration is the highest in the chloroplasts (1-4 mM) (May *et al.* 1998).

Tocopherols

Tocopherols (α, β, γ and δ) are lipophilic antioxidants involved in scavenging of $^1O_2$, $O_2^{\bullet-}$, $OH^{\bullet}$ and lipid peroxy radicals (Hollander-Czytko *et al.* 2005; Kruk *et al.* 2005). Among them, the most abundant is α-tocopherol, which is a lipid soluble antioxidant associated with biological membranes of cells, especially with the thylakoid membranes of chloroplasts. It is synthesized only by photosynthetic organisms in chloroplasts and present only in green parts of the plant. Incorporated into the lipid portion of cell membranes α-tocopherol protects them from oxidative damage and prevents the propagation of lipid peroxidation (Fryer, 1992; Kamal-Eldin and Appelqvist, 1996; Ivanov and Khorobrykh, 2003).

Carotenoids

Carotenoids belong to the group of lipophilic antioxidants able to detoxify many forms of ROS (Young, 1991). They are pigments found in plants and microorganisms, which are able to absorb light (400 to 550 nm) and pass the captured energy to chlorophyll, as well as to directly scavenge singlet oxygen and quench triplet state of chlorophyll preventing $^1O_2$ formation and, thus, oxidative damage (Young, 1991; Sieferman-Harms, 1987; Xiao *et al.* 2011).

Phenolic compounds

Phenolic compounds include diverse secondary metabolites possessing antioxidant properties, such as flavonoids, tannins, hydroxycinnamate esters and lignins. They are abundant in plant tissue, where they play a role in the defence against oxidative stress (Grace and Logan, 2000; Agati *et al.* 2012). Under stressful conditions, they chelate transition metals preventing $OH^{\bullet}$ generation, directly scavenge $O_2^{\bullet-}$ and H_2O_2 and inhibit lipid peroxidation by trapping the lipid alkoxyl radical (Arora *et al.* 2000; Grace and Logan, 2000; Agati *et al.* 2012).

1.2 Expression regulation of genes encoding chloroplast antioxidant enzymes

Regulation of gene expression is fundamental for coordination of synthesis, assembly and localization of many biologically active molecules in plant cells, such as DNA, RNA and proteins. It is achieved by complicated multi-step process regulated at diverse levels (Figure 1-4) (Mata *et al.* 2005; Halbeisen *et al.* 2008).

Gene expression process starts in the nucleus, where transcription factors (TFs) bind to the specific DNA sequences located upstream to the transcription start codon (promoters). Bound to DNA, TFs recruit RNA polymerase and regulate the synthesis of the mRNA precursors (pre-mRNAs). As soon as these precursors are formed, mRNA-binding proteins associate with them and mediate their processing. The processing reactions include 5'-end capping, splicing, editing and polyadenylation of 3'-end of pre-mRNAs, and result in the formation of mature mRNAs (transcripts). These mRNAs are then exported through nuclear pores to the cytosol, where undergo either degradation (mRNA decay) or translation. For translation, mRNAs assemble with specific RNA-binding proteins (RBPs) and ribosomes forming large polyribosome

complexes (polysomes). As soon as the protein synthesis is finished, the proteins are folded and subjected to numerous post-translational modifications (e.g. glycosylation, phosphorylation and acetylation), directed for degradation or targeted to the particular subcellular organelles (Kuhlemeier, 1992; Mata *et al.* 2005; Halbeisen *et al.* 2008; Agne and Kessler, 2009).

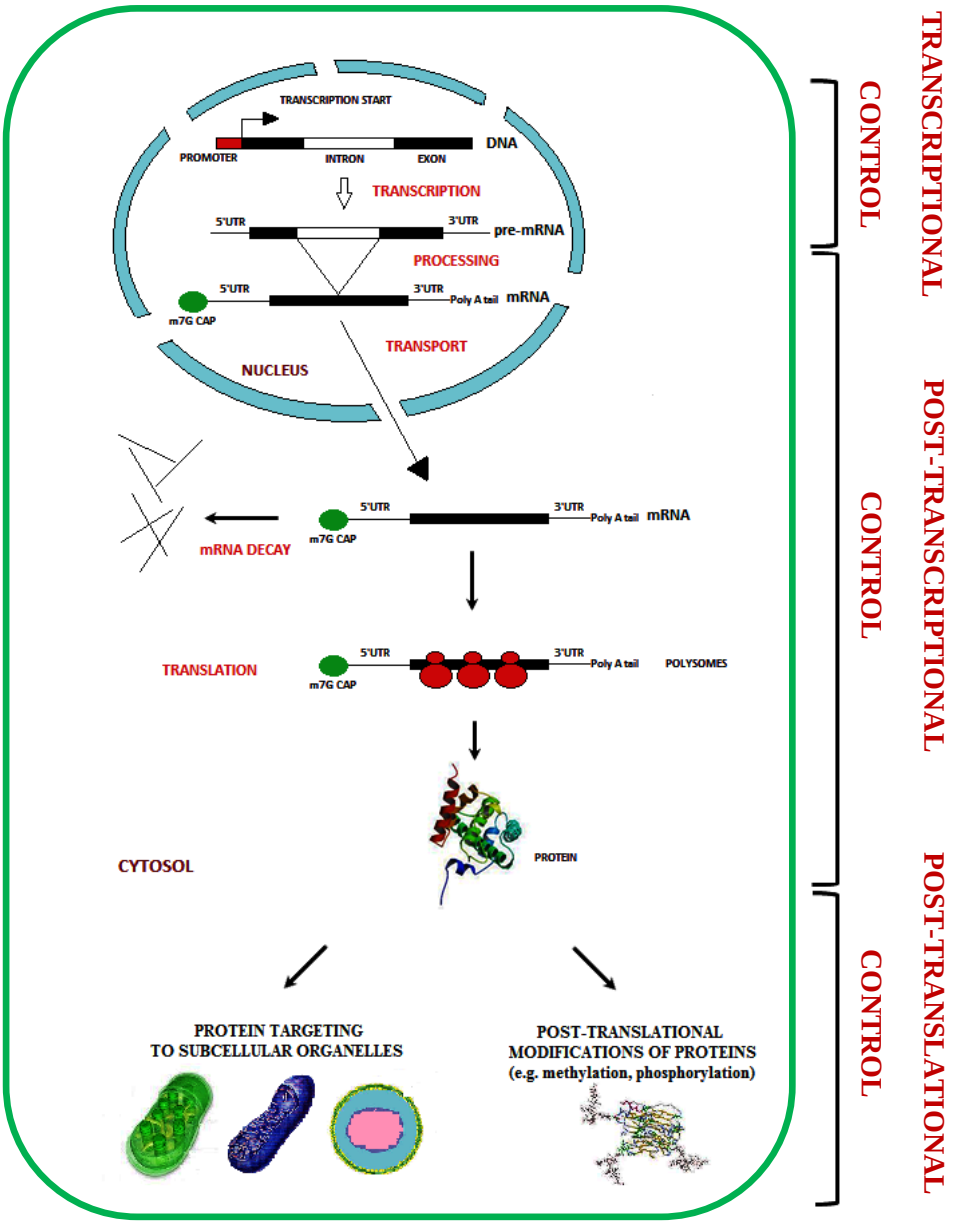

Figure 1-4. Schematic representation of gene expression process regulated at diverse levels and consisting of various well interconnected steps. The scheme was drawn according to Agne and Kessler (2009) and Kuhlemeier (1992).

To properly exert the biological functions, plant cells need to appropriately coordinate the regulation of diverse steps in the gene expression process. It is done by different regulatory mechanisms acting at all, transcriptional, post-transcriptional and post-translational levels. The transcriptional regulation includes transcription of DNA to pre-mRNA and is mediated by a large number of transcription factors (TFs) controlling the expression of various genes in many metabolic processes and signal transduction cascades (Zhou, 1999; Vanderpoele *et al.* 2009; Wu and Gallagher, 2012). As soon as the pre-mRNAs are formed, the gene expression process undergoes the post-transcriptional control, which is more complex than transcription and includes many processes, such as processing, export, translation and degradation of mRNA (Kuhlemeier, 1992; Kawaguchi and Bailey-Serres, 2002; Mata *et al.* 2005; Halbeisen *et al.* 2008). All of these processes are well coordinated and mediated by various combinations of RNA-binding proteins (RBPs), which facilitate mRNA export and determine the fate of its degradation and translation (Kawaguchi and Bailey-Serres, 2002; Gebauer and Hentze, 2004; Ambrosone *et al.* 2012). Moreover, the degradation and translation of transcripts are also influenced by structural properties of their untranslated regions (3'UTRs and 5'UTRs for degradation and translation, respectively) as well as by small interfering RNAs (RNAi) and microRNAs (miRNA), which, together with protein-effector complexes, control the degradation of their target transcripts (Mignone *et al.* 2002; Hulzink *et al.* 2003; Gebauer and Hentze, 2004; Kruszka *et al.* 2012). Following translation, proteins undergo multiple post-translational modifications (e.g. glycosylation, phosphorylation and acetylation), are degraded or targeted to the specific subcellular compartments (Huber and Hardin, 2004; Miura and Hasegawa, 2010; Olszewski *et al.* 2010). Since all chloroplast antioxidant enzymes are encoded by the nuclear genes and post-translationally targeted to the chloroplasts (Kitagawa *et al.* 1991; Baier and Dietz, 1997; Jespersen *et al.* 1997; Asada, 1999), from all of the post-translational modifications the control of protein import into chloroplasts may be especially important in the expression regulation of chloroplast antioxidant system. In plants chloroplast protein import is guided by two translocon machineries present in the outer and inner envelope membranes, TOC (translocon in the outer envelope of chloroplasts) and TIC (translocon in the inner envelope of chloroplasts), respectively (Inaba and Schnell, 2008; Jarvis, 2008; Benz *et al.* 2009). From them, TIC proteins seem to be important in linking import of chloroplast antioxidant enzymes with

the chloroplast redox state. Thus, they have been shown to sense at least two types of signals, calcium (Chigri *et al.* 2006) and redox signals ($NADP^+$/NADPH ratio and possibly also the redox state of thioredoxins and glutathione) (Bartsch *et al.* 2008; Oreb *et al.* 2008), which might provide information about the metabolic state of the chloroplasts (Gomez *et al.* 2004; Chigri *et al.* 2006).

Although so many regulatory mechanisms of the gene expression process have been discovered and characterized, the expression regulation of genes encoding chloroplast antioxidant enzymes have been so far intensively studied at the transcript level (AtGenExpress, Baier *et al.* 2004; Klein *et al.* 2012) and only exemplarily at the post-transcriptional levels (Sunkar *et al.* 2006). Nevertheless, these studies already showed extreme differences in the relevance of transcriptional and post-transcriptional regulation. For example, the expression of 2CPA (Baier *et al.* 2004) and sAPX (Klein *et al.* 2012) is predominantly transcriptionally regulated, while that of chloroplast Cu/Zn-SOD (CSD2) remains under post-transcriptional control by miR398 (Sunkar *et al.* 2006).

1.2.1 Transcriptional regulation of 2-Cys peroxiredoxins A (2CPA)

In plants, 2CPA is one of the most abundant chloroplasts proteins with 0.6 ± 0.2 % of total soluble proteins in barley leaves (König *et al.* 2003). Intensive analyses of its expression under various growth conditions and in different plant backgrounds, such as liverwort *Riccia fluitans*, monocot barley and dicot *Arabidopsis thaliana*, revealed for this gene the transcriptional, redox- and photosynthesis-dependent regulation (Baier and Dietz, 1996; Baier and Dietz, 1997; Horling *et al.* 2003; Baier *et al.* 2004). Moreover, it was shown that the expression of this gene is strongly linked to the chloroplast development (Baier and Dietz, 1996; Baier *et al.* 2004; Peña-Ahumada*et al.* 2006) and is induced in response to insufficient chloroplast antioxidant protection (in double mutants lacking both chloroplast APXs) (Kangasjarvi *et al.* 2008).

Regulation of *2CPA* expression has been analysed using several approaches, such as application of signal transduction cascade inhibitors as well as mapping of *cis*-regulatory elements in the *2CPA* promoters and screening for their *trans*-regulators (Baier and Dietz, 1996; Baier and Dietz, 1997; Horling *et al.* 2001; Baier *et al.* 2004; Shaikhali *et al.* 2008). The first analyses revealed that exogenously applied ascorbate strongly decreases the *2CPA* transcript abundance in liverwort *Riccia fluitans* and

Arabidopsis thaliana. However, this suppression could be blocked by the application of serine/threonine-kinase inhibitor staurosporin, indicating that kinase-mediated signalling mechanisms control the ascorbate-dependent regulation of *2CPA* expression (Horling *et al.* 2001; Baier *et al.* 2004). Later, by the use of promoter deletion constructs, the *cis*-regulatory element of the *2CPA* mapped to a 216 bp domain called "redox box" was discovered (Baier *et al.* 2004). A subsequent yeast-one-hybrid screen resulted in the identification of a redox-sensitive transcription factor, Rap2.4a, which binds in its dimeric form to a CE3-like element within the "redox-box" (Shaikhali *et al.* 2008). The analyses of Rap2.4a-KO lines demonstrated that Rap2.4a controls tolerance against natural environmental fluctuation via the expression regulation of the nuclear genes encoding components of the chloroplast antioxidant and photosynthetic systems. It is activated by moderate redox imbalances, but inactivated upon severe stress, consistent with decreased transcript levels for nuclear genes encoding chloroplast antioxidants after ROS-application (Shaikhali *et al.* 2008). Beside Rap2.4a, the mutant screen for promoter activators indicated at least six additional regulators of *2CPA* expression, designed *rimb* for the redox-imbalance of gene expression regulation. The genes affected in these mutants might be either, *trans*-regulators of Rap2.4a or independent *2CPA* regulators (Heiber *et al.* 2007).

1.2.2 Transcriptional regulation of ascorbate peroxidases (sAPX and tAPX)

Ascorbate peroxidases (APXs) play a key role in H_2O_2 neutralization (Asada, 1999). It is estimated that their activity reaches approximately 60 % of total hydrogen peroxide decomposition capacity in these organelles (Dietz *et al.* 2006). However, sAPX and tAPX are also prone to inhibition by H_2O_2 when the ascorbate concentration is low (Miyake and Asada, 1996). Thus, when APXs are inactivated, the activity of chloroplast antioxidant system decreases. Therefore, to cope with demand for antioxidant capacity, *de novo* synthesis of these enzymes must be activated. It is suggested that such activation is due to retrograde signals from chloroplasts, which stimulate the expression of nuclear genes encoding sAPX and tAPX (Heiber *et al.* 2007; Oelze *et al.* 2012).

In *A. thaliana* sAPX and tAPX are encoded by separate nuclear genes, *sAPx* and *tAPx*, respectively (Jespersen *et al.* 1997). The transcripts accumulation of these genes was shown to be dependent on the developmental stage of the leaves (Peña-Ahumada*et al.* 2006) and influenced by environmental stimuli, such as light intensity (Oelze *et al.*

2012). Moreover, the analyses of mutant lines with suppressed expression of 2-Cys peroxiredoxins, double knockout mutants of tAPX and sAPX (*sapx*/*tapx*), *rimb* mutants and knockout mutant of Rap2.4a revealed that decreased expression of *APxs* or *2CPs* is compensated by enhanced accumulation of *2CPs* and *APxs* transcripts, respectively (Baier *et al.* 2000; Heiber *et al.* 2007; Kangasjarvi *et al.* 2008; Shaikhali *et al.* 2008). However, the mechanisms responsible for the expression regulation of these genes at the transcript level are still poorly characterized. So far, only for *sAPx* a transcription factor (TF) binding to the promoter of this gene and influencing its transcription has been identified (Klein *et al.* 2012). This TF, ANAC089, belongs to the plant-specific NAC transcription factors family, which comprises of about 110 members in *A. thaliana* (Jensen *et al.* 2010). The members of this family are involved in developmental processes, hormonal signalling and responses to abiotic and biotic stresses (Hu *et al.* 2010). Moreover, several TFs from this family belong also to the group of membrane-tethered transcription factors (MTTFs). When associated to the membrane they are inactive and only the proteolytic processing releases the functional TF (Kim *et al.* 2007). Such cleavage is often stimulated under abiotic stress conditions or in the presence of hormones (Kim *et al.* 2006). ANAC089, being one of the members of MTTF family, was shown to be released from the membrane upon treatment with reductant DTT. The active ANAC089 bound then to the promoter of *sAPx* repressing the transcription of this gene. It is proposed that such regulation of *sAPX* expression might be a mechanism causing down-regulation of chloroplast antioxidant system and, thus, saving resources for other important synthetic activities when the production of ROS in chloroplast is low e.g. when the leaves are permanently shaded or the glutathione system is in the reduced state. This hypothesis was confirmed by analyses of *sAPx* and *ANAC089* expression in *A. thaliana* plants acclimated to normal (NL; 80 µmol m^{-2} s^{-1}) and low light (LL; 8 µmol m^{-2} s^{-1}). The transcript levels of *sAPx* were higher under NL than under LL, while the *ANAC089* mRNA levels behaved in the inverse manner, showing that higher light intensities stimulates ANAC089-mediated repression of *sAPx* expression (Klein *et al.* 2012).

1.2.3 Post-transcriptional regulation of chloroplast superoxide dismutase (Csd2)

The expression of chloroplast Cu/Zn-SOD (CSD2) is regulated post-transcriptionally in micro-RNA-dependent manner (Sunkar *et al.* 2006). Micro-RNAs (miRNAs) are

single-stranded, small RNA molecules consisting of approximately 21-24 nucleotides. In plants, their biogenesis includes several, well-coordinated steps, such as transcription of encoding them genes (*miRNAs*), processing of their precursors and incorporation of mature miRNAs to RNA-induced silencing complexes (RISC) (Bartel, 2004; Jung and Niyogi, 2009). The first step, transcription of *miRNAs* genes, is driven by the RNA polymerase II (Pol II) and results in the formation of micro-RNA precursors (pri-miRNAs), which are much longer than the mature miRNAs and possess the characteristic stem-loop structure (Lee *et al.* 2004; Xie *et al.* 2005). These pri-mRNAs are then subjected to the stepwise maturation process catalyzed by having RNase III activity Dicer-like protein 1 (DCL1) assisted by two other proteins, HYPONASTY LEAVES1 (HYL1) and SERRATE (SE) (Kurihara and Watanabe, 2004; Kurihara *et al.* 2006). The whole processing takes place in the nuclear Dicing bodies (D-bodies) and results in the formation of a duplex consisting of the miRNA and the antisense (miRNA*) strands (Fang and Spector, 2007; Kurihara and Watanabe, 2004). These miRNA/miRNA* duplexes are then methylated at the 2'OH of their 3'ends by a small methylotransferase HUA ENHANCER1 (HEN1) and subsequently exported from the nucleus to the cytosol by the exportin HASTY (Bollman *et al.* 2003; Yu *et al.* 2005). Finally, in the cytosol miRNA* from the miRNA/miRNA* duplex is degraded and the remaining mature miRNA is incorporated to the RISC complex containing ARGONAUTE (AGO) protein (Llave *et al.* 2002; Bartel, 2004). Such miRNA-loaded RISC regulates the expression of miRNA-target genes by two post-transcriptional mechanisms: mRNA cleavage or translational repression (Bartel, 2004). A key determinant of mechanism used is the degree of miRNA-mRNA complementarity - the perfect one induces RNA cleavage, while central mismatches trigger translational repression. Since, in most plants, unlike animals, miRNAs have near-perfect or perfect complementarity to their targets mRNA cleavage is predominant mechanisms of miRNA-dependent expression regulation of their genes (Bartel, 2004; Jones-Rhoades *et al.* 2006). Such cleaved mRNA are then released from RISC complex and degraded by 5'-3' exonuclease XRN4 (Souret *et al.* 2004).

1.2.3.1 The post-transcriptional regulation of Csd2 expression depends on the miR398

Computational and experimental analyses revealed that the post-transcriptional regulation of *Csd2* expression depends on the miR398 (Sunkar and Zhu, 2004; Sunkar

et al. 2006; Jones-Rhoades and Bartel, 2004). In the genome of *Arabidopsis thaliana* this conserved miRNA is encoded by three loci: *MIR398a*, *MIR398b* and *MIR398c*. miR398b and c are identical in their sequences, whereas miR398a differs from them by a single nucleotide at 3' end. Moreover, the expression of miR398b/c is much higher than that of miR398a giving miR398b/c a stronger regulatory impact (Sunkar and Zhu, 2004; Jones-Rhoades and Bartel, 2004; Yamasaki *et al.* 2007).

Beside chloroplast Cu/Zn-SOD (CSD2), miR398 targets also cytosolic Cu/Zn-SOD (CSD1), mitochondrial cytochrome c oxidase 5b (COX5b-1) and the copper chaperone for SOD (CCS1) (Bonnet *et al.* 2004; Sunkar and Zhu, 2004; Sunkar *et al.* 2006; Beauclair *et al.* 2010). However, the miR398 complementary sites in these genes differ in their location. In *Csd2* and *CCS1* mRNAs miR398 binds within the coding sequence (Jones-Rhoades and Bartel, 2004; Beauclair *et al.* 2010), while in the *Csd1* and *COX5b-1* transcripts miR398 complementary sites are located in the 5' UTRs (Sunkar and Zhu, 2004). Furthermore, some differences were observed also between the conservancy of the miR398 binding sites. Thus, in *Csd1* and *Csd2* transcripts these sites are highly conserved between Arabidopsis, pea, poplar and rice, while the conservation of the miR398 binding site within *COX5b* is lower, indicating less conserved post-transcriptional regulation (Dugas and Bartel, 2008).

1.2.3.2 miR398-mediated regulation of Csd2 expression is affected by stressful stimuli and Cu availability

In plants, miR398 has been reported to be directly involved in the response to various stress conditions, such as oxidative stress, drought stress, UV-B stress and copper deficiency (Figure 1-5) (Zhu *et al.* 2011a).

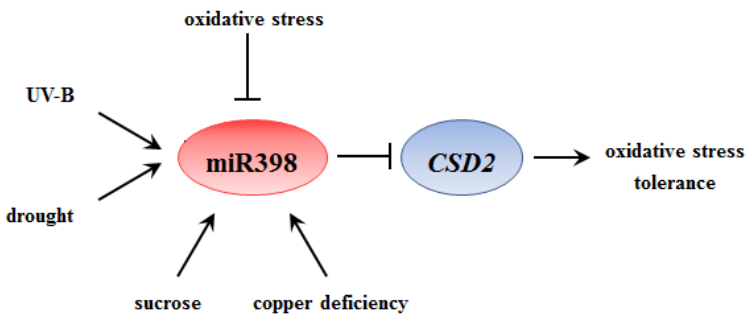

Figure 1-5. miR398-dependent regulation of *Csd2* transcript abundance in response to different stressful stimuli. The scheme was drawn according to Zhu *et al.* (2011a).

Among tested stressful stimuli, oxidative stress promoted the decrease in the miR398 level (Sunkar *et al.* 2006) and enhancement in the *Csd2* transcript accumulation, while drought stress, high UV-B radiation, copper deficiency and high sucrose levels resulted in up-regulation of *miR398* and the down-regulation of *Csd2* expression (Yamasaki *et al.* 2007; Dugas and Bartel, 2008; Jia *et al.* 2009; Beauclair *et al.* 2010; Trinidade *et al.* 2010).

The mechanisms of *Csd2* and *miR398* expression regulation under copper deficiency and oxidative stress conditions have been studied in details (Sunkar *et al.* 2006; Yamasaki *et al.* 2007). It was shown that when copper is below a critical threshold, even under oxidative stress conditions, the expression of *miR398* is highly up-regulated (Abdel-Ghany and Pilon, 2008). Consequently, *Csd2* transcripts are degraded and CSD2 protein is not synthesized. Such inhibition of CSD2 allows copper to be preferentially transferred to the plastocyanin, being the indispensable copper-containing protein in chloroplasts involved in photosynthetic electron transport and expressed independently on miR398. Meanwhile, to prevent the oxidative damage, the function of CSD2 is replaced by another chloroplast SOD, Fe-SOD (Yamasaki *et al.* 2007; Abdel-Ghany and Pilon, 2008). In contrast, under oxidative stress conditions and sufficient copper availability, the level of miR398 decreases, permitting post-transcriptional induction of *Csd2* expression, and thereby helping plants to cope with stressful conditions (Sunkar *et al.* 2006). Thus, plastocyanin via sensing Cu availability controls the ratio between self-determined photosynthetic activity and ROS neutralization (Abdel-Ghany and Pilon, 2008).

1.3. Influence of environmental stimuli on the plant metabolism, ROS production and expression of chloroplast antioxidant enzymes

Environmental stresses play a key role in determining the evolutionary history of populations and the geographical distribution of plants and animals. The impacts of stresses are especially critical among plants, since they are sessile and unable to move from stressful environments (Huey *et al.* 2002; Nicotra *et al.* 2010). Thus, plants are often exposed in natural habitats to adverse or stress conditions that disturb their normal growth and development. For protection, they have developed sophisticated mechanisms acting at all, anatomical, morphological, cellular and molecular levels. Such mechanisms need to be well-coordinated and strictly controlled to guarantee the

survival in stress environments (Boyer, 1982; Huey *et al.* 2002; Nicotra *et al.* 2010). To coordinate these mechanisms, plants have evolved finely adjustable gene regulatory systems with some common signals and responding pathways. Such systems can be activated either temporarily or permanently depending on the duration of stress (Chinnusamy *et al.* 2004). Thus, temporal activation of these systems is a result of spatial fluctuations in the environmental parameters (e.g. drought stress caused by low precipitation and high temperatures in summers), while the permanent one results from the climatic changes that emerges across generations. The processes are referred to as acclimation and adaptation, respectively. Among them, acclimation results the most often from the temporal alternation in the transcriptional or translational activity of genes encoding specific components of this system, while adaptation rather from stable mutations or epigenetic changes in these genes that are inherited over generations (Whitehead, 2011; Grativol *et al.* 2012).

Below, responses of plants on the metabolic and genetic levels (with emphasis on the expression regulation of chloroplast antioxidant system) to the most common stressful parameters (water deficit, elevated UV-B radiation as well as high and low temperature) limiting plant growth, productivity and geographical distribution, will be discussed.

1.3.1 Drought

Drought imposes an osmotic stress that leads to turgor loss, disorganization of membranes, loss of enzymatic activities and excess production of ROS. As a consequence, inhibition of photosynthesis, metabolic dysfunction, and damage of cellular structures occur. To prevent these damages, plants have evolved efficient mechanisms controlling their water status. The first response of virtually all plants under drought stress conditions is ABA-mediated stomata closure. It prevents the transpirational water loss, but aside leads also to CO_2 deficiency and, thus, decline in rate of photosynthesis. As a result, more electrons are transferred to molecular oxygen causing ROS generation. Therefore, in addition to drought-responsive genes, the expression of antioxidant genes under drought stress needs to be also tightly controlled (Mahajan and Tuteja, 2005; Krasensky and Jonak, 2012).

The response of plants to water deficit is mediated by drought-responsive signalling cascade, in which DREB (DRE binding protein) transcription factors play a central role. They belong to the family of AP2/EREBP (APPETALA2/ETHYLENE-RESPONSIVE

ELEMENT BINDING PROTEIINS) transcription factors, which bind to the DRE elements in the promoters of genes encoding drought responsive proteins. By binding to the promoters of these genes DREB activate their expression and confer the tolerance to water deficit (Mahajan and Tuteja, 2005; Yamaguchi-Shinozaki and Schinozaki, 2006). However, so far such DRE elements have been not identified in the promoters of any gene encoding chloroplast antioxidant enzymes, indicating that they are regulated independently from DREB. Consistent with this hypothesis, array experiments performed by the AtGenExpress Consortium (Kilian *et al.* 2007) revealed that upon drought stress, when the DREB levels are elevated, the transcripts of all chloroplast antioxidant enzymes are accumulated in lower amounts than in control plants, excluding DREB-driven expression activation of these genes.

1.3.2 Cold stress

Cold stress is another major environmental factor limiting plant productivity and geographical distribution. Plants exposed to low temperatures poorly germinate, have decreased fertility (usually due to the pollen sterility) and show several other phenotypic symptoms, such as reduced leaf expansion, wilting, and chlorosis. Moreover, the cold-induced changes appear also at the cellular level. Thus, extremely low temperatures cause the damage of biological membranes (due to alternations in their lipid composition), enhance synthesis and accumulation of compatible solutes (e.g. proline, soluble sugars and glycinebetaine), change the carbohydrate metabolism and boost the ROS scavenging potential. Further, cold stress disrupts the integrity of organelles leading to the loss of compartmentalization as well as impairs photosynthesis, protein assembly and general metabolic processes (Thomashow, 1999; Heidarvand and Amiri, 2010).

The response of plants to cold stress is controlled and integrated by cold-stress signal transduction cascades, in which cold-induced CBF (C-REPEAT BINDING FACTOR) transcription factors and CBF-regulated COR (COLD RESPONSIVE) proteins play a key role (Thomashow, 1999). Low temperature is sensed by so far unknown sensors or changes in the membrane rigidification, which might induce also Ca^{2+} influx and activation of calcium-dependent kinases necessary for cold acclimation. Such kinases via phosphorylation activate the ICE1 (INDUCER OF CBF EXPRESSION), a MYC-type basic helix-loop-helix transcription factor, which binds to MYC

recognition elements in the promoters of CBF genes leading to the activation of their expression. CBF subsequently activate the expression of COR proteins, which confers the cold tolerance. However, CBFs regulate only approximately 12 % of the cold responsive transcriptome, therefore also other transcription factors (e.g. G-box binding factor 1) have to be involved in the activation of *COR* expression (Thomashow, 1999; Chinnusamy *et al.* 2007).

Beside the COR, CBFs regulate also the expression of genes involved in phosphoinositide metabolism, transcription, membrane transport, hormone metabolism and ROS detoxification (Fowler and Thomashow, 2002; Maruyama *et al.* 2004). However, among chloroplast antioxidant enzymes the cold induction of gene expression was shown only for *GPx1* (Soitamo *et al.* 2008).

1.3.3 Heat stress

Heat-stress is one of the major abiotic stress limiting the growth and development of plants. It leads to the inhibition of photosynthesis, damage of cell membranes, senescence and programmed cell death (Karim *et al.* 1999; Iba, 2002; Larkindale and Huang, 2004; Qu *et al.* 2013). To avoid these damages, plants have developed sophisticated heat-stress defence mechanisms, in which heat-shock proteins (HSPs) play a key role (Iba, 2002).

In plants, HSPs are divided according to their approximate molecular weight in kDa into five classes: HSP100, HSP90, HSP70, HSP60 and small heat-shock proteins (sHSPs) with molecular masses of 15-30 kDa. All of them are regulated through the action of heat-stress transcription factors (HSFs) and function as molecular chaperones essential for maintenance of protein homeostasis (Iba, 2002). Thus, the basic heat-stress signalling cascade in plants consists of HSFs, HSPs and several cellular temperature sensors including a plasma membrane calcium channel, a nuclear histone sensor and two unfolded protein sensors in the endoplasmic reticulum and the cytosol (Mittler *et al.* 2012). Among them, HSFs can be activated either by Ca^{2+} or ROS (Pnueli *et al.* 2003; Mittler *et al.* 2012). In response, they bind to the heat stress elements (HSE) present in the promoters of genes encoding all HSPs and at least one antioxidant enzyme. Such HSE were identified in the promoter of cytosolic ascorbate peroxidase APX1, but not in the chloroplast tAPX, indicating that tAPX is less essential in response to high temperatures than APX1 (Storozhenko *et al.* 1998). Indeed, a knockout mutant of

A. thaliana lacking tAPX was shown to be less sensitive to the heat stress than the wild type, while the lack of cytosolic APX1 resulted in the stunted growth and enhanced sensitivity to both, heat and oxidative stress (Pnueli *et al.* 2003; Miller *et al.* 2007).
Recently, HSE was also identified in the promoter of gene encoding one of the miRNA responsible for post-transcriptional regulation of *Csd2* expression, miR398b (Guan *et al.* 2013). It was shown that two heat-shock transcription factors, HSFA1b and HSFA7b bind directly to the promoter of *mir398b* leading to the enhanced synthesis of this miRNA. In response, the expression of miR398b-target genes (*Csd1*, *Csd2* and *CCS1*) is down-regulated resulting in higher sensitivity to ROS and, thus, heat stress damages (Guan *et al.* 2013).

1.3.4 UV-B stress

UV-B is a toxic sunlight fraction (280 to 315 nm), which is known to damage DNA, inhibit photosynthesis and arrest the cell cycle (Tohge *et al.* 2011). Since plants are dependent on sunlight for photosynthesis, they cannot avoid exposure to the UV-B radiation and, therefore, needed to adapt to its deleterious effects. Thus, they have developed a wide range of strategies, which allow them to survive under high UV-B radiation. Such strategies include: changes in the cellular levels of UV-B absorbing metabolites (flavonoids, ascorbate, carotenoids, tocopherol and vitamin B6), increase in the leaf thickness and UV-B reflective properties (Jenkins, 2009). All of these alternations are activated by specific UV-B signalling cascade consisting of several well characterized components, such as bZIP transcription factor HY5 (ELONGATED HYPOCOTYL 5), HY5-homolog HYH, multi-functional E3-ubiquitin ligase COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1) and UV-B receptor UVR8. Within this cascade, UVR8 absorbs UV-B and sends the signal to COP1, HYH and HY5 inducing the changes in the expression of encoding them genes, such as down-regulation of *COP1* and up-regulation of both, *HYH* and *HY5* (Ulm *et al.* 2004; Oravecz *et al.* 2006; Brown and Jenkins, 2008; Jenkins, 2009; Rizzini *et al.* 2011). The up-regulation of *HY5* expression results in the enhanced synthesis of these transcription factor and, thus, subsequent activation of its target genes including *ELIP1*, *PAP1* and *MYB12*. Among them, *ELIP1* encodes EARLY LIGHT-INDUCIBLE PROTEIN 1 known to be one of the major light responsive genes involved in the induction of tolerance to photoinhibition and photooxidative stress (Rossini *et al.* 2006), while *PAP1* and *MYB12*

code for proteins involved in the flavonol/anthocyanin pathway (Tohge *et al.* 2005; Stracke *et al.* 2010). Similar to HY5, HYH is also able to induce the expression of several genes, such as *SIG5* for sigma factor 5 and *GPx7* encoding chloroplast glutathione peroxidase 7 (Tohge *et al.* 2011). Since high UV-B radiation is known to decrease the photosynthetic light-saturated CO_2 assimilation and limited CO_2 assimilation leads to the excessive production of ROS, the activation of chloroplast antioxidant system is not a surprise. Besides GPX7, the induction of this system was shown in *A. thaliana* also for other chloroplast peroxidases (GPX1, sAPX and tAPX) and low molecular weight antioxidant, ascorbate (Rao *et al.* 1996; Gao and Zhang, 2008).

1.4. Aim of the present studies

The present work aims to understand the expression regulation of chloroplast antioxidant enzymes in response to fluctuations in environmental parameters (e.g. growth temperature, average monthly precipitation and UV radiation). For such studies the accessions of *A. thaliana* broadly distributed among Northern hemisphere and exposed across their latitudinal range (from mountains in Tanzania and Kenya to North Scandinavia) to various climatic conditions are widely exploited (Koornneef *et al.* 2004). Here, seven of them originating from contrasting habitats (from Russia to Cape Verde Islands) are used to demonstrate the genetically controlled dynamic, acclimatory importance and restrictions in the expression regulation of chloroplast antioxidant system. The studies focus on the main antioxidant enzymes (CSD2, PRXs, GPXs, APXs, MDHAR, DHAR and GR) and aim to characterize the regulatory mechanisms acting at every level (transcript, polysomal RNA and protein) of their expression process.

Chapter 2

MATERIALS AND METHODS

2.1 Plant material

Accessions of *Arabidopsis thaliana* used in all studies were selected to cover a wide range of habitats (from 15 to 62° N latitude) with contrasting temperature and precipitation conditions (see Table 2-1 for a list). Seeds of them were obtained either from the Nottingham Arabidopsis Stock Centre (NASC, United Kingdom) or from the Versailles nested core collections (INRA, France).

2.2 Growth conditions and stress treatments

2.2.1 Cultivation of mature Arabidopsis thaliana plants on soil

Following three days of stratification at 4 °C, seeds of *Arabidopsis thaliana* accessions were germinated and grown on soil [50 % potting soil containing four volumes garden compost, one volume Toresa wood fibers (Franz Kranzinger GmbH, Austria), one volume Floratron 1 (Floragrad GmbH, Germany); 50 % Toresa wood fibers; 1.5 g l^{-1} Osmocote extract standard (Scott Celaflor, Germany); 0.5 g l^{-1} Dolomite lime (Deutsche Raiffeisen-Warenzentrale, Germany); 0.5 g l^{-1} Axoris Insekten-frei (COMPO, Germany)] for 6 weeks in a climate-controlled chamber at a day / night temperature of 20 °C / 18 °C and 120 µmol m^{-2} s^{-1} light (L36W/840 Lumilux Cool White bulbs, OSRAM, Germany) with a 10 h light/14 h dark photoperiod.

2.2.2 Different temperature treatments

After three days of stratification seeds of *A. thaliana* were germinated and grown on potting soil composed of one volume Terreau Professionell Gepac Einheitserde Typ T (Einheitserde, Germany), one volume Terreau Professionell Gepac Einheitserde Typ T (Einheitserde, Germany) and one volume Perligran G (Knauf Perlite, Germany)

Table 2-1. *Arabidopsis thaliana* accessions used for analyses of natural variation in the chloroplast antioxidant system. Geographical and climatological data were obtained from the websites of Versailles Biological Resource Centre (http://dbsgap.versailles.inra.fr/vnat/) and NASC European Arabidopsis Stock Centre (http://arabidopsis.info/EcoForm).

Accession	NASC ID	Versailles ID	Geographical origin	Latitude and longitude	Altitude [m]	Average temperature [°C]		Average monthly precipitation [mm]	
						April to September	October to March	April to September	October to March
Col-0	N1092	186 AV	Gorzów Wielkopolski (Poland)	N52 E15	1-100	7-18	(-1)-9	40-80	30-50
Kas-1	N1264	-	Kashmir (India)	N34-N36 E74-E80	1580	0-12	(-12)-2	0-50	10-50
Cvi-0	N1096	166 AV	Cape Verde Islands	N15-N17 W23-W25	1100-1200	23-27	22-26	0-50	1-80
Ms-0	N1376	93 AV	Moscow (Russia)	N56 E38	100-200	4-18	(-2)-(-11)	40-60	40-60
WS	N1602	84 AV	Wassilewskija (Belarus)	N52-N53 E30	100-200	6-19	(-7)-1	40-80	30-40
C24	N906	183 AV	Unknown	-	-	-	-	-	-
Van-0	N1584	161 AV	Vancouver (Canada)	N49 W123	1-100	7-15	(-1)-8	20-60	100-170
Can-0	-	163 AV	Canary Islands (Spain)	N28 W15	1200-1300	8-9	8-9	0-14	2-10
Sah-0	-	233 AV	Sierra Alhambra (Spain)	N38 E3	1300	7-8	6-7	7-37	26-56
N13	-	266 AV	Konchezero (Russia)	N62 E34	1-100	1-16	(-11)-3	41-77	32-59
N14	-	267 AV	Sampo Mountain (Russia)	N61-N62 E34	500	(-1)-17	(-10)-3	27-77	17-58

for two weeks in a climate-controlled chamber (CU-41L4X; Percival Scientific Inc., United States) at 120 µmol m^{-2} s^{-1} light with a 10 h light / 14 h dark photoperiod and a day / night temperature of 20 °C / 18 °C. After two weeks, the plants were transferred for one additional week either to 10, 20 or 30 °C and 120 µmol m^{-2} s^{-1} light in a 10 h light / 14 h dark photoperiod.

2.2.3 Cold stress treatments

2.2.3.1 Short-term cold stress treatment

Seeds of *A. thaliana* accessions were germinated and grown on potting soil containing one volume Terreau Professionell Gepac Einheitserde Typ T (Einheitserde, Germany), one volume Terreau Professionell Gepac Einheitserde Typ T *(*Einheitserde, Germany) and one volume Perligran G (Knauf Perlite, Germany) for four weeks in a climate-controlled chamber at 20 °C and 120 µmol m^{-2} s^{-1} light (L36W/840 Lumilux Cool White bulbs, OSRAM, Germany) with 10 h light / 14 h dark photoperiod. After four weeks plants were subjected to the priming stimulus (24 h at 4 °C and 90 µmol m^{-2} s^{-1} light in a 10 h light / 14 h dark photoperiod). Afterwards, plants were transferred for five days to 20 °C ("lag phase") and subsequently subjected to the triggering stimulus (24 h at 4 °C) followed by three days at 20 °C.

2.2.3.2 Long-term cold stress treatment

Eleven *Arabidopsis thaliana* accessions were grown for six weeks in the greenhouse of the Max Planck Institute of Molecular Plant Physiology (Golm, Germany) at a day / night temperature of 20 °C / 18 °C and 200 µmol m^{-2} s^{-1} light with a 16 h light / 8 h dark photoperiod. After six weeks, the plants were transferred to a growth chamber for cold stress treatment (two weeks at 4 °C and 90 µmol m^{-2} s^{-1} light in a 16 h light / 8 h dark photoperiod), followed by three days of de-acclimation phase in the greenhouse.

2.3 RNA isolation and analyses

2.3.1 Total RNA isolation (ready-to-use kit)

For total RNA isolation 100 mg of leaf tissue was ground in liquid nitrogen using the Retsch Mixer Mill MM400 (Retsch, Germany). RNA was isolated with the GeneMATRIX Universal RNA Purification Kit (EURx, Poland) according to manufacturer's instructions. To ensure that isolated RNA was not contaminated with genomic DNA on-column digestion with RNase-free DNaseI (Fermentas, Germany) was performed. RNA samples were stored at -80 °C.

2.3.2 Total RNA isolation (manual method)

For RNA isolation from cold-stressed plants, five rosettes of each accession were pooled and immediately ground in liquid nitrogen. Total RNA was extracted from 200-300 mg of frozen plant material using 500 µl lysis buffer (100 mM Tris-HCl, pH 8.5-9.0; 25 mM EDTA; 25 mM EGTA; 2 % SDS; 100 mM β-mercaptoethanol), 500 µl phenol and 300 µl chloroform/isoamyl alcohol (24:1). After centrifugation (Centrifuge 5415R, Eppendorf, Germany) for 10 minutes at 16100 x g and 4 °C, the upper (aqueous) phase was transferred to a fresh 1.5 ml reaction tube and subsequently extracted twice: first with 500 µl phenol and 500 µl chloroform/isoamyl alcohol (24:1) and second with 1 ml chloroform/isoamyl alcohol (24:1). Following the extraction steps total nucleic acids were precipitated from the supernatant in 500 µl 2-propanol for 1 hour at -20 °C and sedimented by centrifugation for 15 minutes at 16100 x g and 4 °C. Afterwards, the nucleic acid pellet was dissolved in 200 µl 0,1 % SDS (w/v) in DEPC-treated water, 20 µl 3 M sodium acetate (pH 5.2) and 600 µl of 96 % ethanol were added and an overnight precipitation of nucleic acids was performed at -20 °C. After sedimentation of nucleic acids by centrifugation for 15 minutes 16100 x g and 4 °C, pellets were dissolved in 40 µl water and treated with 20 µl of 7.5 M lithium chloride to remove sugar contaminations. Following the incubation with LiCl for 1 hour at -20 °C, the nucleic acids were sedimented by centrifugation for 15 minutes at 16100 x g and 4 °C. Pellets obtained after sedimentation were dissolved in 200 µl LG Buffer (EURx, Poland) and 100 µl RL Buffer (EURx, Poland), 300 µl of 96 % ethanol was added and the whole mixture was loaded on RNA binding column from the GeneMATRIX

Universal RNA Purification Kit (EURx, Poland). The final purification of isolated RNA including on-column digestion of DNA with RNase-free DNaseI (Fermentas, Germany) was performed according to manufacturer's instructions.

2.3.3 Polysomal RNA isolation

Polysomes were purified as described by Kahlau and Bock (2008) with slight modifications. 200 mg of leaf tissue was treated with 1 ml of freshly prepared extraction buffer (200 mM Tris-HCl, pH 9.0; 200 mM KCl; 35 mM $MgCl_2$; 25 mM EGTA; 200 mM sucrose; 100 mM β-mercaptoethanol; 1 % (v/v) Triton X-100; 2 % (v/v) polyoxyethylene-10-tridecylether; 1 mg ml^{-1} heparin; 100 µg ml^{-1} chloramphenicol; 25 µg ml^{-1} cycloheximide) in conditions maintaining the integrity of the polysomes. After removal of cell debris by centrifugation (Centrifuge 5415R, Eppendorf, Germany) for 5 minutes at 13200 x g and 4 °C, the soluble material was supplemented with 1/20 volume of 10 % (w/v) sodium deoxycholate, incubated for 5 minutes on ice and centrifuged (Centrifuge 5415R, Eppendorf, Germany) for 5 minutes at 13200 x g and 4 °C. 500 µl of supernatant was loaded on the continuous sucrose density gradients (15-56 % sucrose in 40 mM Tris-HCl, pH 8.5; 20 mM KCl; 10 mM $MgCl_2$; 100 µg ml^{-1} chloramphenicol; 500 µg ml^{-1} heparin) and separated by ultracentrifugation (rotor 60Ti; Ultracentrifuge Optima L90K, Beckman-Coulter, Switzerland) for 80 minutes at 200000 x g and 4 °C. After centrifugation 10 fractions, 410 µl each, were stepwise collected. Control gradients, on which 500 µl of supernatant supplemented with 20 mM puromycin (Kahlau and Bock, 2008) was loaded, were used to identify fractions containing polysomes and lacking free mRNA and ribosomes. The polysomes-enriched fractions (five lowest fractions) were pooled and subjected to RNA isolation. Pooled fractions were supplemented with 0.7 volume of DEPC-treated water, 0.12 volume of 5 % SDS in 0.2 M EDTA pH 8.0 and 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1), incubated for 12 minutes at room temperature and centrifuged (Centrifuge 5810, Eppendorf, Germany) for 15 minutes at 5500 x g and 4 °C. Following the centrifugation, the upper (aqueous) phase was transferred to a fresh 1.5 ml reaction tube, supplemented with 0.1 volume of 3 M sodium acetate pH 4.8 and subjected to RNA precipitation in 2.5 volume of 96 % ethanol. Afterwards, RNA was sedimented by centrifugation (Centrifuge 5415R, Eppendorf, Germany) for 20 minutes at 16100 x g and 4 °C and subsequently subjected

to 2.5 M LiCl treatment to remove heparin. Following the purification with LiCl, pellets obtained after centrifugation were dissolved in 200 µl LG Buffer (EURx, Poland) and 100 µl RL Buffer (EURx, Poland), 300 µl of 96 % ethanol was added and the whole mixture was loaded on RNA binding columns from GeneMATRIX Universal RNA Purification Kit (EURx, Poland). The final purification of isolated RNA including on-column digestion of DNA with RNase-free DNaseI (Fermentas, Germany) was performed according to manufacturer's instructions.

2.3.4 RNA quantification

RNA quantity and purity was assessed spectrophotometrically using NanoPhotometer P300 (Implen, United States) by measuring absorbance at 230, 260 and 280 nm. The absorbance measured at 260 nm gives information about RNA concentration, whereas the calculated A_{260}/A_{230} and A_{260}/A_{280} ratios indicate phenol/sugars and protein contaminations, respectively. For further expression analyses only RNA samples possessing both ratios, A_{260}/A_{230} and A_{260}/A_{280}, higher than 2.0 were chosen.

2.3.5 Electrophoretic separation of RNA

Integrity of RNA samples was checked by electrophoresis on a 1 % (w/v) agarose gel in 1 x MOPS buffer (20 mM MOPS, pH 7.0; 5 mM sodium acetate; 1 mM EDTA). After melting 1.5 g agarose in 150 ml 1 x MOPS buffer and subsequent cooling down to around 60 °C, formaldehyde was added to final concentration of 0.9 % (v/v) and gels were cast in horizontal gel trays. Samples were prepared by mixing 3 µl isolated RNA with 3 µl loading dye (10 mM Tris-HCl, pH 7.6; 60 % (v/v) glycerol; 60 mM EDTA; 0,03 % (w/v) bromophenol blue) and 4.8 µl loading buffer (3 mM MOPS, pH 7.0; 0.75 mM sodium acetate; 0.15 mM EDTA; 6.5 % (v/v) formaldehyde, 57.5 % (v/v) formamide; 0.12 mg ml^{-1} ethidium bromide) and subsequent denaturation for 15 minutes at 65 °C. After denaturation samples were immediately transferred on ice and then loaded into gel pockets. RNA was separated for 20 minutes at constant voltage (90 V) in 1 x MOPS buffer. Ethidium bromide-stained bands were visualized at UV light using INTAS Gel Imager (INTAS, Germany).

2.4 First strand cDNA synthesis

2.4.1 First strand cDNA synthesis for gene expression analyses

cDNA was synthesized from DNase-treated total and polysomal RNAs using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, United States). cDNA synthesis was performed in 20 µl total volume from 500 ng of RNA in sample mixture containing 1 x RT Buffer, 10 mM dNTP Mix, 25 U MultiScribe^{TM} Reverse Transcriptase and 1 x RT Random Primers or 5 µM Oligo(dT) Primers (Sigma-Aldrich, Germany). Samples were incubated for 10 minutes at 25 °C to anneal the primers to RNA template, followed by cDNA synthesis for 120 minutes at 37 °C and reverse transcriptase de-activation for 5 minutes at 85 °C. Prior to use for quantitative real-time PCR analyses obtained cDNA samples were diluted 1:2 with RNase-free water.

2.4.2 First strand cDNA synthesis for micro-RNAs analyses

For quantification of mature micro-RNAs total RNA was reverse-transcribed according to Chen *et al.* (2005) using micro-RNA-specific stem-loop primers (see Table 2-2 for a list) and MultiScribe^{TM} Reverse Transcriptase (Applied Biosystems, United States).

Table 2-2. Stem-loop primers used for synthesis of cDNA encoding micro-RNAs.

micro-RNA	stem-loop primer [5'→3']
miR398a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGG
miR398b	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGGGT
miR168a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCCC

500 ng of total RNA was mixed with 0.5 µl 10 mM dNTPs and 0.5 µl 2.5 µM micro-RNA-specific stem-loop primer (Sigma-Aldrich, Germany), filled with RNase-free water to a final volume of 21.25 µl, incubated for 5 minutes at 65 °C and subsequently chilled on ice. Afterwards, 2.5 µl of 10x reverse transcriptase buffer (Applied Biosystems, United States), 1 µl 100 mM DTT, 0.25 µl 12.5 U/µl RNase inhibitor (EURx, Poland) and 0.5 µl MultiScribe^{TM} Reverse Transcriptase (Applied Biosystems, United States) were added. The reaction mixture was incubated for 30 minutes at 16 °C, followed by 30 minutes at 42 °C and 5 minutes at 85 °C.

2.5 Quantitative real-time PCR (qRT-PCR) analyses

2.5.1 Primer design

Primers used in qRT-PCR analyses were designed using QuantPrime software (Arvidsson *et al.* 2008) (see Table 2-3 for a list). To prevent the amplification of genomic DNA at least one primer of the pair spanned an exon-intron border. The optimal annealing temperature for each primer pair was determined to ensure that the primers would bind efficiently to their targets and to prevent both non-specific annealing and primer-dimer formation. The specificity of the primers was verified via gel electrophoresis and resulted in a single product of desired length. Additionally, the analyses of the melting curves generated in the CFX96 thermocycler (BioRad, United States) were performed. The melting curve for each PCR product of interest displayed a single sharp peak indicating high specificity of the tested primers. Moreover, the efficiency of amplification was verified for each primer pair in a qRT-PCR reaction performed for 10-fold dilution series of cDNA synthesized using total RNA isolated from *Arabidopsis thaliana* var. Col-0.

Table 2-3. Primers used for gene expression analyses by qRT-PCR (primer pair marked with asterisk was used for amplification of *sAPx* only in Kas-1).

Gene	Gene code	Name of the primer pair	Forward primer Reverse primer	Annealing temperature [°C]
Act2	At3g18780	qPCR_Act2	AATCACAGCACTTGCACCAAGC CCTTGGAGATCCACATCTGCTG	60
Csd2	At2g28190	qPCR_Csd2_1	ATGACACACGGAGCTCCAGAA ATTGTTGTTTCTGCCACGCCA	60
Csd2	At2g28190	qPCR_Csd2_2	CTCAACAGGACCACATTTCAACCC ATTGTTGTTTCTGCCACGCCA	60
sAPx	At4g08390	qPCR_sAPx_1	AGAATGGGATTAGATGACAAGGAC TCCTTCTTTCGTGTACTTCGT	60
sAPx*	At4g08390	qPCR_sAPx_2	CTACTGCTATAGAGGAAGCTGG TGGAAGCCTTCCTTCTTCTG	60
tAPx	At1g77490	qPCR_tAPx	GCTAGTGCCACAGCAATAGAGGAG TGATCAGCTGGTGAAGGAGGTC	60
GPx1	At2g25080	qPCR_GPx1	TCCCTGCAATCAGTTTGGTTTCC TGGTCCATTCACGTCAACCTTATC	60
GPx7	At4g31870	qPCR_GPx7	CGTTAACGTTGCGTCAAGATGTGG TGACCTCCAAATTGATTGCAAGGG	60
2CPA	At3g11630	qPCR_2CPA	CCCAACAGAGATTACTGCCT ATAGTTCAGATCACCAAGCCC	60

Table 2-3 (continued). Primers used for gene expression analyses by qRT-PCR.

Gene	Gene code	Name of the primer pair	Forward primer Reverse primer	Annealing temperature [°C]
2CPB	At5g06290	qPCR_2CPB_1	TCATACCCTCTTCCTCGGCATC ACCGACCAGTGGTAAATCATCAGC	60
2CPB	At5g06290	qPCR_2CPB_2	TTCTCTCTGTTCCGGGTTCTCCAG ACCGACCAGTGGTAAATCATCAGC	60
PrxIIE	At3g52960	qPCR_PrxIIE	CAGTAACCGTCTCATCCCTAAC TGAGTTCTCCGGCTTTGGATACG	60
PrxQ	At3g26060	qPCR_PrxQ	AGATGACTCTGCTTCTCACAAGGC TCCCTGGCAATGCTCCAAACAG	60
MDHAR	At1g63940	qPCR_MDHAR	TGGGAGAAACAGTGGAGGTTGG TGGTAGAAGCTGGAACTCCTCAG	60
DHAR	At1g19550	qPCR_DHAR	CTCGAGGAGAAGTATCCTGATC CCATCGTGACTCTTGAGATGGTTC	60
GR	At3g54660	qPCR_GR	GAAATTCCGCAAAGACTCCTC CAGACACAATGTTCTCCTTATCAG	60
CCS1	At1g12520	qPCR_CCS1	GAGCCATGCCTCAGCTTCTTAC TCACAGCATTAACACAACCCTCAC	60
Ago1	At1g48410	qPCR_Ago1	GCACACGCTCAGTTTCAATTGTTC ATGCTCCCACTAGCCATTGAGC	60
PetE2	At1g20340	qPCR_PetE2	TCACCGGCCTTAAAGCCTCAAC TGATGCAACAGCAGCAGTTTGG	60
miR398a	At2g03445	qPCR_miR398	CTCGACGTGTGTTCTCAGGTCAC CCAGTGCAGGGTCCGAGGT	60
miR398b	At5g14545	qPCR_miR398	CTCGACGTGTGTTCTCAGGTCAC CCAGTGCAGGGTCCGAGGT	60
miR398c	At5g14565	qPCR_miR398	CTCGACGTGTGTTCTCAGGTCAC CCAGTGCAGGGTCCGAGGT	60
miR168a	At4g19395	qPCR_miR168a	GCTATCGCTTGGTGCAGGTC CCAGTGCAGGGTCCGAGGT	60
COR15A	At2g42540	qPCR_COR15A	AACGAGGCCACAAAGAAAGC CAGCTTCTTTACCCAATGTATCTGC	60
GAPDH	At1g13440	qPCR_GAPDH	TTGGTGACAACAGGTCAAGCA AAACTTGTCGCTCAATGCAATC	60
EXPRS	At2g32170	qPCR_EXPRS	ATCGAGCTAAGTTTGGAGGATGTAA TCTCGATCACAAACCCAAAATG	60
PDF2	At1g13320	qPCR_PDF2	TAACGTGGCCAAAATGATGC GTTCTCCACAACCGCTTGGT	60

2.5.2 Verification of primers specificity via DNA gel electrophoresis

The specificity of the primers used in qRT-PCR analyses was verified via DNA gel electrophoresis. Obtained after qRT-PCR reaction products were separated electrophoretically on 1 % (w/v) agarose gel in 1 x TAE buffer (0.8 mM Tris-acetate, pH 7.5; 0.02 mM EDTA). After melting 1.5 g agarose in 150 ml 1 x TAE buffer and

subsequent cooling down to approximately 60 °C, ethidium bromide was added to a final concentration of 0.4 µg ml^{-1} and gels were cast in horizontal gel trays. Samples were prepared by mixing five volumes of PCR product with 1 volume of loading dye (10 mM Tris-HCl, pH 7.6; 60 % (v/v) glycerol; 60 mM EDTA; 0,03 % (w/v) bromophenol blue), loaded into gel pockets and separated for 20 minutes at constant voltage (120 V) in 1 x TAE buffer. For estimation of the fragment sizes GeneRuler 100 bp DNA Ladder (Fermentas, Germany) was separated in parallel. Ethidium bromide-stained bands were visualized at UV light using INTAS Gel Imager (INTAS, Germany).

2.5.3 Fluorometric detection and quantification of PCR product

Quantification of transcript abundances was performed according to MIQE standards (Bustin *et al.* 2009) in a CFX96 thermocycler (BioRad, United States) using SYBR Green qPCR Master Mix and gene specific primers (for a list see Table 2-3). SYBR Green is a fluorescent dye intercalating into double-stranded DNA and after excitation with blue light (λ = 497 nm) emitting fluorescent green light (λ = 521 nm). The ability of SYBR Green to emission of green light enables fluorometrical determination of PCR product amount after each polymerization cycle.

Each PCR reaction was performed in a total volume of 10 µl and consisted of tested cDNA, 1 x SYBR Green Master Mix [Brilliant II SYBR® Green QPCR Master Mix (Agilent Technology, United States), Maxima SYBR Green qPCR Master Mix (Fermentas, Germany) or SG qPCR Master Mix (EURx, Poland)] and mixture of specific forward and reverse primer (0.6 µM each). Two-step cycling protocol involving amplification of PCR product and generation of melting curves was used for all qRT-PCR analyses. The amplification step includes 3 minutes of polymerase activation at 95 °C and 40 cycles of PCR product synthesis, each consisting of 15 seconds of DNA template denaturation at 95 °C followed by 30 seconds of primers annealing at 60 °C and 30 seconds of product extension at 72 °C. Afterwards PCR products were denatured for 15 seconds at 95 °C followed by temperature decrease to 60 °C and subsequent increase from 60 °C to 95 °C at a ramp speed of 0.5 °C s^{-1}. While the temperature increased, fluorescent data were continuously collected to create melting curves for PCR product.

All analyses were performed for at least two biological replicates representing independent RNA isolations and analyzed in three technical replicates. In all qRT-PCR analyses, a non-template control was included to test the purity of the used reagents and assess for primer-dimer formation.

2.5.4 Standardization and quantification of qRT-PCR results

Transcripts levels of all analyzed genes were normalized to the transcript level of *Act2* gene (At3g18780), which was shown to be constitutively expressed in all vegetative tissues at all developmental stages (An *et al.* 1996). In expression analyses performed at the Max Planck Institute of Molecular Plant Physiology (Golm, Germany) for plants subjected to long-term cold stress, transcripts of four genes, *Act2* (At3g18780), *GAPDH* (At1g13340), *EXPRS* (At2g32170) and *PDF2* (At1g13320), were used for normalization. The expression level of all analyzed genes was quantified using $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001). In $2^{-\Delta\Delta Ct}$ equation, ΔCt corresponds to the difference in Ct (threshold cycle) values between the gene of interest and gene used for normalization e.g. *Act2* ($\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{Act2}$), whereas ΔΔCt is the difference in ΔCt between the analyzed (e.g. Kas-1) and reference (Col-0) *A. thaliana* accession ($\Delta\Delta Ct = \Delta Ct_{Kas\text{-}1} - \Delta Ct_{Col\text{-}0}$).

2.5.5 Statistical analyses

The significance of results obtained by qRT-PCR analyses was analyzed using the ANOVA or Student's t-test. In addition, the expression ratio results were tested for significance using the randomization test provided in the REST software (Pfaffl *et al.* 2002).

2.6 Protein isolation and analyses

2.6.1 Protein isolation

Total protein extracts were isolated as described by Martínez-García *et al.* (1999). 100 mg of plant material was ground in 180 µl buffer E (125 mM Tris-HCl, pH 8.8; 1 % (w/v) SDS; 10 % (v/v) glycerol; 50 mM sodium metabisulfite) using plastic pestles until the mixture was homogenous and immediately placed on ice. When all extracts

were prepared, the tubes were warmed up to room temperature to resolubilize SDS and subsequently centrifuged for 10 minutes at 13000 x g to remove cell debris. An aliquot of supernatant (10 µl) was used to determine the protein concentration using a DC Protein Assay (BioRad, United States). The reminder was diluted with 1/10 volume of buffer Z (125 mM Tris-HCl, pH 6.8; 12 % (w/v) SDS; 10 % (v/v) glycerol; 22 % (v/v) β-mercaptoethanol; 0.001 % (w/v) bromophenol blue) and either directly used for SDS-PAGE separation or stored at -80 °C.

2.6.2 Protein quantification

Total protein concentration was estimated using the DC Protein Assay (BioRad, United States). This modified Lowry assay is based on the reaction of proteins with an alkaline copper tartrate solution and the subsequent reduction of the Folin reagent by the copper-treated protein. The reduction of Folin reagent results in the production of several reduced species which have a characteristic blue colour with maximum absorbance at 750 nm (Peterson, 1979). In the assay, 10 µl of standard BSA samples (0-15 µg) or protein samples were mixed with 25 µl of buffer E (for recipe see 2.6.1.), 125 µl of Reagent A supplemented with Reagent S and 1 ml of Reagent B. The absorbance was measured spectrophotometrically (Ultrospec 2100 pro, Biochrom Ltd, England) at 750 nm after 15 minutes of incubation at room temperature. The protein concentration in the isolated samples was estimated from the standard curve of BSA.

2.6.3 Electrophoretic separation of proteins (SDS-PAGE)

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Schägger and von Jagow (1987). During SDS-PAGE, proteins are separated according to their molecular mass. The electrophoresis was performed in the presence of sodium dodecyl sulfate (SDS), a negatively charged detergent binding to proteins (one anion of SDS per two amino acid residues), masking their intrinsic electrical charge and giving them negative charge. The negative charge is proportional to the length of polypeptide chain, therefore it is possible to separate proteins approximately according to their molecular mass, not to the electrical charge. The electrophoresis was performed in a discontinuous gel system, in which proteins due to the differences in pH between gel and running buffer in the tank were firstly

concentrated at the border of stacking and resolving gels in the sharp narrow band and then separated in the resolving gel according to their molecular weight.

Freshly prepared 12 % resolving gel solution (375 mM Tris-HCl, pH 8.8; 12 % (w/v) acrylamide, 0.32 % (w/v) bis-acrylamide; 0.1 % (w/v) SDS, 0.1 % (w/v) APS; 0.04 % (v/v) TEMED) was filled between two glass plates and subsequently overlaid with water to avoid contact with molecular oxygen, which can inhibit the gel polymerization. After polymerization initiated by the tertiary-amine (TEMED) and catalyzed by ammonium persulfate (APS) water was discarded and the 5 % stacking gel solution (6 mM Tris-HCl, pH 6.8; 5 % (w/v) acrylamide; 0.13 % (w/v) bis-acrylamide; 0.1 % (w/v) SDS, 0.1 % (w/v) APS; 0.09 % (v/v) TEMED) was poured on the top of resolving gel. Samples loaded into individual lanes of polyacrylamide gel were prepared by mixing 4 volumes of protein sample with 1 volume of SDS-PAGE loading buffer (650 mM Tris-HCl, pH 6.8; 50 % (v/v) glycerol, 20 % (w/v) SDS; 0.025 % (w/v) bromophenol blue; 25 % (v/v) β-mercaptoethanol) and subsequent protein denaturation in presence of reducing agents (DTT and β-mercaptoethanol) for 15 minutes at 56 °C. Afterwards, proteins were separated in 1x SDS-PAGE running buffer (25 mM Tris-HCl, pH 8.3; 200 mM glycine; 1 % (w/v) SDS) for approximately 2 hours at 100 V. For estimation of the molecular mass of separated proteins PageRuler^{TM} Prestained Protein Ladder (Fermentas, Germany) was loaded in parallel with the samples.

2.6.4 Electrophoretic transfer of proteins

In order to make the separated by SDS-PAGE proteins accessible to antibody detection they were transferred from the polyacrylamide gel onto the nitrocellulose membrane (Whatman, Germany) in FastBlot B44 (Biometra, Germany) using a semi-dry blotting method. Equilibrated for 5 minutes in the transfer buffer (25 mM Tris-HCl, pH 8.3; 150 mM glycine; 10 % methanol) gel and membrane were assembled between six layers of filter paper (Schleicher & Schuell GmbH, Germany) soaked in transfer buffer. The transfer was performed for 1 hour at constant current of 2 mA per cm^2 of the membrane. The efficiency of the transfer was checked by staining of the membrane with 0.2 % (w/v) PonceauS in 3 % (v/v) acetic acid.

2.6.5 Immunodetection with specific antibodies

Proteins bound to the nitrocellulose membrane were detected immunologically using specific primary antibodies. Afterwards membrane was probed with secondary antibodies able to recognize primary antibodies and conjugated to an enzyme (e.g. horseradish peroxidase, alkaline peroxidase), which cleaves a chemiluminescent substrate producing the luminescence in the proportion to the amount of protein. After the semi-dry transfer, membrane was washed for 10 minutes with TBS buffer (20 mM Tris-HCl, pH 7.6; 0.8 % (w/v) NaCl) and subsequently blocked by overnight incubation in 5 % (w/v) fat-free milk powder in TBS to avoid unspecific binding of antibodies. Afterwards, membrane was washed three times for 10 minutes with TBS buffer supplemented with 0.1 % (v/v) Tween-20 (TBS-T buffer) and incubated for 1 hour with polyclonal primary antibodies in TBS buffer (see Table 2-4 for a list).

Table 2-4. List of primary antibodies used for protein analyses.

Target	Antibody Supplier	Dilution (with TBS buffer)
2CPs	**anti-2CPs** (Baier and Dietz, 1996)	1:5000
Csd2	**anti-CSD2** (AS06 170) Agrisera, Sweden	1:2000
APxs	**anti-APXs** (AS08 368) Agrisera, Sweden	1:2000
GPxs	**anti-GPXs** (AS04 055) Agrisera, Sweden	1:5000
PrxQ	**anti-PrxQ** (AS05 093) Agrisera, Sweden	1:5000
GR	**anti-GR** (AS06 181) Agrisera, Sweden	1:2000
α-tubulin	**anti-TUA** (AS10 680) Agrisera, Sweden	1:1000

After incubation with primary antibodies the membrane was washed once for 15 minutes and three times for 5 minutes with TBS-T buffer to remove unbound antibodies and subsequently probed for 45 minutes at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Sigma-Aldrich, Germany) diluted with TBS buffer in a ratio of 1:10 000.

2.6.6 Chemiluminescent detection of proteins

After incubation with the secondary antibodies, the membranes were washed once for 15 minutes and three times for 5 minutes with TBS-T buffer to remove unbound antibodies and subsequently subjected to the chemiluminescent detection of proteins. Detection was performed using the Western Lighting® Plus-ECL Enhanced Chemiluminescence Substrate (PerkinElmer, United States) and chemiluminescence imager (ImageQuant™ LAS 4000 Mini Biomolecular Imager; GE Healthcare, United States). In this assay horseradish peroxidase (HRP) conjugated to secondary antibodies catalyzes the oxidation of luminol to 3-aminophthalate by hydrogen peroxide. As oxidized luminol decays to its ground state, emits low-intensity light at 428 nm, which can be detected by chemiluminescence imagers. Since horseradish peroxidase is complexed on the membrane with the protein of interest, the amount of light which is emitted during reaction catalyzed by HRP is directly correlated with the amount of protein on the membrane.

2.6.7 Quantification of protein levels

The optical density of the bands detected by the chemiluminescent imager was quantified using the LabImage 1D software (Intas, Germany). Levels of the analyzed proteins were normalized to large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) level detected in the same lane due to the background activity of the primary antibodies (Agrisera, personal communication). Moreover, to verify the accuracy of the quantification, analyses of twofold dilution series of the respective protein sample were performed and the precision of the samples loading was controlled by detection of α-tubulin levels.

2.7 Pigment analyses

2.7.1 Determination of chlorophyll content

Total chlorophyll (Chl) was extracted from 10-20 mg of frozen plant material in 1 ml 80 % (v/v) acetone. Following overnight incubation of plant extracts at -20 °C, samples were centrifuged (Microcentrifuge 5424, Eppendorf, Germany) for 5 minutes at 10000 x g to remove cell debris. Clear supernatants obtained after centrifugation were

analyzed spectrophotometrically (Ultrospec 2100 pro, Biochrom Ltd, England) at 647 and 664 nm. Chlorophyll a (Chl a) and b (Chl b) contents were calculated from the absorbance at given wavelengths according to Porra (2002) by the following equations and standardized on fresh weight:

$$\textbf{Chl a } [\mu g/ml] = 12{,}25 \times A_{664} - 2{,}55 \times A_{647}$$

$$\textbf{Chl b } [\mu g/ml] = 20{,}31 \times A_{647} - 4{,}91 \times A_{664}$$

$$\textbf{Chl a + b } [\mu g/ml] = 17{,}76 \times A_{647} + 7{,}34 \times A_{664}$$

2.7.2 Determination of anthocyanin content

Anthocyanin content was determined spectrophotometrically in samples obtained by extraction of 50 mg frozen plant material in methanol containing 1 % (v/v) HCl. After incubation for an hour in the darkness, samples were centrifuged (Microcentrifuge 5424, Eppendorf, Germany) for 5 minutes at 16100 x g to remove cell debris. Clear supernatants obtained after centrifugation were analyzed spectrophotometrically (Ultrospec 2100 pro, Biochrom Ltd, England) at 530 and 657 nm. Anthocyanin content was calculated according to Mancinelli *et al.* (1991) by the following equation and standardized on fresh weight:

$$\mathbf{A_{530\ corr.}} = A_{530} - 0{,}2 \times A_{657}$$

2.8 Histological detection of ROS

2.8.1 Detection of superoxide radical anion (NBT staining)

Histochemical detection of superoxide radical anion ($O_2^{\bullet-}$) was performed *in situ* with nitroblue tetrazolium (NBT) according to Kawai-Yamada *et al.* (2004). Firstly the leaves were vacuum-infiltrated for 10 minutes with 10 mM sodium azide in PBS buffer (73 mM sodium chloride; 3 mM potassium chloride; 10 mM disodium hydrogen phosphate; 2 mM monopotassium phosphate; pH 7.8) to inhibit the activity of peroxidases present in plant cells and then again vacuum-infiltrated with 1 mg ml^{-1} NBT in PBS for 4 to 8 hours in the darkness. Superoxide detection is based on the reaction of NBT reduction by $O_2^{\bullet-}$ to dark blue, water-insoluble formazan. The amount of formed formazan is correlated with the content of superoxide produced in the plant tissue.

Finally, to improve the visibility of the blue precipitate, chlorophyll was removed by incubation of leaves in a boiling mixture of acetic acid, glycerol and ethanol (1:1:3).

2.8.2 Detection of hydrogen peroxide (DAB staining)

Hydrogen peroxide was detected *in situ* by overnight vacuum-infiltration of leaves in a solution of 1 mg ml^{-1} 3,3'-diaminobenzidine (DAB) in PBS buffer according to Vanacker *et al.* (2000). The assay is based on the reaction of DAB oxidation by hydrogen peroxide leading to the formation of dark brown precipitate. To better visualize obtained precipitate chlorophyll was removed by incubation of leaves in a boiling mixture of acetic acid, glycerol and ethanol (1:1:3).

2.8.3 Quantification of ROS levels

Before the quantification of ROS levels the photos of histologically stained with either NBT or DAB leaves were taken using Nikon D3100 camera (Nikon, Japan). Afterwards the photos were subjected to the digital processing using GIMP 2.6.11 software including contrast improvement and the background colour correction. The processed photos were saved as 32-bit black-and-white images and used for quantification of ROS levels using ImageJ software (Schneider *et al.* 2012). Firstly, the lower and upper threshold values were set to segment the grayscale picture into the stained area and background, secondly the whole leaf area was marked and finally the stained area of the leaf was calculated in [%].

2.9 Superoxide dismutase activity assay

2.9.1 Protein isolation

Total protein extracts were prepared by homogenization of 200 mg of *A. thaliana* leaves in 1.2 ml of ice-cold extraction buffer (50 mM K_2HPO_4, pH 7.6; 10 % (v/v) glycerol; 0.1 % (v/v) Triton X-100; 1 mM EDTA; 1 mM EGTA; 1 mM PMSF; 0.05% β-mercaptoethanol; 0.1 mg ml^{-1} BSA). The homogenization was followed by centrifugation (Centrifuge 5415R, Eppendorf, Germany) for 30 minutes at 16000 x g and 4 °C. The supernatant obtained after centrifugation was directly used for separation of proteins by native polyacrylamide gel electrophoresis.

2.9.2 Protein quantification

Total protein concentration was estimated using the BioRad assay (BioRad, United States) based on the method of Bradford (Bradford, 1976). This assay uses the ability of the acidic solution of Coomassie Brilliant Blue G-250 to shift the absorbance from 465 nm to 595 nm when binding to the protein occurs.

For protein quantification 10 µl of standard samples of BSA (0-20 µg) or protein samples were diluted with 790 µl of water and 200 µl of BioRad Protein Assay reagent was added. The absorbance was measured spectrophotometrically (Ultrospec 2100 pro, Biochrom Ltd, England) at 595 nm after 10 minutes of incubation at room temperature. The protein concentration in isolated samples was estimated from a BSA standard curve.

2.9.3 Native polyacrylamide gel electrophoresis

Proteins were separated by native polyacrylamide gel electrophoresis (Native-PAGE). In contrast to SDS-PAGE (see 2.6.3.), the Native-PAGE includes no negatively charged detergent (SDS), which masks the intrinsic charge of proteins. Therefore in this type of electrophoresis the migration distance of proteins depends on their charge, size and shape (Wittig and Schägger, 2005). As in the case of SDS-PAGE, Native-PAGE was performed in a discontinuous gel system.

All gels were freshly prepared at 4 °C. 12 % resolving gel solution (375 mM Tris-HCl, pH 8.8; 12 % (w/v) acrylamide, 0.32 % (w/v) bis-acrylamide; 10 % (v/v) glycerol, 0.1 % (w/v) APS; 0.04 % (v/v) TEMED) was filled between two glass plates and subsequently overlaid with water. After polymerization initiated by the tertiary-amine (TEMED) and catalyzed by the ammonium persulfate (APS) water was discarded and the 5 % stacking gel solution (6 mM Tris-HCl, pH 6.8; 5 % (w/v) acrylamide; 0.13 % (w/v) bis-acrylamide; 10 % (v/v) glycerol, 0.1 % (w/v) APS; 0.09 % (v/v) TEMED) was poured on the top of resolving gel. Samples loaded into polyacrylamide gel were prepared by mixing four volumes of protein sample containing 50 µg of proteins with one volume of loading buffer (650 mM Tris-HCl, pH 6.8; 50 % (v/v) glycerol; 0.025 % (w/v) bromophenol blue) on ice to prevent protein denaturation. Native-PAGE was performed at 4 °C in 1x Native-PAGE running buffer (25 mM Tris-HCl, pH 8.3; 200 mM glycine) for approximately 3 hours at 35 mA.

2.9.4 SOD activity detection

Superoxide dismutase (SOD) activity assay was performed according to Beauchamp and Fridovich (1971). The assay is based on the ability of superoxide dismutase to inhibit the reduction of nitroblue tetrazolium (NBT) to dark blue formazan by photochemically generated from riboflavin superoxide.

Following the Native-PAGE, gels were stained for 20 minutes in 50 ml of SOD assay buffer (50 mM KH_2PO_4/K_2HPO_4, pH 7.8; 0.1 mM EDTA; 0.32 mg ml^{-1} NBT; 20 µg ml^{-1} riboflavin; 0.4 % (v/v) TEMED) in the darkness. Afterwards, gels were exposed to light to observe development of white bands corresponding to the sites, where superoxide dismutase was active. Different SOD isoforms were identified in inhibitor studies performed as by Kanematsu and Asada (1990). Two different inhibitors of SOD activity, potassium cyanide or hydrogen peroxide, were applied. For identification of Mn-SOD SOD assay buffer was supplemented with 5 mM H_2O_2, an inhibitor of Cu/Zn-SOD and Fe-SOD activities, whereas Fe-SOD was identified after incubation in SOD assay buffer containing 2 mM KCN inhibiting Cu/Zn-SODs.

2.10 Sequence analyses

Single nucleotide polymorphisms (SNPs) in genes encoding enzymes and regulators of chloroplast water-water cycle in different accessions of *Arabidopsis thaliana* were identified using the GE Browser 3.0 (http://signal.salk.edu/atg1001/3.0/gebrowser.php). The nucleic acids sequences of genes from different accessions were clustered based on the sequence similarity using the DNA Identity Matrix/Unity Matrix of ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The following settings of multiple sequence alignment were chosen: GAP OPEN – 10; GAP EXTENSION – 0.20; GAP DISTANCE – 5, CLUSTERING – NJ (Neighbour Joining). Afterwards alignments were edited with JalView (http://www.jalview.org).

Chapter 3

RESULTS

3.1 Expression regulation of chloroplast antioxidant system among *Arabidopsis thaliana* accessions

The model plant *Arabidopsis thaliana* is a small annual weed of the mustard family widely distributed throughout western and central Europe, south-east Asia, as well as in the mid-Atlantic and the Pacific north-western United States into south-western Canada (Hoffmann, 2002). Across its latitudinal range (from 68 °N to equator) it is exposed to a wide range of climatic conditions. In response to this substantial environmental variation many adaptive traits evolved (Koornneef *et al.* 2004). So far, considerable natural genetic variation among *A. thaliana* accessions for such traits as flowering time (Alonso-Blanco *et al.* 1998), seed size (Alonso-Blanco *et al.* 1999), circadian rhythms (Swarup *et al.* 1999), as well as tolerance to biotic (Kunkel, 1996) and abiotic (Hannah *et al.* 2006; Bouchabke *et al.* 2008; Lefebvre *et al.* 2009; Katori *et al.* 2010; Bai *et al.* 2012; Zuther *et al.* 2012) stresses, has been described.

Although the differences in the stress responses and acclimation observed among *A. thaliana* accessions have been studied, very little is known about natural variation in the regulation of chloroplast antioxidant system. Since, this system has an essential role in neutralization of reactive oxygen species (ROS) and protection of plant cells from caused by them damage of proteins, nucleic acids and lipids (Asada, 1999; Temple *et al.* 2005), it is very important to check whether its regulation differs among *A. thaliana* accessions and, if yes, whether the differences were introduced to this system during evolution process to ease adaptation of accessions to various climatic conditions at their natural habitats. It is known that in such adaptation the regulation of gene expression plays a key role (Stone and Wray, 2001; Vuylsteke *et al.* 2005; Delker *et al.* 2010). Therefore, based on the set of transcript, polysomal RNA and protein data generated for population of seven *A. thaliana* accessions (see Figure 3-1 for photos), namely Col-0 (N1092), Kas-1 (N1264), Cvi-0 (N1096), Ms-0 (N1376), WS (N1602), C24 (N906) and

Van-0 (N1584), natural variation in expressional dynamic of the chloroplast antioxidant system will be discussed.

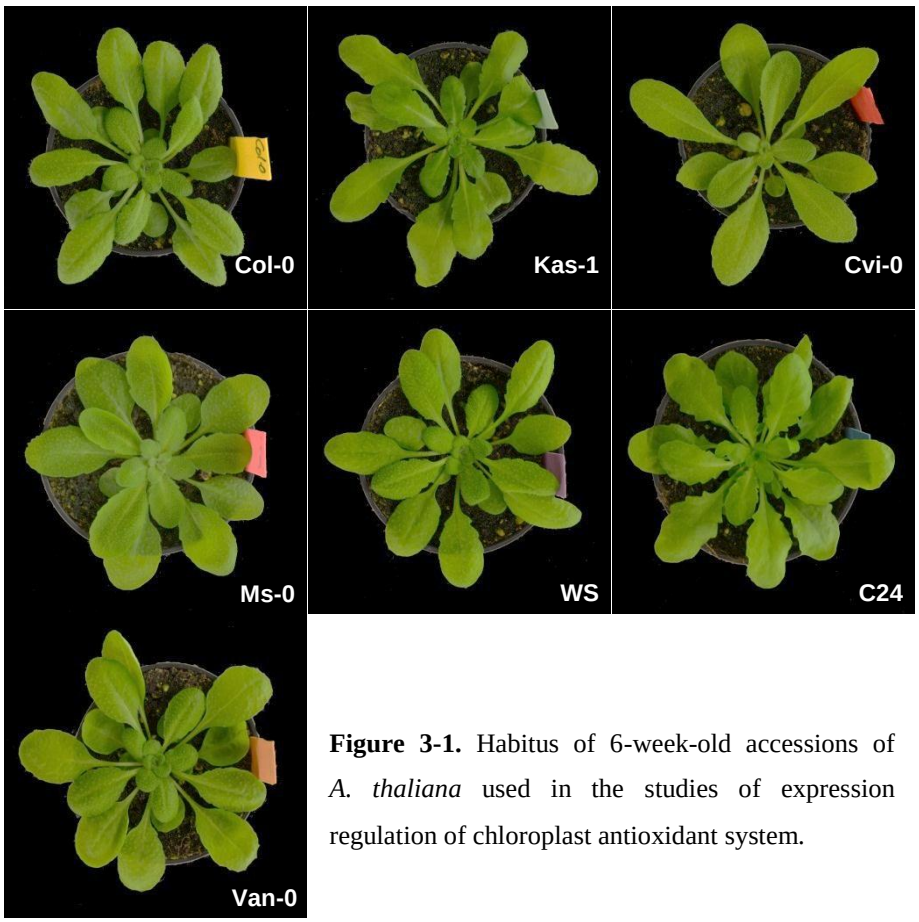

Figure 3-1. Habitus of 6-week-old accessions of *A. thaliana* used in the studies of expression regulation of chloroplast antioxidant system.

The accessions were selected to cover a wide range of northern habitats with contrasting temperature and precipitation conditions (see Figure 3-2 and Table 2-1 for details on the origin and natural habitats). Among all of them, Cvi-0 origins from the hottest and driest climate (average monthly temperature between 22 and 27 °C during entire year and average monthly precipitation higher than 10 mm only between August and September). On the contrary, Kas-1 comes from the coldest region with the average monthly temperature between -12 °C in winter and 12 °C in summer. All of the remaining accessions (Col-0, Ms-0, WS and Van-0) origin from continental climates, but more extreme changes in the temperature between summer and winter are detected at the natural habitats of Ms-0 and WS (cold winters and relatively warm summers) than for Col-0 and Van-0 (relatively warm winters and summers).

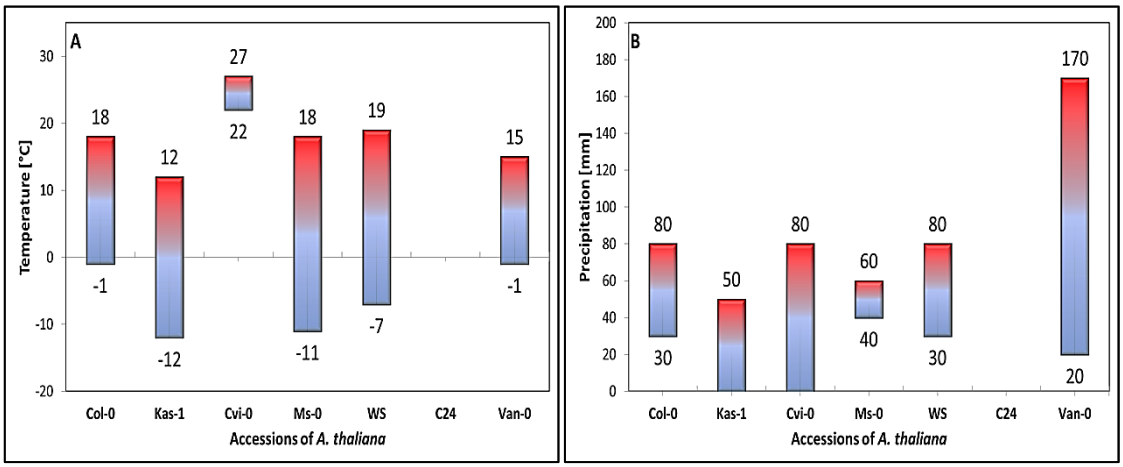

Figure 3-2. Average monthly temperature (**A**) and precipitation (**B**) at the natural habitats of selected *A. thaliana* accessions.

Growing such different accessions under the same environmental conditions enables studies of the genetic variation in many traits. Here, the expression regulation of chloroplast antioxidant system will be analyzed in accessions cultivated under growth conditions optimized for Col-0 (day / night temperature of 20 °C / 18 °C and 120 µmol m^{-2} s^{-1} light with a 10 h light / 14 h dark photoperiod).

3.1.1 Regulation at the transcript level

Variation in the transcript abundance, termed expression level polymorphism (ELP), influences quantitative phenotypic variation (Doerge, 2002). To study such variation at the transcript level under different developmental and physiological conditions microarrays and quantitative real-time PCR (qRT-PCR) analyses are routinely used (Hannah *et al.* 2006; van Leeuwen *et al.* 2007; Zuther *et al.* 2012). Here, natural variation in the chloroplast antioxidant system at the transcript level among seven *A. thaliana* accessions was analyzed using qRT-PCR.

Special attention was given to the genes encoding key ROS-neutralizing enzymes of this system, namely *Csd2* (At2g28190), *2CPA* (At3g11630), *2CPB* (At5g06290), *PrxQ* (At3g26060), *PrxIIE* (At3g52960), *sAPx* (At4g08390), *tAPx* (At1g77490), *GPx1* (At2g25080), *GPx7* (At4g31870), *MDHAR* (At1g63940), *DHAR* (At1g19550) and *GR* (At3g54660). The enzymes encoded by selected genes are responsible for dismutation of superoxide radical anion to hydrogen peroxide (*Csd2*), reduction of H_2O_2 to H_2O in ascorbate-independent (*2CPA*, *2CPB*, *PrxQ*, *PrxIIE*, *GPx1* and *GPx7*) or ascorbate-

dependent manner (*sAPx* and *tAPx*), as well as for regeneration of oxidized ascorbate generated in the reaction catalyzed by ascorbate peroxidases (*MDHAR*, *DHAR* and *GR*) (Asada, 1999; Baier and Dietz, 1996).

The transcripts of selected genes were detected using qRT-PCR and gene-specific primers (see Table 2-3 for a list). The specificity of these primers was verified after each qRT-PCR reaction by analyses of melting curves generated during this reaction in the CFX96 thermocycler (BioRad, United States) and via electrophoretic separation of obtained PCR products in 1% (w/v) agarose gel. In all cases melting curves displayed a single sharp peak and gel electrophoresis resulted in a single product of desired length, indicating that used primers were highly specific (Figure 3-3).

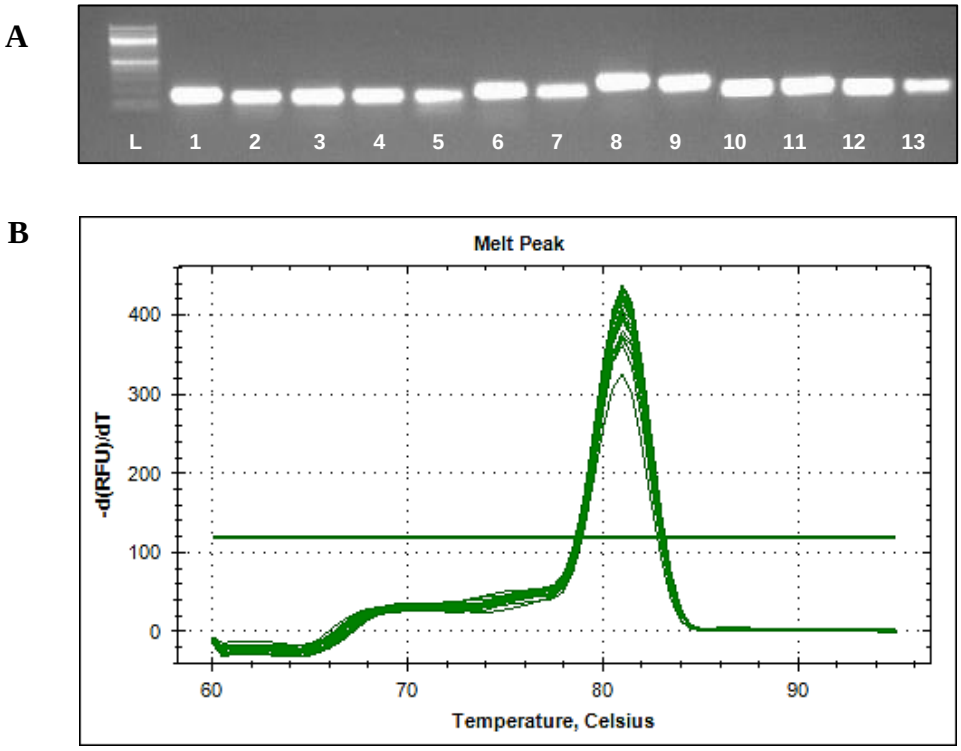

Figure 3-3. Specificity of the primers used in qRT-PCR analyses. **A.** Agarose gel electrophoresis of PCR products amplified with 1. qPCR_2CPA, 2. qPCR_2CPB, 3. qPCR_PrxQ, 4. qPCR_PrxIIE, 5. qPCR_sAPx_1, 6. qPCR_tAPx, 7. qPCR_MDHAR, 8. qPCR_DHAR, 9. qPCR_Csd2_1, 10. qPCR_GR, 11. qPCR_Act2, 12. qPCR_GPx1, 13. qPCR_GPx7 pairs of primers (in parallel with PCR products PageRuler 100 bp DNA ladder (L) was separated) and **B.** Melting curve obtained for PCR product amplified with qPCR_Act2 primers pair.

Moreover, standard curves obtained for each tested primer pair after qRT-PCR reaction performed for 10-fold dilution series of cDNA synthesized from total RNA of Col-0 had

an efficiency of 100 % ± 10 % and R^2 value higher than 0.99, indicating that mRNAs encoding all analyzed chloroplast antioxidant enzymes were amplified in qRT-PCR reactions with high efficiencies (Figure 3-4).

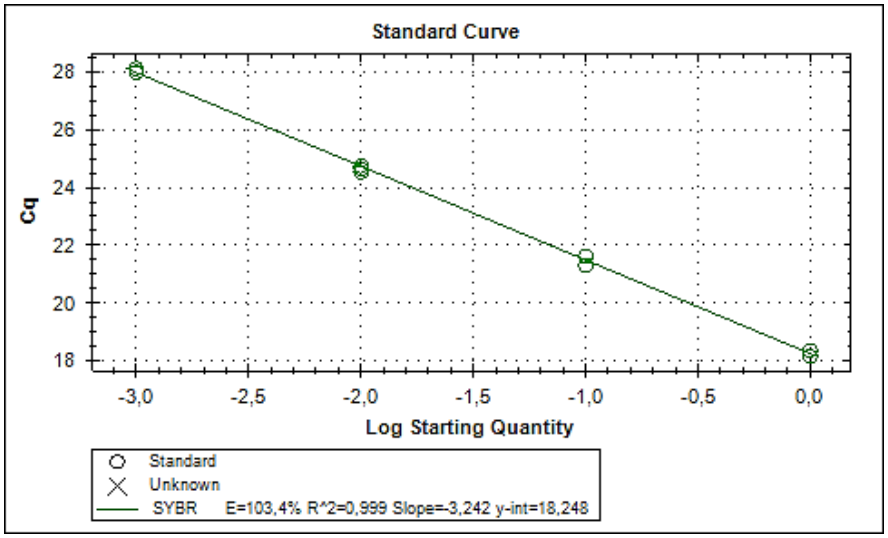

Figure 3-4. Standard curve generated for 10-fold dilution series of cDNA (synthesized from total RNA of Col-0) amplified in a qRT-PCR reaction using qPCR_Act2 primers pair.

As soon as the sequence data for all analyzed genes and accessions became available in the 1001 genome project (Cao *et al.* 2011), the binding efficiency of the primers designed for Col-0 to the gene of interest was also tested in accessions other than Col-0. The comparison of sequences recognized by the used primers in the gene of interest of all selected accessions revealed single nucleotide polymorphisms (SNPs) in *Csd2* of Cvi-0, *2CPB* of Kas-1, Ms-0, WS and C24, *PrxIIE* of Kas-1, *PrxQ* of WS, *MDHAR* of Cvi-0, Ms-0, C24 and Van-0 and in *DHAR* of Kas-1, Cvi-0 and WS (Figure 3-5). Such sequence variation might affect the binding affinity of used primers to the gene of interest and lead to the lack of amplicon. Therefore, it was verified whether *Csd2*, *2CPB*, *PrxIIE*, *PrxQ*, *MDHAR* and *DHAR* in accessions, which show sequence variation, could be synthesized. The analyses showed that tested genes were amplified even in the accessions containing SNPs (Figure 3-5). However, the efficiency of amplification in these accessions might be affected. Therefore, as soon as the sequence data for all selected accessions became available, new primers binding in the regions without SNPs were designed.

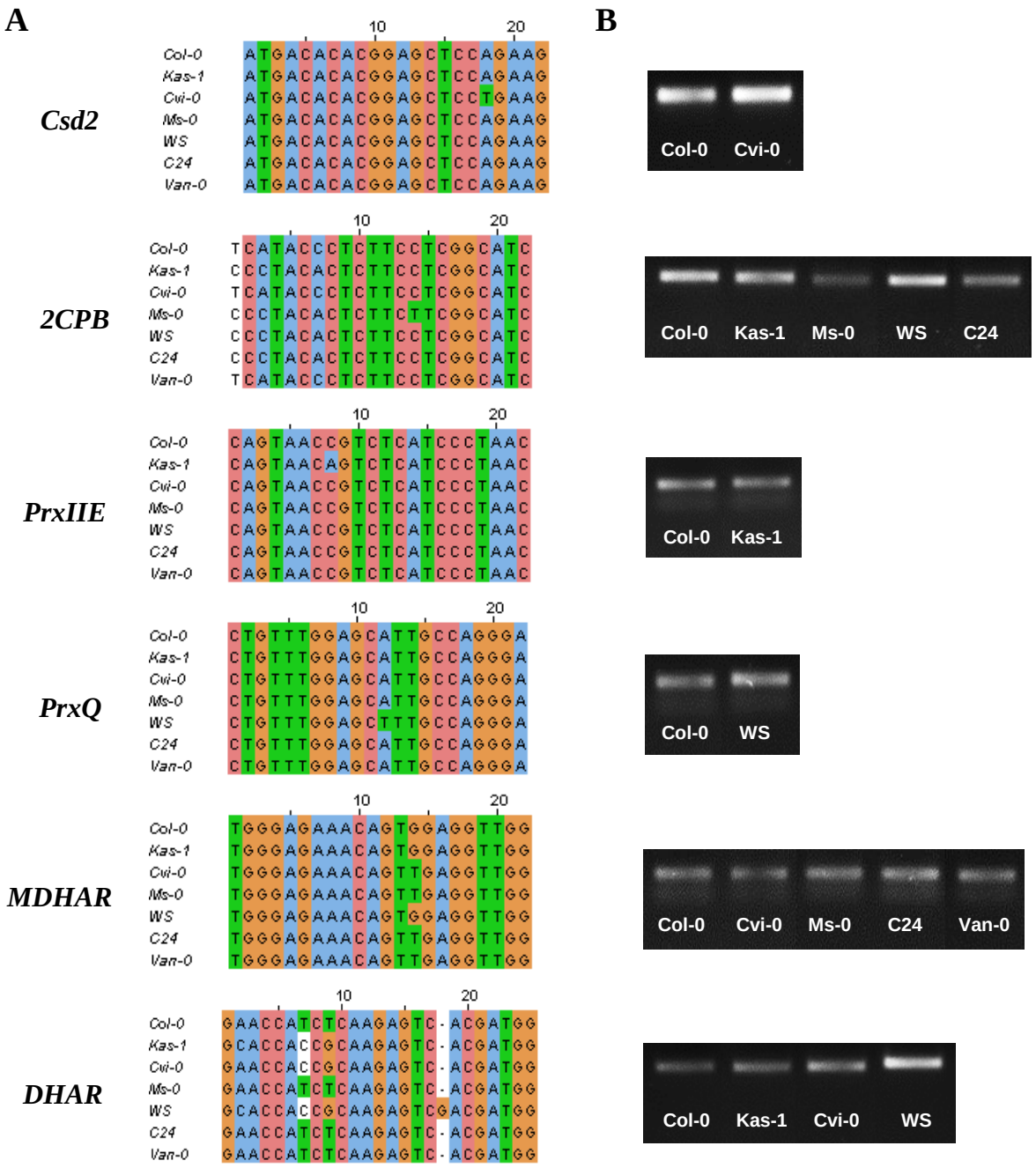

Figure 3-5. Primers used in qRT-PCR analyses. **A.** Comparison of sequences recognized by forward or reverse primers in genes encoding chloroplast antioxidant enzymes, in which SNPs were detected; **B.** Agarose gel electrophoresis of PCR products amplified in accessions containing SNPs in the sequences recognized by primers designed for Col-0.

All qRT-PCR reactions were performed for RNA samples isolated out of 6-week-old rosettes of *A. thaliana* accessions grown on soil (for description of growth conditions see 2.2.1). At this developmental stage all plants used for analyses did not show

symptoms of senescence, had well expanded leaves and no visible inflorescence (see Figure 3-1 for photos).

The transcript abundances of all genes encoding key chloroplast ROS-neutralizing enzymes in selected *A. thaliana* accessions were calculated relatively to the expression of the same gene in the reference accession Col-0 (the relative transcript levels of all genes of interest in Col-0 were set as 1) and standardized on the transcript level of *Act2* gene (At3g18780), which was shown to be constitutively expressed in all vegetative tissues at all developmental stages (An *et al.* 1996).

For each accession, three biological replicates, each representing an independent RNA isolation from single, independently grown plant, were analyzed. All analyses were performed in three technical replicates.

3.1.1.1 Copper-zinc superoxide dismutase (Csd2)

Copper-zinc superoxide dismutase (Cu/Zn-SOD, CSD2) is a key element of the chloroplast antioxidant system. It catalyzes the dismutation of superoxide radical anion to hydrogen peroxide, a first step in the defence against many environmental stimuli leading to the oxidative stress. So far, natural variation in the *Csd2* expression was studied only for *A. thaliana* var. Col-0, Cvi-0 and Ler (Abarca *et al.* 2001). The analyses revealed that Cu/Zn-SOD in Cvi-0 is encoded by different *Csd2* allele (*Csd2-2*) than that of Col-0 and Ler (*Csd2-1*). Moreover, it was shown that the single nucleotide polymorphisms (SNPs) detected in *Csd2-2* (in comparison with *Csd2-1*) led to two amino acids changes in CSD2 of Cvi-0 and thus, an increase in the isoelectric point and reduction of its mobility in Native PAGE (Abarca *et al.* 1999). Here, the pattern of different SOD isoenzymes and the expression of chloroplast *Csd2* at the transcript level were analyzed in seven accessions of *A. thaliana*.

Different SOD isoenzymes, namely Mn-SOD, Fe-SOD and both, chloroplastic and cytosolic Cu/Zn-SODs (Kliebenstein *et al.* 1998), were visualized by NBT staining of native polyacrylamide gels (Beauchamp and Fridovich, 1971). The same patterns in isoenzyme migration as that described for Col-0 and Ler (Abarca *et al.* 2001) were revealed in Kas-1, Ms-0, WS, C24 and Van-0, while the accession-specific isoform of chloroplastic Cu/Zn-SOD (Cu/Zn-SOD3) (Abarca *et al.* 2001) was detected only in Cvi-0 (Figure 3-6). Moreover, compared to Col-0, the activity of CSD2 was higher in

Ms-0 and WS, slightly lower in Kas-1 and almost not detectable in C24 and Van-0 (Figure 3-6).

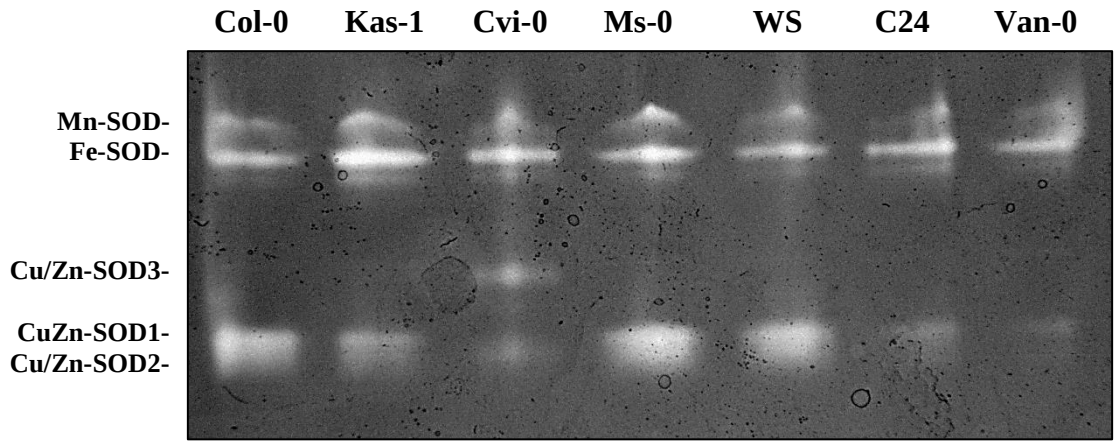

Figure 3-6. SOD activity assay. Total protein extracts from leaves of seven accessions of *Arabidopsis thaliana* were separated by native polyacrylamide gel electrophoresis. SOD activity assay was performed as described in materials and methods. Cu/Zn-SOD1 – chloroplastic Cu/Zn-SOD detected in all accessions excluding Cvi-0; Cu/Zn-SOD2 – cytosolic Cu/Zn-SOD; Cu/Zn-SOD3- chloroplastic Cu/Zn-SOD detected in Cvi-0; Mn-SOD – manganese SOD; Fe-SOD-iron SOD.

The subsequent analyses of *Csd2* expression also revealed significant differences in relative *Csd2* transcript levels between almost all analyzed *A. thaliana* accessions, except Kas-1, C24 and Van-0, in which similar amounts of *Csd2* mRNAs were accumulated (Figure 3-7).

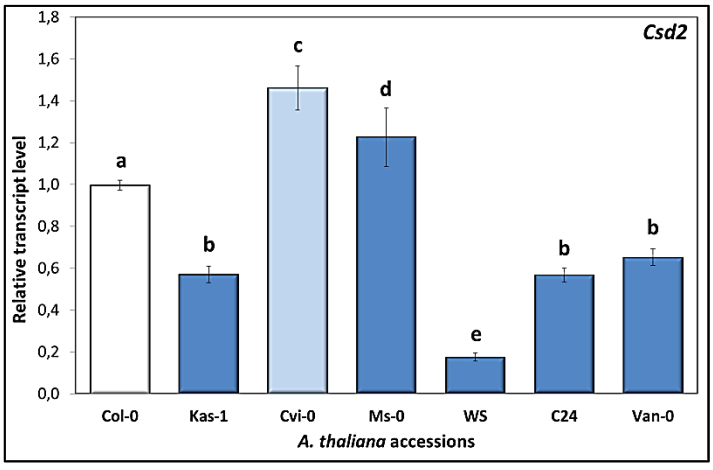

Figure 3-7. Transcript level of *Csd2* in six accessions of *A. thaliana* relative to Col-0. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Light blue bar corresponds to the *Csd2* transcript level in the accession, in which the sequences recognized by the qPCR_Csd2_1 primers pair contain SNPs. Data are mean ±SD from three independent biological replicates, each analyzed in triplicates.

In comparison with reference accession (Col-0), lower *Csd2* transcript levels were observed for WS (0.18 ± 0.02 relative to Col-0), Kas-1 (0.57 ± 0.04 relative to Col-0), C24 (0.57 ± 0.03 relative to Col-0) and Van-0 (0.65 ± 0.04 relative to Col-0). Among these four accessions, the lowest level of this transcript was detected for WS, in which the *Csd2* mRNA abundance was less than 20 % of that observed in Col-0, showing more than fivefold reduction. On the contrary, *Csd2* transcript level in Cvi-0 and Ms-0 was about 50 % and 20 % higher than in Col-0, respectively. Since the sequence recognized by used primers in *Csd2* of Cvi-0 contains SNPs and thereby binding of this primers pair might be affected, the relative *Csd2* transcript level in this accession could be even slightly higher than that measured in the following analyses. It indicated that the accumulation of *Csd2* mRNA in Cvi-0 was the strongest among all tested *A. thaliana* accessions.

Further comparison of selected accessions revealed that in almost all of them (except WS) the transcript levels of *Csd2* were well correlated with the activity of encoded by this mRNA protein (Figures 3-6 and 3-7). Thus, CSD2 was more active than in Col-0 only in Ms-0, which also accumulated high amounts of *Csd2* mRNA. In contrast, lower than in Col-0 activity of CSD2 was detected in Kas-1, Van-0 and C24, in which also *Csd2* transcript abundances were lower than in Col-0. Only in WS, low levels of *Csd2* transcript were correlated with high activity of protein, indicating accession-specific regulation of *Csd2* expression and CSD2 activity.

3.1.1.2 Enzymes of the ascorbate-independent water-water cycle

In chloroplasts of *Arabidopsis thaliana* four peroxiredoxins (2CPA, 2CPB, PrxQ and PrxIIE) and two glutathione peroxidases (GPX1 and GPX7) reduce hydrogen peroxide to water in ascorbate-independent manner (Dietz, 2011; Foyer and Shigeoka, 2011). So far, the expression regulation of peroxiredoxins (PRXs) and glutathione peroxidases (GPXs) in *A. thaliana* was studied only in Col-0 background (Baier and Dietz, 1997; Horling *et al.* 2003; Soitamo *et al.* 2008). Here, the transcript levels of all chloroplast *Prxs* and *GPxs*, namely *2CPA, 2CPB, PrxQ, PrxIIE, GPx1* and *GPx7*, were analyzed and compared in seven accessions of *A. thaliana*.

Peroxiredoxins (*2CPA*, *2CPB*, *PrxQ* and *PrxIIE*)

The analyses of transcript levels of genes encoding chloroplast peroxiredoxins, namely *2CPA*, *2CPB*, *PrxQ* and *PrxIIE*, revealed that all of them were expressed in all selected *A. thaliana* accessions (Figure 3-8). Among them, *2CPA* transcript was accumulated at the highest level in almost all tested accessions, except Ms-0, in which accumulation of mRNA encoding *PrxQ* dominated. In contrast, in all accessions the abundance of *PrxIIE* transcript was the lowest among tested *Prxs.*

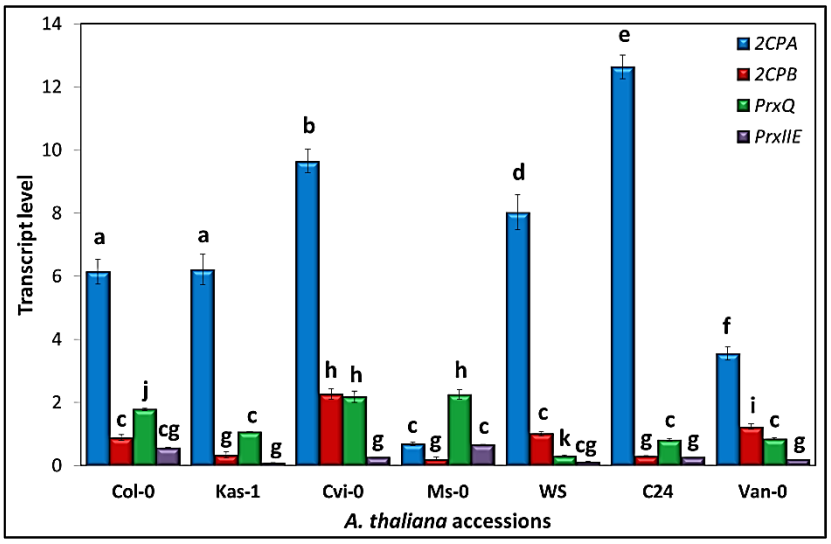

Figure 3-8. Comparison of *2CPA* (blue bars), *2CPB* (red bars), *PrxQ* (green bars) and *PrxIIE* (violet bars) transcript levels in seven accessions of *A. thaliana*. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are mean ±SD from three independent biological replicates, each analyzed in triplicates.

Since two of the analyzed chloroplast peroxiredoxins, namely 2CPA and 2CPB, belong to the family containing two cysteine residues (2-Cys peroxiredoxins), the paralogue variation in their transcript levels (*2CPA* vs. *2CPB*) could be analyzed (Figure 3-8). The analyses revealed that all tested accessions accumulated more mRNA of *2CPA* than that of *2CPB*, but *2CPA* / *2CPB* ratio strongly differed between accessions (Figure 3-8 and Table 3-1). It indicated that the expression of *2CPB* is regulated at the transcript level independently from that of *2CPA*.

	Col-0	**Kas-1**	**Cvi-0**	**Ms-0**	**WS**	**C24**	**Van-0**
2CPA/*2CPB* ratio	6.90	18.12	4.25	3.33	7.85	42.47	2.89

Table 3-1. *2CPA*/*2PB* ratio in selected accessions of *A. thaliana*.

Strong variation observed in the transcript levels of all chloroplast peroxiredoxins in selected accessions prompted for further analyses of the accumulation of mRNAs encoding particular PRXs in these accessions (Figure 3-9).

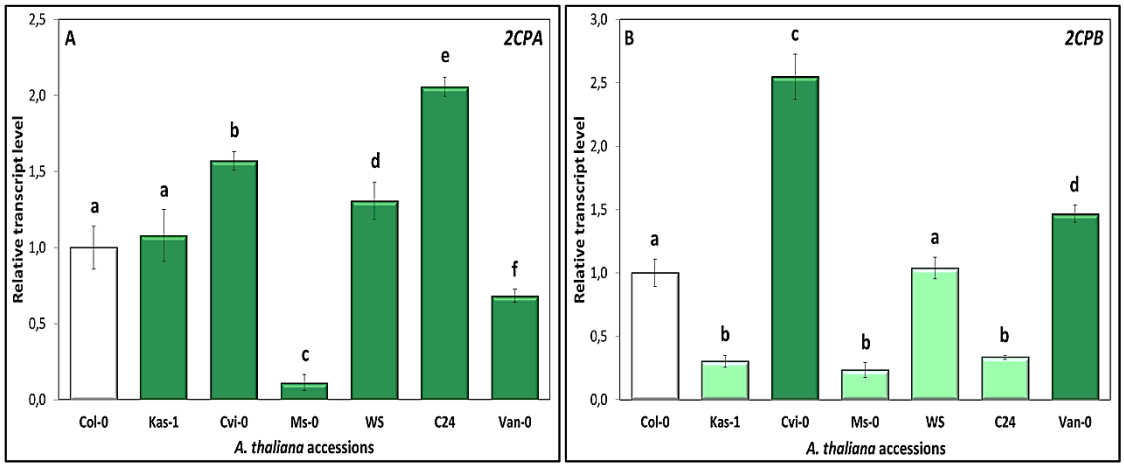

Figure 3-9. Transcript levels of **A.** *2CPA* and **B.** *2CPB* in six accessions of *A. thaliana* relative to Col-0. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Light green bars correspond to the *2CPB* transcript level in the accessions, in which the sequences recognized by the qPCR_2CPB_1 primers pair contain SNPs. Data are mean ±SD from three independent biological replicates, each analyzed in triplicates.

Relative to reference accession (Col-0), significantly higher transcript accumulation of *2CPA* was detected in Cvi-0 (1.57 ± 0.06 relative to Col-0), WS (1.31 ± 0.12 relative to Col-0) and C24 (2.06 ± 0.06 relative to Col-0) (Figure 3-9). In contrast, the level of *2CPA* mRNA in Ms-0 (0.11 ± 0.05 relative to Col-0) was approximately tenfold lower than that in Col-0 (Figure 3-9).

The accumulation of the transcript encoding the second 2-Cys peroxiredoxin *(2CPB*) in comparison with Col-0, was significantly higher in Cvi-0 (2.55 ± 0.18 relative to Col-0) and Van-0 (1.47 ± 0.07 relative to Col-0), similar in WS (1.04 ± 0.08 relative to Col-0) and lower in Kas-1 (0.30 ± 0.05 relative to Col-0), Ms-0 (0.23 ± 0.06 relative to Col-0), and C24 (0.33 ± 0.02 relative to Col-0) (Figure 3-9). However, the sequence of *2CPB* recognized by used primers in Kas-1, Ms-0, WS and C24 contains SNPs, which could affect the binding efficiency of the primers. Therefore, relative *2CPB* transcript level in these accessions might have been higher.

Moreover, the variation in the total transcript levels of *2CPs* (*2CPA* + *2CPB*) was analyzed among all tested accessions (Figure 3-9). The analyses revealed that, in comparison with Col-0 (7.03 ± 0.24), *2CPs* were accumulated at significantly higher

level in Cvi-0 (11.91 ± 0.27), C24 (12.93 ± 0.20) and WS (9.04 ± 0.31). In contrast, the total transcript levels of *2CPs* in Ms-0 (0.90 ± 0.05) and Van-0 (4.79 ± 0.14) were approximately seven- and twofold lower than that detected in Col-0, respectively. Since Ms-0 and WS, as well as Col-0 and Van-0 origin from similar climates, it indicated that the expression regulation of *2CPs* at the transcript level is not specifically modulated by environmental parameters at the natural habitats of tested accessions.

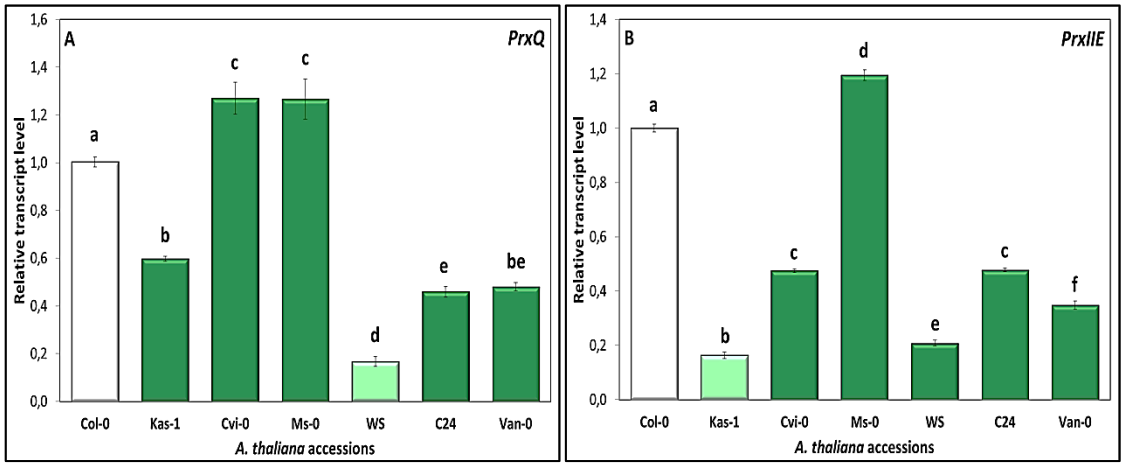

Figure 3-10. Transcript levels of **A.** *PrxQ* and **B.** *PrxIIE* in six accessions of *A. thaliana* relative to Col-0. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Light green bars correspond to the *PrxQ* and *PrxIIE* transcript levels in the accessions, in which the sequences recognized by the qPCR_PrxQ and qPCR_PrxIIE primers pairs contain SNPs. Data are mean ±SD from three independent biological replicates, each analyzed in triplicates.

Among other analyzed peroxiredoxins (*PrxQ* and *PrxIIE*), slightly higher transcript accumulation than in Col-0 was observed only for *PrxQ* in Cvi-0 (1.27 ± 0.07 relative to Col-0) and Ms-0 (1.27 ± 0.08 relative to Col-0) and for *PrxIIE* in Ms-0 (1.20 ± 0.02 relative to Col-0), while in other tested accessions the transcript levels of these genes were at least 40 % lower than in Col-0 (Figure 3-10).

Glutathione peroxidases (*GPx1* and *GPx7*)

Genome of *A. thaliana* encodes two chloroplast glutathione peroxidases (GPX1 and GPX7), both reducing hydrogen peroxide to water in ascorbate independent manner. To check how the expression of these genes is regulated at the transcript level among selected *A. thaliana* accessions, the *GPx1* and *GPx7* mRNA abundances were compared between all of them (Figure 3-11).

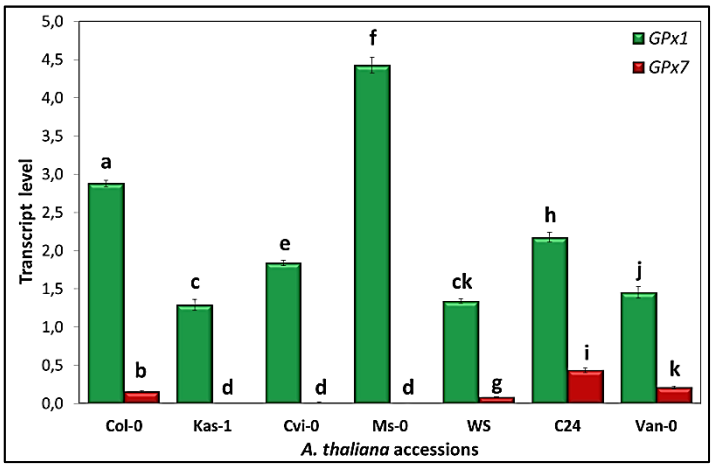

Figure 3-11. Comparison of *GPx1* (green bars) and *GPx7* (red bars) transcript levels in seven accessions of *A. thaliana*. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are mean ±SD from three independent biological replicates, each analyzed in triplicates.

The comparison revealed that all accessions accumulated significantly higher amount of *GPx1* transcript than that of *GPx7*, suggesting that in reducing H_2O_2 GPX1 play a more important role than GPX7. Moreover, the transcript levels of *GPx1* were generally more variable between tested accessions than that of *GPx7* (Figure 3-11). Thus, *GPx1* mRNA was accumulated in different amounts in almost all accessions (except WS), while the level of *GPx7* mRNA was strongly variable only between Col-0, WS, C24 and Van-0. In Kas-1, Cvi-0 and Ms-0 the transcript of *GPx7* was undetectable or expressed at really low level.

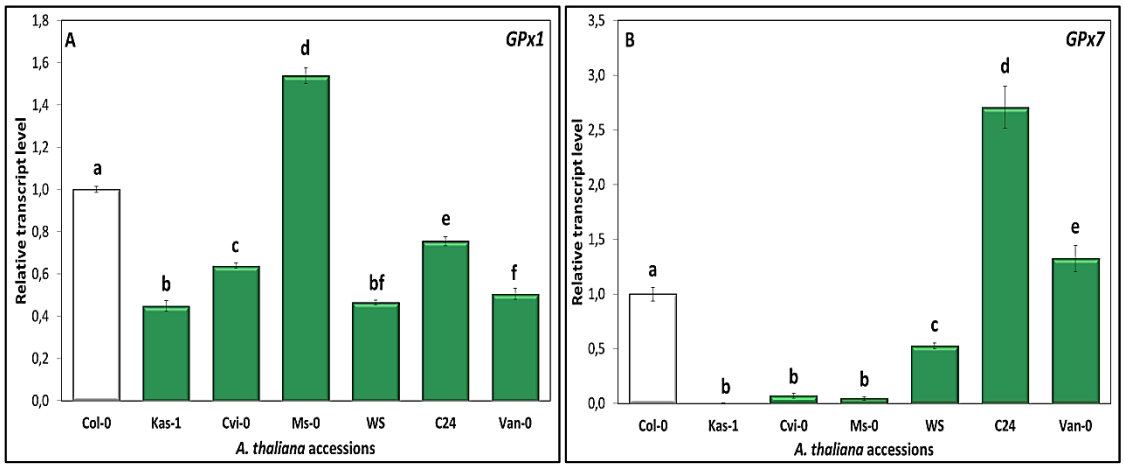

Figure 3-12. Transcript levels of **A.** *GPx1* and **B.** *GPx7* in six accessions of *A. thaliana* relative to Col-0. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are mean ±SD from three independent biological replicates, each analyzed in triplicates.

In comparison with the reference accession (Col-0), twofold lower *GPx1* mRNA abundance was detected for Kas-1 (0.45 ± 0.03 relative to Col-0), WS (0.47 ± 0.01 relative to Col-0) and Van-0 (0.51 ± 0.03 relative to Col-0). On the contrary, the strongest accumulation of the *GPx1* transcript was observed in Ms-0 (1.54 ± 0.04 relative to Col-0) (Figure 3-12).

The steady-state mRNA abundance measured for *GPx7* was lower than that for Col-0 in almost all tested accessions, except C24 (2.70 ± 0.19 relative to Col-0) and Van-0 (1.32 ± 0.11 relative to Col-0). Among them, the strongest reduction in the accumulation of this mRNA was detected for Kas-1 (undetectable level), Cvi-0 (0.07 ± 0.02 relative to Col-0) and Ms-0 (0.04 ± 0.02 relative to Col-0) (Figure 3-12).

2-Cys peroxiredoxins vs. glutathione peroxidases

Among genes encoding the enzymes of chloroplast ascorbate-independent water-water cycle, the strongest differences between selected *A. thaliana* accessions were observed for transcript levels of *2CPs* and *GPxs* (Figures 3-8 and 3-11). Since, enzymes encoded by these genes have in chloroplast of *A. thaliana* overlapping functions, the paralogue variation in their expression regulation at the transcript level was studied (Figure 3-13).

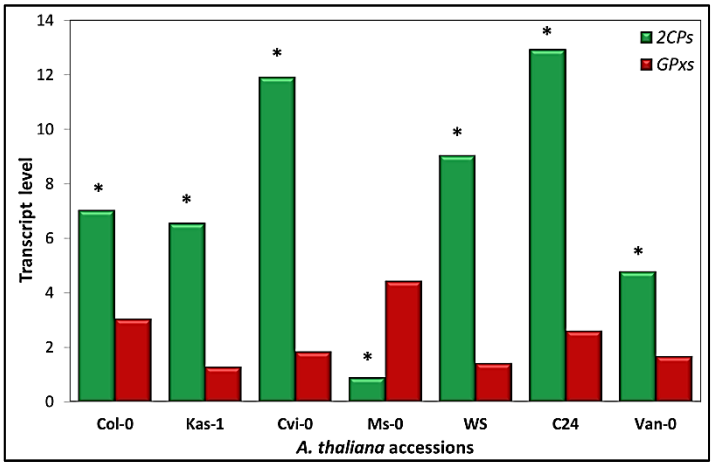

Figure 3-13. Comparison of total *2CPs* (green bars) and *GPxs* (red bars) transcript levels in seven accessions of *A. thaliana*. The accessions, in which *2CPs* are accumulated at significantly different level than *APxs* are marked with asterisks (t-test; $p < 0.01$).

The analyses revealed that *2CPs* in almost all tested accessions (except Ms-0) were accumulated at higher level than *GPxs*. Among them the highest differences between *2CPs* and *GPxs* were detected for Kas-1, Cvi-0, WS and C24, in which the quotient *2CPs* / *GPxs* was higher than five. The remaining accessions, Col-0 and Van-0,

accumulated *2CPs* at approximately threefold higher level than *GPxs*. In contrast, the abundance of *2CPs* mRNAs was lower than that of *GPxs* only in Ms-0. Moreover, among all of the tested accessions, Ms-0 accumulated the lowest level of *2CPs* transcripts, but the highest level of *GPxs.* It indicated that the low expression of *2CPs* at the transcript level might be compensated in this accession by elevated levels of *GPxs* transcripts.

3.1.1.3 Enzymes of the ascorbate-dependent water-water cycle

Besides GPXs and PRXs, two chloroplast ascorbate peroxidases (sAPX and tAPX) are involved in reduction of hydrogen peroxide to water. They use ascorbate as an electron donor and are much more active than thiol peroxidases, GPXs and PRXs (Asada, 1999). *A. thaliana* is one of the species, in which two different isoforms of chloroplast APXs are not generated, as in spinach (Ishikawa *et al.* 1997), pumpkin (Mano *et al.* 1997) and tobacco (Yoshimura *et al.* 2002), from a single gene by alternative splicing, but are encoded by two different nuclear genes (*sAPx* and *tAPx*) of ancestral origin (Jespersen *et al.* 1997; Pitsch *et al.* 2010). So far, the analyses of expression regulation of these genes at the transcript level in *A. thaliana* were performed only in Col-0 and C24 backgrounds (Panchuk *et al.* 2002; Shigeoka *et al.* 2002). Here, the variation in the expression regulation of *sAPx* and *tAPx* at the transcript level will be analyzed among seven accessions of *A. thaliana* (Figure 3-14).

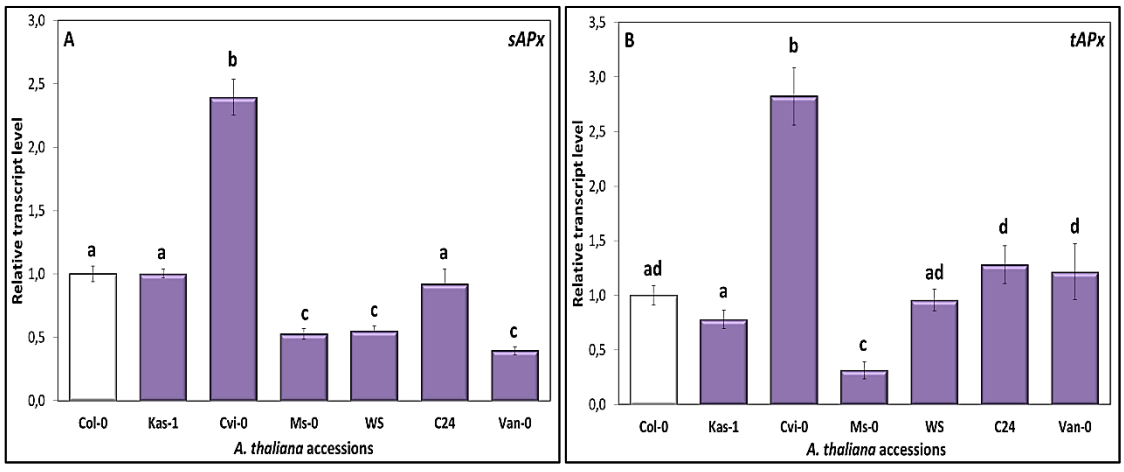

Figure 3-14. Transcript levels of **A.** *sAPx* and **B.** *tAPx* in six accessions of *A. thaliana* relative to Col-0. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are mean ±SD from three independent biological replicates, each analyzed in triplicates.

The analyses revealed that the transcript levels of both, *sAPx* and *tAPx*, were weakly variable among selected accessions of *A. thaliana* (Figure 3-14). The amount of *sAPx* did not significantly differ between Col-0, Kas-1 and C24, as well as between Ms-0, WS and Van-0. Similarly, the transcript level of *tAPx* was not significantly different between Col-0, Kas-1 and WS, as well as between Col-0, WS, C24 and Van-0. Among all tested accessions only Cvi-0 accumulated higher and different than any other accession amount of both, *sAPx* and *tAPx*, transcripts, indicating that the expression of chloroplast *APxs* at the transcript level in this accession is regulated in unique accession-specific manner.

Moreover, the comparison of *sAPx* and *tAPx* transcript levels among selected *A. thaliana* accessions revealed that almost all of them accumulated similar or lower amount of both analyzed transcripts than the reference accession Col-0 (Figure 3-14). The strongest reduction in the transcript accumulation was detected for *sAPx* in Ms-0 (0.53 ± 0.04 relative to Col-0), WS (0.55 ± 0.04 relative to Col-0) and Van-0 (0.40 ± 0.03 relative to Col-0) and for *tAPx* in Ms-0 (0.31 ± 0.08 relative to Col-0). In contrast, higher levels of *sAPx* and *tAPx* transcripts than in Col-0 were measured only for Cvi-0, in which both of these genes were expressed at more than twofold higher level than in Col-0.

Since ascorbate peroxidases are encoded in the genome of *A. thaliana* by two genes, paralogue variation in the expression of *sAPx* and *tAPx* at the transcript level could be studied (Figure 3-15).

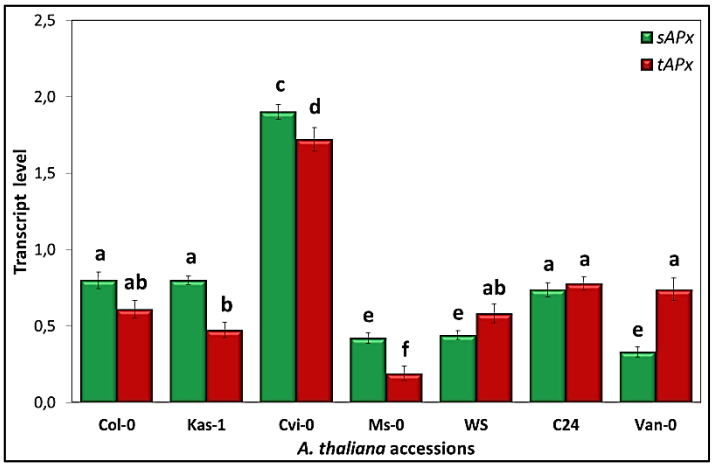

Figure 3-15. Comparison of *sAPx* (green bars) and *tAPx* (red bars) transcript levels in seven accessions of *A. thaliana*. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$).

The analyses revealed that Col-0, Kas-1, Cvi-0 and Ms-0 accumulated significantly higher amounts of *sAPx* mRNA than that of *tAPx*, while in WS and Van-0 higher levels of *tAPx* mRNA than that of *sAPx* were detected. Since, Cvi-0 origins from hot, Kas-1 from cold and the remaining accessions from intermediate continental climates, it indicates that the paralogue variation in the expression of *APxs* is not regulated in habitat-dependent manner.

3.1.1.4 Ascorbate-independent vs. ascorbate-dependent water-water cycle

Baier *et al.* (2000) showed that in transgenic *A. thaliana* plants with reduced expression of 2-Cys peroxiredoxins the transcripts encoding APXs were accumulated at high levels. Later, experiments performed by Kangasjarvi *et al.* (2008) revealed that the deficiency of tAPX and sAPX in plants exposed to high-light stress was also partially compensated by 2-Cys peroxiredoxins. It suggests that APXs and 2CPs are redundant and when one of them is absent the second one is accumulated in higher amounts to take the function of the missing one.

To check, whether such compensatory mechanisms exist also in selected accessions of *A. thaliana* in all of them the total transcript levels of *APxs* (*sAPx* + *tAPx*) were compared with total transcript levels of *2CPs* (*2CPA* + *2CPB*) (Figure 3-16).

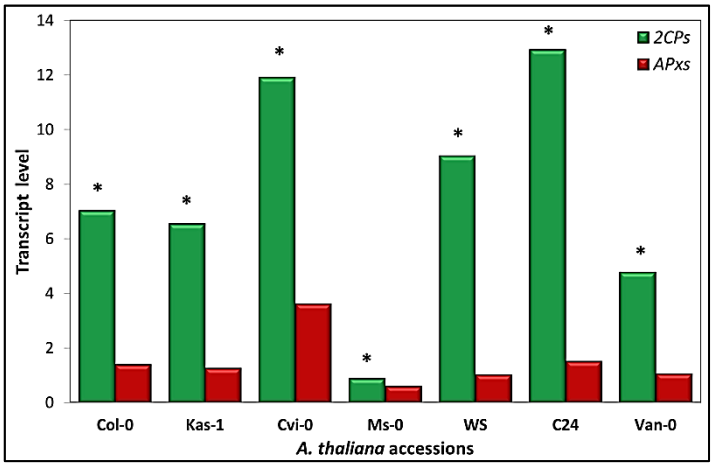

Figure 3-16. Comparison of *2CPs* (green bars) and *APxs* (red bars) transcript levels in seven accessions of *A. thaliana*. The accessions, in which *2CPs* are accumulated at significantly different level than *APxs* are marked with asterisks (t-test; $p < 0.01$).

Among all of the tested accessions, the lowest transcript levels of *2CPs* were detected in Ms-0 (Figure 3-16). However, low amounts of these transcripts were not compensated

by up-regulation of *APxs* expression. Similar situation was observed in Van-0, in which lower levels of *2CPs* and *APxs* mRNAs than Col-0 were also accumulated. In contrast, the transcript levels of both, *2CPs* and *APxs*, in Cvi-0 were significantly higher than in Col-0. It indicated that the expression of both analyzed genes at the transcript level in almost all selected accessions is regulated in parallel (both genes were up-regulated or down-regulated). Only in WS lower accumulation of mRNAs encoding *APxs* led to higher expression of *2CPs*. It showed that the compensation mechanisms described by Baier *et al.* (2000) and Kangasjarvi *et al.* (2008) for Col-0 among tested accessions of *A. thaliana* is conserved only in WS.

3.1.1.5 Enzymes of the ascorbate-recycling system

Monodehydroascorbate (MDA) produced in the reaction catalyzed by APXs is either rapidly reduced to ascorbate (AsA) by the action of monodehydroascorbate reductase (MDHAR) or spontaneously converted to AsA and dehydroascorbate (DHA). From DHA ascorbate is regenerated at the expense of glutathione (GSH) by dehydroascorbate reductase (DHAR). The reaction leads to the oxidation of GSH to GSSG, which is then reduced to GSH by glutathione reductase (GR) (Asada, 1999; Foyer and Shigeoka, 2011).

MDHAR, DHAR and GR are postulated to be the key players in maintaining ascorbate homeostasis in chloroplasts. Such control is of great importance, because under low concentration of AsA, the chloroplast ascorbate peroxidases are rapidly inactivated in the presence of H_2O_2 (Ishikawa and Shigeoka, 2008).

So far, natural variation in the expression regulation of genes encoding reductases of ascorbate-recycling system has been studied only in *A. thaliana* var. Col-0 and Cvi-0 (Zhu et al. 2011b). Here, transcript levels of *MDHAR*, *DHAR* and *GR* will be compared between seven accessions originating from various habitats. (Figure 3-17).

Among analyzed enzymes, the strongest variation was revealed for *MDHAR* transcript, which level significantly differed between almost all analyzed accessions, except WS and C24 (Figure 3-17). Despite the presence of SNPs in the sequence recognized by used for amplification primers, the strongest *MDHAR* transcript accumulation among all of the analyzed accessions was observed for Cvi-0 (2.20 ± 0.09 relative to Col-0) and Ms-0 (1.81 ± 0.08 relative to Col-0). In contrast, the amounts of *MDHAR* transcripts in

WS (0.28 ± 0.01 relative to Col-0) and C24 (0.28 ± 0.01 relative to Col-0) were more than threefold lower than that of Col-0 (Figure 3-17).

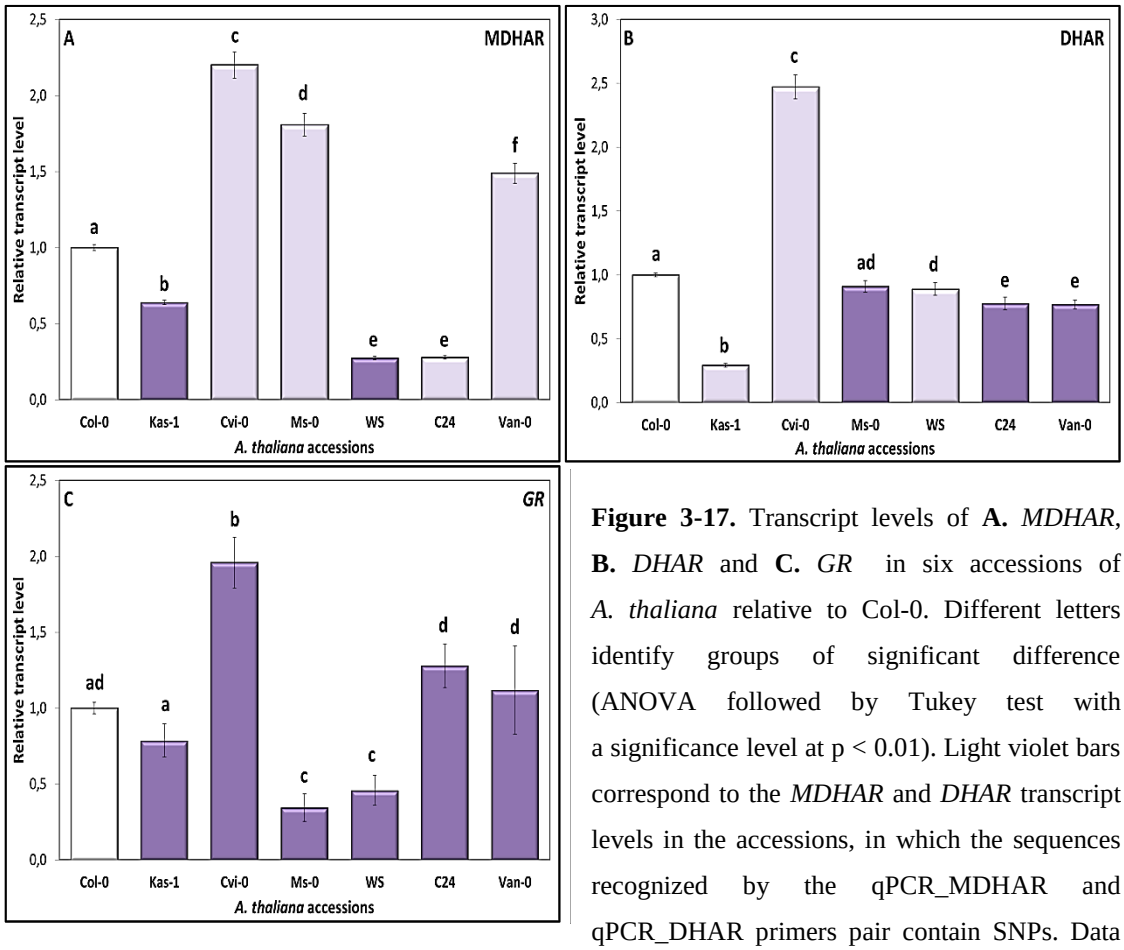

Figure 3-17. Transcript levels of **A.** *MDHAR*, **B.** *DHAR* and **C.** *GR* in six accessions of *A. thaliana* relative to Col-0. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Light violet bars correspond to the *MDHAR* and *DHAR* transcript levels in the accessions, in which the sequences recognized by the qPCR_MDHAR and qPCR_DHAR primers pair contain SNPs. Data are means ±SD from three independent biological replicates, each analyzed in triplicates.

Lower expression amplitude, than for *MDHAR*, was observed for *DHAR* (Figure 3-17). The highest and the lowest level of this transcript was measured for Cvi-0 (2.47 ± 0.09 relative to Col-0) and Kas-1 (0.29 ± 0.01 relative to Col-0), respectively. Since the sequences recognized qPCR_DHAR primers in both, Kas-1 and Cvi-0, contain the same SNPs, it is concluded that the low level of this transcript in Kas-1 is not due to the in-efficient primer binding to the gene of interest (high level of *DHAR* transcript in Cvi-0 containing the same SNPs as Kas-1) but rather due to the accession-specific regulation of *DHAR* expression.

Similar to *DHAR*, weak variation among selected accessions was observed also for transcript encoding glutathione reductase (Figure 3-17). The highest accumulation of

GR mRNA was detected, as for *MDHAR* and *DHAR*, in Cvi-0 (1.96 ± 0.17 relative to Col-0) and the lowest in Ms-0 (0.34 ± 0.09 relative to Col-0).

In general, the analyses of expression regulation of genes encoding reductases of ascorbate-recycling system revealed that in almost all tested accessions (except Cvi-0) the transcript levels of *MDHAR*, *DHAR* and *GR* were hardly correlated. It demonstrates that there is no common pathway controlling the steady-state mRNA levels of ascorbate-recycling enzymes.

3.1.1.6 Accession-specific variation

In the comparison of 6-week-old *A. thaliana* accessions, the strongest variation in the levels of transcripts encoding enzymes of chloroplast antioxidant system was detected for Kas-1 and Cvi-0 (Figure 3-18). In Kas-1 mRNA abundances of seven out of twelve analyzed genes were lower than the transcript levels of the same genes in Col-0. In contrast, Cvi-0 expressed the highest number of genes encoding the enzymes of chloroplast water-water cycle (nine out of twelve analyzed) at higher level than Col-0. The transcript abundances patterns of WS, C24 and Van-0 largely resembled that of Kas-1, in which most of the analyzed mRNAs were accumulated in lower amounts than in Col-0 (Figure 3-18). Ms-0, which origins from Moscow in Russia, showed the most distinct pattern in comparison to Col-0. In this accession, the expression of half of the analyzed genes was up-regulated and half down-regulated (Figure 3-18).

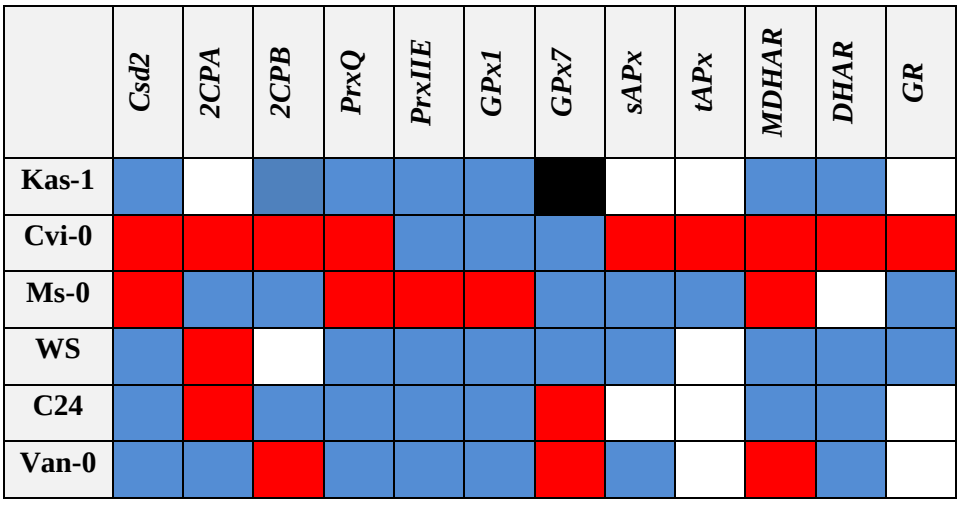

Figure 3-18. Comparison of expression levels of genes encoding enzymes of chloroplast antioxidant system at the transcript levels in six accessions of *A. thaliana* relative to Col-0. With blue, red and white colours are marked down-regulated, up-regulated and expressed at the same level as in Col-0 genes, respectively. The black region corresponds to not-expressed gene.

3.1.2. Regulation at the protein level

Eukaryotic gene expression is a complex process regulated at multiple levels, including transcription (Vandepoele *et al.* 2009; Kaufmann *et al.* 2010), nuclear export (Lipshitz and Smibert, 2000), mRNA decay (Narsai *et al.* 2007), translation (Horiguchi *et al.* 2012), protein targeting (Lam *et al.* 2010) and protein degradation (Vierstra, 1996; Kirschner, 1999; Voges *et al.* 1999). Since, all of these regulatory mechanisms are well coordinated, understanding gene expression requires combining data from different aspects of its regulation (Kawaguchi and Bailey-Serres, 2002). Therefore, the analyses of natural variation in the expression regulation of chloroplast antioxidant system at the transcript level were followed by the studies of such variation in this system at the protein level. In order to perform such analyses, total protein extracts were isolated from the same, as used for extraction of total RNA, rosettes of 6-week-old *A. thaliana* accessions and separated in SDS-PAGE. Following the electrophoresis and transfer of proteins from polyacrylamide gels to nitrocellulose membranes, chloroplast antioxidant enzymes were detected by Western-blotting using specific antibodies (see Table 2-4 for a list).

For detection of CSD2, 2CPs, PrxQ and GR the antibodies raised against heterologously expressed particular protein were used (Baier and Dietz, 1999; Mittova *et al.* 2003; Petersson *et al.* 2006; Abdel-Ghany and Pilon, 2008). In all analyzed accessions they gave a clear, single signal of approximately 22 kDa (CSD2), 29 kDa (2CPs), 16 kDa (PrxQ) or 54 kDa (GR). The anti-2CPs antibodies reacted with both 2CPs, but, due the small differences in the molecular weight between 2CPA (29 kDa) and 2CPB (29.6 kDa), only single signal reflecting the total amount of 2CPs was revealed. In contrast, GPXs and APXs were detected using peptide-specific antibodies (Kangasjarvi *et al.* 2008; Chang *et al.* 2009). Anti-GPXs were raised against a 58-amino acid region between Ser175 and the C-terminal Ala of GPX7, conserved in all tested accessions. The antibodies recognized both GPXs, but, due to the high amino acid and molecular weight identity between GPX1 and GPX7, gave in Western blots only one signal of approximately 20 kDa, reflecting the total amount of chloroplast GPXs (Chang *et al.* 2009). Anti-APXs were raised against a highly conserved region among tAPX, sAPX and cytoplasmic APXs, corresponding to amino acids 204-215 of tAPX (EEGRLPDAGPPS) (Kangasjarvi *et al.* 2008). However, the comparison of amino acids sequences of tAPX among all analyzed accessions revealed that the epitope

recognized by the anti-APXs in tAPX is not conserved in all of them. Thus, in Kas-1 a glycine residue (G) presented in the epitope of Col-0 was replaced by an arginine residue (R) (Figure 3-19). Such replacement of small, aliphatic amino acid to the bigger and basic one might affect the protein folding and, thus the affinity between tAPX and antibodies in this accession.

Figure 3-19. Comparison of amino acids sequences of epitope recognized by anti-APX antibodies in tAPX of Col-0 and Kas-1.

The levels of tested chloroplast antioxidant enzymes were normalized to the level of also nuclear encoded and targeted to chloroplast large subunit of 1,5-bisphosphate carboxylase oxygenase (RuBisCO) detected in the same line due to the background activity of used antibodies (personal communication with Agrisera). Moreover, to ensure that the same amount of protein sample was loaded in each lane and all proteins were transferred from the gel to the membrane with equal efficiency among different lanes, tubulin α detected with anti-TUA was used as a loading control (Park *et al.* 2004; Chen *et al.* 2010).

For each accession, three biological replicates, each representing an independent protein isolation from single, independently grown plant, were analyzed. All analyses were performed in three technical replicates.

3.1.2.1 Copper-zinc superoxide dismutase (CSD2)

Superoxide dismutases (SODs) are metalloenzymes catalyzing dismutation of superoxide radical anion to hydrogen peroxide and molecular oxygen (Beyer *et al.* 1991). By a specific metal ligand required for their activity, they are classified as copper-zinc SOD (Cu/Zn-SOD), iron SOD (FeSOD) and manganese SOD (Mn-SOD) (McCord and Fridovich, 1969; Bowler, 1992). *Arabidopsis thaliana* has three Cu/Zn-SODs (CSDs), one of which (CSD2) resides in chloroplasts, where is attached to the thylakoid membrane (Kliebenstein *et al.* 1998; Asada, 1999).

The analyses of SOD isoenzymes patterns in selected accessions of *A. thaliana* (see chapter 3.1.1.1) revealed that among all of them only Cvi-0 had different than in any

other accession isoform of chloroplast Cu/Zn-SOD (Cu/Zn-SOD3). It is known that this Cvi-0-specific SOD isoenzyme is encoded by *Csd2-2* gene allele which differs from the one in Col-0 (*Csd2*-1) by several SNPs. These SNPs lead to two amino acids changes in CSD2 of Cvi-0 and thus, an increase in the isoelectric point and reduction of its mobility in Native PAGE (Abarca *et al.* 1999; Abarca *et al.* 2001). Moreover, current comparison of selected accessions revealed that CSD2 in Cvi-0 showed a slightly higher mobility in SDS-PAGE gels than CSD2 in other accessions, suggesting a truncated isoform (Figure 3-20).

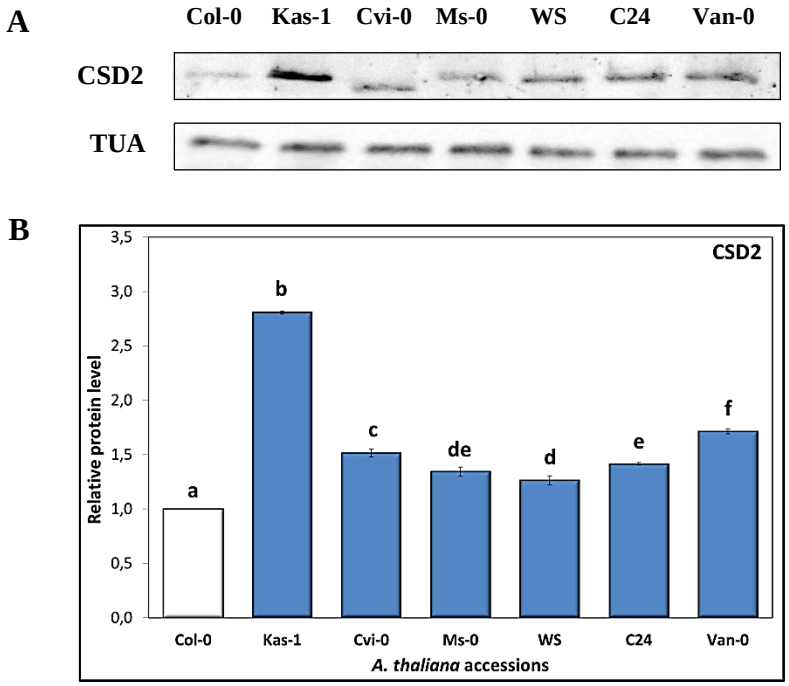

Figure 3-20. Cu/Zn-superoxide dismutase. **A.** Total protein extracts from leaves of seven accessions of *Arabidopsis thaliana* were separated by 12 % SDS-PAGE, transferred to the nitrocelulose membrane and probed with antibodies raised against superoxide dismutase (CSD2). **B.** Level of CSD2 in six accessions of *A. thaliana* relative to Col-0. Tubulin α (TUA) was used as a loading control. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.05$). Data are means ±SD from three independent protein isolations.

The subsequent comparison of CSD2 protein levels in selected accessions of *A. thaliana*, namely Col-0, Kas-1, Cvi-0, Ms-0, WS, C24 and Van-0, revealed significant variation, indicating that *Csd2* expression is regulated in accession-specific manner not only at the transcript, but also at the protein level (Figure 3-20). Moreover, it was shown that all of the tested accessions accumulated more CSD2 than reference accession, Col-0 (Figure 3-20). Among them the highest level of CSD2 was detected in

Kas-1 (2.80 ± 0.02 relative to Col-0) and Cvi-0 (1.51 ± 0.04 relative to Col-0), in which the amount of analyzed protein was almost three- and twofold higher than in Col-0, respectively. Since, the NBT staining of native polyacrylamide gels for activity of CSD2 revealed that this enzyme was more active than in Col-0 only in Ms-0 and WS (Figure 3-6), it might indicate that in remaining accessions (Kas-1, Cvi-0, C24 and Van-0) CSD2 is accumulated mainly in inactive state.

3.1.2.2 Enzymes of the ascorbate-independent water-water cycle

In chloroplasts of *A. thaliana* four peroxiredoxins (PRXs) and two glutathione peroxidases (GPXs) reduce hydrogen peroxide to water in ascorbate independent manner (Dietz *et al.* 2002; Chang *et al.* 2009). Among them, 2-Cys peroxiredoxins (2CPA, 2CPB) switch between stromal soluble dimeric and thylakoid-attached isoforms (König *et al.* 2003), atypical peroxiredoxin Q (PrxQ) is localized in the thylakoid lumen (Petersson *et al.* 2006), type II peroxiredoxin (PrxIIE) in the chloroplast stroma (Zybailov *et al.* 2008), GPX1 can reside on the thylakoid membrane (Ferro *et al.* 2003; Peltier *et al.* 2004) or in stroma and the localization of GPX7 is unclear (Zybailov *et al.* 2008). So far, all Prxs and GPXs have been characterized and studied only in barley and *A. thaliana* var. Col-0 (Baier and Dietz, 1996; Baier and Dietz, 1997; Baier and Dietz, 1999; Baier *et al.* 2000; Petersson *et al.* 2006). Here, the natural variation in the protein levels of 2CPA, 2CPB, PrxQ, GPX1 and GPX7 was analyzed in seven accessions of *A. thaliana* using western blotting and polyclonal antibodies (Figure 3-21).

Among all analyzed enzymes of ascorbate-independent water-water cycle the weakest and the strongest variations in the protein levels were observed for 2CPs and GPXs, respectively (Figure 3-21), demonstrating that 2CPs levels are very similar and that of GPXs very variable in various accessions of *A. thaliana*. The level of 2CPs was slightly lower than in Col-0 only in WS (0.88 ± 0.02 relative to Col-0) and C24 (0.91 ± 0.06 relative to Col-0), while in Kas-1, Cvi-0, Ms-0 and Van-0 did not significantly differ from that of Col-0. In contrast, GPXs were accumulated in higher amount than in Col-0 in almost all tested accessions, except Kas-1.

The protein level of atypical peroxiredoxin Q (PrxQ) was less variable than the level of GPXs but more than that of 2CPs (Figure 3-21). The protein was accumulated in higher than in Col-0 and different than in any other tested accessions amount only in Kas-1 (1.75 ± 0.04 relative to Col-0) and Ms-0 (2.13 ± 0.04 relative to Col-0). Among

remaining accessions, the level of PrxQ was similar to Col-0 in WS and C24 and slightly lower than in Col-0 in Cvi-0 (0.90 ± 0.05 relative to Col-0) and Van-0 (0.86 ± 0.03 relative to Col-0).

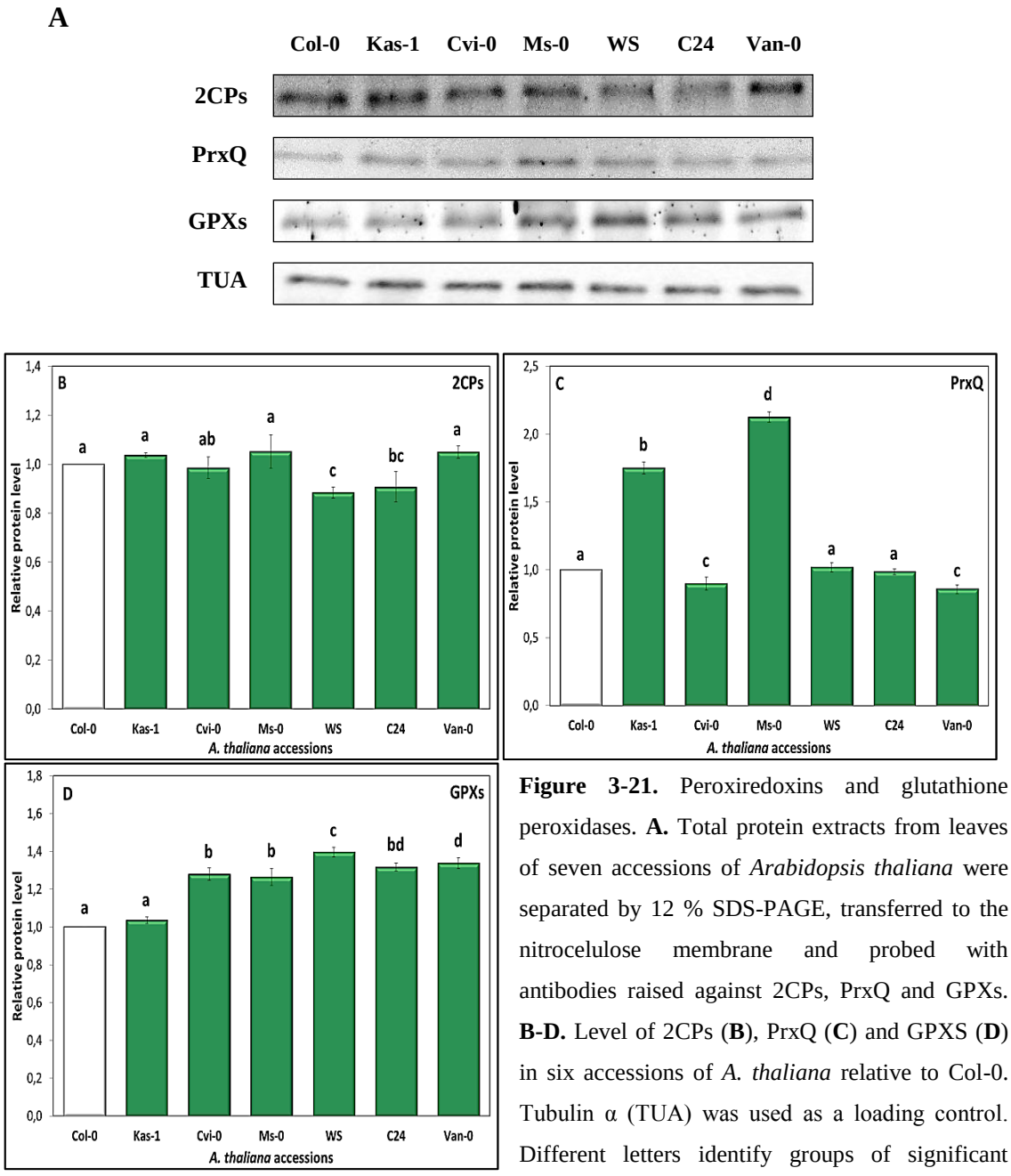

Figure 3-21. Peroxiredoxins and glutathione peroxidases. **A.** Total protein extracts from leaves of seven accessions of *Arabidopsis thaliana* were separated by 12 % SDS-PAGE, transferred to the nitrocelulose membrane and probed with antibodies raised against 2CPs, PrxQ and GPXs. **B-D.** Level of 2CPs (**B**), PrxQ (**C**) and GPXS (**D**) in six accessions of *A. thaliana* relative to Col-0. Tubulin α (TUA) was used as a loading control. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.05$). Data are means ±SD from three independent protein isolations.

3.1.2.3 Enzymes of the ascorbate-dependent water-water cycle

Ascorbate peroxidases (APXs) are haem-binding enzymes reducing hydrogen peroxide to water in ascorbate-dependent manner (Asada, 1999). *Arabidopsis thaliana* has several APX isoenzymes localized in different cellular compartments, two of which reside in chloroplasts (Jespersen *et al.* 1997; Jimenez *et al.* 1997). A 38 kDa tAPX is anchored by hydrophobic domain to the thylakoid membrane, while a 33 kDa sAPX is found in the stroma (Jespersen *et al.* 1997; Asada, 1999). So far, natural variation in the expression regulation of genes encoding chloroplast APXs at the protein level has been studied in *A. thaliana* only in Col-0 and C24 backgrounds (Panchuk *et al.* 2002; Kangasjarvi *et al.* 2008; Maruta *et al.* 2010). Here, sAPX and tAPX protein levels were compared between seven accessions of *A. thaliana* (Figure 3-22).

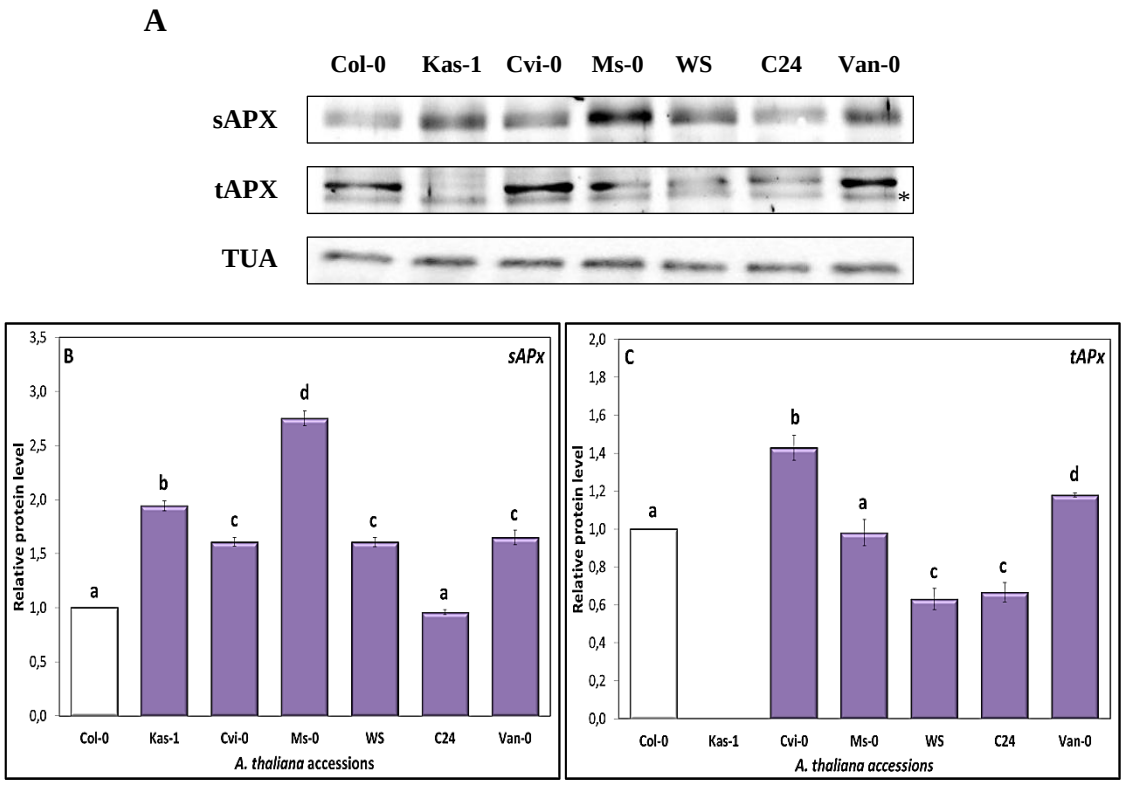

Figure 3-22. Ascorbate peroxidases. **A.** Total protein extracts from leaves of seven accessions of *Arabidopsis thaliana* were separated by 12 % SDS-PAGE, transferred to the nitrocelulose membrane and probed with antibodies raised against stromal (sAPX) and thylakoid-bound (tAPX, * corresponds to unspecific band) ascorbate peroxidases. **B-C.** Level of sAPX (**B**) and tAPX (**C**) in six accessions of *A. thaliana* relative to Col-0. Tubulin α (TUA) was used as a loading control. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.05$). Data are means ±SD from three independent protein isolations.

The comparison revealed that all analyzed accessions significantly differed in the protein levels of both chloroplast APXs (Figure 3-22), indicating that the expression of genes encoding these enzymes is regulated in the accession-specific manner not only at the transcript level, but also at the protein level. Among these accessions, the strongest accumulation of sAPX and tAPX was detected in Ms-0 and Cvi-0, respectively. In contrast, the level of sAPX was the lowest in Col-0 and C24, while that of tAPX in WS and C24.

Moreover, the analyses of APXs levels among selected *A. thaliana* accessions, revealed that almost all of them (except C24) accumulated higher amounts of sAPX than the reference accession, Col-0 (Figure 3-22). In contrast, the level of tAPx was higher than in Col-0 only in Cvi-0 (1.43 ± 0.07 relative to Col-0) and Van-0 (1.18 ± 0.01 relative to Col-0) and lower in WS (0.63 ± 0.06 relative to Col-0) and C24 (0.67 ± 0.05 relative to Col-0).

3.1.2.4 Glutathione reductase – the enzyme of ascorbate-recycling system

Glutathione reductase (GR) is a 54 kDa enzyme localized in the chloroplast stroma responsible for regeneration of GSH from its oxidized form (GSSG) (Asada, 1999). So far, GR has been characterized and studied only in *A. thaliana* var. Col-0 and Cvi-0 (Zhu *et al.* 2011b). Here, its level was analyzed and compared between seven *A. thaliana* accessions (Figure 3-23).

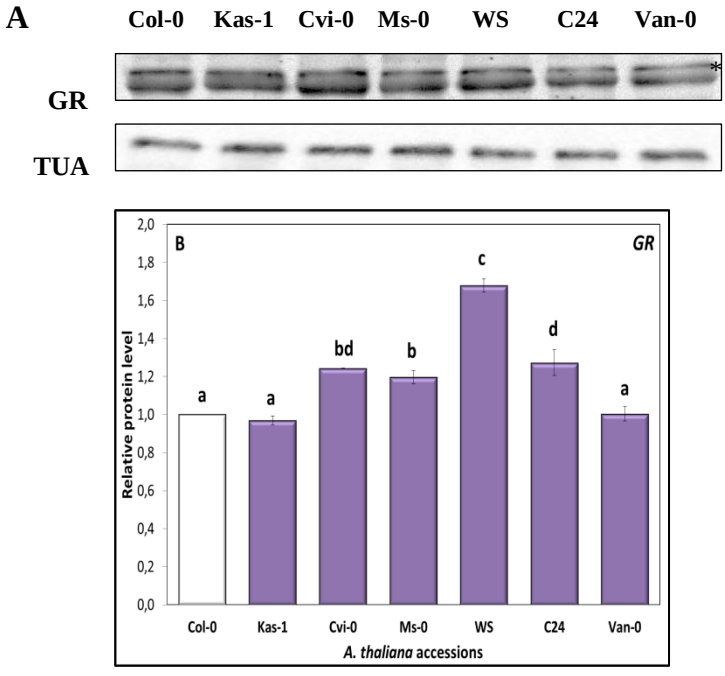

Figure 3-23. Glutathione reductase. **A.** Total protein extracts from leaves of seven accessions of *A. thaliana* were separated by 12 % SDS-PAGE, transferred to the nitrocelulose membrane and probed with antibodies raised against GR. **B.** Level of GR (* corresponds to unspecific band) in six accessions of *A. thaliana* relative to Col-0. Tubulin α (TUA) was used as a loading control. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.05$). Data are means ±SD from three independent protein isolation.

The comparison of GR levels between selected *A. thaliana* accessions revelaed that most of them accumulated significantly different amounts of this protein (Figure 3-23). Among all of them, the highest GR level was detected in WS and the lowest in Col-0, Kas-1 and Van-0. The remaining acessions, Cvi-0, Ms-0 and C24, accumulated approximately 25 % more GR than reference accession (Col-0) and 40 % less than WS. It suggests that the glutathione metabolism in chloroplasts might be different in all analyzed accessions.

3.1.2.5 Accession-specific variation

Among all tested *A. thaliana* accessions the strongest variation in expression regulation of chloroplast antioxidant enzymes at the protein level were observed for Kas-1, Cvi-0 and Ms-0, in which five out of seven analyzed enzymes were accumulated at higher level than in Col-0 (Figure 3-24). On the contrary, the least changes were observed for C24. In this accession the level of sAPX and all analyzed peroxiredoxins (2CPs and PrxQ) were not significantly different than the levels of these proteins in Col-0 (Figure 3-24). However, most of these observed at the protein level variation was not consistent with variation detected among analyzed accessions at the transcript level (Figure 3-24), suggesting that also post-transcriptional level or the protein stability are regulated in accession-specific manner.

	CSD2		2CPs		PrxQ		GPXs		sAPX		tAPX		GR	
	TRANSCRIPT	PROTEIN	TRANSCRIPT	PROTEIN	TRANSCRIPT	PROTEIN	TRANSCRIPT	PROTEIN	TRANSCRIPT	PROTEIN	TRANSCRIPT	PROTEIN	TRANSCRIPT	PROTEIN
Kas-1												?		
Cvi-0														
Ms-0														
WS														
C24														
Van-0														

Figure 3-24. Comparison of expression regulation of chloroplast antioxidant system at the transcript and protein levels in six accessions of *A. thaliana* relative to Col-0. With blue, red and white colours are marked down-regulated, up-regulated and expressed at the same level as in Col-0 genes, respectively.

3.1.3 Regulation of the translation initiation

Previous analyses revealed that the patterns of variation in the expression regulation of genes encoding chloroplast antioxidant enzymes among *A. thaliana* accessions significantly differed at the transcript and the protein levels. Such discrepancies between these two patterns might result from post-transcriptional regulation of gene expression, including processing, export, localization, turnover and translation of mRNAs, as well from protein turnover (Pradet-Balade *et al.* 2001; Mata *et al.* 2005). Among them, the efficiency of mRNA binding to ribosomes at the level of translation initiation was often shown to be responsible for the lack of correlation between mRNA and protein levels in various plant species (Berry *et al.* 1988; Crosby and Vayda, 1991; Kahlau and Bock, 2008). To check whether the discrepancies between the amounts of transcripts and proteins encoding chloroplast antioxidant enzymes in tested *A. thaliana* accessions also resulted from differential regulation of translation initiation, all of these accessions were tested for the effectiveness of transcript usage for protein synthesis.

For the analyses, polysomal RNA (pRNA) was isolated from the same plant material as that used for extraction of total RNA and proteins. To describe the efficiency of translation initiation, the levels of mRNA bound to the ribosomes determined in pRNA samples by qRT-PCR were divided by total mRNA levels measured for the same gene and the same accession in total RNA samples (Hesling *et al.* 2007; Piechota *et al.* 2010). Calculated in this way pRNA / total RNA quotients determine the efficiency of mRNA binding to ribosomes normalized to the steady-state mRNA levels, but do not give information about the actively translated portion of total RNA (total RNA and pRNA were isolated separately). The quotients calculated for all analyzed genes in particular accessions were standardized on the pRNA / total RNA level detected for the same gene in the reference accession, Col-0.

For each accession, two biological replicates, each representing an independent RNA isolation from single, independently grown plant, were analyzed. All analyses were performed in three technical replicates.

3.1.3.1 Copper-zinc superoxide dismutase (Csd2)

When the efficiency of *Csd2* mRNA binding to ribosomes was compared between selected accessions of *A. thaliana*, strong differences between all of them were observed

(Figure 3-25), indicating that the amount of *Csd2* mRNA used for translation is regulated in accession-specific manner.

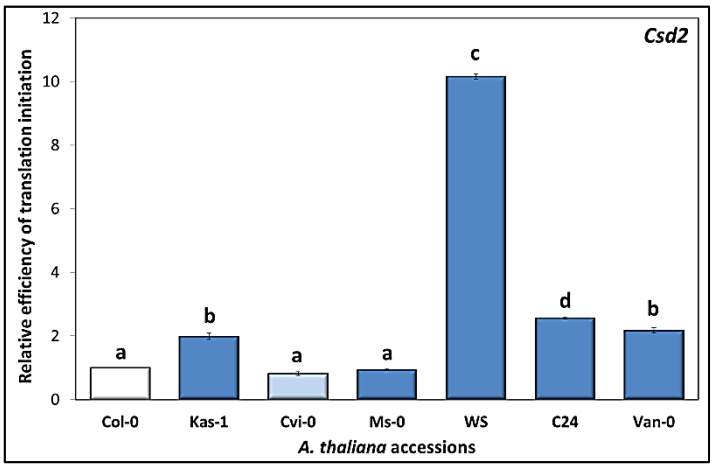

Figure 3-25. Efficiency of *Csd2* translation initiation in six accessions of *A. thaliana* relative to Col-0. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Light blue bar corresponds to the *Csd2* transcript level in the accession, in which the sequences recognized by the qPCR_Csd2_1 primers pair contain SNPs. Data are means ±SD from two independent RNA isolation, each representing three technical replicates.

Among all of the tested accessions, the strongest use of *Csd2* mRNA in translation process was shown for WS (10.15 ± 0.08 relative to Col-0), in which *Csd2* transcript was bound to ribosomes with tenfold higher efficiency than in Col-0, Cvi-0 and Ms-0 (the accessions with the least effective translation initiation of *Csd2*). In the remaining accessions (Kas-1, C24 and Van-0) the efficiency of *Csd2* mRNA binding to ribosomes was approximately fivefold lower than in WS and twofold higher than in the reference accession, Col-0 (Figure 3-25).

3.1.3.2 Enzymes of the ascorbate-independent water-water cycle

Similar analyses of translation efficiency, as for *Csd2*, were also performed for genes encoding chloroplast peroxiredoxins (*2CPA*, *2CPB*, *PrxQ* and *PrxIIE*) and glutathione peroxidases (*GPx1* and *GPx7*), both reducing hydrogen peroxide in ascorbate-independent manner.

Peroxiredoxins (*2CPA*, *2CPB*, *PrxQ* and *PrxIIE*)

The analyses of translation initiation performed for genes encoding peroxiredoxins, namely *2CPA*, *2CPB*, *PrxQ* and *PrxIIE*, revealed that all of them were translated with variable efficiencies in all tested *A. thaliana* accessions (Figure 3-26). Among them, the strongest use of mRNA in the translation process was shown in almost all accessions (except Kas-1 and Ms-0) for *PrxIIE* (Figure 3-26). Since, *PrxIIE* transcript was accumulated in all of these accessions at the lowest level among analyzed peroxiredoxins (Figure 3-8), it might indicate that the expression of this gene is regulated mainly at the post-transcriptional level. In contrast, the lowest translation efficiency was shown in almost all accessions (except Ms-0 and Van-0) for *2CPA* (Figure 3-26), which mRNA level was the highest among analyzed peroxiredoxins (Figure 3-8). It indicates that the expression of *2CPA* is controlled transcriptionally.

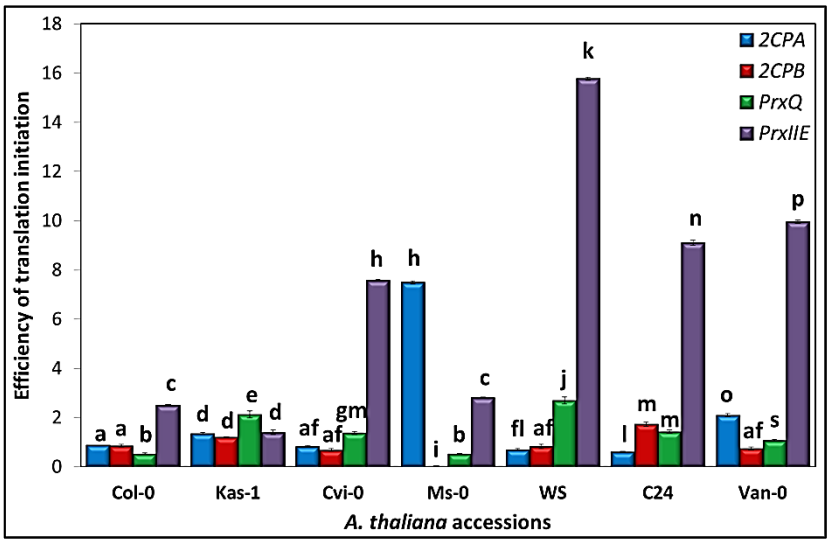

Figure 3-26. Comparison of *2CPA* (blue bars), *2CPB* (red bars), *PrxQ* (green bars) and *PrxIIE* (violet bars) translation efficiency in seven accessions of *A. thaliana*. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are mean ±SD from two independent biological replicates, each analyzed in triplicates.

Since two out of the four analyzed peroxiredoxins, namely 2CPA and 2CPB, belong to the same family of 2-Cys peroxiredoxins (2CPs), the paralogue variation in their translation efficiency (*2CPA* vs. *2CPB*) could be analyzed (Figure 3-26). The analyses revealed that in most of the tested accessions *2CPA* and *2CPB* mRNAs were used in the translation process with low and similar efficiencies (Figure 3-26), indicating that the expression of these genes is regulated mainly at the level of transcription, at which

independent control of *2CPA* and *2CPB* accumulation was shown (Figure 3-8). Among remaining accessions, *2CPA* (0.60 ± 0.02 relative to Col-0) was bound to the ribosomes with lower efficiency than that of *2CPB* (1.72 ± 0.01 relative to Col-0) only in C24, showing stronger support of *2CPB* translation (Figure 3-26). In contrast, in Ms-0 and Van-0 stronger use of mRNA in translation process was detected for *2CPA* than for *2CPB* (Figure 3-26). Moreover, *2CPB* (0.04 ± 0.01 relative to Col-0) in Ms-0 was translated with the lowest efficiency among all analyzed accessions and genes. However, the low efficiency of *2CPB* binding to ribosomes in this accession was accompanied by very strong support of *2CPA* translation (8.63 ± 0.03 relative to Col-0), indicating a compensatory regulation in the *2CPs* expression.

High variability among selected *A. thaliana* accessions was shown also for the translation efficiency of the last from analyzed peroxiredoxins, PrxQ (Figure 3-26). In general, the use of *PrxQ* mRNA in all accessions was stronger than in the reference accession (Col-0), with the highest efficiency of this process detected for Kas-1 (2.13 ± 0.14 relative to Col-0) and WS (2.69 ± 0.15 relative to Col-0).

Glutathione peroxidases (*GPx1* and *GPx7*)

The comparison of the translation initiation efficiency of genes encoding glutathione peroxidases (*GPx1* and *GPx7*) revealed that in almost all selected accessions ribosomes bound significantly more *GPx1* transcript than that of *GPx7* (Figure 3-27). Only in Ms-0, higher efficiency of translation was observed for *GPx7* (21.37 ± 0.05 relative to Col-0) than for *GPx1* (1.11 ± 0.09 relative to Col-0) (Figure 3-27). Since *GPx1* mRNA was accumulated in Ms-0 at the highest level among all tested accessions and that of *GPx7* at the lowest (Figure 3-11), it indicated that the expression of *GPxs* (*GPx1* vs. *GPx7)* is inversely regulated at both, transcription and translation levels. Moreover, low transcription efficiency of highly accumulated *GPx1* transcript and strong use in the translation process of low abundant *GPx7* mRNA suggested that the expression of *GPx1* is controlled mainly transcriptionally, whereas that of *GPx7* post-transcriptionally.

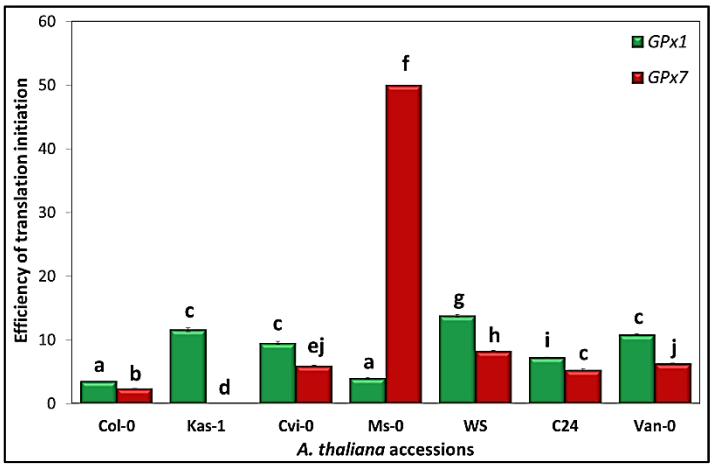

Figure 3-27. Comparison of *GPx1* (green bars) and *GPx7* (red bars) translation efficiencies in seven accessions of *A. thaliana*. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are mean ± SD from three independent biological replicates, each analyzed in triplicates.

3.1.3.3 Enzymes of ascorbate-dependent water-water cycle

The analyses of translation efficiency performed for genes encoding chloroplast ascorbate peroxidases *(sAPx* and *tAPx*) indicated that in almost all tested accessions (except Kas-1 and Ms-0) lower amount of *tAPx* than *sAPx* was bound to the ribosomes (Figure 3-28). Since in most of the accessions (except WS and Van-0) also less mRNA encoding *tAPx* than *sAPx* was accumulated, it indicated stronger support of *sAPx* than *tAPx* expression at both analyzed levels, transcription and translation initiation.

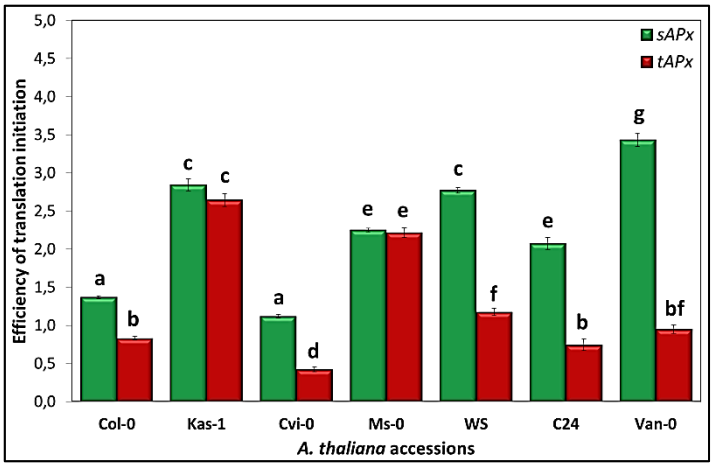

Figure 3-28. Comparison of *sAPx* (green bars) and *tAPx* (red bars) translation efficiencies in seven accessions of *A. thaliana*. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$).

Moreover, the analyses revealed that all analyzed accessions significantly differed in the efficiency of translation initiation of both, *sAPx* and *tAPx* (Figure 3-28). Among them, the highest use of mRNA in translation process was shown for *sAPx* in Van-0 (2.51 ± 0.16 relative to Col-0) and for *tAPx* in Kas-1 (3.19 ± 0.01 relative to Col-0) and Cvi-0 (2.68 ± 0.16 relative to Col-0). On the contrary, the lowest translation efficiency was detected for *tAPx* in Cvi-0 (0.51 ± 0.02 relative to Col-0).

3.1.3.4 Ascorbate-independent vs. ascorbate-dependent water-water cycle

Strong differences in the accumulation of *2CPs* and *APxs* transcripts observed in almost all (except Ms-0) tested accessions (Figure 3-16), prompted for comparison of the efficiencies of their translation (Figure 3-29).

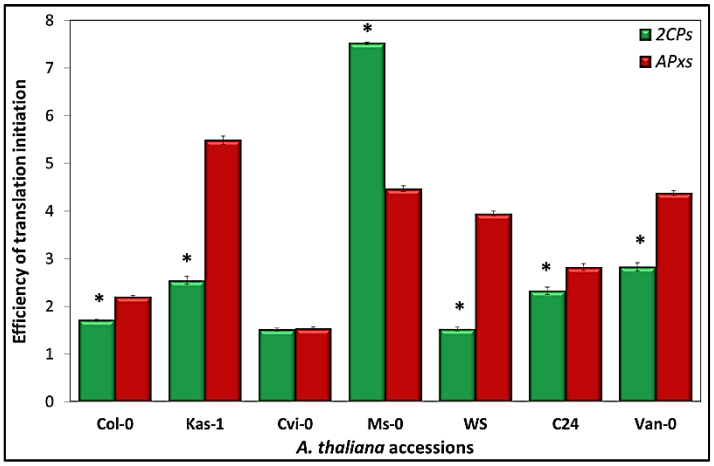

Figure 3-29. Comparison of *2CPs* (green bars) and *APxs* (red bars) translation efficiency in seven accessions of *A. thaliana*. The accessions, in which *2CPs* are accumulated at significantly different level than *APxs* are marked with asterisks (t-test; $p < 0.01$).

The analyses revealed that Col-0, Kas-1, WS, C24 and Van-0 stronger supported translation of accumulated at lower levels *APxs* mRNAs than that of highly abundant *2CPs* (Figure 3-16 and 3-29), indicating compensation of low transcription activity at the post-transcriptional level and inverse regulation of *2CPs* and *APxs* expression at these levels. Similarly, low transcriptional activity of *2CPs* and *APxs* in Ms-0 was also compensated at the translation level by enhancement of mRNA binding to the ribosomes.

3.1.3.5 Enzymes of the ascorbate-recycling system

The comparison of translation initiation efficiency of genes encoding reductases of chloroplast ascorbate-recycling system (*MDHAR*, *DHAR* and *GR*) revealed the strongest variations among tested accessions for *GR* (Figure 3-30). As for ascorbate peroxidases, similar or higher amount of *GR* transcript was bound to ribosomes in almost all tested accessions than in the reference accession, Col-0. Moreover, for some of the selected *A. thaliana* accessions positive correlation between translation efficiency of ascorbate peroxidases and enzymes of ascorbate-recycling system was observed. The strongest correlation was detected for Kas-1 and Cvi-0, in which binding of *APxs* and ascorbate-recycling reductases (*MDHAR*, *DHAR* and *GR*) to ribosomes was regulated in the same direction (e.g. high binding efficiency for all, *APxs*, *MDHAR*, *DHAR* and *GR*).
However, as at the transcript level (Figure 3-17) the translation efficiencies of *MDHAR*, *DHAR* and *GR* were also hardly correlated, indicating that at both, transcription and translation initiation, levels there is no common pathway controlling *MDHAR*, *DHAR* and *GR* mRNA binding to ribosomes.

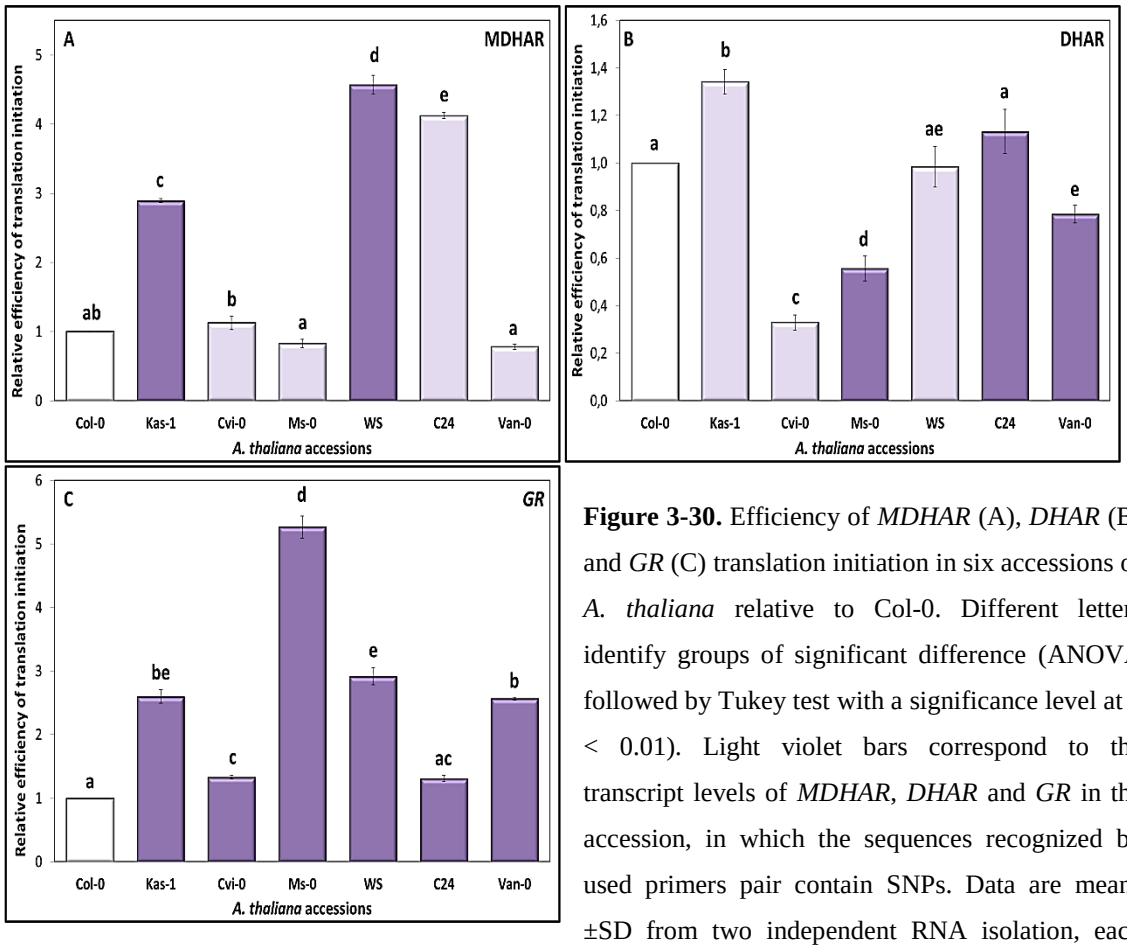

Figure 3-30. Efficiency of *MDHAR* (A), *DHAR* (B) and *GR* (C) translation initiation in six accessions of *A. thaliana* relative to Col-0. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Light violet bars correspond to the transcript levels of *MDHAR*, *DHAR* and *GR* in the accession, in which the sequences recognized by used primers pair contain SNPs. Data are means ±SD from two independent RNA isolation, each representing three technical replicates.

3.1.3.6 Accession-specific variation

Among all selected *A. thaliana* accessions, the highest translation efficiency of genes encoding chloroplast antioxidant enzymes was shown for Kas-1, WS, C24 and Van-0, in which eight to nine out of twelve analyzed transcripts were overrepresented in pRNA. On the contrary, Cvi-0 and Ms-0 showed the lowest efficiency of mRNA binding to ribosomes with only five out of twelve transcripts overrepresented in polysomal RNA fraction (Figure 3-31).

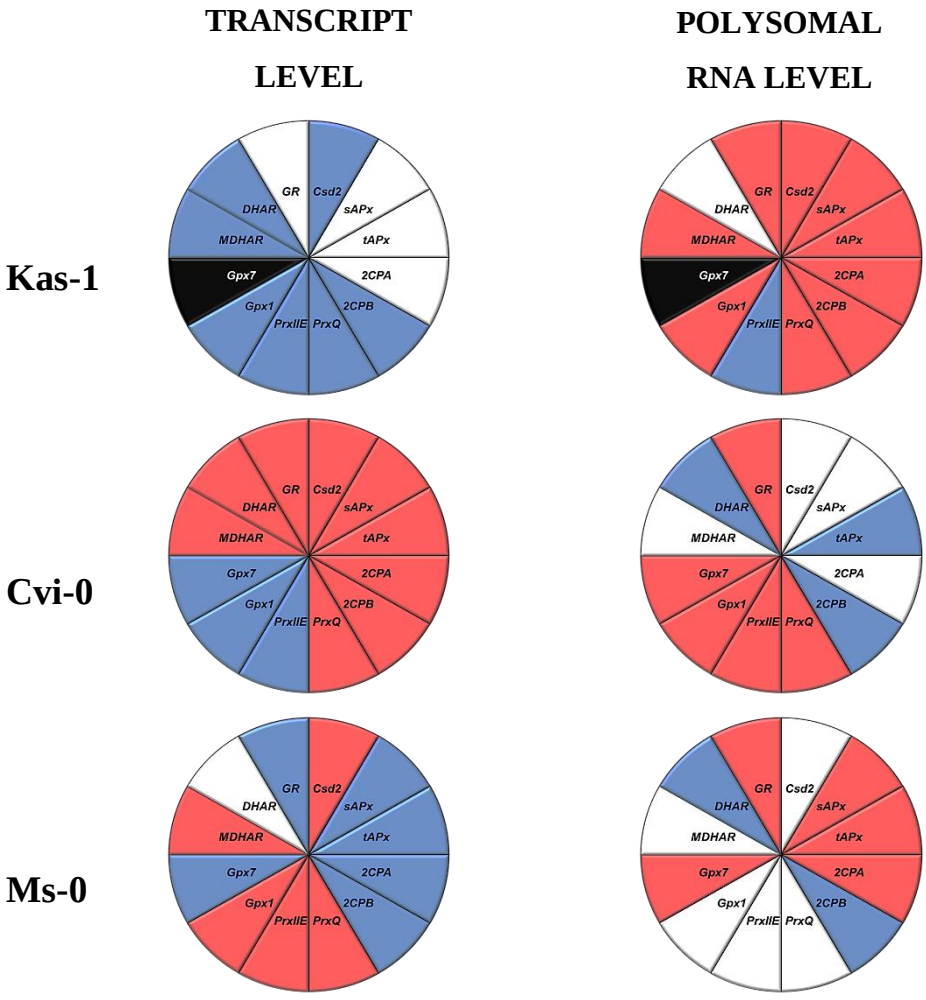

Figure 3-31. Comparison of expression level of genes encoding chloroplast antioxidant enzymes at the transcript and polysomal RNA levels in six accessions of *A. thaliana* relative to Col-0. Blue, red and white regions of the pie charts represent down-regulated, up-regulated and expressed at the same level as in Col-0 genes, respectively. The black region responds to not-expressed gene.

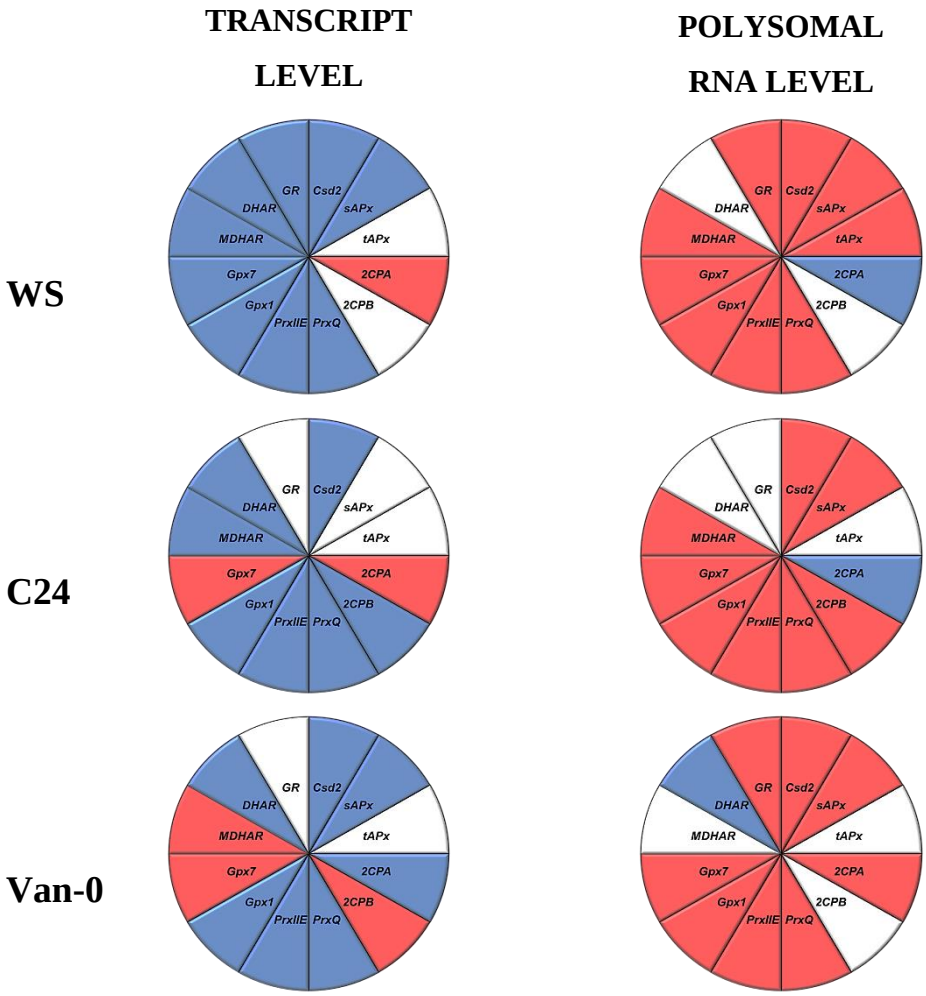

Figure 3-31 (continued). Comparison of expression level of genes encoding chloroplast antioxidant enzymes at the transcript and polysomal RNA levels in six accessions of *A. thaliana* relative to Col-0. Blue, red and white regions of the pie charts represent down-regulated, up-regulated and expressed at the same level as in Col-0 genes, respectively. The black region responds to not-expressed gene.

Moreover, the comparison of total and polysomal data depicted in Figure 3-31 indicated that in almost all tested *A. thaliana* accessions the reduction in transcriptional activity is compensated at the translation level by enhancing the efficiency of mRNA binding to ribosomes. The exceptions from this compensatory regulation are *PrxIIE* in Kas-1, *2CPB* in Ms-0 and *DHAR* in Van-0, for which in comparison with Col-0 lower level of transcripts and lower efficiency of translation initiation were observed.

On the contrary, in most of the tested accessions high transcriptional activity was accompanied by reduction in the efficiency of mRNA binding to ribosomes. However, there are also some exceptions from this regulatory mechanism, namely *PrxQ* and *GR*

in Cvi-0, as well as *GPx7* in C24 and Van-0, for which high activity was observed at both, transcription and translation levels.

It is known that the differences in the activity of translation initiation might be a result of changes in the structural properties of the untranslated regions of analyzed genes (UTRs), especially in the 5'UTRs, which are known to have a major role in the control of mRNA translation (Mignone *et al.* 2002). Examples of such structural properties are length (Gallie *et al.* 2000), presence of secondary structures (Klaff *et al.* 1996; Gallie *et al.* 2000) or upstream open reading frames (Lukaszewicz *et al.* 1998), as well as the composition of the sequence that surrounds the translation initiation codon (Geballe and Morris, 1994; Joshi *et al.* 1997). Therefore, to check, whether observed between *A. thaliana* accessions differences in the activity of initiation translation resulted from such changes in the structural properties of untranslated regions, the 5'UTRs sequences of each analyzed gene were compared between tested accessions (for the analyses the publicly available sequencing data from 1001 genome project were used).

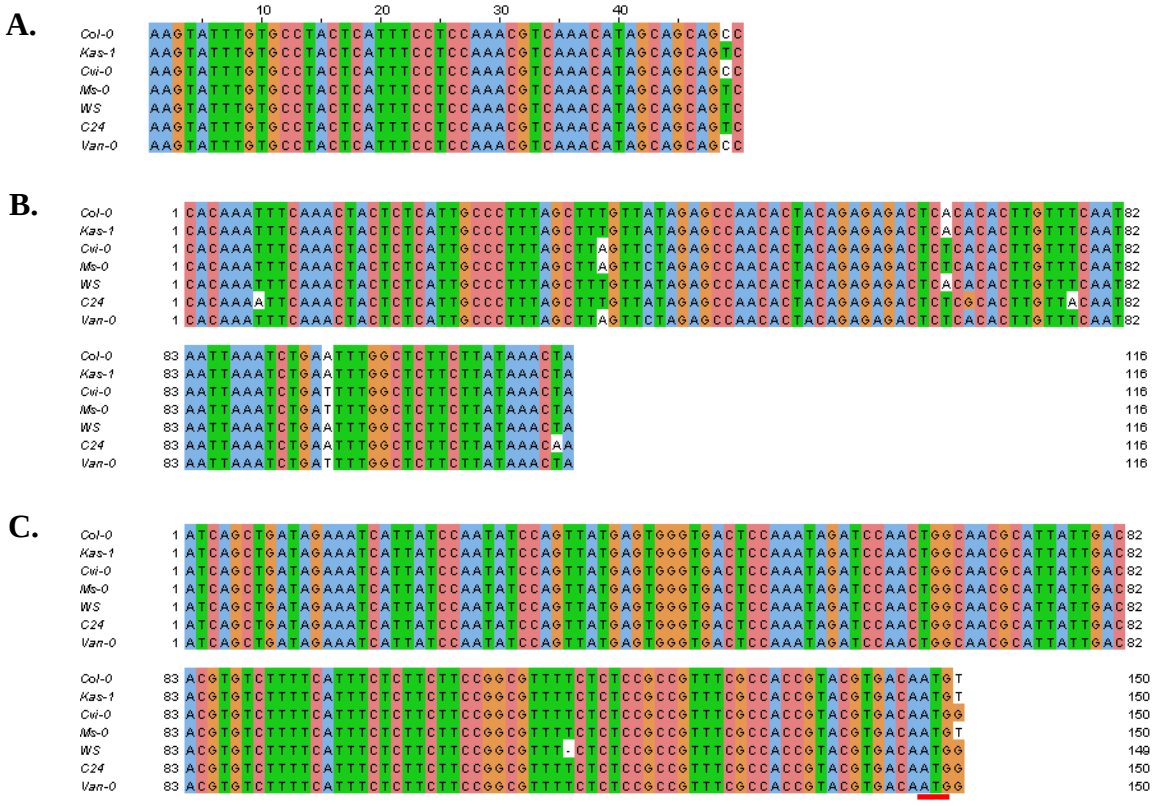

Figure 3-32. Comparison of 5'UTRs preceding **A.** *Csd2*, **B.** *MDHAR* and **C.** *tAPx* in tested accessions of *A. thaliana* (the sequence of *tAPx* besides 5'UTR contains underlined translation initiation site and following it nucleotide +4).

The comparison revealed that 5'UTRs of *2CPA* and *GPx1* were identical in all accessions, while in 5'UTRs of other analyzed genes several single nucleotide polymorphisms (SNPs) were detected (Figures 3-32 and A1 in Appendix). These SNPs did not change any structural properties of 5'UTRs preceding genes encoding *sAPx, 2CPB, PrxQ* and *PrxIIE* (see Figure A1 in Appendix) or introduced the modifications, which might have an influence on the activity of translation initiation of *Csd2*, *tAPx* and *MDHAR* (Figure 3-32). Thus, in the 5'UTRs of the last three genes SNPs were detected in the region flanking translation initiation site (TIS), which sequence in *Arabidopsis thaliana* was identified as aaaaaaa(A/G)(A/C)aATGGcgaataata (underlined part corresponds to the translation initiation site) (Rangan *et al.* 2008). It is known that modifications in the sequence of TIS, especially in its conserved positions (G at the position +4 and A or C at the position -2), might change the efficiency, with which the translation initiation codon is recognized by translation initiation complex (Rangan *et al.* 2008).

Among analyzed accessions, cytosine (C) was found in 5'UTR of *Csd2* only in Col-0, Cvi-0 and Van-0, while in Kas-1, Ms-0, WS and C24 cytosine was exchanged into thymine (C→T) (Figure 3-32). Consistently, in almost all (except Ms-0) accessions with thymine at position -2 translation initiation was more efficient (Figure 3-25), indicating positive influence of C→T exchange on the translation activity. In contrast, 5'UTR of *MDHAR* in almost all tested accessions contained thymine at the position -2 (Figure 3-32). Only, in C24, in which one of the highest efficiency of translation initiation (beside WS), this thymine was exchanged into adenine.

Besides the changes at the position -2, modifications of the TIS sequences at the position +4 were detected for *tAPx* in all of the tested accessions. Among them, in Col-0, Kas-1 and Ms-0 conserved at this position A or C were exchanged into thymine (T), while in Cvi-0, WS, C24 and Van-0 the exchange into guanine (G) was detected (Figure 3-32). Since, the highest *tAPx* translation efficiency was observed in Kas-1 and Ms-0, it suggests that T at the position +4 promotes mRNA binding to ribosomes.

3.1.4 Reactive oxygen species (ROS) production

Antioxidant enzymes, such as superoxide dismutases (SODs), ascorbate peroxidases (APXs), peroxiredoxins (PRXs) and glutathione peroxidases (GPXs), by catalyzing ROS detoxification are involved in maintaining redox balance of plant cells (Asada, 1999). The capacity of these enzymes for ROS detoxification was shown to be balanced with regard to ROS production (Foyer and Shigeoka, 2011). Therefore, determination of the ROS levels may serve as a read-out for total antioxidant protection of the cell.

Here, the ROS production was determined in young, middle-age and mature rosette leaves of 6-week-old *A. thaliana* accessions using two different ROS staining methods. In these stainings, superoxide radical anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) can be specifically trapped by NBT and DAB, respectively. The reactions lead to accumulation of either, purple (NBT staining) or brownish (DAB staining) precipitates in the sites of ROS production. To quantify the ROS levels in the leaves of analyzed *A. thaliana* accessions, the size of these stained by NBT or DAB sites of ROS production relative to the area of the whole leaf was determined using digital image analyzer ImageJ.

The amount of superoxide radical anion ($O_2^{\bullet-}$) differed between analyzed *A. thaliana* accessions (Figure 3-33). Among them, the lowest levels of the $O_2^{\bullet-}$ were detected for C24. Moreover, in this accession no obvious differences in the $O_2^{\bullet-}$ accumulation between leaves of various ages were observed. In Kas-1, WS and Van-0 the lowest levels of superoxide were detected in the middle-age leaves. In contrast, the middle-age leaves of Cvi-0 accumulated more $O_2^{\bullet-}$ than two other tested leaf stages. In Ms-0 and Col-0 different patterns of $O_2^{\bullet-}$ production than in any other accession were observed. In Ms-0 the level of superoxide gradually decreased with the age of the leaf, while in Col-0 the old and middle-age leaves accumulated similar and lower amounts of $O_2^{\bullet-}$ than the youngest leaf.

Variation in the accumulation of superoxide observed between analyzed accessions might indicate differences in the activity of superoxide dismutases, enzymes responsible for reduction of $O_2^{\bullet-}$ to H_2O_2. It was expected that the activity of SODs would be lower in accessions accumulating high amounts of $O_2^{\bullet-}$ (Kas-1, Ms-0 and WS) than in the ones, in which low levels of $O_2^{\bullet-}$ were detected (C24 and Col-0). However, SOD activity was higher in Kas-1, Ms-0 and WS than in Col-0 and C24 (Figure 3-6). Thus, it indicated that enhancement of SOD activity was more likely a consequence of ROS levels than the cause of ROS accumulation.

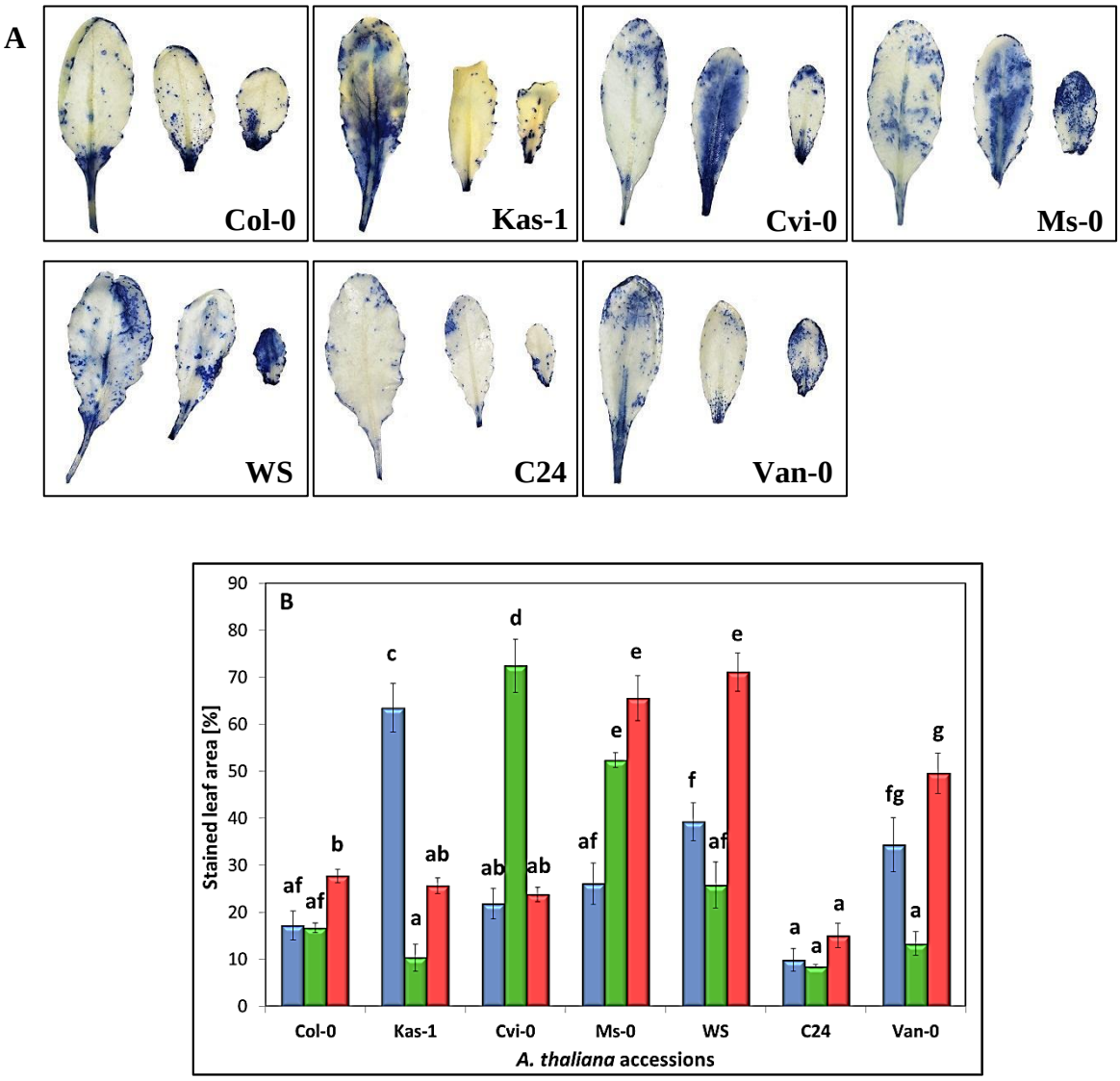

Figure 3-33. A. Endogenous production of superoxide radical anion ($O_2^{\bullet-}$) in 6-week-old *A. thaliana* accessions; **B.** Quantification of $O_2^{\bullet-}$ levels in mature (blue bars), middle-age (green bard) and young (red bars) leaves of selected accession. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.05$).

Differences between analyzed accessions were observed also in the accumulation of hydrogen peroxide (Figure 3-34). Among all of them, the lowest level of H_2O_2 was detected in the middle-age leaves of Kas-1 and the highest in the oldest leaves of Col-0, Ms-0 and C24. Moreover, differences between accessions were observed in the pattern of hydrogen peroxide accumulation. In Col-0, Ms-0 and C24 the level of H_2O_2 increased with the age of the leaves, while in Cvi-0 a decrease with the age was observed. In WS only very slight differences between analyzed leaf stages were shown. In Kas-1 and Ms-0, the significant differences between analyzed leaf stages were

detectable only for middle leaf (the levels of H_2O_2 in oldest and the youngest leaves were the same), in which either, the lowest (Kas-1) or the highest (Van-0) amounts of H_2O_2 were accumulated.

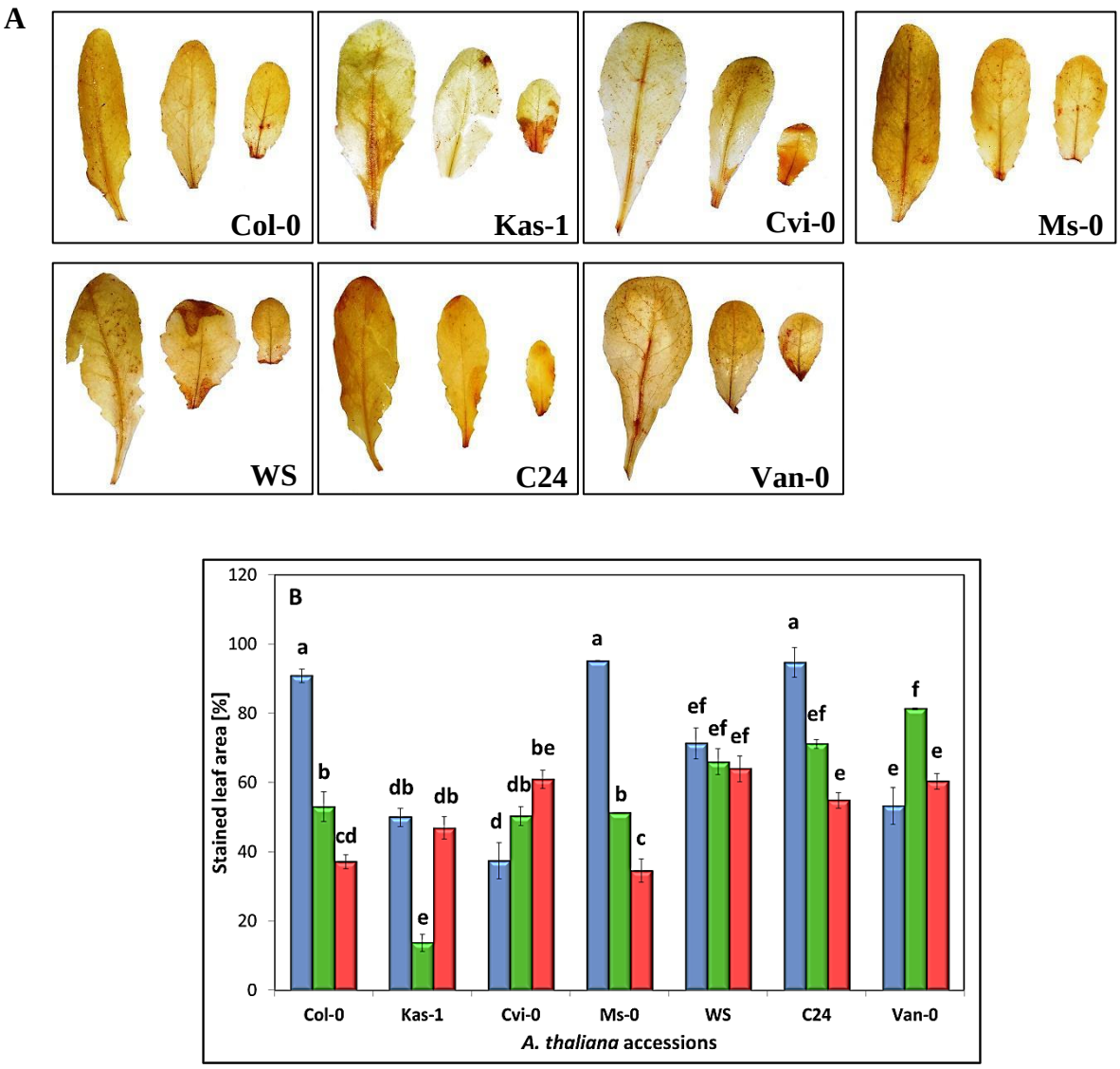

Figure 3-34. A. Endogenous production of hydrogen peroxide (H_2O_2) in 6-week-old *A. thaliana* accessions; **B.** Quantification of H_2O_2 levels in mature (blue bars), middle-age (green bard) and young (red bars) leaves of selected accession. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.05$).

3.2 The expression regulation of chloroplast antioxidant system depends on the developmental stage of analyzed *Arabidopsis thaliana* accessions

It is known that the expression of many genes is influenced by developmental stage of plants. The genes encode proteins involved in various cellular processes, such as cell cycle, metabolism and intracellular signalling (Kaufmann *et al.* 2010). However, only a little is known about developmental control of ROS-scavenging in chloroplasts (Baier *et al.* 2000; Rodriguez Milla *et al.* 2003; Peña-Ahumada*et al.* 2006). Here, the expression of chloroplast antioxidant system at the transcript level was compared between seven 3-week-old and 6-week-old *A. thaliana* accessions.

For the comparison, the steady-state mRNA abundances of genes encoding chloroplast antioxidant enzymes, namely *Csd2*, *2CPA*, *2CPB*, *PrxQ*, *PrxIIE*, *GPx1*, *GPx7*, *sAPx*, *tAPx*, *MDHAR*, *DHAR* and *GR*, were measured in RNA samples extracted from the rosette leaves of selected 3-week-old or 6-week-old *A. thaliana* accessions using qRT-PCR and gene specific primers (see Table 2-3 for a list).

The mRNA levels obtained for all analyzed genes were standardized on the transcript abundance of *Act2* gene (At3g18780), which was shown to be constitutively expressed at all developmental stages (An *et al.* 1996).

For each accession, two biological replicates, each representing an independent RNA isolation from single, independently grown plant, were analyzed. All analyses were performed in three technical replicates.

3.2.1 Copper-zinc superoxide dismutase (Csd2)

When *Csd2* transcript level was compared between 6-week-old and 3-week-old accessions, less variation was observed for younger than for older plants (Figure 3-35). Moreover, almost all tested accessions (except WS) significantly differed in the accumulation of *Csd2* mRNA at both analyzed developmental stages (Figure 3-35). In general, older plants accumulated higher amounts of this transcript than the younger ones, suggesting higher importance of CSD2 in the neutralization of ROS at later developmental stage of selected accessions. The strongest differences between 3-week-old and 6-week-old plants were detected for Cvi-0 and Ms-0, in which the level of *Csd2* transcript in younger plants was respectively nineteen- and twelvefold higher than in older ones. Among remaining accessions, the 3-week-old Col-0, Kas-1, C24 and

Van-0 accumulated three- to sixfold more *Csd2* mRNA than the 6-week-old ones. Only in WS, the level of this transcript was similar at both analyzed developmental stages.

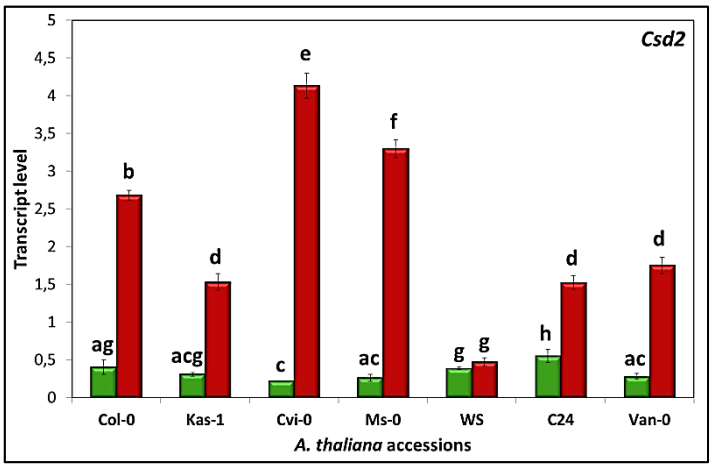

Figure 3-35. Comparison of *Csd2* transcript levels between 3-week-old (green bars) and 6-week-old (red bars) *A. thaliana* accessions. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ±SD from two independent RNA isolation, each representing three technical replicates.

3.2.2 Enzymes of the ascorbate-independent water-water cycle

The subsequent comparison of the transcript levels of genes encoding peroxiredoxins and glutathione peroxidases between 6-week-old and 3-week-old *A. thaliana* accessions also revealed significant differences (Figures 3-36 and 3-37), demonstrating that the expression regulation of chloroplast antioxidant enzymes is strongly age-dependent.

Peroxiredoxins (*2CPA*, *2CPB*, *PrxQ* and *PrxIIE*)

Among all analyzed chloroplast peroxiredoxins stronger differences in the transcript accumulation between younger and older plants were detected for genes encoding 2-Cys peroxiredoxins (*2CPA* and *2CPB*) than for that of *PrxQ* and *PrxIIE* (Figure 3-36).
In general, the mRNA abundances of both *2CPs* in almost all accessions were higher in 6-week-old than in 3-week-old plants (Figure 3-36), indicating stronger support of *2CPA* and *2CPB* transcripts accumulation at later from analyzed developmental stages. Only in Ms-0 the amount of *2CPA* mRNA was approximately fourfold lower in 6-week-old than in 3-week-old plants. Moreover, *2CPB* transcript was accumulated in this accession at both analyzed developmental stages at the lowest level, suggesting that in Ms-0 both 2-Cys peroxiredoxins are of lower importance. In contrast, in Cvi-0 the total

amount of *2CPs* mRNAs was the highest among tested accessions, indicating that this accession strongly support expression of *2CPA* and *2CPB* at all developmental stages.

The accumulation of transcripts encoding remaining peroxiredoxins was also age-dependent (Figure 3-36). Higher abundance of *PrxQ* mRNA was detected in older than in younger plants for Col-0, Cvi-0 and Ms-0, while Kas-1, WS and C24 accumulated this transcript in higher amounts at earlier from tested developmental stages. In contrast, the level of *PrxIIE* mRNA did not significantly differ between 3-week-old and 6-week-old plants for Col-0, Kas-1 and Ms-0, while for Cvi-0, WS, C24 and Van-0 its higher accumulation was detected in younger than in older plants.

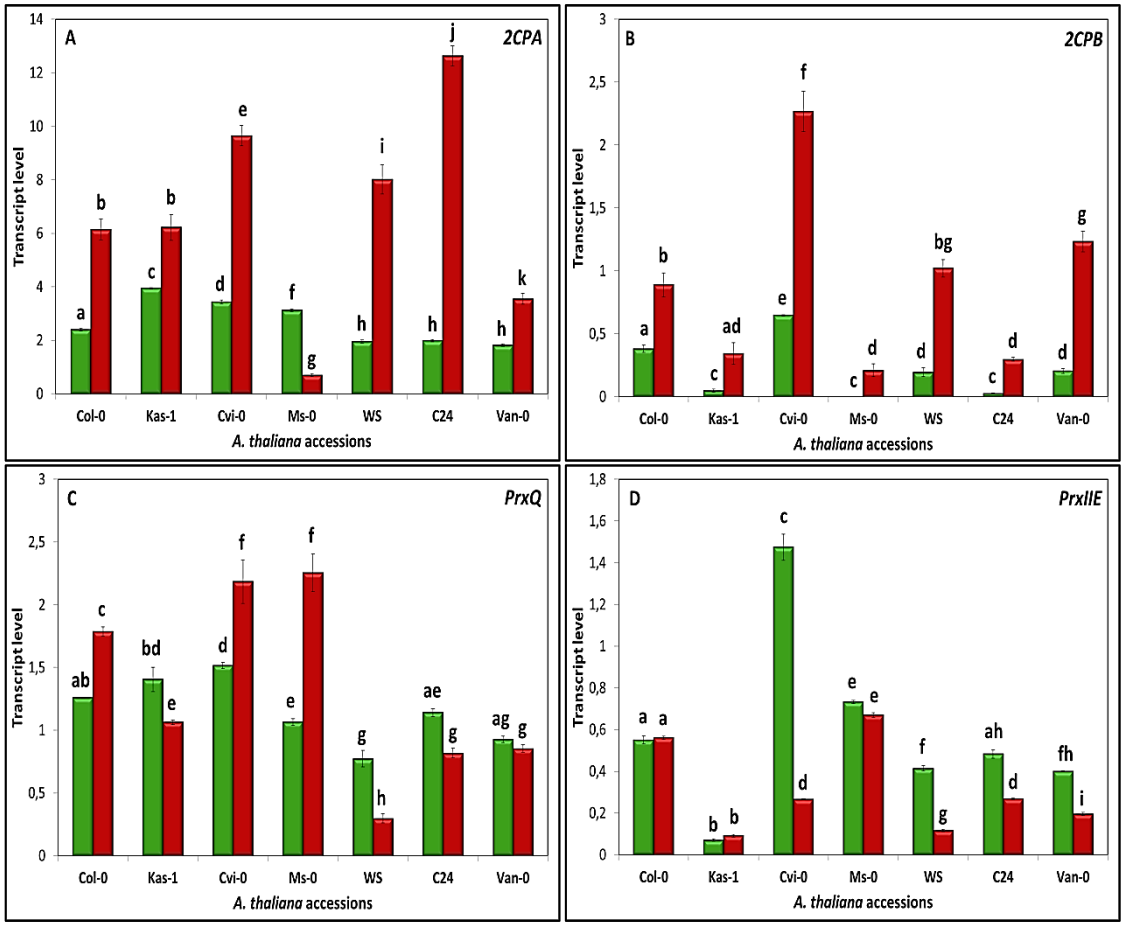

Figure 3-36. Comparison of **A.** *2CPA*, **B.** *2CPB*, **C.** *PrxQ*, **D.** *PrxIIE* transcript levels between 3-week-old (green bars) and 6-week-old (red bars) *A. thaliana* accessions. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ±SD from two independent RNA isolation, each representing three technical replicates.

Glutathione peroxidases (*GPx1* and *GPx7*)

Significant differences between 3-week-old and 6-week-old *A. thaliana* accessions were detected also in the accumulation of transcripts encoding both chloroplast glutathione peroxidases, *GPx1* and *GPx7* (Figure 3-37). In general, the abundance of *GPx1* mRNA was higher in older than in younger plants for all tested accessions with the highest difference between analyzed developmental stages detected for Ms-0. In contrast, the transcript of *GPx7* was accumulated at higher levels in 6-week-old than in 3-week-old plants only in Col-0, WS, C24 and Van-0, while in Kas-1, Cvi-0 and Ms-0 the level of this transcript was not significantly different and low at both analyzed developmental stages.

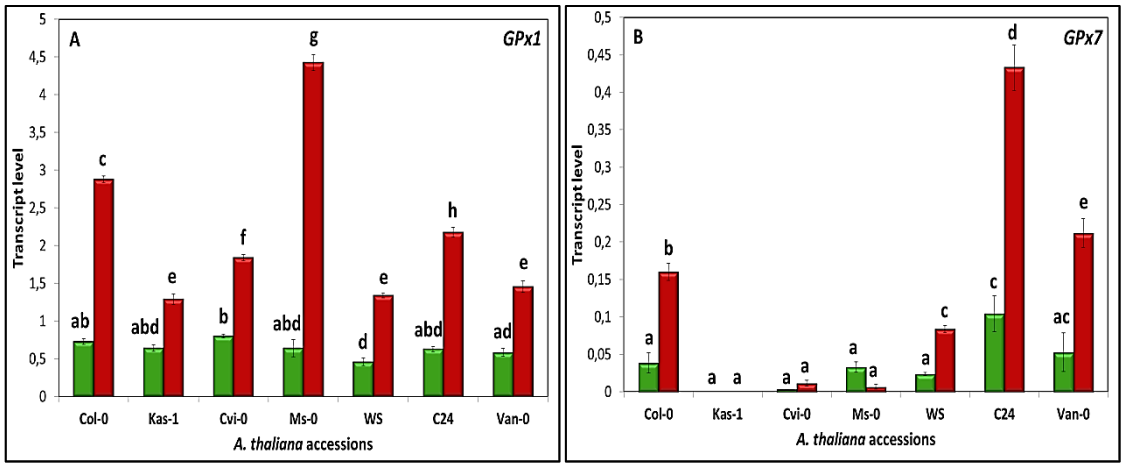

Figure 3-37. Comparison of **A.** *GPx1* and **B.** *GPx7* transcript levels between 3-week-old (green bars) and 6-week-old (red bars) *A. thaliana* accessions. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ±SD from two independent RNA isolation, each representing three technical replicates.

3.2.3 Enzymes of the ascorbate-dependent water-water cycle

When the levels of transcripts encoding *sAPx* and *tAPx* were compared between 3-week-old and 6-week-old plants, significant differences for all tested accessions were observed (Figure 3-38). In general, in all of them mRNAs of both *APxs* were accumulated at later developmental stage at significantly higher level than at the earlier one, except *tAPx* in Ms-0, which amount was similar in younger and in older plants. Among all tested accessions, the strongest differences in the levels of *APxs* transcripts between 3-week-old and 6-week-old plants were observed for Cvi-0, in which *sAPx* and

tAPx were accumulated in older plants at respectively thirty- and twelvefold higher level than in younger ones (Figure 3-38). Moreover, the amounts of both, *sAPx* and *tAPx*, mRNAs in Cvi-0 were the highest among all tested 6-week-old accessions and low in 3-week-old ones, indicating that ascorbate peroxidases in this accession are of higher importance at later from analyzed developmental stages.

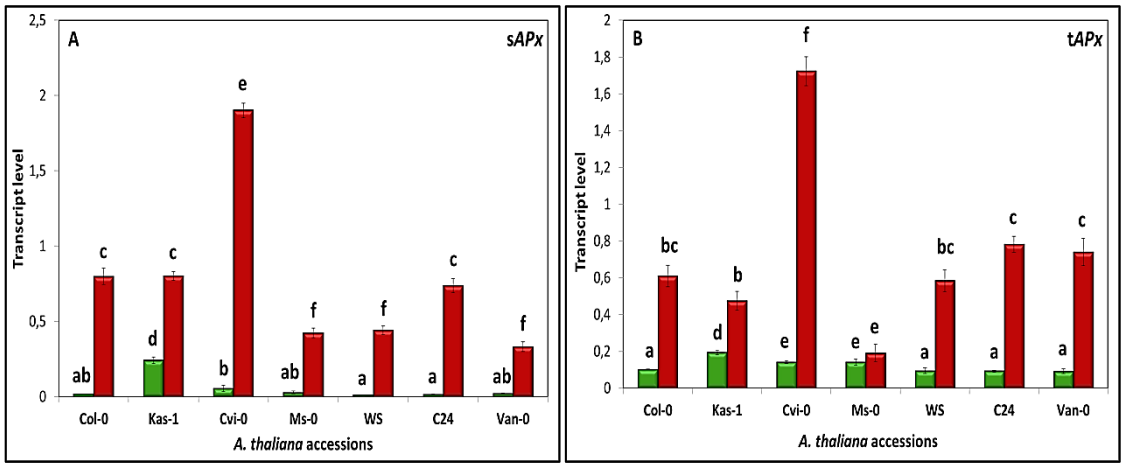

Figure 3-38. Comparison of **A.** *sAPx* and **B.** *tAPx* transcript levels between 3-week-old (green bars) and 6-week-old (red bars) *A. thaliana* accessions. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ±SD from two independent RNA isolation, each representing three technical replicates.

3.2.4 Enzymes of the ascorbate-recycling system

The comparison of *MDHAR*, *DHAR* and *GR* transcript levels among 3-week-old and 6-week-old accessions (Figure 3-39) revealed the strongest differences between these two developmental stages for *DHAR*. In general, the amount of *DHAR* mRNA was almost not detectable in all younger accessions and dramatically increased (between one and three hundredfold) at later from analyzed developmental stages, indicating higher importance of DHAR in older than in younger plants. In contrast, the transcript levels of *MDHAR* were in almost all tested accessions either lower in older than in younger plants or similar at both developmental stages (Figure 3-39). Only Cvi-0 and Ms-0 accumulated this transcript in 6-week-old plants at more than twofold higher level than in 3-week-old ones, demonstrating that these accessions stronger support expression of *MDHAR* at later developmental stages, while the others rather at earlier ones.

Significant variation was observed among tested accessions also in the accumulation of *GR* transcript, indicating that the expression of this gene is also regulated in age-

dependent manner (Figure 3-39). In general, the level of *GR* mRNA was higher in 6-week-old plants than in 3-week-old ones for those accessions, which accumulated also in older plants the highest amount of *DHAR* transcripts (Cvi-0 and Col-0). In contrast, in accessions accumulating lower levels of *DHAR* mRNA than Cvi-0 and Col-0 in older plants also the level of *GR* was either lower in older than younger plants or similar at both analyzed developmental stages. It indicated common pattern in the developmental regulation of both, *DHAR* and *GR*.

Moreover, the highest levels of transcripts encoding all enzymes of ascorbate-recycling system among tested accessions at the later from analyzed developmental stages were detected for Cvi-0, which also accumulated in older plants the highest amounts of *APxs* (Figures 3-38 and 3-39). It suggested that this accession in response to high activation of APxs strongly support the expression of all, *MDHAR*, *DHAR* and *GR*. In contrast, in other tested accessions such strong co-regulation of *APxs* and enzymes of the ascorbate-recycling system expression was not observed.

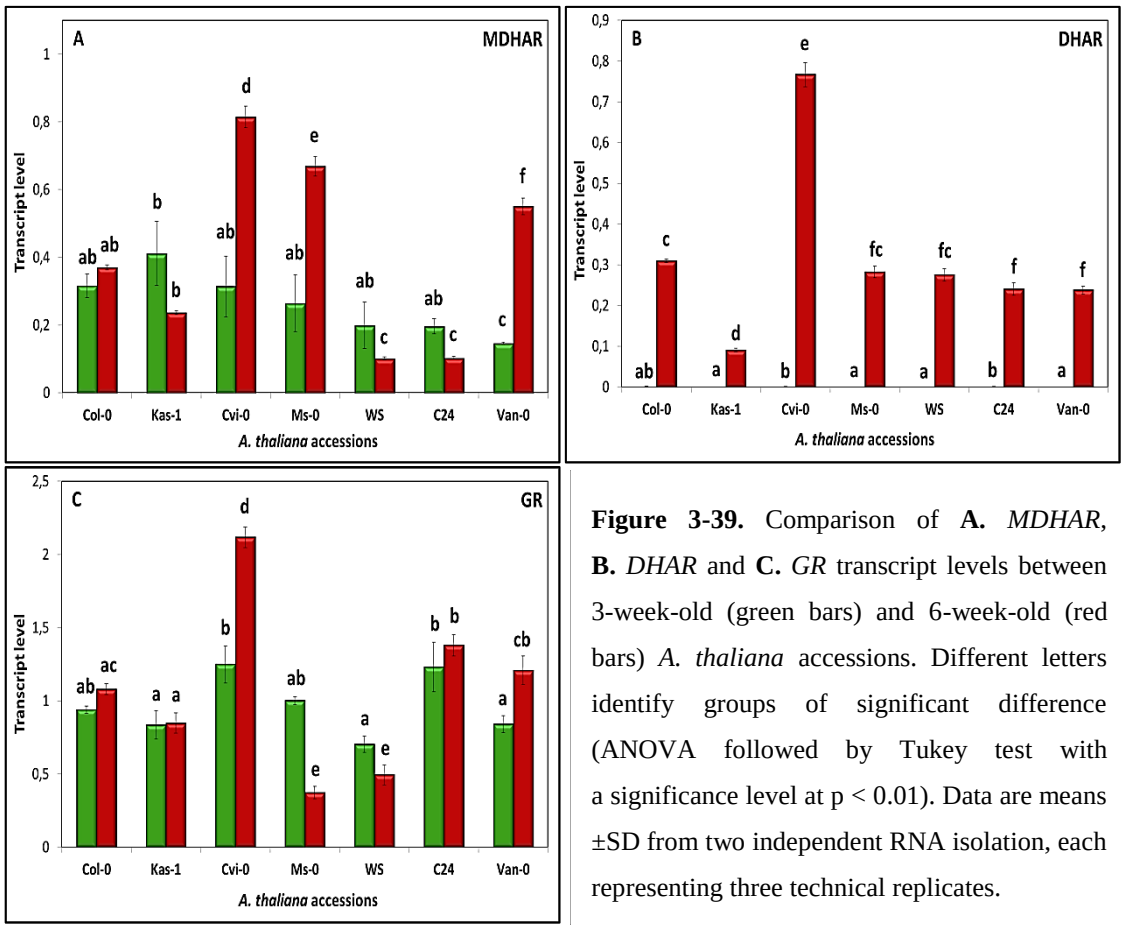

Figure 3-39. Comparison of **A.** *MDHAR*, **B.** *DHAR* and **C.** *GR* transcript levels between 3-week-old (green bars) and 6-week-old (red bars) *A. thaliana* accessions. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ±SD from two independent RNA isolation, each representing three technical replicates.

3.2.5 Accession-specific variation

The comparison of 3-week-old and 6-week-old *A. thaliana* accessions revealed that the expression of genes encoding all chloroplast antioxidant enzymes is regulated at the transcript level in age-dependent manner. In general, almost all transcripts encoding these enzymes were accumulated in older plants in higher amounts than in younger ones, indicating greater importance of antioxidant enzymes at later developmental stages. Among selected accessions, the strongest support of chloroplast antioxidant enzymes expression in older plants was observed for Cvi-0 and Van-0, in which ten out of twelve transcripts were accumulated in 6-week-old plants at significantly higher level than in 3-week-old ones. In contrast, WS, with the level of four out of twelve analyzed transcripts higher in younger than in older plants, invested the most from the tested accessions into the expression regulation of chloroplast antioxidant enzymes at earlier from analyzed developmental stages.

3.3 The expression regulation of chloroplast antioxidant system in analyzed *Arabidopsis thaliana* accessions depends on the growth temperature

Since the selected *A. thaliana* accessions originate from habitats with different temperatures and temperature amplitudes, the influence of growth temperature on the regulation of the chloroplast antioxidant system at the transcript level was analyzed in 3-week-old plants acclimated for one week to 10, 20 or 30 °C (for description of growth conditions see 2.2.2).

The transcript levels of all selected genes were standardized on the mRNA abundance of *Act2* gene (At3g18780) and normalized to the expression of the same gene in Col-0 cultivated at its optimal temperature, 20 °C.

For each accession, two biological replicates, each representing an independent RNA isolation from pools of six rosettes, were analyzed. All analyses were performed in three technical repeats.

3.3.1 Copper-zinc superoxide dismutase (Csd2)

The analyses of *Csd2* transcript levels in plants acclimated to different growth temperatures revealed, that the steady-state mRNA abundance of this gene gradually increased with the growth temperature in almost all tested accessions (Figure 3-40). Only in Cvi-0 *Csd2* transcript was accumulated at 10 and 30 °C in significantly higher amount than in plants cultivated at 20 °C, demonstrating accession-specific adaptation to temperature fluctuations.

Moreover, the adaptive changes in temperature responsiveness may be indicated for *Csd2* regulation (Figure 3-40). Among all tested accessions, the lowest *Csd2* transcript level at 10 °C was detected for Kas-1 and Van-0, but in response to elevated temperature (30 °C) stronger increase in *Csd2* mRNA abundance was observed for Kas-1 than for Van-0. It suggests that Van-0, which sometimes faces 30 °C in summer can invest in the regulation of *Csd2* expression less than Kas-1, which is hardly subjected to such high temperatures at altitude of 1580 m in Kashmir Mountains.

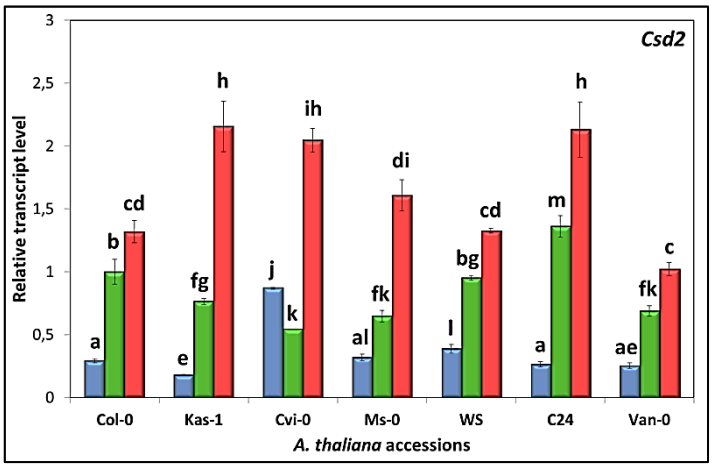

Figure 3-40. Transcript level of *Csd2* in six accession of *A. thaliana* relative to Col-0. All plants were cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (blue bars), 20 °C (green bars) or 30 °C (red bars), for low, control or high temperature treatment, respectively. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ±SD from two biological replicates, each representing an independent RNA isolation from pools of six rosettes. Each biological replicate was analyzed in three technical repeats.

3.3.2 Enzymes of the ascorbate-independent water-water cycle

The same analyses as for *Csd2*, were performed for transcripts encoding enzymes of ascorbate-independent water-water cycle, namely *2CPA*, *2CPB*, *PrxQ*, *PrxIIE*, *GPx1* and *GPx7* (Figures 3-41 and 3-42).

Peroxiredoxins (*2CPA*, *2CPB*, *PrxQ* and *PrxIIE*)

The comparison of *Prxs* transcripts accumulation between selected *A. thaliana* accessions acclimated to different temperatures revealed variation for each analyzed gene (Figure 3-40). Among them, the strongest differences between the patterns of temperature responsiveness were observed for *2CPA* (Figure 3-41). In Cvi-0 and Ms-0 lower level of this transcript was detected at 30 °C than at 10 and 20 °C, while in Van-0 *2CPA* mRNA was accumulated in lower amounts at 10 and 20 °C and significantly higher at 30 °C. Among remaining accessions, in Kas-1 the expression of *2CPA* in response to changing temperature was regulated by disturbance effect (the highest level of transcript at 20 °C and significantly lower at both, 10 and 20 °C), while in WS and C24 no obvious differences between plants acclimated to different temperatures were detected.

In contrast, in response to different temperatures less variation between tested accessions was observed in the accumulation of mRNAs encoding remaining peroxiredoxins (Figure 3-41). Among them, for *2CPB* and *PrxIIE* gradual variation with temperature was observed in almost all accessions, while changes in the same direction (e.g. increase in the transcript level in response to both, decreased and increased temperature) dominated for *PrxQ*. However, for all transcripts encoding peroxiredoxins different patterns of mRNA accumulation in response to temperature fluctuations were observed in accessions coming from similar habitats and the same patterns in that originating from contrasting climates (Figure 3-41). Therefore, no obvious adaptive changes in temperature responsiveness for *Prxs* transcripts are indicated.

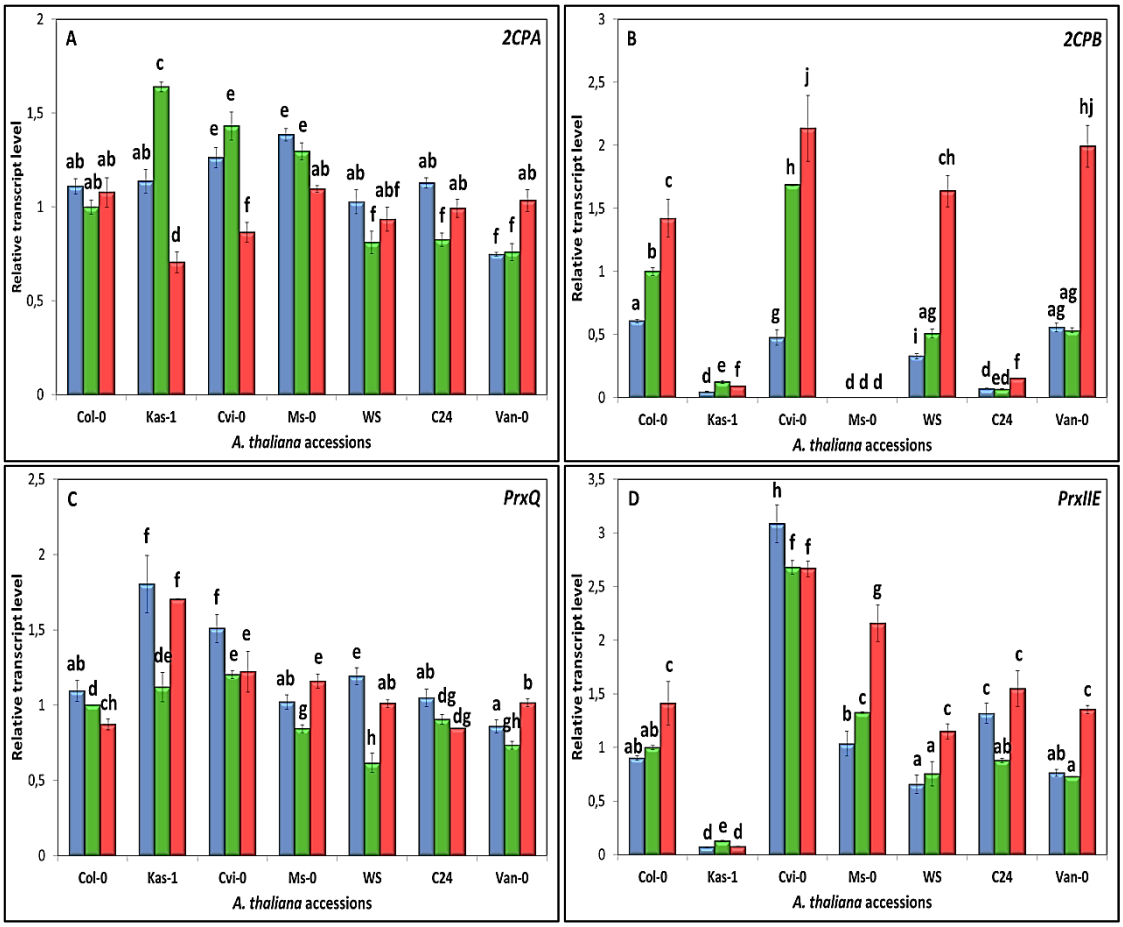

Figure 3-41. Transcript levels of **A.** *2CPA*, **B.** *2CPB*, **C.** *PrxQ*, **D.** *PrxIIE* in six accession of *A. thaliana* relative to Col-0. All plants were cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (blue bars), 20 °C (green bars) or 30 °C (red bars), for low, control or high temperature treatment, respectively. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ± SD from two biological replicates, each representing an independent RNA isolation from pools of six rosettes. Each biological replicate was analyzed in three technical repeats.

Glutathione peroxidases (*GPx1* and *GPx7*)

Among chloroplast glutathione peroxidases, different patterns of variation in response to temperature fluctuations were revealed for *GPx1* than for *GPx7* (Figure 3-42). It was shown that in all of the tested accession the level of *GPx1* gradually increased with growth temperature, while the mRNA abundance of *GPx7* was regulated by disturbance effect (increase in the transcript level with both, decreased and increased growth temperature). It indicated that in *A. thaliana* accessions there was no common mechanism regulating the accumulation of *GPxs* transcripts in response to different growth temperatures.

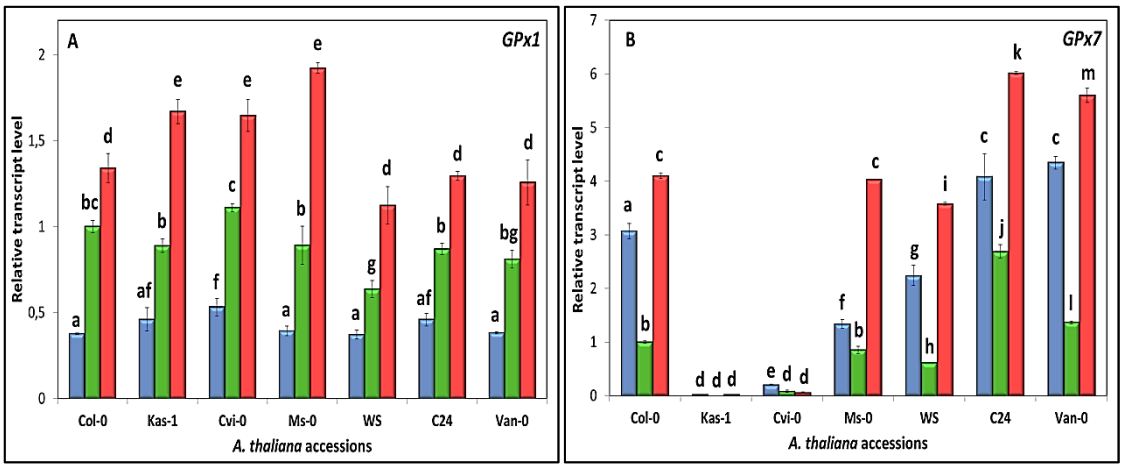

Figure 3-42. Transcript levels of **A.** *GPx1* and **B.** *GPx7* in six accession of *A. thaliana* relative to Col-0. All plants were cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (blue bars), 20 °C (green bars) or 30 °C (red bars), for low, control or high temperature treatment, respectively. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ±SD from two biological replicates, each representing an independent RNA isolation from pools of six rosettes. Each biological replicate was analyzed in three technical repeats.

3.3.3 Enzymes of the ascorbate-dependent water-water cycle

Subsequent comparison of the response of *sAPx* and *tAPx* transcripts levels to the temperature acclimation revealed that mRNA abundance of both genes is regulated by disturbance effect (e.g. decrease in the transcript level in response to both, decreased and increased temperature) in almost all tested accessions (Figure 3-43). For *sAPx* only in Kas-1 and Cvi-0 and for *tAPx* in WS, C24 and Van-0 different patterns of transcript

accumulation (the same mRNA levels at two out of three or at all tested temperatures) were observed.

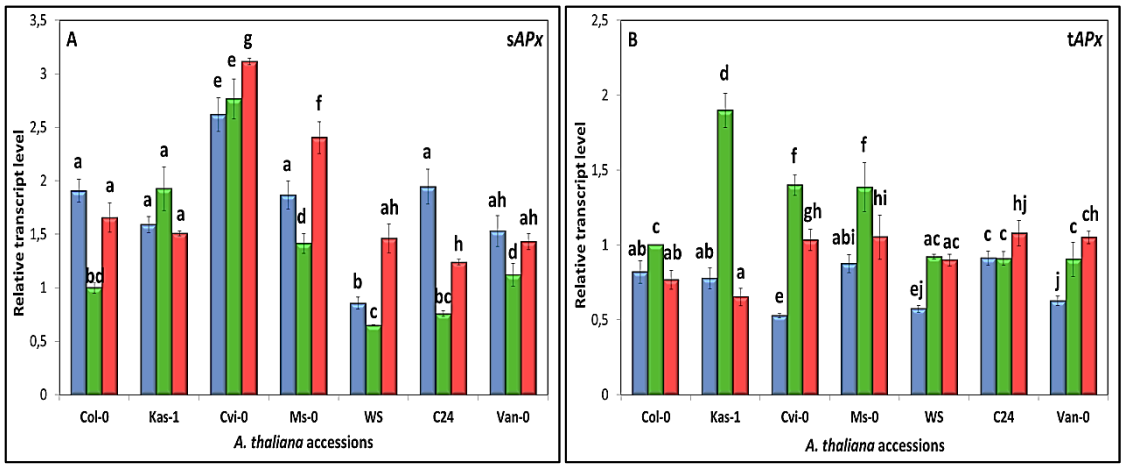

Figure 3-43. Transcript levels of **A.** *sAPx* and **B.** *tAPx* in six accession of *A. thaliana* relative to Col-0. All plants were cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (blue bars), 20 °C (green bars) or 30 °C (red bars), for low, control or high temperature treatment, respectively. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ±SD from two biological replicates, each representing an independent RNA isolation from pools of six rosettes. Each biological replicate was analyzed in three technical replicates.

3.3.4 Enzymes of the ascorbate-recycling system

The analyses of transcript levels encoding all ascorbate-recycling reductases in response to temperature acclimation (Figure 3-44), showed that in almost all selected accessions the abundance of *DHAR* mRNA is regulated by disturbance effect (increase in the level of transcript with both, increased and decreased growth temperature), whereas level of *GR* mRNA gradually decreased with the increase of growth temperature.

In contrast, stronger variability between tested accessions was observed in the accumulation of *MDHAR* transcripts (Figure 3-44). In Kas-1 and Ms-0, their levels gradually decreased with the growth temperature, while in WS, C24 and Van-0 were regulated by disturbance effect (the lowest abundance of mRNA encoding *MDHAR* was detected for plants cultivated at 10 °C). In remaining accessions, Col-0 and Cvi-0, significantly higher *MDHAR* transcript levels were observed in plants acclimated to 30 °C than in those cultivated at 10 or 20 °C (similar level of this transcript at both,

10 and 20 °C), demonstrating strong responsiveness of *MDHAR* expression to low temperatures.

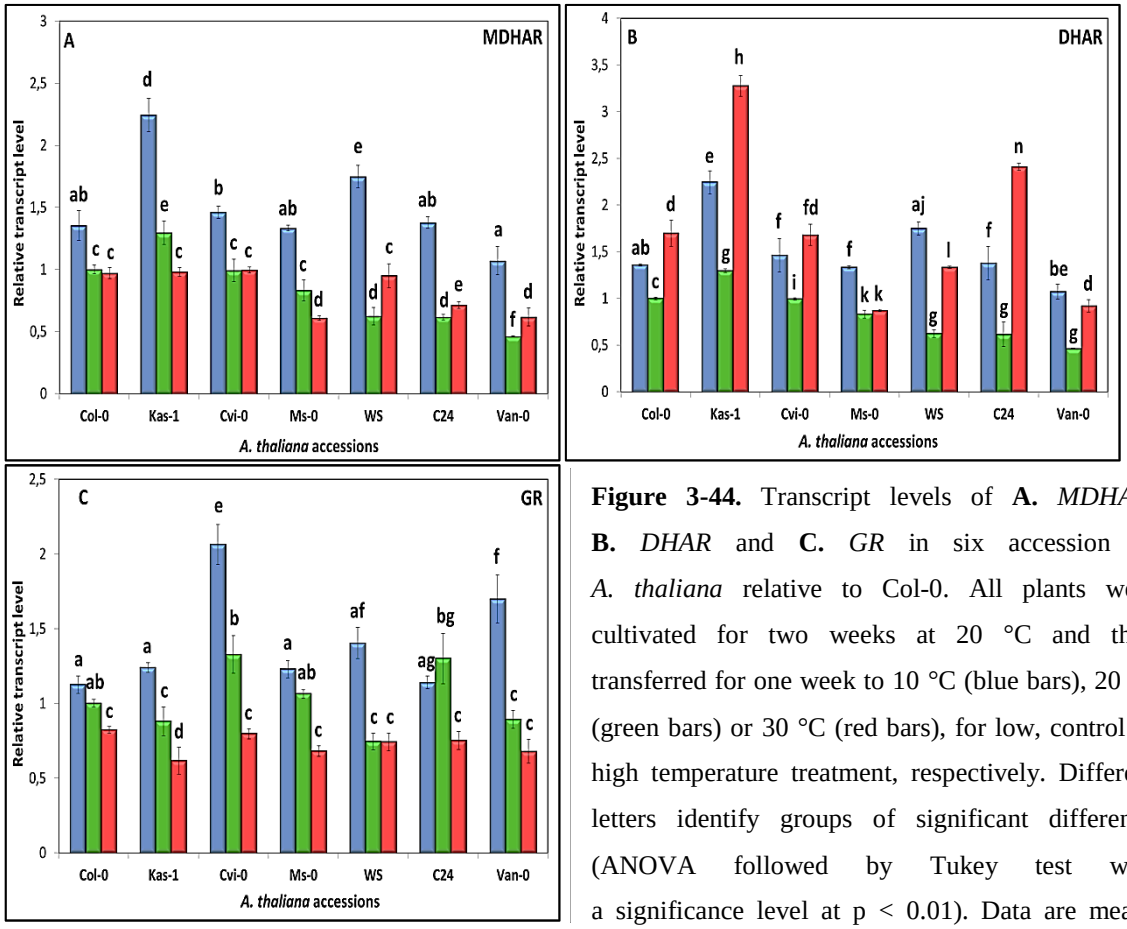

Figure 3-44. Transcript levels of **A.** *MDHAR*, **B.** *DHAR* and **C.** *GR* in six accession of *A. thaliana* relative to Col-0. All plants were cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (blue bars), 20 °C (green bars) or 30 °C (red bars), for low, control or high temperature treatment, respectively. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ±SD from two biological replicates, each representing an independent RNA isolation from pools of six rosettes. Each biological replicate was

3.3.5 Accession-specific variation

The analyses of temperature influence on the transcript levels of genes encoding chloroplast antioxidant enzymes in *A. thaliana* accessions originating from habitats with different temperatures and temperature amplitudes did not show general habitat temperature-related characteristics in gene expression regulation (Figure 3-45). Among all analyzed genes the adaptive changes in temperature responsiveness could be indicated only in plants acclimated to 10 °C for transcripts encoding *Csd2* and *2CPB*, as well as at 30 °C for *PrxQ* mRNA and at 20 °C for *2CPB* and *tAPx* (Figure 3-45). At 10 °C the highest accumulation of *Csd2* and *2CPB* was shown for Cvi-0, which in its natural environment is never subjected to such low temperatures, while in accessions frequently facing at their natural habitats the temperatures of 10 °C the amount of these

transcripts was significantly lower (Figures 3-40 and 3-41). In contrast, at 30 °C the highest abundance of *PrxQ* mRNA was detected in Kas-1, which is never subjected to such high temperatures in its natural environment, while WS, Van-0, Col-0 and Cvi-0 facing 30 °C in summer accumulated significantly lower level of this transcript (Figure 3-41).

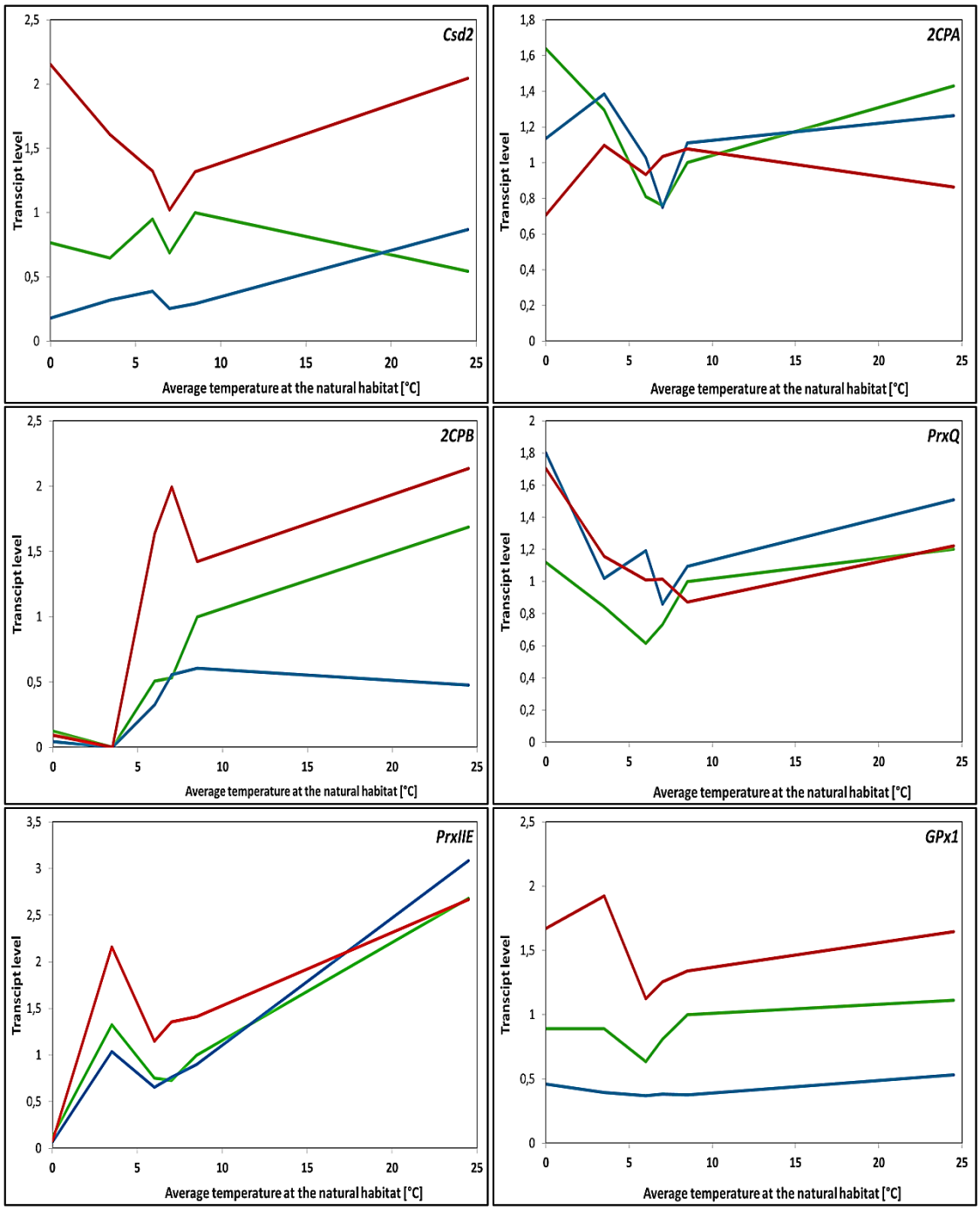

Figure 3-45. Dependency of the transcript levels of genes encoding chloroplast antioxidant enzymes on the average temperature at the natural habitats of tested accessions. The plants used for transcript analyses were cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (blue lines), 20 °C (green lines) or 30 °C (red lines), for low, control or high temperature treatment, respectively.

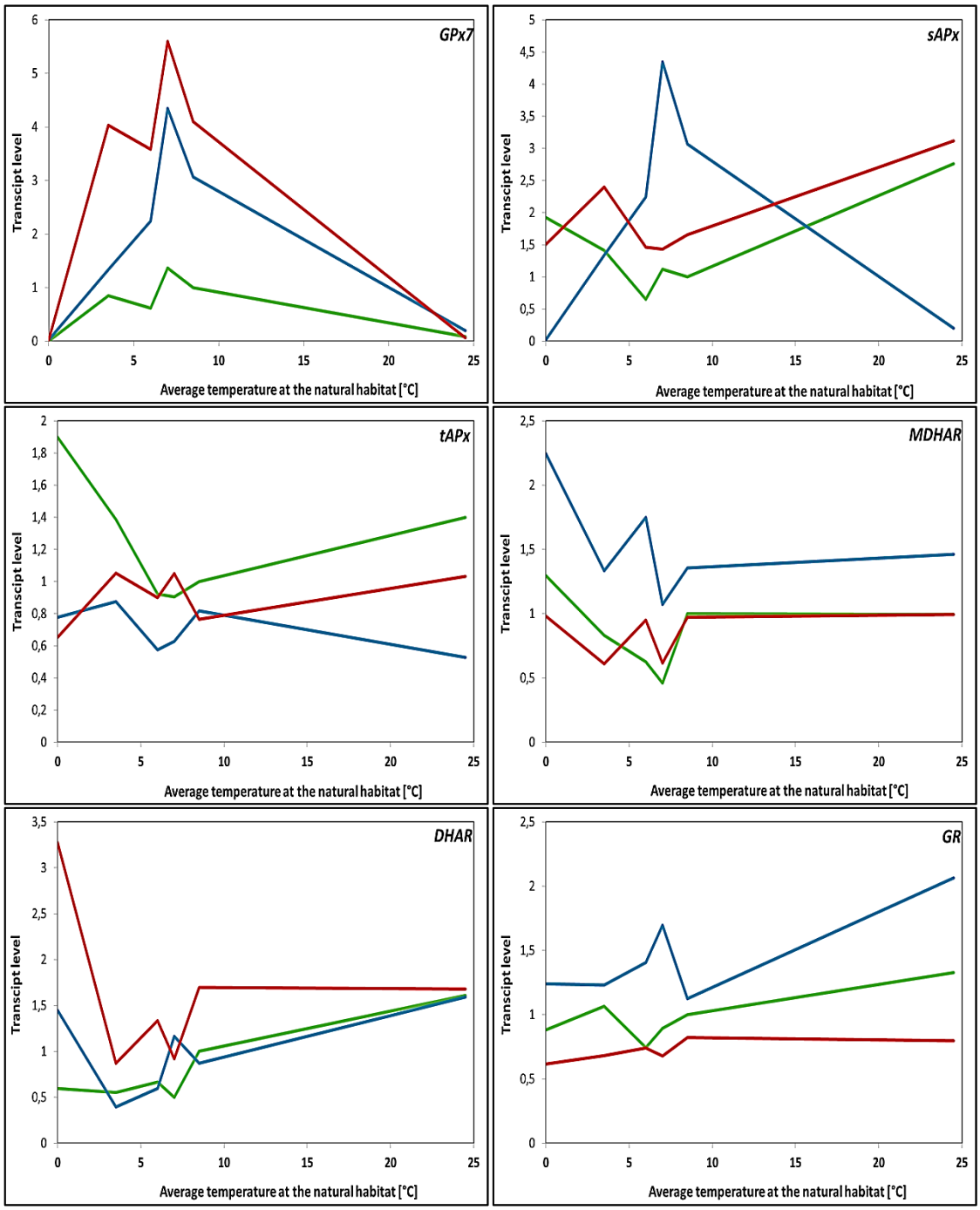

Figure 3-45 (continued). Dependency of the transcript levels of genes encoding chloroplast antioxidant enzymes on the average temperature at the natural habitats of tested accessions. The plants used for transcript analyses were cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (blue lines), 20 °C (green lines) or 30 °C (red lines), for low, control or high temperature treatment, respectively.

3.4 Main chloroplast copper-containing proteins and their expression regulation in different *Arabidopsis thaliana* accessions

Previous analyses of natural variation in the expression regulation of chloroplast antioxidant enzymes among selected accessions of *A. thaliana* revealed strong variability in the accumulation of *Csd2* mRNA upon temperature acclimation. Moreover, it was shown that the expression of this gene at the transcript level was strongly age-dependent. These observations prompted for further investigations of *Csd2* expression regulation to find which one from upstream regulators of this process led to observed accession- and age-dependent variation.

Since, the expression of *Csd2* is known to be regulated in micro-RNA dependent manner by miR398, it was analyzed whether detected variation depends on miR398 regulation itself, on the regulation of its interaction partner Ago1 or on its upstream regulator miR168a. Moreover, the correlation between variation in *Csd2* mRNA abundance and accession-specific regulation of the transcript levels of other main chloroplast copper-containing proteins, PetE2 and CCS1, was studied.

3.4.1 The levels of transcripts encoding the main chloroplast copper-containing proteins

To check whether upon temperature acclimation selected accessions of *A. thaliana* differ in the expression regulation of main chloroplast copper-containing proteins, namely Cu/Zn-superoxide dismutase (CSD2), the copper chaperone for SOD (CCS1) and plastocyanin (PetE2), the levels of their transcripts were measured in RNA samples isolated from pools of six 3-week-old rosettes of these accessions by quantitative real-time PCR (qRT-PCR). Plants used in these studies were cultivated for two weeks on soil containing 30 mg/kg copper at a day / night temperature of 20 °C / 18 °C and then acclimated for one additional week to 10, 20 or 30 °C. These temperatures span the temperature range at the natural habitats of selected accessions.

The transcript abundances of all analyzed genes were standardized on the transcript level of *Act2* gene (At3g18780) and compared to the expression of the same gene in the reference accession Col-0 grown at its optimal temperature, 20 °C.

All qRT-PCR reactions were performed for two biological replicates, each analyzed in three technical repeats.

3.4.1.1 Copper-zinc superoxide dismutase (Csd2)

The analyses of *Csd2* expression regulation in plants cultivated at different temperatures revealed that its mRNA levels responded to temperature acclimation (Figure 3-46), what was a pre-requisite for transcript correlation studies. In general, the amounts of *Csd2* mRNA gradually increased with growth temperature in almost all tested accessions, except Cvi-0, in which *Csd2* transcript level at 20 °C (0.48 ± 0.07 relative to Col-0) was lower than that observed at 10 °C (0.79 ± 0.09 relative to Col-0) and 30 °C (2.01 ± 0.07 relative to Col-0). It indicated Cvi-0-specific adaptation to temperature fluctuations.

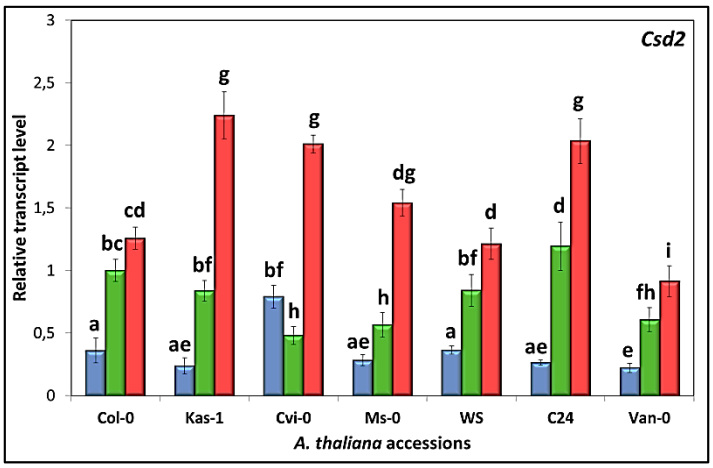

Figure 3-46. Transcript level of *Csd2* in six accession of *A. thaliana* relative to Col-0. All plants were cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (blue bars), 20 °C (green bars) or 30 °C (red bars), for low, control or high temperature treatment, respectively. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ±SD from two biological replicates, each representing an independent RNA isolation from pools of six rosettes. Each biological replicate was analyzed in three technical repeats.

Moreover, the adaptive changes in temperature responsiveness could be indicated for *Csd2* regulation (Figure 3-46). Among all tested accessions, the highest *Csd2* transcript level at 10 °C was detected for Cvi-0 (0.79 ± 0.09 relative to Col-0), which is never subjected to such low temperatures at its natural habitats, while remaining accessions (facing 10 °C in winter) accumulated the *Csd2* mRNA at significantly lower levels than in Cvi-0. On the contrary, in response to elevated temperature (30 °C) the weakest and the strongest increase in *Csd2* mRNA abundance was observed for Cvi-0 and Kas-1, respectively. It showed that Cvi-0 often facing 30 °C in summer induced the regulation

of *Csd2* expression less than Kas-1, which is hardly subjected to such high temperatures at altitude of 1580 m in Kashmir Mountains.

3.4.1.2 Copper chaperone of Csd2 (CCS1)

Similar to *Csd2*, the comparison of *CCS1* expression regulation between plants acclimated to different temperatures revealed that the level of encoding this gene transcript increased with the growth temperature in all of the selected accessions (Figure 3-47).

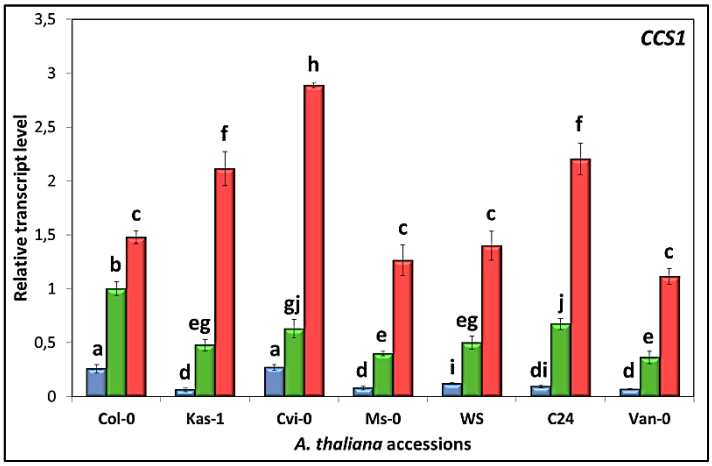

Figure 3-47. Transcript level of *CCS1* in six accession of *A. thaliana* relative to Col-0. All plants were cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (blue bars), 20 °C (green bars) or 30 °C (red bars), for low, control or high temperature treatment, respectively. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ±SD from two biological replicates, each representing an independent RNA isolation from pools of six rosettes. Each biological replicate was analyzed in three technical replicates.

Moreover, adaptive changes in temperature responsiveness were also indicated for the regulation of *CCS1* expression (Figure 3-47). Similar to *Csd2*, at 10 °C the highest levels of these transcripts were detected in Cvi-0 (0.27 ± 0.02 relative to Col-0), which is never subjected to such low temperatures at its natural habitat, while Ms-0 (0.08 ± 0.02 relative to Col-0), Van-0 (0.07 ± 0.01 relative to Col-0) and Kas-1 (0.06 ± 0.02 relative to Col-0) coming from much colder climates than Cvi-0 accumulated *CCS1* mRNA in significantly lower amounts. In contrast, stronger induction of *CCS1* accumulation in response to 30 °C was detected for Kas-1, Ms-0 and Van-0 than for Cvi-0.

3.4.1.3 Plastocyanin

In the case of plastocyanin the focus was set on *PetE2*, which is more prominently expressed than *PetE1*, responds to copper availability and affects the regulation of *Csd2* and *CCS1* (Abdel-Ghany, 2009).

The analyses of *PetE2* expression regulation in plants acclimated to different temperatures revealed that the abundances of these transcripts were more variable among tested accessions of *A. thaliana* than those observed for *Csd2* and *CCS1* (Figure 3-48).

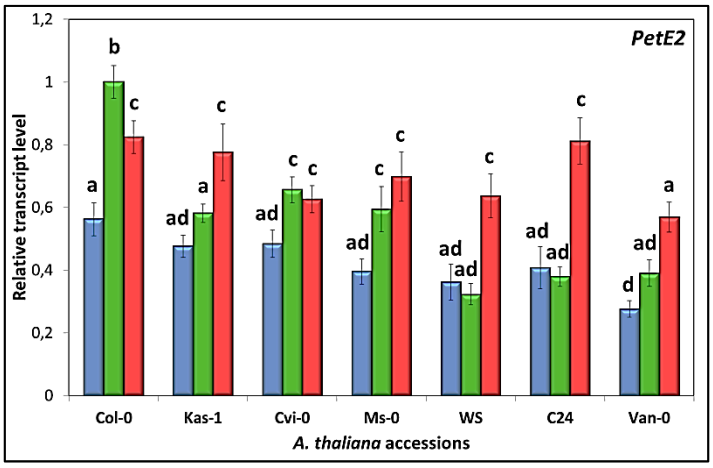

Figure 3-48. Transcript level of *PetE2* in six accession of *A. thaliana* relative to Col-0. All plants were cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (blue bars), 20 °C (green bars) or 30 °C (red bars), for low, control or high temperature treatment, respectively. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ±SD from two biological replicates, each representing an independent RNA isolation from pools of six rosettes. Each biological replicate was analyzed in three technical repeats.

However, the adaptive changes in temperature responsiveness could not be indicated for *PetE2* regulation (Figure 3-48). Thus, in originating from contrasting climates Ms-0 and Cvi-0 the same pattern of *PetE2* transcript accumulation in response to temperature fluctuations (significantly lower level of this transcript at 10 °C than at 20 °C and 30 °C) was detected. In contrast, in Kas-1 and WS coming from similar habitats to Ms-0, significantly higher amounts of *PetE2* mRNA were measured at 30 °C than at 10 °C and 20 °C. Among remaining accessions, gradual increase in *PetE2* transcript level with the growth temperature was observed for originating from cold continental habitat Van-0, while in coming from similar climate as Van-0, Col-0, the accumulation

of *PetE2* was regulated by disturbance effect (decreased upon elevated and decreased growth temperature).

Despite no adaptive changes in *PetE2* transcript accumulation upon temperature treatment, further analyses revealed that the levels of this mRNA positively correlated in all accessions with both, *Csd2* and *CCS1* transcript abundances. Among them, the weakest and the highest correlation were detected for pair *Csd2*/*PetE2* in Cvi-0 ($r_s = 0.165$) and Kas-1 ($r_s = 0.998$), respectively (Table 3-1).

3.4.2 miR398, its upstream regulators and their influence on the expression of main chloroplast copper-containing proteins

The expression of both, *Csd2* and *CCS1*, is regulated post-transcriptionally by miR398 (Sunkar *et al.* 2006; Beauclair *et al.* 2010). In the genome of *A. thaliana* miR398 is encoded by three loci: *MIR398a*, *MIR398b* and *MIR398c*. miR398b and c are identical in their sequence, whereas miR398a differs from them by a single nucleotide at 3' end (Sunkar *et al.* 2006). However, the expression of all of them is known to be controlled by upstream regulators, Ago1 and miR168a (Vaucheret *et al.* 2006). Moreover, *miR398s* expression is linked to *PetE2* expression intensity via sensing the copper availability (Abdel-Ghany, 2009). Taken together, it indicates that *Csd2* and *CCS1* transcript levels might remain not only under control of miR398, but may also depend on the expression of *PetE2* and upstream regulators of miR398.

To test this hypothesis, the abundances of *miR398a*, *miR398b/c*, *miR168a* and *Ago1* were quantified using qRT-PCR in RNA samples isolated from the same plants as that used for analyses of transcript levels of genes encoding the main chloroplast copper containing proteins.

The mRNA abundances of all selected genes were standardized on the transcript level of *Act2* gene (At3g18780) and normalized to the expression of the same gene in Col-0.

All qRT-PCR analyses were performed for at least two biological replicates, each representing an independent RNA isolation from pools of six 3-week-old rosettes of *A. thaliana* accessions acclimated to different growth temperatures (10 , 20 or 30 °C).

3.4.2.1 Correlation of Csd2 and CCS1 transcript levels with miR398 level

Comparison of *Csd2* and *CCS1* mRNAs accumulation is selected accessions of *A. thaliana* revealed that at 20 °C in all of them the levels of both transcripts were

strongly correlated (r_s > 0.95; Table 3-1), indicating that post-transcriptional regulation of *Csd2* and *CCS1* expression by miR398 is widely conserved.

To test this hypothesis, the levels of *miR398a* and *miR398b/c* were analyzed in plants acclimated to different growth temperatures (10, 20 or 30 °C) using qRT-PCR and subsequently compared with the transcript levels of *Csd2*, *CCS1* and *PetE2* measured in the same accession.

The comparison of plants acclimated to 10, 20 or 30 °C indicated significant differences in the accumulation of *miR398a* and *miR398b/c* at different temperatures in all selected accessions (Figure 3-49). In general, the gradual decrease in the level of both of these micro-RNAs with the increase in the growth temperature was observed. Moreover, in accordance with the results obtained by Yamasaki *et al.* (2007), *miR398b/c* was stronger expressed than *miR398a* (Figure 3-49). Among tested accessions, the highest *miR398a* level was detected in Cvi-0 acclimated to 10 °C (36.62 ± 0.40) and the lowest in Ms-0 at 30 °C (28.25 ± 0.93), whereas the level of *miR398b/c* was the highest in Kas-1 acclimated to 10 °C (43.96 ± 0.28) and the lowest in Cvi-0 at 30 °C (37.30 ± 0.13).

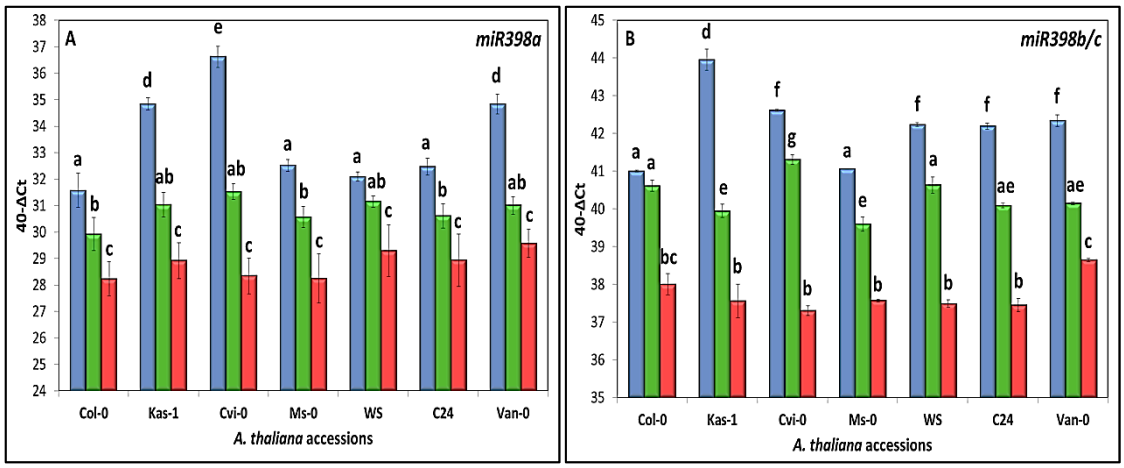

Figure 3-49. Mature **A.** *miR398a* and **B.** *miR398b/c* levels in seven accessions of *A. thaliana* cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (blue bars), 20 °C (green bars) or 30 °C (red bars) for low, control or high temperature treatment, respectively. Expression levels are given on a logarithmic scale expressed as 40 – ΔCt, where ΔCt is the difference in qRT-PCR threshold cycle number of the *miR398* and the reference gene *Act2*, whereas 40 is the cycle number when qRT-PCR reaction ends. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.05$). Data are means ±SD from two biological replicates.

To check whether the post-transcriptional regulation of *Csd2* and *CCS1* expression by miR398 is conserved among *A. thaliana* accessions, the *miR398a* and *miR398b/c*

abundances (Figure 3-49) were compared with *Csd2* and *CCS1* transcript levels (Figures 3-46 and 3-47). The comparison revealed that in each of the tested 3-week-old accessions high and low levels of *miR398* were accompanied by low or high abundances of *Csd2* and *CCS1* mRNAs, respectively. This indicated that the post-transcriptional regulation of *Csd2* and *CCS1* expression by miR398 is conserved among all selected accessions.

Since the levels of *miR398a*, *miR398b/c*, *Csd2* and *CCS1* were quantified in each accession at three different temperatures, it was possible to assess for every of them how well the decrease in the abundance of *miR398s* with the growth temperature correlated with the increase in *Csd2* and *CCS1* steady-state mRNA levels. The analyses were performed using Spearman's rank correlation coefficients (r_s) calculated for every pair of genes (Table 3-1).

The comparison of accessions revealed that the decrease in *miR398a* and *miR398b/c* abundance with the growth temperature was almost perfectly correlated ($r_s < -0.95$) with the increase in *Csd2* and *CCS1* transcripts levels in Ms-0, WS and C24 (Table 3-1). In Kas-1, *miR398a* and *miR398b/c* levels were better correlated with the expression of *Csd2* ($r_s < -0.92$) than *CCS1* ($r_s > -0.9$). In Van-0, almost perfect correlation was shown for pairs *miR398a/Csd2* ($r_s = -0.978$), *miR398bc/Csd2* ($r_s = -0.999$) and *miR398bc/CCS1* ($r_s = -0.937$), while the levels of *miR398a* and *CCS1* were correlated slightly worse ($r_s = -0.866$). Nevertheless, the strongest accession specific regulation of *Csd2* and *CCS1* expression by *miR398s* was observed for Cvi-0 and Col-0 (Table 3-1). In Cvi-0, the Spearman's rank correlation coefficients were lower for pairs *miR398a/Csd2* ($r_s = -0.660$) and *miR398a/CCS1* ($r_s = -0.862$) than for *miR398bc/Csd2* ($r_s = -0.908$) and *miR398bc/CCS1* ($r_s = -0.994$), suggesting that *Csd2* and *CCS1* expression in this accession is preferentially regulated by *miR398b/c*. On the contrary, in Col-0 the transcript levels of *Csd2* and *CCS1* were better correlated with the abundance of *miR398a* ($r_s < -0.97$) than *miR398b/c* ($r_s > -0.9$).

Table 3-1. Spearman's rank correlation coefficients (r_S) for analyzed pairs of genes

Accession	*Csd2 miR398a*	*Csd2 miR398b/c*	*CCS1 miR398a*	*CCS1 miR398b/c*	*petE2 miR398a*	*petE2 miR398b/c*	*Ago1 miR168a*	*petE2 Csd2*	*petE2 CCS1*	*Csd2 CCS1*	*Ago1 Csd2*	*Ago1 CCS1*	*miR168a Csd2*	*miR168a CCS1*
Col-0	-0.971	-0.800	-0.992	-0.863	-0.595	-0.231	0.278	0.769	0.691	0.993	0.354	0.245	-0.800	-0.963
Kas-1	-0.925	-0.931	-0.881	-0.888	-0.946	-0.951	0.849	0.998	0.987	0.995	-0.983	-0.997	-0.931	-0.888
Cvi-0	-0.660	-0.908	-0.862	-0.994	-0.850	-0.564	0.672	0.165	0.467	0.949	-0.299	-0.584	-0.908	-0.994
Ms-0	-0.967	-0.977	-0.978	-0.986	-0.974	-0.963	0.797	0.883	0.904	0.999	-0.649	-0.684	-0.977	-0.986
WS	-0.951	-0.968	-1.000	-0.999	-0.924	-0.900	0.382	0.761	0.919	0.955	-0.603	-0.339	-0.968	-0.999
C24	-1.000	-0.996	-0.960	-0.982	-0.818	-0.869	-0.738	0.818	0.947	0.960	0.672	0.853	-0.996	-0.982
Van-0	-0.978	-0.999	-0.866	-0.937	-0.920	-0.973	0.633	0.982	0.993	0.993	-0.597	-0.323	-0.973	-0.937

3.4.2.2 Sequence analyses of miR398s and their target sites in Csd2 and CCS1

The transcript levels of *Csd2* and *CCS1* were strongly correlated with *miR398s* abundance in some accessions, but only loosely in the others (Table 3-1). Such differences between tested accessions could be a result of either, specific individual control of *Csd2* and *CCS1* expression or mutations in the sequences of the micro-RNA or in its binding site in target gene. To distinguish between these two possibilities, the sequences of *miR398s* and their target sites in *Csd2* and *CCS1* were compared among tested *A. thaliana* accessions.

The comparison revealed no variation in the analyzed sequences (Figure 3-50). Thus, it can be assumed that the binding affinity of *miR398s* to the transcripts of *Csd2* and *CCS1* is the same in all accessions and the regulatory force of the tested micro-RNA depends only on its level.

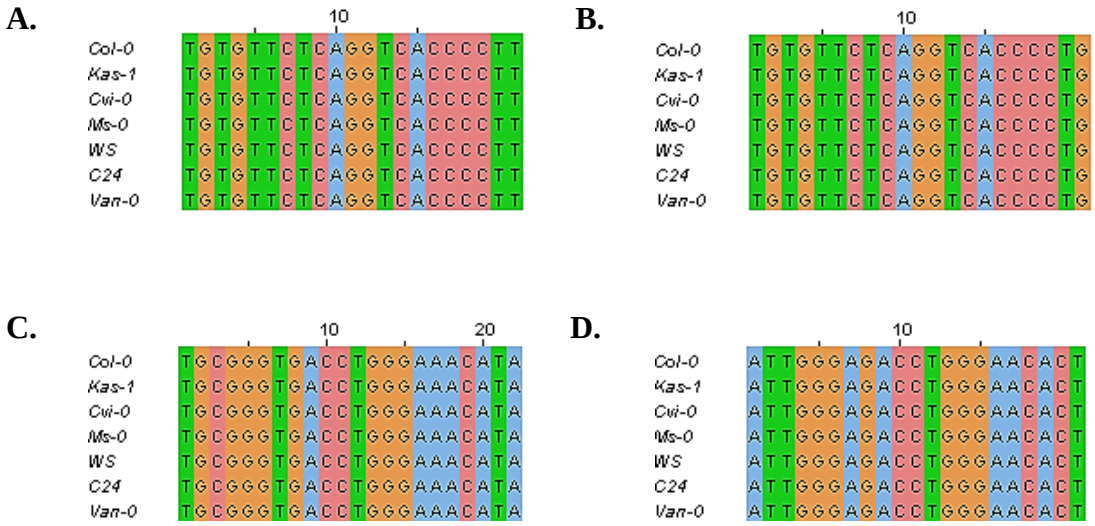

Figure 3-50. Multiple sequence alignments of **A.** *miR398a*, **B.** *miR398b/c* and sequences recognized by them in **C.** *Csd2* and **D.** *CCS1* transcripts in selected accessions of *A. thaliana*.

To test the second possibility and to check what stabilizes or breaks the miR398-dependent regulation of *Csd2* and *CCS1* mRNA levels, the correlation analyses of *miR398s* amounts with the transcript abundances of their upstream regulators, *PetE2*, *Ago1* and *miR168a*, were performed.

3.4.2.3 Correlation of PetE2 transcript level with abundance of miR398s

The comparison of tested accessions acclimated to different temperatures revealed that the levels of *PetE2* transcript were well correlated with the abundances of *miR398a* and *miR398b/c* (r_s < -0.9) only in Kas-1, Ms-0, WS and Van-0 (Table 3-1). Among all of them, the strongest negative correlation for pair *miR398a/PetE2* was indicated in Ms-0 (r_s = -0.974) and for that of *miR398bc/PetE2* in Van-0 (r_s = -0.973), demonstrating almost perfect link of *PetE2* and *miR398s* expression. In contrast, the levels of *miR398a* and *PetE2* were weakly correlated in Col-0 (r_s = -0.595), while that of *miR398b/c* and *PetE2* in Col-0 (r_s = -0.231) and Cvi-0 (r_s = -0.564). Among remaining accessions, Spearman's rank correlation coefficients were low for *miR398a/PetE2* (r_s = -0.818) and *miR398bc/PetE2* (r_s = -0.869) in C24, and for *miR398a/PetE2* (r_s = -0.850) in Cvi-0.

3.4.2.4 miR168a-Ago1 auto-regulon

To check whether the abundance of *miR398s* is controlled by their upstream regulators, the levels of *Ago1* and *miR168a* were analyzed in 3-week-old *A. thaliana* accessions acclimated to different temperatures and pairwise compared with the amounts of *miR398s* (Figure 3-51).

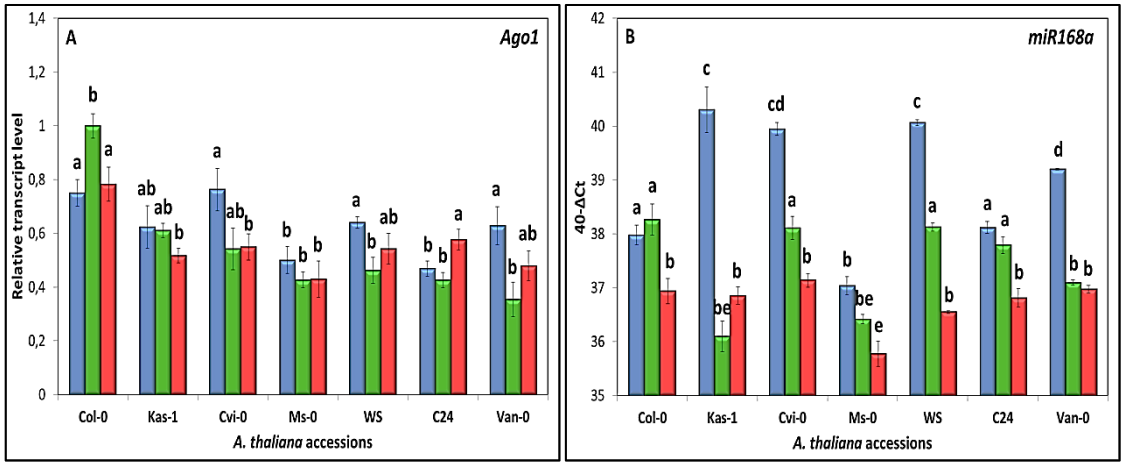

Figure 3-51. The level of **A.** *Ago1* and **B.** *miR168a* in seven accessions of *A. thaliana*. All accessions were cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (blue bars), 20 °C (green bars) or 30 °C (red bars), respectively. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$ for *Ago1* or $p < 0.05$ for *miR168a*). Data are means ±SD from two independent replicates.

The analyses revealed that the levels of both mRNAs, *Ago1* and *miR168a*, significantly differed between selected accessions and temperature treatments (Figure 3-51). The transcript abundance of *Ago1* increased or decreased in parallel upon elevated and decreased growth temperature in Col-0, WS and Van-0, was similar at all tested temperatures in Kas-1 and C24 or was the highest at 10 °C and the lowest at 30 °C in Cvi-0 (Figure 3-51). Similar variability between tested accessions was observed also for accumulation of *miR168a* (Figure 3-51). The level of this micro-RNA gradually decreased with the growth temperature in Cvi-0, Ms-0 and WS, was significantly lower at 30°C than at 10 and 20 °C in Col-0 and C24 or the highest at 10 °C and similar at 20 and 30 °C in Kas-1 and Van-0. However, despite high variability in the accumulation of *Ago1* and *miR168a* between tested accessions, no adaptive changes in temperature responsiveness could be indicated for expression regulation of these genes.

Since *Ago1* transcript level is post-transcriptionally regulated by *miR168a*, also the correlation analyses for this pair of genes were performed (Table 3-1). The calculated Spearman's rank correlation coefficients showed positive correlation between *Ago1* and *miR168a* for all tested 3-week-old accessions acclimated to different temperatures, except C24 (r_s = -0.74), in which the levels of both analyzed RNAs were correlated negatively. However, in most of the selected accessions the correlation of *Ago1/miR168a* was weak, indicating that miR168a-Ago1 auto-regulon described by Vaucheret *et al.* (2006) is generally weaker than that controlled by miR398.

3.4.2.5 Influence of Ago1 and miR168a transcripts abundance on the expression of Csd2 and CCS1

To analyze the impact of *miR168a* and *Ago1* abundances on the post-transcriptional regulation of *Csd2* and *CCS1* expression, their transcript levels were pairwise compared in selected accessions (Table 3-1). Among all of them, the abundance of *miR398s* was strongly correlated with the level of *Ago1* only in Kas-1 (r_s < -0.93), while in other accessions for tested pairs of genes either, weak negative or positive correlation, was observed. It indicated that *Ago1* expression only weakly impacts the transcript levels of *Csd2* and *CCS1*. On the contrary, the abundance of *miR168a* was well to almost perfectly negatively correlated with *Csd2* and *CCS1* transcript levels, suggesting that *miR168a* might modulate the strength of miR398-controlled regulon.

3.4.3 The miR398-Csd2-CCS1 regulon is developmentally controlled

Significant differences in *Csd2* transcript accumulation observed between 3-week-old and 6-week-old *A. thaliana* accessions, encouraged to check whether the expression of other copper-containing proteins and their upstream regulators is also developmentally controlled. Therefore, the levels of *Csd2*, *CCS1*, *PetE2* and *Ago1* mRNAs as well as abundances of mature *miR398a*, *miR398b/c* and *miR168a* were measured in selected 6-week-old *A. thaliana* accessions using qRT-PCR and subsequently compared with the levels of the same gene obtained for 3-week-old ones acclimated to 20 °C.

Main chloroplast copper-containing proteins (*Csd2*, *CCS1* and *PetE2*)

The comparison of *Csd2*, *CCS1* and *PetE2* transcript levels between 3-week-old and 6-week-old *A. thaliana* accessions revealed that the expression of all genes encoding main chloroplast copper-containing proteins is regulated in age-dependent manner (Figure 3-52).

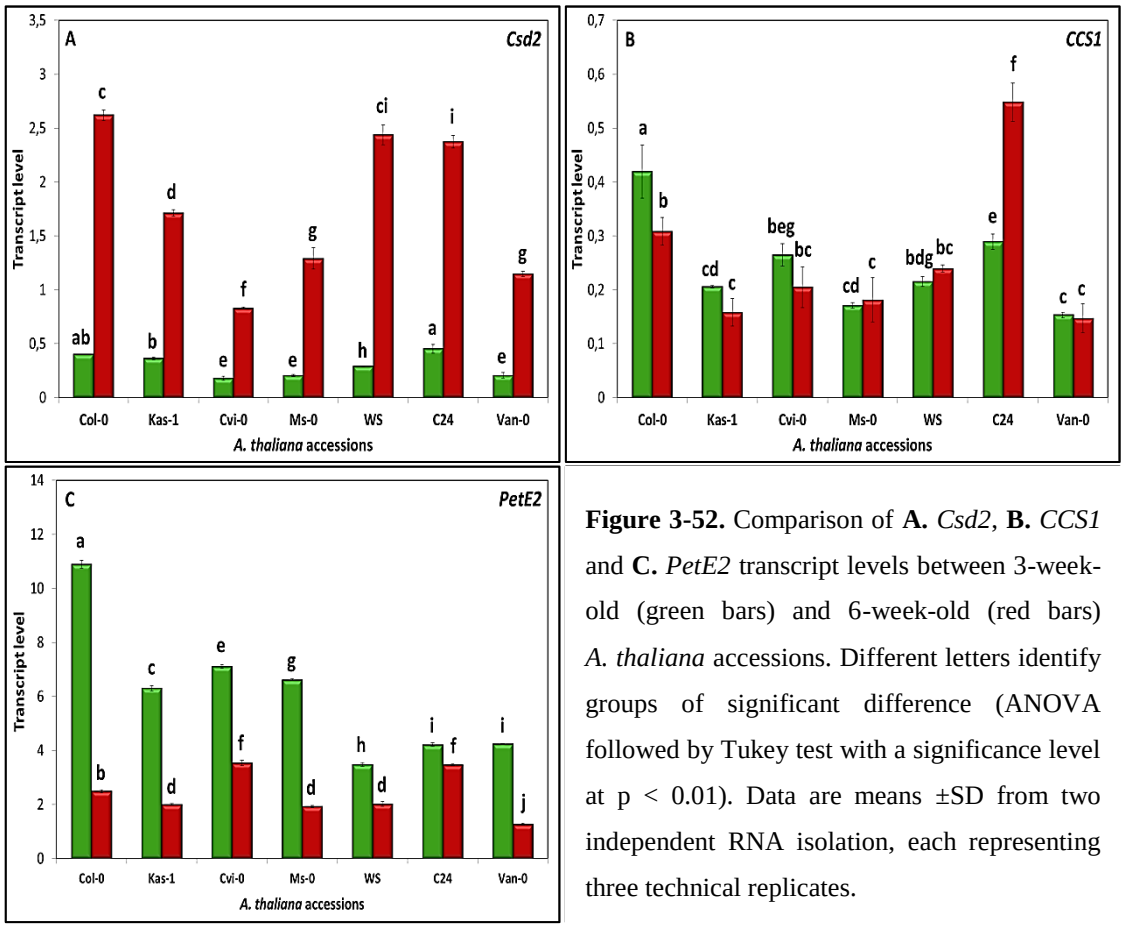

Figure 3-52. Comparison of **A.** *Csd2*, **B.** *CCS1* and **C.** *PetE2* transcript levels between 3-week-old (green bars) and 6-week-old (red bars) *A. thaliana* accessions. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$). Data are means ±SD from two independent RNA isolation, each representing three technical replicates.

Among all analyzed genes encoding copper-containing proteins, expression regulation of *CCS1* was the weakest influenced by developmental stage in all analyzed accessions (Figure 3-52). In general, the level of this transcript differed between 3-week-old and 6-week-old plants only in Col-0 and C24, in which respectively decrease or increase with the age of plants was observed. In contrast, no differences were detected in the accumulation of *CCS1* between younger and older Kas-1, Cvi-0, Ms-0, WS and Van-0.
For remaining genes, stronger than for *CCS1* influence of developmental stage on their expression was observed (Figure 3-52). In general, the level of *Csd2* transcript increased with the age of plant in all selected accessions, while *PetE2* mRNA was accumulated in higher amounts in younger than in older plants. Among all tested accessions, the highest increase in *Csd2* transcript level with the age of plants (more than eightfold) was indicated for WS, whereas the amount of *PetE2* transcript was the strongest reduced in older plants of Col-0.

miR398s (*miR398a* and *miR398b/c*)

The same analyses as for genes encoding main chloroplast copper-containing proteins were performed for the levels of mature *miR398a* and *miR398b/c* (Figure 3-53). They revealed that expression of genes encoding these micro-RNAs in almost all tested accessions (except Kas-1) is strongly age-dependent (Figure 3-53).

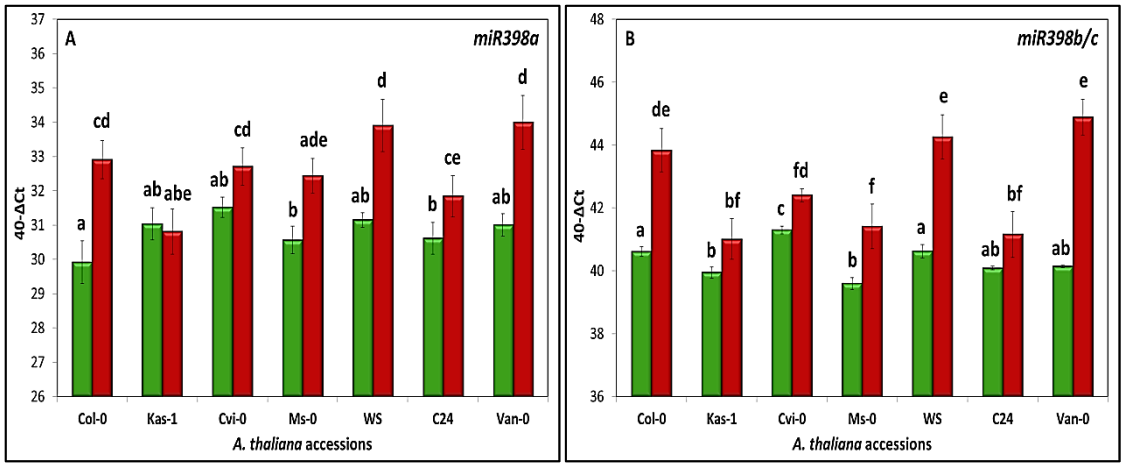

Figure 3-52. Comparison of mature **A.** *miR398a* and **B.** *miR398b/c* abundances between 3-week-old (green bars) and 6-week-old (red bars) *A. thaliana* accessions. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.05$). Data are means ±SD from two independent RNA isolation, each representing three technical replicates.

In general, increase in the levels of both *miR398s* with the age of plants was observed in all of the tested accessions (Figure 3-53). Since, miR398 negatively regulates the expression of *Csd2* and *CCS1*, consistently with the results obtained for the accumulation of *Csd2* and *CCS1* transcripts (Figure 3-52), lower level of transcripts encoding these copper-containing proteins should be observed in older than in younger plants. However, such pattern was indicated only for accumulation of *CCS1* transcript in almost all tested accessions (Figure 3-52), what shows that miR398s preferentially regulate the expression of copper chaperon for *Csd2* (*CCS1)* than that of *Csd2*.

Upstream regulators of miR398s (*Ago1* and *miR168a*)

Similar to *miR398s*, the variation in the expression of their upstream regulators, *miR168a* and *Ago1*, was also influenced by developmental stage of the plant in almost all tested accessions (Figure 3-54). In general, younger plants accumulated more *Ago1* transcript than the older ones, while the levels of *miR168a* were higher at later than at earlier from analyzed developmental stages. Such inverse patterns observed in the accumulation of *Ago1* and *miR168a* for all tested accession, indicated that Ago1-miR168a auto-regulon is active during the whole development of *A. thaliana*.

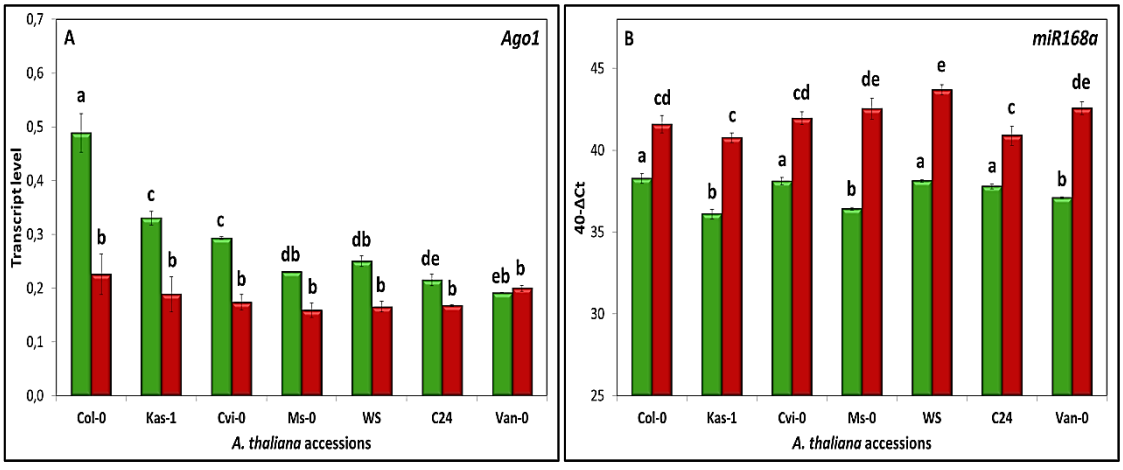

Figure 3-54. Comparison of **A.** *Ago1* transcript and **B.** mature *miR168a* abundances between 3-week-old (green bars) and 6-week-old (red bars) *A. thaliana* accessions. Different letters identify groups of significant difference (ANOVA followed by Tukey test with a significance level at $p < 0.01$ for *Ago1* and at $p < 0.05$ for *miR168a*). Data are means ±SD from two independent RNA isolation, each representing three technical replicates.

3.5 Regulation of chloroplast antioxidant system upon long-term cold stress conditions among *Arabidopsis thaliana* accessions

The temperature acclimation studies of chloroplast antioxidant system among *A. thaliana* accessions originating from distinct habitats of the northern hemisphere revealed that the levels of transcripts encoding all enzymatic components of this system were regulated in accession-specific manner and responded to growth temperature (see chapter 3.3). Such observation of different strategies in the adjustment of chloroplast antioxidant enzymes expression during evolutional adaptation of selected accessions to their natural habitats and acclimation to stressful environmental stimuli, suggests that the control of this system might also have an important role in plant adaptation to unfavourable growth conditions.

Since low temperature has a huge impact on the survival and geographical distribution of plants, the response of chloroplast antioxidant system to a long-term (2-week-long) cold stress was studied among eleven, originating from distinct habitats accessions of *A. thaliana*, namely Col-0 (186 AV), Kas-1 (N1264), Cvi-0 (166 AV), Ms-0 (93 AV), WS (84 AV), C24 (183 AV), Van-0 (161 AV), Can-0 (163 AV), Sah-0 (233 AV), N13 (266 AV) and N14 (267 AV) (see Table 2-1 for a list of selected accessions and characterization of their natural habitats). For these analyses, the basic set of accessions investigated before (Col-0, Kas-1, Cvi-0, Ms-0, WS, C24 and Van-0) was extended by additional accessions (N14, N13, Can-0 and Sah-0), which have been intensively studied for induction of cold tolerance (Zuther *et al.* 2012). For the analyses, all of these accessions were grown for six weeks on soil in a greenhouse at the MPI for Plant Physiology (Golm; collaboration with D. Hincha and E. Zuther) at a day / night temperature of 20 °C / 18 °C (NA plants) and then transferred for additional two weeks of cold acclimation to a 4 °C growth chamber (ACC plants). The cold treatment was followed by three days of de-acclimation in a greenhouse (DeA1, DeA2 and DeA3 plants).

3.5.1 Expression regulation at the transcript level upon long-term cold stress

The influence of long-term cold stress treatment on the accumulation of transcripts encoding chloroplast antioxidant enzymes, namely *Csd2* (At2g28190), *2CPA* (At3g11630), *2CPB* (At5g06290), *PrxQ* (At3g26060), *PrxIIE* (At3g52960), *sAPx*

(At4g08390), *tAPx* (At1g77490), *GPx1* (At2g25080), *GPx7* (At4g31870), *MDHAR* (At1g63940), *DHAR* (At1g19550) and *GR* (At3g54660), was analyzed using qRT-PCR in RNA samples isolated out of pool of five rosettes harvested at each experimental time point (NA, ACC, DeA1, DeA2 and DeA3 plants).

C_t values obtained for all selected genes were normalized by subtracting the mean of four reference genes, *Act2* (At3g18780), *GAPDH* (At1g13340), *EXPRS* (At2g32170) and *PDF2* (At1g13320).

All expression studies were performed for two biological replicates, each analyzed in two technical repeats. However, the results obtained for the first and the second biological replicate significantly differed (Figure 3-55). It was suggested that these differences might be due to a different light regimes in greenhouse during cultivation of the first (light on between 6 am and 10 pm during the whole experiment) and the second (for NA plants the light was all the day off, whereas during the de-acclimation phase light was on only between 6 – 10 am and 6 – 10 pm) set of plants.

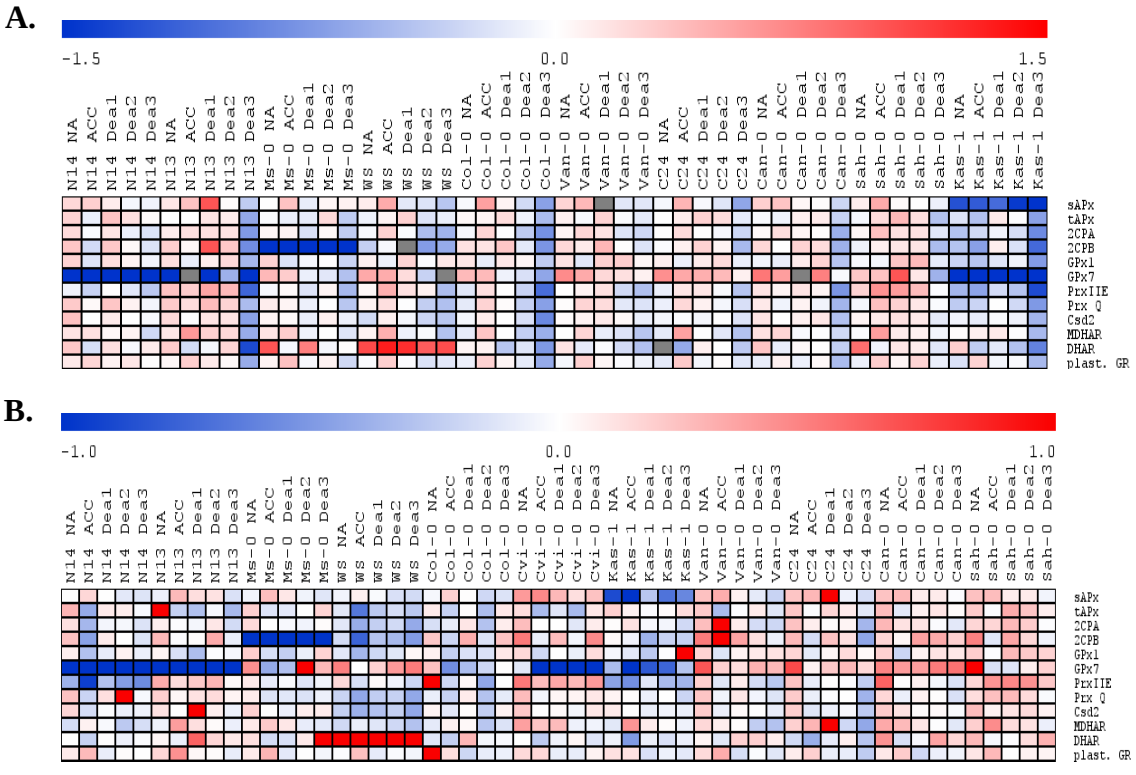

Figure 3-55. Expression of genes encoding chloroplast antioxidant enzymes in selected accessions of *A. thaliana* before (NA plants) and after (ACC plants) 14 days of cold acclimation at 4 °C, as well as during de-acclimation phase (DeA1-3 plants). Panels A and B show the results obtained for the first and the second biological replicate, respectively. The accessions are ordered from the lowest LT_{50} after cold acclimation on the left to the highest on the right.

In general, for the plants harvested in a greenhouse (NA and DeA1-3 plants) stronger activation of chloroplast antioxidant system at the transcript level in the second than in the first biological replicate was observed (Figure 3-55), indicating that the plants from the second biological replicate were cultivated under more severe growth conditions.

However, in the second biological replicate NA and DeA1-3 plants were subjected to different stress stimulus (high light stress) than ACC plants (cold stress), what makes the effects of cold and light on the expression regulation of chloroplast antioxidant enzymes hard to distinguish. Therefore, only the results obtained for the first biological replicate, in which the regulation of chloroplast antioxidant system was affected by single stimulus (cold), will be analyzed in details.

In all of the graphs presented below, selected accessions are ordered according to the increasing LT_{50} (temperature at which 50 % freezing damage occurred) measured for ACC plants (Figure 3-56; data kindly provided by E. Zuther and D. Hincha, MPIMP, Golm, Germany). The freezing damage in these accessions was determined as electrolyte leakage after freezing of detached leaves to temperatures ranging from -1 to -25 °C at 4 °C h^{-1} followed by measurement of conductivity in the water (Rohde *et al.* 2004; Hannah *et al.* 2006). The obtained values of LT_{50} give the information about the cold acclimation capacity of tested accessions and, moreover, are correlated with the latitude of their geographical origin (Zuther *et al.* 2012).

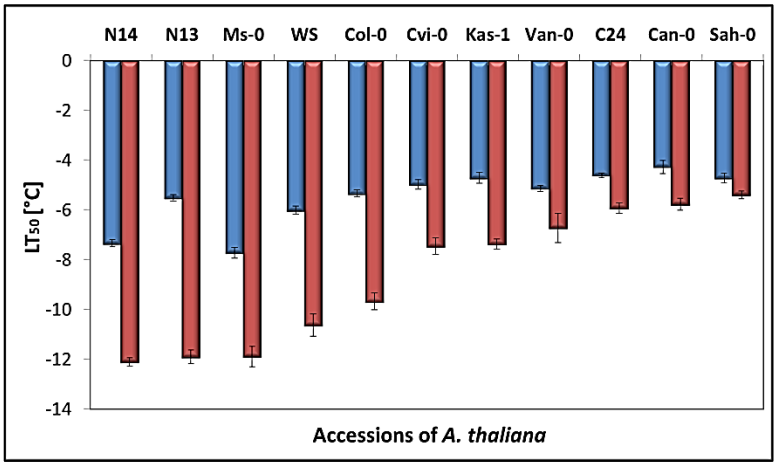

Figure 3-56. LT_{50} measured for leaves of selected *A. thaliana* accessions before (NA plants; blue bars) and after (ACC plants; red bars) 14 days of cold acclimation at 4 °C.

The analyses of LT_{50} revealed that originating from Russia N14 and N13 were the most freezing tolerant accessions (the lowest values of LT_{50}), while coming from Spain Sah-0

and Can-0 were characterized by the lowest tolerance to the freezing temperatures (Figure 3-56).

3.5.1.1. Copper-zinc superoxide dismutase (Csd2)

The comparison of *Csd2* mRNA levels between selected accessions of *A. thaliana* subjected to long-term cold stress treatment revealed that the accumulation of this transcript at each experimental time-point was controlled in accession-specific manner (Figure 3-57), indicating that tested accessions in acclimation to low temperatures used different strategies of gene expression regulation.

For NA plants, there seemed to be a tendency towards higher *Csd2* transcript levels in the most (N14, N13) and the least (Can-0 and Sah-0) freezing tolerant accessions than in the ones characterized by middle tolerance to freezing temperatures (Figure 3-57). It indicated that at growth temperature optimized for Col-0 (day / night temperature of 20 °C / 18 °C) *Csd2* expression is strongly activated only in the accessions adapted at their natural habitats to lower (N14, N13 and Ms-0) or higher temperature (Can-0 and Sah-0) than that for Col-0.

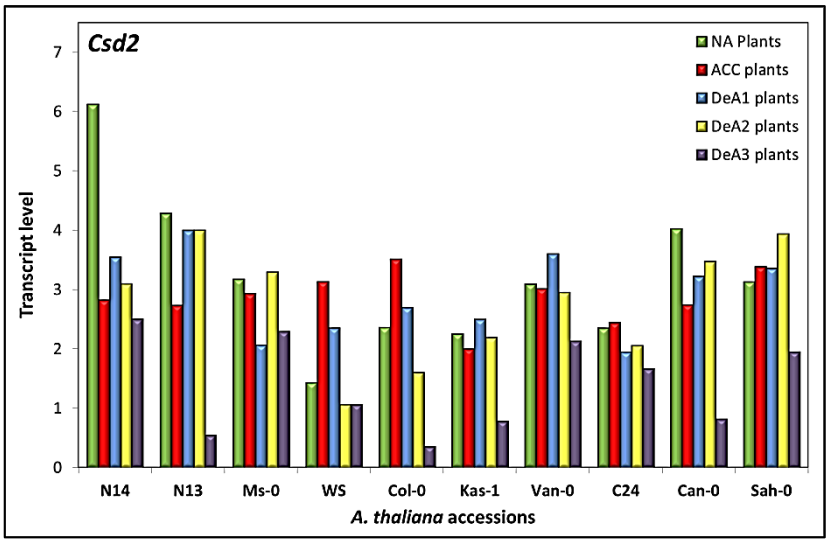

Figure 3-57. Transcript levels of *Csd2* in ten accessions of *A. thaliana* upon long-term cold stress treatment. The mRNA abundances of this gene were measured in NA plants (green bars), ACC plants (red bars), DeA1 plants (blue bars), DeA2 plants (yellow bars) and DeA3 plants (violet bars) using qRT-PCR. All analyses were performed for one biological replicate representing RNA isolated from pools of five rosettes.

Correlation of *Csd2* expression with LT_{50} could be shown also for some of the tested accessions upon cold acclimation (Figure 3-57). In general, the most freezing tolerant ones (N14 and N13) accumulated at 4 °C (ACC plants) less *Csd2* transcript than non-acclimated plants (NA plants), while in almost all accessions (except Can-0) with lower freezing tolerance than N14 and N13 either, similar or higher amounts of *Csd2* mRNA than in NA plants were measured for ACC plants. Moreover, the strongest differences in *Csd2* transcript accumulation between NA and ACC plants were detected for N14 (twofold decrease) and WS (twofold increase), indicating that in both of them cold treatment had the strongest impact on the *Csd2* expression among all tested accessions.

In contrast, no correlation of *Csd2* transcript accumulation with LT_{50} of selected accessions could be observed during de-acclimation phase (Figure 3-57). On the first day of this phase (DeA1 plants), the level of *Csd2* mRNA was higher than in ACC plants for N14, N13, Kas-1, Van-0 and Can-0, suggesting that the switch from lower to higher temperatures might lead in these accessions to the strongest induction of ROS production and, in response to high ROS levels, strong activation of chloroplast antioxidant enzymes. In contrast, lower *Csd2* transcript level in DeA1 than in ACC plants were observed for Col-0, Ms-0 and WS, the accessions, which at their natural habitats are subjected to such changes from lower to higher temperatures at least during season change from winter to spring.

On the second and the third day of de-acclimation phase (DeA2-3 plants), decrease in *Csd2* mRNA abundance was shown for all of the tested accessions (Figure 3-57). However, as for NA plants, higher levels of this transcript were generally observed for the most (DeA2 plants of N14, N13, Ms-0, as well as DeA3 plants of N14 and Ms-0) and the least (DeA2 plants of Can-0 and Sah-0, as well as DeA3 plants of Sah-0) freezing tolerant accessions than for that with the middle tolerance to low temperatures (WS, Col-0, Kas-1, Van-0, C24).

3.5.1.2 Enzymes of the ascorbate-independent water-water cycle

The same analyses as for *Csd2* were performed also for peroxiredoxins (*2CPA*, *2CPB*, *PrxQ* and *PrxIIE*) and glutathione peroxidases (*GPx1* and *GPx7*). They revealed that the accumulation of transcripts encoding all of these enzymes at all experimental

time-points was regulated by cold in accession- and gene-specific manner (Figures 3-58 and 3-59).

Peroxiredoxins (*2CPA, 2CPB, PrxQ* and *PrxIIE*)

As for *Csd2*, the highest levels of transcripts encoding almost all analyzed peroxiredoxins (except *PrxIIE*) were detected in NA plants of the most freezing tolerant accessions (N14 and N13), while in the less tolerant ones for each *Prx* different trends in the accumulation of mRNAs were observed (Figure 3-58). In general, lower levels of *2CPA* and *PrxQ* than in N13 and N14 were detected for all remaining accession, indicating that these genes are the most active in the plants originating from cold habitats (N13 and N14). In contrast, no correlation between natural habitat of tested accessions and accumulation of transcripts encoding *2CPB* and *PrxIIE* was observed (Figure 3-58). The highest levels of *PrxIIE* were detected in N13, WS, and Sah-0, while accumulation of *2CPB* was the strongest in N13, N14, Van-0 and Can-0.

In ACC plants higher levels of *2CPA* transcript than in NA plants were detected for almost all tested accessions, except N14 (Figure 3-58). Among them, the strongest induction of *2CPA* expression in response to cold acclimation was observed in originating from continental climates Col-0 and WS, as well as in coming from dry and hot habitat Sah-0. In contrast, in ACC plants higher levels of *2CPB* transcript than in NA plants were detected only for WS, C24 and Sah-0, while in ACC plants of other tested accessions *2CPB* was accumulated in lower amounts than in NA plants (Figure 3-58).

Moreover, it was shown that upon cold treatment Col-0, Kas-1, Van-0, Can-0 and N13 controlled the transcription of *2CPA* and *2CPB* inversely (increase and decrease in accumulation of *2CPA* and *2CPB*, respectively), while in other accessions changes in the same direction dominated (increase in the level of *2CPs* for WS, C24 and Sah-0 or decrease for N14). Since different trends in the accumulation of *2CPs* were observed in accessions originating from similar climates (e.g. Can-0 and Sah-0 or Col-0 and WS), it indicated that the expression of 2-Cys peroxiredoxins in plants subjected to cold stress is regulated rather in accession than in habitat-dependent manner.

The expression of remaining peroxiredoxins (*PrxQ* and *PrxIIE*) upon cold treatment was regulated in similar way to that observed for *2CPB* (Figure 3-58). In ACC plants the levels of both analyzed transcripts increased (in comparison with NA plants) in

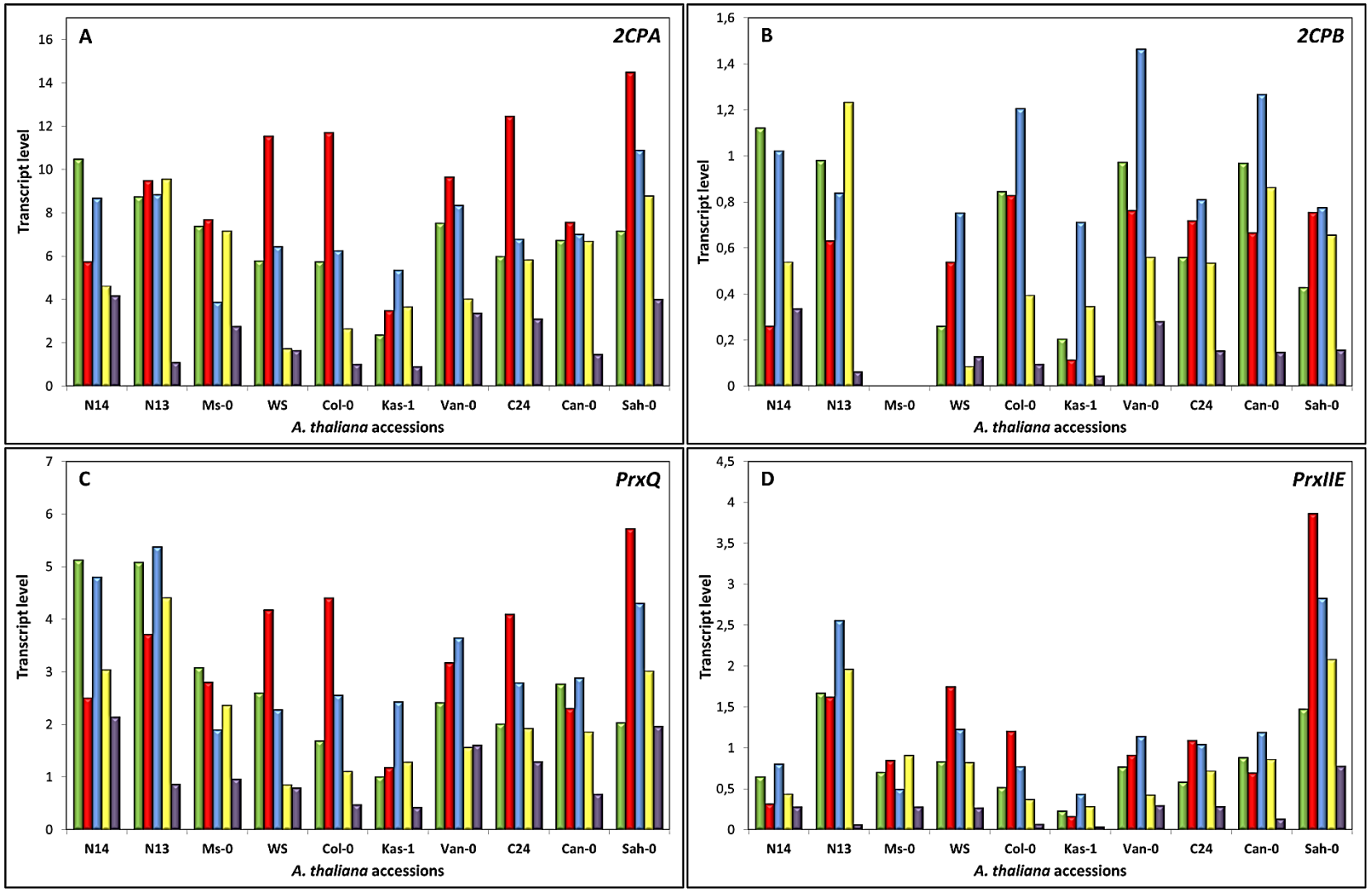

Figure 3-58. Transcript levels of **A.** *2CPA*, **B.** *2CPB*, **C.** *PrxQ* and **D.** *PrxIIE* in ten accessions of *A. thaliana* upon long-term cold stress treatment. The mRNA abundances of these genes were measured in NA plants (green bars), ACC plants (red bars), DeA1 plants (blue bars), DeA2 plants (yellow bars) and DeA3 plants (violet bars) using qRT-PCR. All analyses were performed for one biological replicate representing RNA isolated from pools of five rosettes.

Col-0, WS and Sah-0, while in other accessions, either, slightly increased (Kas-1) or decreased (Ms-0, N13 and N14) levels of *PrxQ* and *PrxIIE* transcripts were detected.
On the first day of de-acclimation phase (DeA1 plants), the levels of transcripts encoding all analyzed *Prxs* in the most freezing tolerant accessions (N14 and N13) were slightly lower than in NA plants and higher than in ACC plants, indicating that the expression of *Prxs* in these accessions is rather activated in response to 20 °C than to lower temperatures (4 °C). In contrast, remaining accessions accumulated the transcripts encoding almost all *Prxs* (except 2*CPB*) at the highest level in ACC plants, showing induction of *Prxs* expression at low temperature (4 °C).
On the second (DeA2 plants) and the third day (DeA3 plants) of de-acclimation phase lower levels of all analyzed *Prxs* than in DeA1 plants were observed in almost all accessions (except *2CPA* and *2CPB* in DeA2 plants of N13, as well as *2CPA*, *PrxQ* and *PrxIIE* in DeA2 plants of Ms-0), demonstrating that activation of *Prxs* expression by low temperature lasted only for a short time.

Glutathione peroxidases (*GPx1* and *GPx7*)

Upon cold stress conditions different expression regulation at the transcript level than that shown for peroxiredoxins was revealed for glutathione peroxidases (Figure 3-59).
In NA plants, there seemed to be a tendency towards stronger *GPx1* transcript accumulation in the more freezing tolerant accessions (N14, N13 and Ms-0), while higher levels of *GPx7* mRNA were observed in almost all accessions (except Sah-0) with low tolerance to cold, namely Van-0, C24 and Can-0 (Figure 3-59). It showed that at growth temperature optimized for Col-0 (day / night temperature of 20 °C / 18 °C) *GPx1* and *GPx7* expression was strongly activated in the accessions originating from respectively colder or warmer habitats than Col-0.
Upon cold treatment (ACC plants), the mRNA abundance of *GPx1* in all tested accessions was lower than in NA plants, but stronger reduction was shown in those originating from colder habitats (N14, N13 and Ms-0) than in that adapted to warmer climates (WS, Col-0, Van-0, C24, Can-0 and Sah-0) (Figure 3-59). This indicated that the expression of *GPx1* at the transcript level is regulated in habitat dependent manner. In contrast, no correlation between mRNA accumulation and natural habitat of tested accessions was detected for *GPx7* (Figure 3-59). In ACC plants the level of this

transcript was either higher (for Col-0 and Sah-0) or lower (for WS and Can-0) than in NA plants for accessions from warmer habitats, while in the ones adapted to colder climates (except Ms-0) the *GPx7* mRNA abundance was below detection level (Figure 3-59).

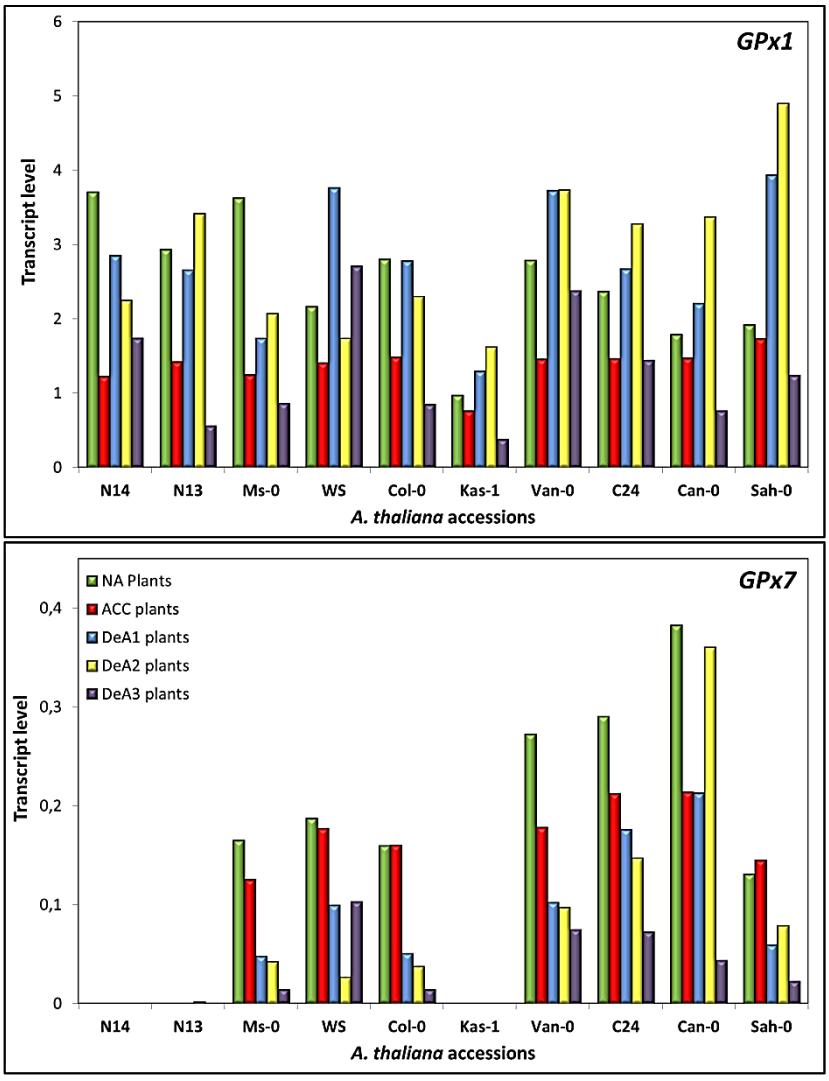

Figure 3-59. Transcript levels of *GPx1* and *GPx7* in ten accessions of *A. thaliana* upon long-term cold stress treatment. The mRNA abundances of these transcripts were measured in NA plants (green bars), ACC plants (red bars), DeA1 plants (blue bars), DeA2 plants (yellow bars) and DeA3 plants (violet bars) using qRT-PCR. All analyses were performed for one biological replicate representing RNA isolated from pools of five rosettes.

Moreover, in *GPx1* and *GPx7* mRNA abundances stronger variation, than for any other analyzed transcript, was shown during de-acclimation phase (Figure 3-59). *GPx1* transcript level was the highest in DeA2 plants and the lowest in DeA3 plants of almost

all tested accessions (except Col-0, WS and N14), showing that, in contrast to *Prxs* (the greatest transcript accumulation in DeA1 plants), expression of *GPx1* is the strongest induced on the second day of de-acclimation phase. On the contrary, mRNA abundance of *GPx7* gradually decreased during de-acclimation phase in Col-0, C24 and Can-0, was the highest in DeA2 plants for Sah-0 and the lowest in DeA2 plants for WS (Figure 3-59).

3.5.1.3 Enzymes of the ascorbate-dependent water-water cycle

Upon long-term cold stress conditions habitat-dependent variation in the expression regulation of chloroplast antioxidant enzymes was observed also for ascorbate peroxidases, *sAPx* and *tAPx* (Figure 3-60).

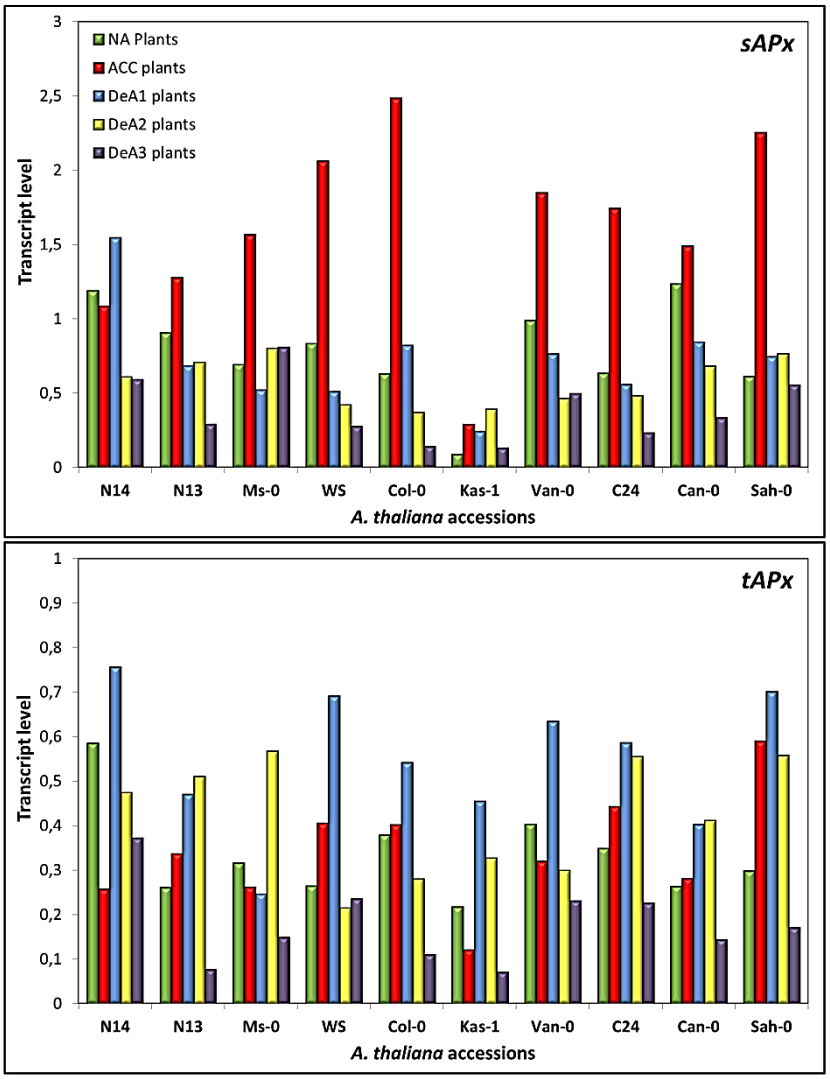

Figure 3-60. Transcript levels of *sAPx* and *tAPx* in ten accessions of *A. thaliana* upon long-term cold stress treatment. The mRNA abundances of these transcripts were measured in NA plants (green bars), ACC plants (red bars), DeA1 plants (blue bars), DeA2 plants (yellow bars) and DeA3 plants (violet bars) using qRT-PCR. All analyses were performed for one biological replicate representing RNA isolated from pools of five rosettes.

For NA plants, the highest levels of transcripts encoding *sAPx* were detected in N14 and Can-0, being the most and one of the least freezing tolerant accessions, respectively (Figure 3-60). Similarly, *tAPx* mRNA was accumulated in the greatest amounts in N14, but, in contrast to *sAPx*, among remaining accessions the highest levels of *tAPx* transcript were detected in Col-0 and Van-0 (Figure 3-60). This indicated that at growth temperature optimized for Col-0 (day / night temperature of 20 °C / 18 °C) the expression of *APxs* is the strongest induced in accessions originating from much colder climates than Col-0, while in the ones from similar or warmer habitats different patterns of transcript accumulation were observed for *sAPx* and *tAPx*.

Upon cold treatment (ACC plants), the accumulation of *sAPx* transcript was induced (in comparison with NA plants) in all tested *A. thaliana* accessions (except N14), but stronger increase was revealed for those originating from cold and continental climates (Ms-0, WS, Col-0 and Kas-1) than for those from warmer habitats (Van-0 and Can-0) (Figure 3-60). In contrast, mRNA abundance of *tAPx* decreased in almost all freezing tolerant accessions (except N13) and increased in the less tolerant ones (except Van-0) (Figure 3-60). This indicated different regulation of both analyzed *APxs* expression in accessions adapted to diverse climates.

Moreover, the habitat-dependent variation was observed for paralogue regulation of *APxs* expression (transcript levels of *sAPx* vs. *tAPx*) (Figure 3-60). Similar to *2CPs*, upon cold treatment almost all less freezing tolerant accessions controlled the transcription of *sAPx* and *tAPx* inversely (increase and decrease in accumulation of *sAPx* and *tAPx*, respectively), while in the more tolerant ones changes in the same direction dominated (increase or decrease in the level of both *APxs*).

In addition, strong differences in the accumulation of *APxs* transcripts were observed in all tested accessions during de-acclimation phase (Figure 3-60). In general, in all of them the level of both *APxs* decreased in the course of this phase, however at these experimental time points (DeA1-3 plants) no habitat-dependent pattern could be revealed (Figure 3-60).

Moreover, strong differences between *sAPx* and *tAPx* were observed in the timing of the strongest induction of their expression. In almost all tested accessions, the highest transcript level of *sAPx* was detected in ACC plants, while that of *tAPx* in DeA1 plants (Figure 3-60). This indicated that the expression of *sAPx* is the strongest activated upon

cold stress (4 °C), while that of *tAPx* right after change from low into higher temperature (4 → 20 °C).

3.5.1.4 Enzymes of the ascorbate-recycling system

Among all enzymes of ascorbate-recycling system, habitat-dependent characteristics in the temperature responsiveness of their expression regulation could be observed upon long-term cold stress conditions only for *MDHAR* (Figure 3-61).

For NA plants, the tendency of slightly higher transcript accumulation in the more freezing tolerant accessions (N14, N13 and Ms-0) was observed for *MDHAR*, while for *DHAR* and *GR* no correlation between mRNA levels and natural habitat of tested accessions could be shown (Figure 3-61). The highest amounts of *DHAR* transcript were accumulated in WS, whereas that of *GR* in N14, Ms-0 and WS (Figure 3-61).

Upon cold stress conditions (ACC plants) all tested accessions accumulated increased amounts (in comparison with NA plants) of *MDHAR* and *GR* transcripts and similar (except WS) of that encoding *DHAR* (Figure 3-61). Among them, the highest differences between NA and ACC plants (more than twofold increase) were detected for *MDHAR* in N13, WS, Kas-1, Col-0, C24 and Sah-0, while for *GR* in N13, WS, C24 and Sah-0 (Figure 3-61).

Strong differences in the accumulation of transcripts encoding ascorbate-recycling enzymes between tested accessions were observed also during de-acclimation phase (Figure 3-61). In general, the highest level of *MDHAR* mRNA for all accessions was detected in DeA1 plants, while for *GR* either in DeA1 (N13, WS, Col-0 and Van-0) or DeA2 (N14, Ms-0, Kas-1, C24, Can-0, and Sah-0) plants. Nevertheless, in the course of this phase the levels of both transcripts decreased to similar or lower levels as in NA plants.

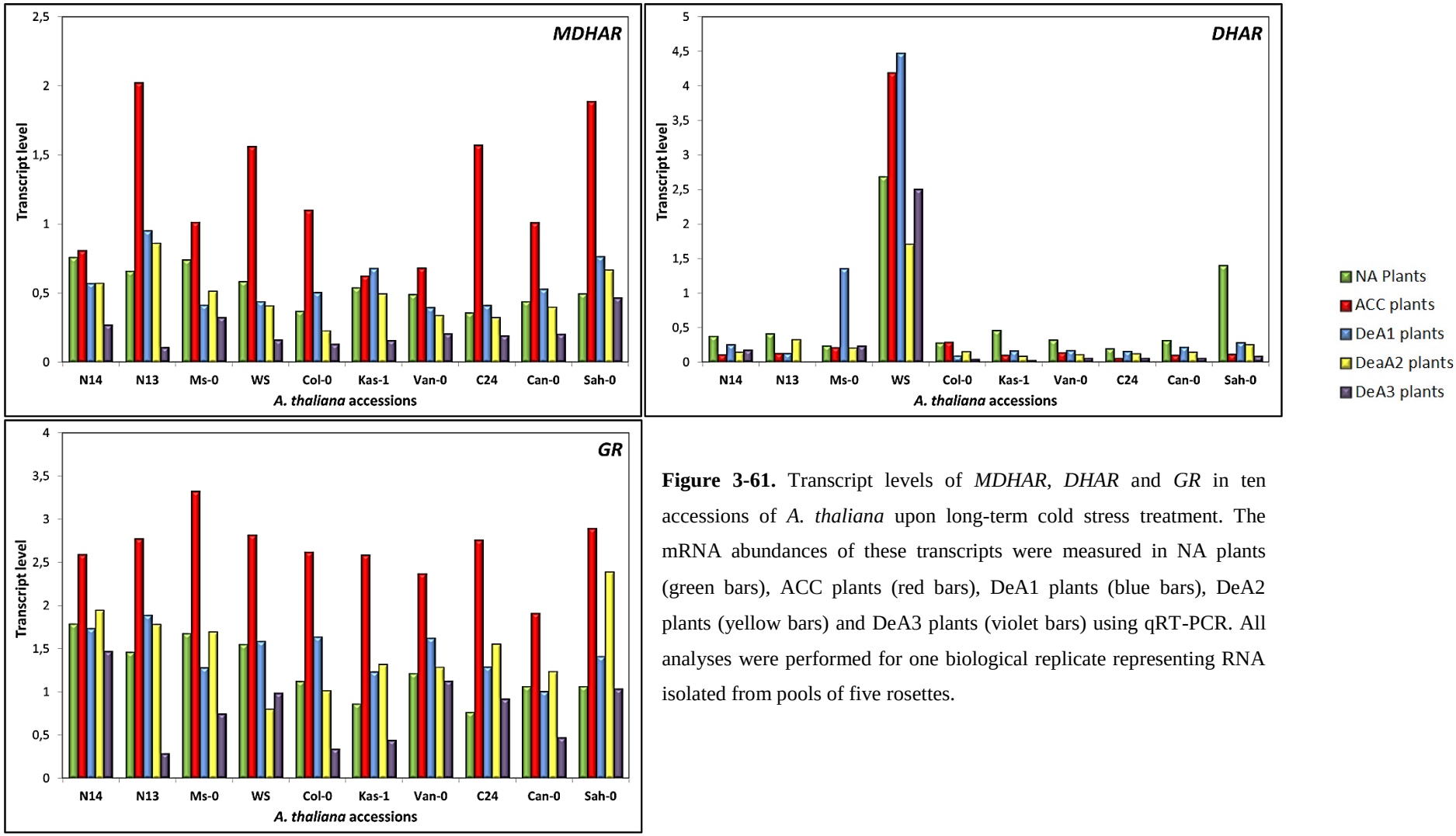

Figure 3-61. Transcript levels of *MDHAR*, *DHAR* and *GR* in ten accessions of *A. thaliana* upon long-term cold stress treatment. The mRNA abundances of these transcripts were measured in NA plants (green bars), ACC plants (red bars), DeA1 plants (blue bars), DeA2 plants (yellow bars) and DeA3 plants (violet bars) using qRT-PCR. All analyses were performed for one biological replicate representing RNA isolated from pools of five rosettes.

3.5.2 Reactive oxygen species (ROS) production upon long-term cold stress

Under a variety of environmental stress conditions, including low temperature, higher amounts of reactive oxygen species are produced (Apel and Hirt, 2004; Mittler *et al.* 2004). In many plants (e.g. maize, rice, Arabidopsis) such increase in ROS production was shown to enhance the transcript accumulation and activity of various ROS-scavenging enzymes, involved in maintaining redox balance of plant cells (Prasad *et al.* 1994; O'Kane *et al.* 1996; Sato *et al.* 2001).

Upon long-term cold stress treatment, strong differences between all tested accessions of *A. thaliana* were detected in the steady-state mRNA levels of genes encoding chloroplast antioxidant enzymes (see chapter 3.5.1). To check, whether this variability was due to different ROS production, superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) levels were determined in rosette leaves of ten *A. thaliana* accessions subjected to 14 days of cold treatment using NBT and DAB staining, respectively. All analyses were performed for mature, middle-age and young leaves harvested before (NA plants) and after (ACC plants) cold acclimation, as well as during first three days of de-acclimation phase (DeA1-3 plants). ROS production in each leaf was quantified by determination of the size of stained by NBT or DAB areas relative to the area of the whole leaf using digital image analyzer ImageJ.

To ease the comparison of quantification results, for each accession and experimental time-point, the ROS production was calculated as average from ROS levels obtained for mature, middle-age and young leaf of this accession.

For determination of ROS generation for each accession and experimental time-point ten independently grown plants were used.

3.5.2.1 NBT staining (determination of superoxide production)

The amount of superoxide radical anion ($O_2^{\bullet-}$) differed between selected *A. thaliana* accessions at each experimental time-point (Figure 3-62; for photos of stained leaves see Figure A2 in Appendix).

For NA plants, the lowest levels of the $O_2^{\bullet-}$ were detected in N14 and C24, while the highest in WS and Van-0 (Figure 3-62). Since previous results indicated that that enhancement of SOD activity was more likely a consequence of ROS levels than the cause of ROS accumulation (see chapter 3.1.4), it was suggested that SODs were the most active in WS and Van-0, and the least in N14 and C24. However, the levels of

mRNA encoding chloroplast superoxide dismutase (*Csd2*) among these accessions were the highest in N14, similar in WS and Van-0, and the lowest in C24 (see chapter 3.5.1.1). This indicated that the amount of accumulated transcripts barely reflects the activity of encoded by them enzymes.

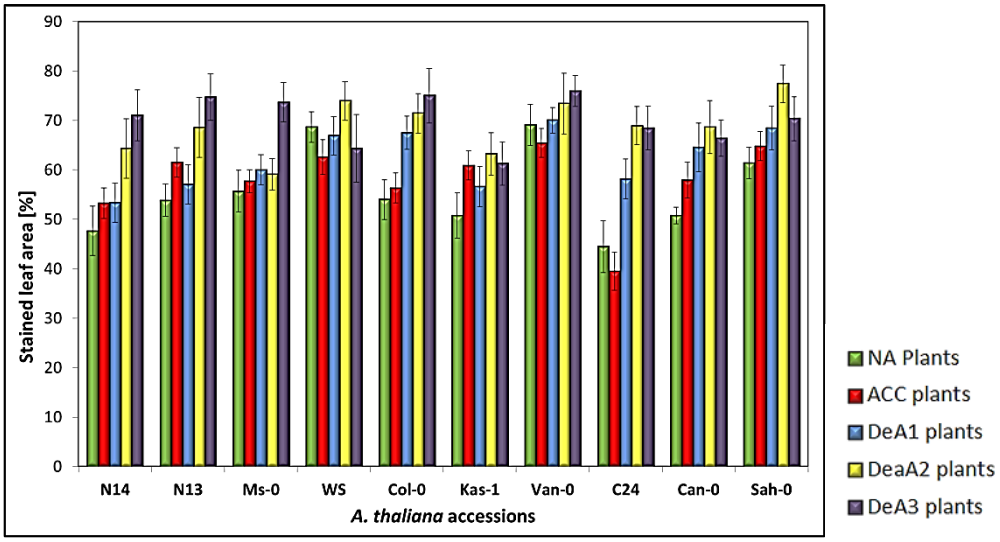

Figure 3-62. Quantification of $O_2^{\bullet-}$ levels in mature (blue bars), middle-age (green bard) and young (red bars) leaves of selected *A. thaliana* accession. For each accession and experimental time-point ten independently grown plants were used to determine superoxide production.

Upon cold acclimation no obvious increase in the $O_2^{\bullet-}$ was observed for almost all tested accessions, except N13, Kas-1 and Can-0, in which superoxide production was slightly enhanced (Figure 3-62). This indicated that in these three accessions SODs might be more active than in the remaining ones.

Differences in superoxide accumulation were observed between tested accessions also during de-acclimation phase (Figure 3-62). In general, for Col-0 and C24 increased $O_2^{\bullet-}$ levels (in comparison with NA and ACC plants) were detected on all three days of this phase (DeA1-3 plants), in N13 and N14 on the second and the third day (DeA2 and DeA3 plants) and in Ms-0 only on the third day (DeA3 plants). In the remaining accessions no obvious differences between plants harvested at all experimental time-points were detected. This indicated that the change from low into higher temperature (4 → 20 °C) is more harmful (leads to increase in superoxide generation) for N13, N14, Ms-0, Col-0 and C24 than for the other accessions. Therefore, in response to this higher in $O_2^{\bullet-}$ accumulation they need to enhance the activity of superoxide dismutases.

3.5.2.2 DAB staining (determination of hydrogen peroxide production)

Differences between tested accessions harvested at different experimental time-points were observed also in the accumulation of hydrogen peroxide (Figure 3-63; for photos of stained leaves see Figure A3 in Appendix).

For NA plants, there seemed to be a tendency towards stronger accumulation of H_2O_2 in the most (N14 and N13) and the least (Sah-0) freezing tolerant accessions than in the ones characterized by middle tolerance to freezing temperatures (Figure 3-63). It suggested that at growth temperature optimized for Col-0 (day / night temperature of 20 °C / 18 °C) H_2O_2-reducing enzymes are the most active in accessions adapted at their natural habitats to extremely low (N13 and N14) or high (Sah-0) temperatures.

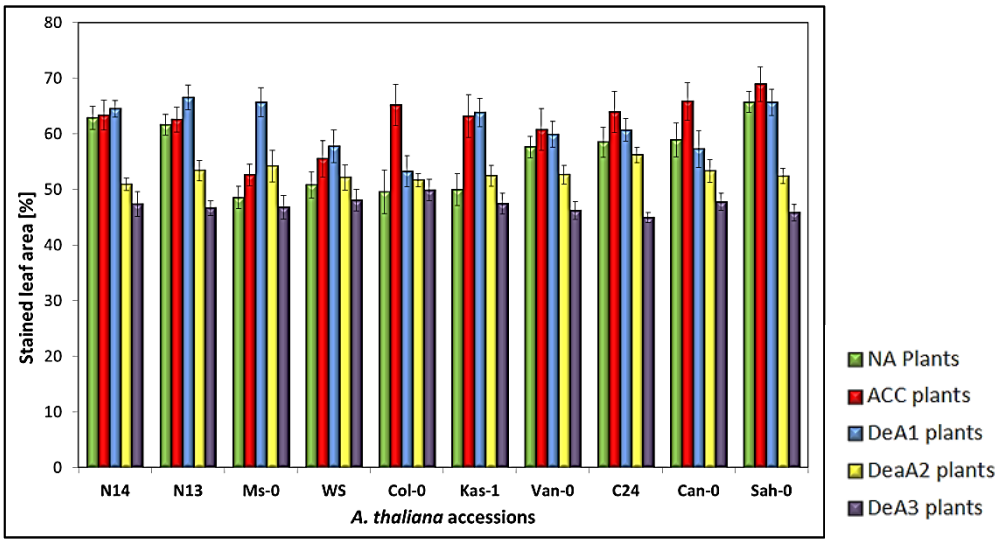

Figure 3-63. Quantification of H_2O_2 levels in mature (blue bars), middle-age (green bard) and young (red bars) leaves of selected *A. thaliana* accession. For each accession and experimental time-point ten independently grown plants were used to determine superoxide production.

Correlation between H_2O_2 accumulation and natural habitat of tested accessions could be observed also for ACC plants (Figure 3-63). In general, in the most (N14 and N13) and the least (Sah-0) freezing tolerant accessions no obvious increase in the levels of hydrogen peroxide was detected, whereas in the accessions characterized by middle tolerance to low temperatures accumulation of H_2O_2 in ACC plants was either, slightly (WS, Van-0, and C24) or much (Col-0, Kas-1) higher than in NA plants. This indicated that accessions from extremely cold or hot habitats can better acclimate to low temperatures than the ones coming from moderate and continental climates.

During de-acclimation phase for Col-0 and Can-0 decreased $O_2^{\bullet-}$ levels (in comparison with ACC plants) were detected on all three days of this phase (DeA1-3 plants), in WS only on the third day (DeA3 plants), while in the remaining accessions on the second and the third day (DeA2 and DeA3 plants) (Figure 3-63). Among all tested accessions, only in DeA1 plants of Ms-0 hydrogen peroxide was accumulated in higher amounts than in ACC plants (Figure 3-63). Moreover, in the most (N14 and N13) and the less (Van-0, C24, Can-0 and Sah-0) tolerant accessions the levels of H_2O_2 after three days of de-acclimation decreased below the levels detected in NA plants, while in those characterized by middle tolerance to low temperatures similar amounts of hydrogen peroxide were accumulated in NA and DeA3 plants. This suggested that cold treatment in accessions originating from extremely cold and warm habitats might lead to the decreased activities of H_2O_2 reducing enzymes.

3.5.3 Anthocyanins accumulation upon long-term cold stress

It is well known that cold stress leads to decrease the rate of photosynthetic electron transport (Hurry *et al.* 2000). As a result of this change, less electrons are directed for photosynthesis and more to molecular oxygen leading to ROS generation (Apel and Hirt, 2004; Mittler *et al.* 2004). Since, anthocyanins are not only plant pigments, but function also as antioxidants, their synthesis is induced by cold stress (Leyva *et al.* 1995).

At low temperature strong differences in ROS accumulation between selected accessions of *A. thaliana* were observed (see chapter 3.5.2). To check, whether these differences in ROS levels were correlated with changes in anthocyanins accumulation, anthocyanins content was measured in all of selected accessions (Figure 3-64).

The analyses revealed that in all tested accessions upon cold treatment higher amounts of anthocyanins were synthesized than in NA plants (Figure 3-64). Among all of them, the strongest induction was detected in WS, Col-0 and Van-0, accessions accumulating at low temperatures high ROS levels (see chapter 3.5.2). In contrast, the lowest increase in anthocyanins content upon cold stress treatment was observed for N14 and Can-0 (Figure 3-64), accessions accumulating at low temperatures slightly lower amounts of ROS than WS, Col-0 and Van-0.

Moreover, the highest accumulation of anthocyanins in non-acclimated plants was observed for coming from extremely hot or cold climates, Sah-0 and N13, respectively,

demonstrating the role of anthocyanins in acclimation of *A. thaliana* accessions to higher or lower temperatures than that at their natural habitats.

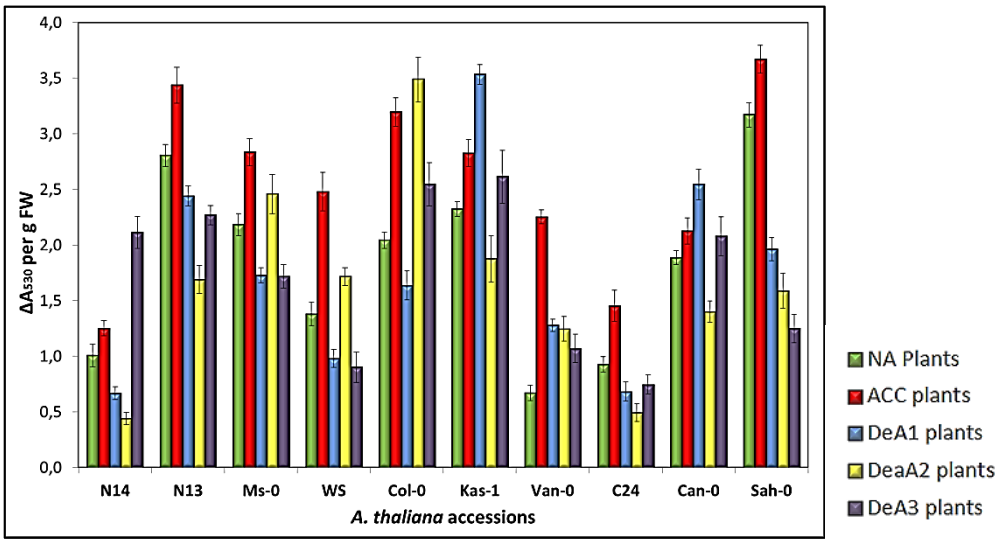

Figure 3-64. Accumulation of anthocyanins in ten accessions of *A. thaliana* upon long-term cold stress treatment at different experimental time-points: NA plants (green bars), ACC plants (red bars), DeA1 plants (blue bars), DeA2 plants (yellow bars) and DeA3 plants (violet bars). All analyses were performed for ten independently grown plants.

3.5.4 Chlorophyll accumulation upon long-term cold stress

Cold stress inhibits sucrose synthesis in the cytosol causing decreased P_i cycling between the cytosol and the chloroplasts. Thus, the chloroplasts become P_i limited impending the synthesis of ATP which is needed for regeneration of RuBisCO. This causes the feedback inhibition of photosynthesis (Hurry *et al.* 2000) and subsequently might lead to the lower chlorophyll synthesis. To test this hypothesis total chlorophyll content was calculated from the amounts of chlorophylls a and b in all selected accessions of *A. thaliana* (Figure 3-65).

The analyses revealed that after 14 days of cold treatment all accessions accumulated lower levels of chlorophyll than NA plants, but stronger decrease was generally observed in the less (WS, Kas-1, Van-0 and Sah-0) than in the most freezing tolerant ones (N14, N13 and Ms-0) (Figure 3-65). Since, ACC plants of N14, N13 and Ms-0 accumulated higher amounts of sucrose than other tested accessions (data kindly provided by E. Zuther and D. Hincha, MPIMP, Golm, Germany), it indicated that indeed decreased sucrose synthesis at low temperatures slows down photosynthesis and leads to the accumulation of lower chlorophyll levels.

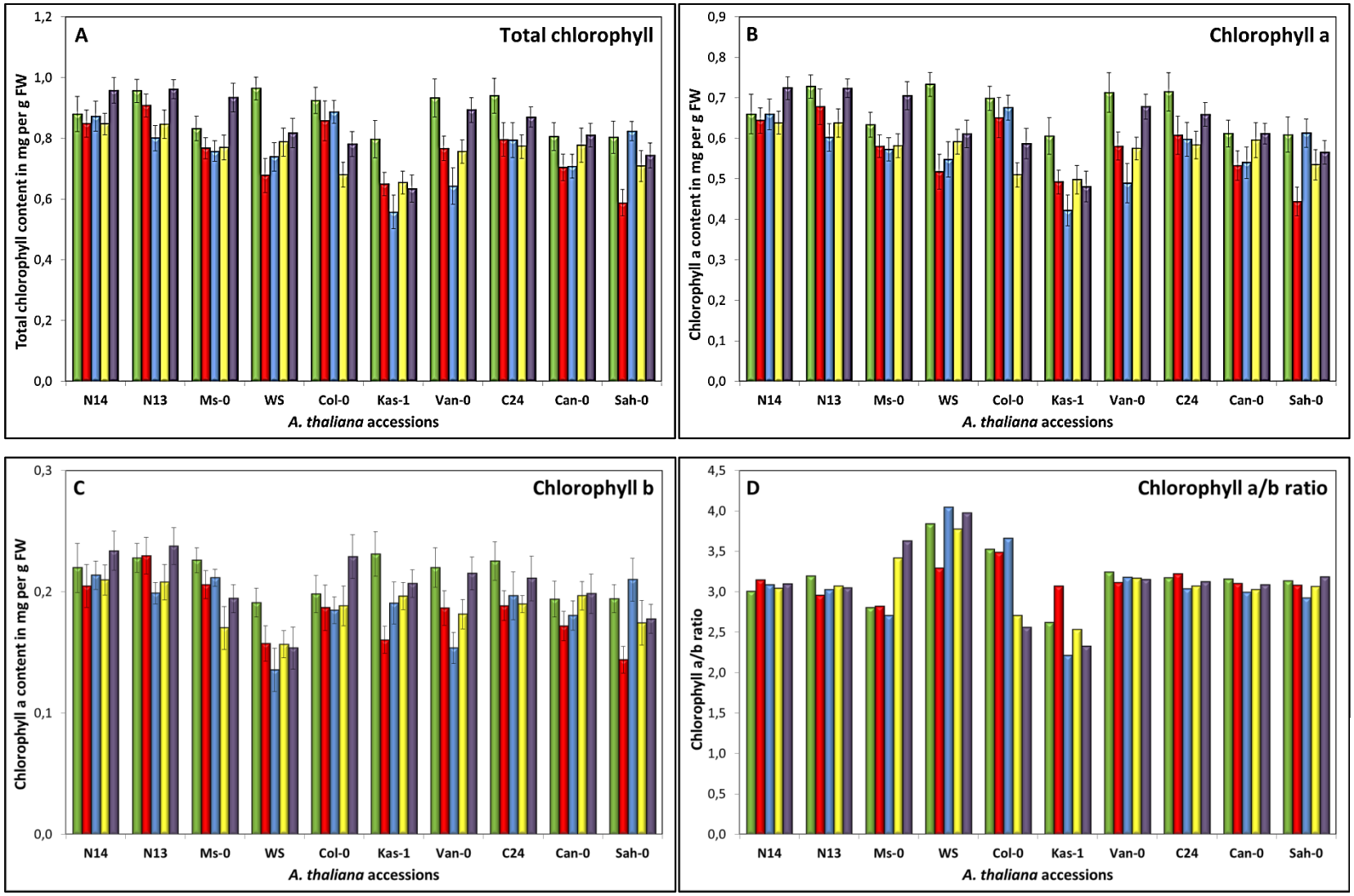

Figure 3-65. Accumulation of **A.** total chlorophyll, **B.** chlorophyll a and **C.** chlorophyll b, as well as chlorophyll a/b ratio **(D)** in ten accessions of *A. thaliana* upon long-term cold stress treatment. All of the chlorophylls were measured in NA plants (green bars), ACC plants (red bars), DeA1 plants (blue bars), DeA2 plants (yellow bars) and DeA3 plants (violet bars). All analyses were performed for ten independently grown plants.

During de-acclimation phase (DeA plants) in the most (N14, N13 and Ms-0) and the less freezing tolerant accessions (Can-0 and Sah-0) similar amounts of chlorophyll as in NA plants were accumulated, while in the accessions characterized by middle tolerance to low temperatures lower levels of chlorophyll were measured for DeA than for NA plants (Figure 3-65). It suggested that the photosynthesis in accessions originating from extremely cold and hot habitats is better acclimated to the changes in the temperatures from low into higher (4 → 20 °C) than that of the accessions from moderate climates.

Moreover, strong differences observed between tested accessions in the chlorophyll a and b contents prompted for comparison of chlorophyll a/b ratio in all of these accessions (Figure 3-65). These analyses revealed that the ratio was similar at all experimental time-points in the most (N14 and N13) and the least (Van-0, C24, Can-0 and Sah-0) freezing tolerant accessions, while in the ones characterized by middle tolerance to low temperatures (Col-0, Ms-0, WS and Kas-1) different chlorophyll a/b ratio was calculated for DeA and NA plants, demonstrating accessions specific regulation of chlorophyll accumulation during de-acclimation phase (Figure 3-65).

3.6 Expression regulation of chloroplast antioxidant system and thermomemory upon short-term cold stress conditions among *Arabidopsis thaliana* accessions

Significant variation in the expression regulation of chloroplast antioxidant system upon long-term cold stress treatment prompted for further analyses of responses, which are generated in this system at the transcript level upon short-term (24-hour-long) cold stress conditions. In contrast to the long-term cold stress, in which freezing tolerance can be induced, in short-term one the redox status is just imbalanced. Therefore, both stresses might lead to different changes in the expression regulation of chloroplast antioxidant enzymes.

The hypothesis was tested by comparison of responses induced in the expression of genes encoding these enzymes between seven out of ten analyzed upon long-term cold stress treatment accession of *A. thaliana*, namely Col-0 (186 AV), Kas-1 (N1264), Cvi-0 (166 AV), Ms-0 (93 AV), WS (84 AV), C24 (183 AV), Van-0 (161 AV). The plants were grown for four weeks in a climate-controlled chamber at a day / night temperature of 20 °C / 18 °C (NA plants) and then, by cultivation for 24 hours in a 4 °C growth chamber, subjected to the cold stress (Primed plants).

Moreover, it is known that the exposure to the stressful stimulus (e.g. cold stress) can make plants more resistant to the future exposure to the same or another stress stimulus. This phenomenon is called priming. For plants the advantage of being primed for particular stress is faster and/or stronger activation of the various defence responses if the stress recurs (Conrath *et al.* 2006). Moreover, it provides benefit of enhanced protection without the costs associated with constitutive expression of stress related genes (van Hulten *et al.* 2006).

Here, it is hypothesized that the chloroplast antioxidant system can act as a memorizing hub, which parameterization by the priming signal and during the lag-phase defines the response to the triggering stimulus. The hypothesis was tested by comparison of the effects caused by priming on the response of chloroplast antioxidant enzymes to the triggering stimulus (24 hours at 4 °C; Trig plants).

Moreover, to monitor the changes in the expression activation of chloroplast antioxidant enzymes initiated by priming and triggering stimuli, the expression regulation of genes encoding these enzymes was analyzed also during the first three days of de-acclimation

phase (in 20 °C / 18 °C growth chamber) following each cold stress stimulus (DAP1-3 and DAT1-3 for plants studied after priming and triggering stimulus, respectively).

3.6.1 Expression regulation at the transcript level upon short-term cold stress

Since, the strongest habitat-dependent variation in the expression regulation of chloroplast antioxidant system upon long-term cold stress treatment was observed in the expression of genes encoding Cu/Zn-superoxide dismutase (*Csd2*), ascorbate peroxidases (*sAPx* and *tAPx*), 2-Cys peroxiredoxins (*2CPA* and *2CPB*) and glutathione peroxidases (*GPx1* and *GPx7*), these genes were chosen for further studies of their expression regulation at the transcript level in *A. thaliana* accessions subjected to short-term cold stress treatment. Moreover, to check whether 24 hours of cold treatment were sufficient for induction of cold responses, the same analyses as for genes encoding chloroplast antioxidant enzymes were performed also for one of the cold responsive genes, *COR15A* (Zuther *et al.* 2012).

The transcript levels of all selected genes were measured using qRT-PCR and gene specific primers, namely qPCR_Csd2_2, qPCR_sAPx_1, qPCR_sAPx_2 (used only for quantification of *sAPx* transcript level in Kas-1), qPCR_tAPx, qPCR_2CPA, qPCR_2CPB_2, qPCR_GPx1, qPCR_GPx7 and qPCR_COR15A (see Table 2-3 for a list). All of these primers were designed to bind in the region of particular gene, where no single nucleotide polymorphisms (SNPs) in all tested accessions were found.

The transcript levels of analyzed genes were standardized on the mRNA abundance of *Act2* gene (At3g18780) and normalized to the expression of the same gene in NA plants of Col-0.

All analyses were performed for one biological replicate representing RNA isolated out of pool of five rosettes harvested at each experimental time point, namely NA, Primed, DAP1-3, Trig and DAT1-3 plants.

3.6.1.1 Cold regulated gene (COR15A)

To check, whether 24 hours of cold treatment were sufficient for induction of cold responses, the expression of cold responsive gene, *COR15A*, which have frequently been associated with the cold acclimation (Thomashow, 1999), was analyzed in selected accessions of *A. thaliana* (Figure 3-66).

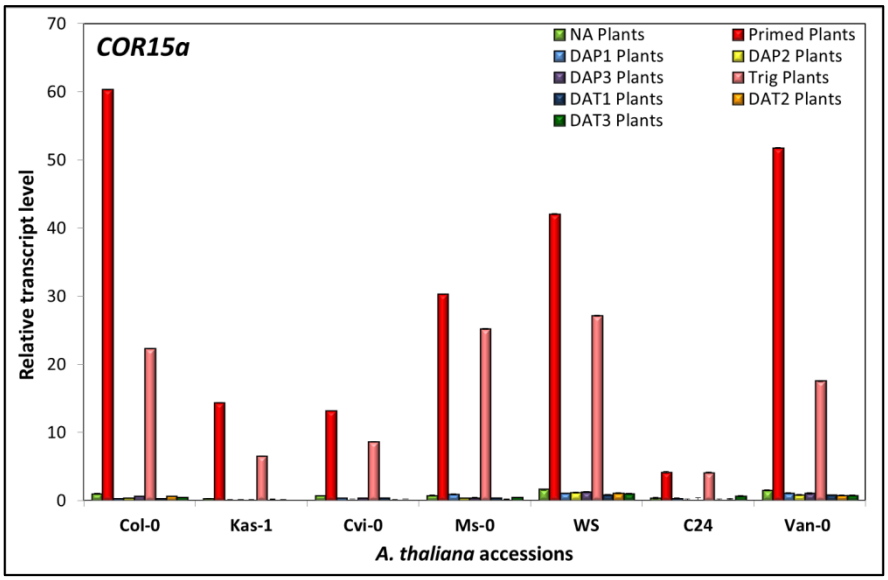

Figure 3-66. Transcript levels of *COR15A* in seven accessions of *A. thaliana* upon short-term cold stress treatment. The mRNA abundances of this gene were measured in NA plants (light green bars), Primed plants (red bars), DAP1 plants (light blue bars), DAP2 plants (yellow bars), DAP3 plants (violet bars), Trig plants (pink bars), DAT1 plants (navy blue bars), DAT2 plants (orange bars) and DAT3 plants (dark green bars) using qRT-PCR. All analyses were performed for one biological replicate representing RNA isolated from pools of five rosettes.

The analyses revealed that the expression of *COR15A* in all tested accessions after application of cold stress stimuli was strongly induced (Figure 3-66), demonstrating that 24 hours of cold treatment were sufficient for activation of cold responsive genes. Moreover, for Primed and Trig plants the correlation between the levels of *COR15a* transcripts and natural habitat of selected accessions could be shown (Figure 3-66). In general, *COR15A* mRNA was accumulated in lower amounts in accessions originating from cold habitats (Kas-1, Ms-0) than in the ones from warmer climates (Col-0, WS, Van-0), suggesting that Kas-1 and Ms-0 are better adapted to cold than Col-0, WS and Van-0. Therefore, subjecting them to cold stress treatment did not lead to high accumulation of transcripts encoding cold responsive genes. However, low levels of *COR15A* transcripts in Primed and Trig plants were also detected in Cvi-0 and C24, accessions characterized by weak tolerance to low temperatures (Figure 3-66). Since, Cvi-0 at its natural habitat is never subjected to temperature of 4 °C, the low activation of *COR15A* transcription is probably not an effect of its evolutional adaptation to low temperatures, but rather indicates low ability of acclimation to cold.

Upon application of triggering stimulus (Trig plants) all tested accessions accumulated lower amounts of *COR15A* mRNA than in Primed plants (Figure 3-66). This indicated that *A. thaliana* accessions when subjected to priming cold stimulus, generated thermomemory, which was "stored" in the course of de-acclimation phase and enabled for faster and more efficient response to triggering cold stimulus. Therefore, as high activation of cold regulated genes as in Primed plants, was not required in Trig plants.

3.6.1.2 Copper-zinc superoxide dismutase (Csd2)

The comparison of *Csd2* transcript levels between *A. thaliana* accessions subjected to short-term cold stress treatment revealed that the accumulation of this transcript was regulated by cold in accession- and habitat-dependent manner (Figure 3-67).

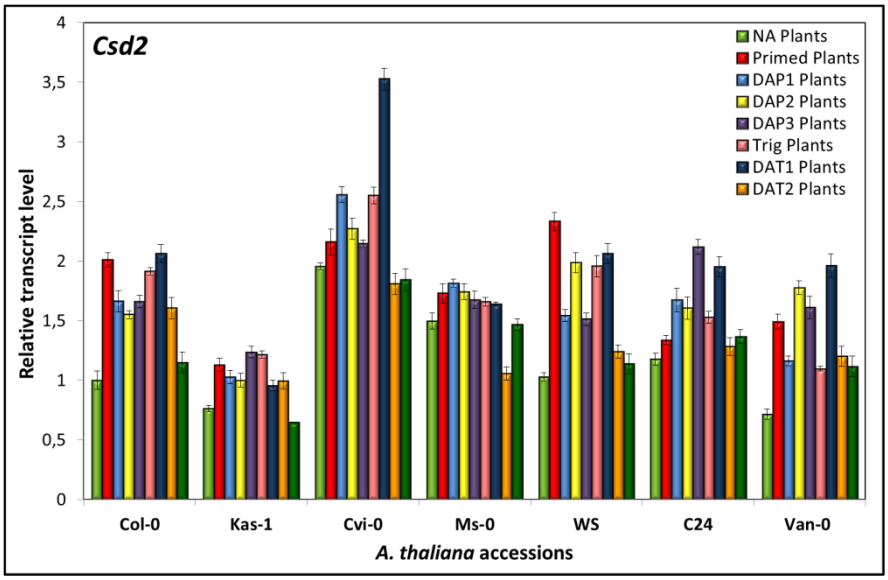

Figure 3-67. Transcript levels of *Csd2* in seven accessions of *A. thaliana* upon short-term cold stress treatment. The mRNA abundances of this gene were measured in NA plants (light green bars), Primed plants (red bars), DAP1 plants (light blue bars), DAP2 plants (yellow bars), DAP3 plants (violet bars), Trig plants (pink bars), DAT1 plants (navy blue bars), DAT2 plants (orange bars) and DAT3 plants (dark green bars) using qRT-PCR. All analyses were performed for one biological replicate representing RNA isolated from pool of five rosettes.

Among all non-acclimated accessions (NA plants) the highest levels of *Csd2* transcript were detected in Cvi-0 and Ms-0, accessions originating from extremely hot and cold habitats, respectively (Figure 3-67). In contrast, Kas-1 and Van-0, coming also from cold climates, accumulated this transcript in the lowest amounts among all tested accessions (Figure 3-67). This demonstrated that under non-stressful conditions the

expression of *Csd2* at the transcript level is regulated rather in accession- than in habitat-dependent manner.

Upon cold treatment (Primed plants), all tested accessions of *A. thaliana* accumulated more *Csd2* transcript than NA plants, but stronger increase in its level was detected for the ones originating from continental habitats (Col-0, WS, Van-0), than for that from extremely hot (Cvi-0) or cold climates (Kas-1, Ms-0) (Figure 3-67). It suggested that this low activation of *Csd2* transcript accumulation in response to cold stress observed in Ms-0, WS and Cvi-0 might be due to either, effective evolutional adaptation to low temperatures (for Ms-0 and WS) or low sensitivity of Cvi-0-specific *Csd2* gene allele to cold.

Strong differences in the accumulation of *Csd2* transcript were observed between tested accessions also in the course of de-acclimation phase following priming stimulus (Figure 3-67). On the first day of this phase (DAP1 plants) similar or lower amounts of *Csd2* mRNA than in ACC plants were detected in almost all accessions, except Cvi-0 and C24, in which the level of this transcript in DAP1 plants was higher than in ACC plants. Since, for Cvi-0 and C24 stronger increase in the amount *Csd2* mRNA was observed after transfer from low to higher temperature (4 → 20 °C; Primed plants) than after that from higher to lower temperature (20 → 4 °C; DAP1 plants), it was suggested that *Csd2* in these accessions is preferably induced by higher temperatures.

On the second day of the lag phase (DAP2 plants) all accessions accumulated generally similar or higher amounts of *Csd2* mRNA, but no habitat-dependent characteristics in the regulation of *Csd2* expression at the transcript level could be revealed on this day (Figure 3-67). In contrast, such characteristics were observed for DAP3 plants (Figure 3-67). In general, DAP3 plants of accessions originating from extremely hot (Cvi-0) or cold climates (Kas-1, Ms-0) accumulated higher or similar amounts of *Csd2* mRNA to ACC plants, while in the ones from continental habitats (except Van-0) the level of *Csd2* transcript was much lower in DAP3 than in ACC plants. The decrease in the amount of *Csd2* mRNA in DAP3 plants (in comparison with ACC plants) observed in Col-0 and WS demonstrated that the activation of *Csd2* expression by priming stimulus in these accessions lasted shorter than in Ms-0, Kas-1, Cvi-0, Van-0 and C24, in which *Csd2* transcript level was similar or higher in DAP3 than in ACC plants. Moreover, it could indicate that Col-0 and WS are not able to generate thermomemory in *Csd2* expression.

Indeed, in Col-0, WS and Van-0 the *Csd2* mRNA abundance detected in Trig plants (subjected to the second cold stimulus) was lower than in ACC plants (Figure 3-67), suggesting that these accessions are not able to generate thermomemory in *Csd2* expression. In contrast, Kas-1, Cvi-0 and C24 accumulated in Trig plants more *Csd2* mRNA than in ACC plants, what indicated that they "remembered" the activation of *Csd2* expression by the priming stimulus and subjecting them to the triggering stimulus led to even stronger accumulation of *Csd2* transcript than in ACC plants and subsequently stronger defence against stressful conditions.

During de-acclimation phase following application of triggering stimulus (DAT1-3 plants) differences in the accumulation of *Csd2* transcript between tested accessions were observed only on the first day of this phase (Figure 3-67). In Col-0, Cvi-0, C24 and Van-0 the level of this mRNA in DAT1 plants increased above that detected in Trig plants, while in the other accessions, either similar or lower *Csd2* amounts were accumulated in DAT1 and Trig plants. Afterwards (DAT2-3 plants), the level of *Csd2* transcript decreased in all tested accessions to similar levels as in NA plants, demonstrating reset of *Csd2* expression to the state before application of cold stress stimuli (NA plants).

3.6.1.3 Ascorbate peroxidases (APxs)

Upon short-term cold stress treatment habitat-dependent characteristics in the responsiveness to temperature acclimation could be also shown for expression regulation of *sAPx* and *tAPx* (Figure 3-68).

In non-acclimated plants (NA plants) the highest amounts of transcripts encoding both chloroplast ascorbate peroxidases (*sAPx* and *tAPx*) were detected in Cvi-0, demonstrating the strongest activation of *APxs* expression at the transcript level among all tested accessions (Figure 3-68). In contrast, Col-0 and Kas-1 accumulated the lowest levels of *sAPx* and C24 that of *tAPx* (Figure 3-68).

After 24 hours at 4 °C (Primed plants) *sAPx* transcript was accumulated at higher levels than in NA plants in all tested accessions, but stronger increase was detected for those coming from continental (Col-0, WS) and hot climates (Cvi-0), than for those from colder habitats (Kas-1, Ms-0) (Figure 3-68). In contrast, *tAPx* transcript levels were lower in Primed than in NA plants for almost all accessions (except Col-0 and C24) but stronger decrease in the accumulation of *tAPx* mRNA was observed in accessions

originating from extremely warm (Cvi-0) or cold (Kas-1, Ms-0) habitats than in the ones from moderate and continental climates (WS and Van-0). Thus, among all tested accessions the strongest response of *APxs* expression to temperature acclimation was demonstrated in originating from extremely hot habitat Cvi-0.

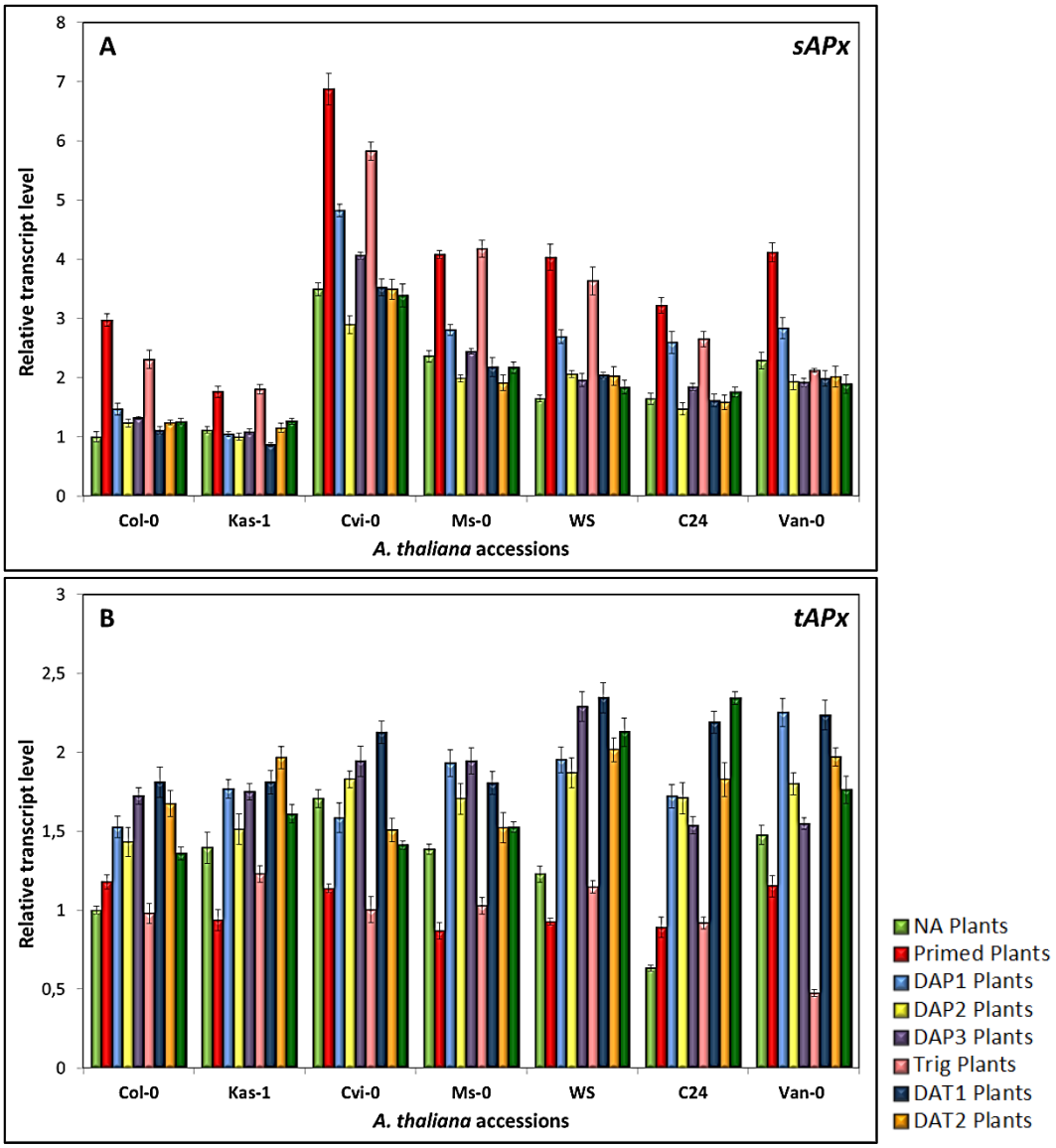

Figure 3-68. Transcript levels of **A.** *sAPx* and **B.** *tAPx* in seven accessions of *A. thaliana* upon short-term cold stress treatment. The mRNA abundances of these genes were measured in NA plants (light green bars), Primed plants (red bars), DAP1 plants (light blue bars), DAP2 plants (yellow bars), DAP3 plants (violet bars), Trig plants (pink bars), DAT1 plants (navy blue bars), DAT2 plants (orange bars) and DAT3 plants (dark green bars) using qRT-PCR. All analyses were performed for one biological replicate representing RNA isolated from pool of five rosettes.

Moreover, paralogue variation in the expression of *APxs* (*sAPx* vs. *tAPx*) upon cold stress conditions seemed to be also controlled in a habitat-dependent manner (Figure 3-68). It was shown, that almost all tested accessions (except Col-0 and C24) regulated the expression of *APxs* at the transcript level inversely (up-regulation of *sAPx* and down-regulation of *tAPx* expression), however the control of this process was modulated in a habitat-dependent manner. Thus, after 24 hours at 4 °C accessions originating from cold habitats (Kas-1, Ms-0) slightly up-regulated *sAPx* expression and strongly down-regulated that of *tAPx*, while the ones from continental climates (Col-0, Van-0) strongly up-regulated the expression of *sAPx* and weakly down-regulated or up-regulated that of *tAPx*.

The analyses of *APxs* expression during lag phase (DAP1-3 plants) revealed for all tested accessions increase and decrease (in comparison with Primed plants) in the accumulation of *sAPx* and *tAPx* transcripts, respectively (Figure 3-68). However, as for *Csd2*, accession-specific pattern in the *APxs* expression could be observed only during the last analyzed day of this phase (DAP3 plants). It was shown that the transcript levels of *sAPx* in DAP3 plants of accessions coming from cold and continental climates (Kas-1, Ms-0, Col-0, WS and Van-0) were either, similar or decreased in comparison with NA plants, while in originating from hot habitat Cvi-0 *sAPx* transcript level in DAP3 plants was slightly higher than in NA plants. This indicated, that the activation of *sAPx* expression among all tested accessions lasted the longest in Cvi-0.

The *tAPx* accumulation pattern in DAP3 plants differed from that observed for *sAPx* (Figure 3-68). In general, in all of the tested accessions the level of *tAPx* transcript was higher in DAP3 than in NA plants, but stronger increase was detected in Col-0, Ms-0, WS and C24 than in Kas-1, Van-0 and Cvi-0. This indicated that the activation of *tAPx* expression among tested accessions lasted the longest in Col-0, Ms-0, WS and C24.

In triggered plants (Trig plants) the transcript levels of *sAPx* were similar or lower than in Primed plants for all selected accessions (Figure 3-68), indicating that they were not able to generate thermomemory in *sAPx* expression. In contrast, *tAPx* mRNA was accumulated at higher levels than in Primed plants in Trig plants of Kas-1, Ms-0 and WS, demonstrating the ability of these accessions to generation of thermomemory in *tAPx* expression.

During de-acclimation phase following triggering stimulus (DAT1-3 plants), as for *Csd2*, reset of expression to the state before subjecting to cold stress stimuli (NA plants) was demonstrated for *sAPx*, while the levels of *tAPx* transcripts in DAT3 plants were

similar to that in NA plants only for Kas-1, Ms-0, and Van-0, lower in Cvi-0 and higher in Col-0, WS and C24 (Figure 3-68).

3.6.1.4 2-Cys peroxiredoxins (2CPs)

Similar to *Csd2* and *APxs*, upon short- term cold treatment expression of *2CPs* at the transcript levels was also regulated in accession and habitat-dependent manner (Figure 3-69).

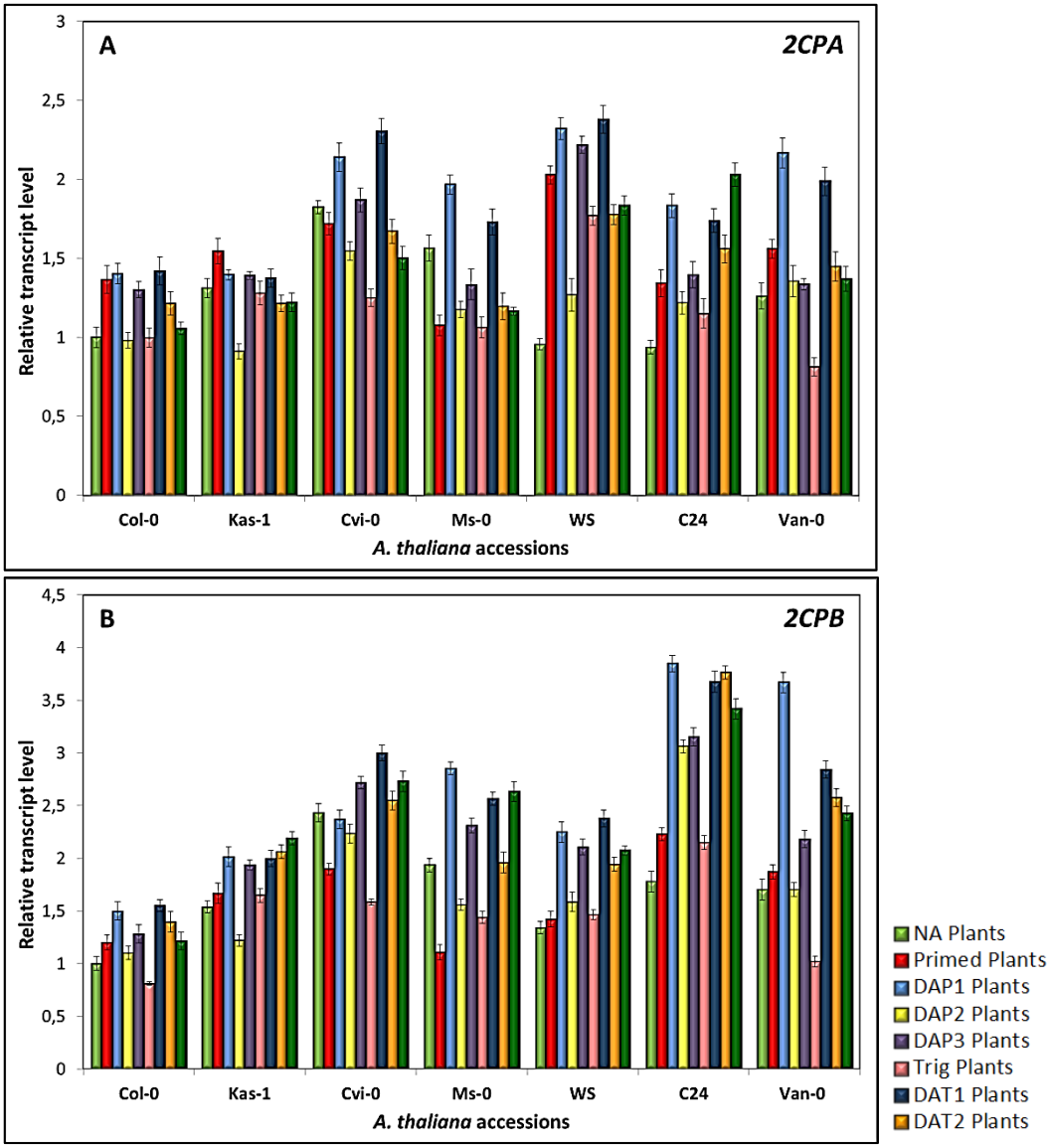

Figure 3-69. Transcript levels of **A.** *2CPA* and **B.** *2CPB* in seven accessions of *A. thaliana* upon short-term cold stress treatment. The mRNA abundances of these genes were measured in NA plants (light green bars), Primed plants (red bars), DAP1 plants (light blue bars), DAP2 plants (yellow bars), DAP3 plants (violet bars), Trig plants (pink bars), DAT1 plants (navy blue bars), DAT2 plants (orange bars) and DAT3 plants (dark green bars) using qRT-PCR. All analyses were performed for one biological replicate representing RNA isolated from pool of five rosettes.

In non-acclimated state (NA plants) among all tested accessions the highest levels of transcripts encoding *2CPs* were detected in Ms-0 and Cvi-0 (Figure 3-69), demonstrating the strongest activation of *2CPA* and *2CPB* expression under non-stressful conditions in accessions originating from extremely cold (Ms-0) or hot (Cvi-0) habitats.

Upon cold treatment (Primed plants) the transcript levels of both, *2CPA* and *2CPB*, were higher than in NA plants for almost all tested accessions, except Ms-0 and Cvi-0, in which lower amounts of *2CPs* mRNAs were accumulated in Primed than in NA plants (Figure 3-69). This indicated that activation of *2CPA* and *2CPB* expression in accessions originating from extremely cold (Ms-0) and hot (Cvi-0) habitats upon cold stress treatment might be less essential than in the ones coming from continental climates (Col-0, WS, Van-0).

In the course of lag phase (DAP1-3 plants) the lowest amounts of *2CPs* transcripts among all tested accessions were detected on the second day of this phase (Figure 3-69). Afterwards (in DAP3 plants), their abundances increased, reaching (for *2CPA* in Kas-1, Cvi-0 and Van-0, and for *2CPB* in Col-0) or exceeding (for *2CPA* in Col-0, WS, C24, and for *2CPB* in Kas-1, Cvi-0, Ms-0, WS, C24 and Van-0) the transcript levels measured for the same gene in NA plants.

After application of triggering stimulus (Trig plants) increase in the accumulation of *2CPs* transcripts (in comparison with Primed plants) was detected only for *2CPB* in Ms-0, while other accessions in Trig plants accumulated similar or lower amounts of *2CPs* mRNAs than Primed plants (Figure 3-69). This indicated that upon cold-treatment only Ms-0 was able to generate thermomemory in the expression of *2CPs*.

During de-acclimation phase following triggering stimulus (DAT1-3 plants) higher levels of *2CPB* transcripts than in NA plants were detected in all tested accessions (Figure 3-69), indicating that the activation of *2CPB* expression at the transcript level in all of them lasted at least three days after application of triggering stimulus. In contrast, in DAT3 plants of almost all tested accessions (except WS and C24) similar or lower levels of *2CPA* transcripts than in NA plants were measured, demonstrating a reset of *2CPs* expression to the state before subjecting to cold stress stimuli (NA plants).

3.6.1.5 Glutathione peroxidases (GPxs)

Upon short-term cold treatment some accession- and habitat-specific characteristics in the responsiveness to temperature acclimation could be also shown for expression regulation of genes encoding chloroplast glutathione peroxidases, *GPx1* and *GPx7* (Figure 3-70).

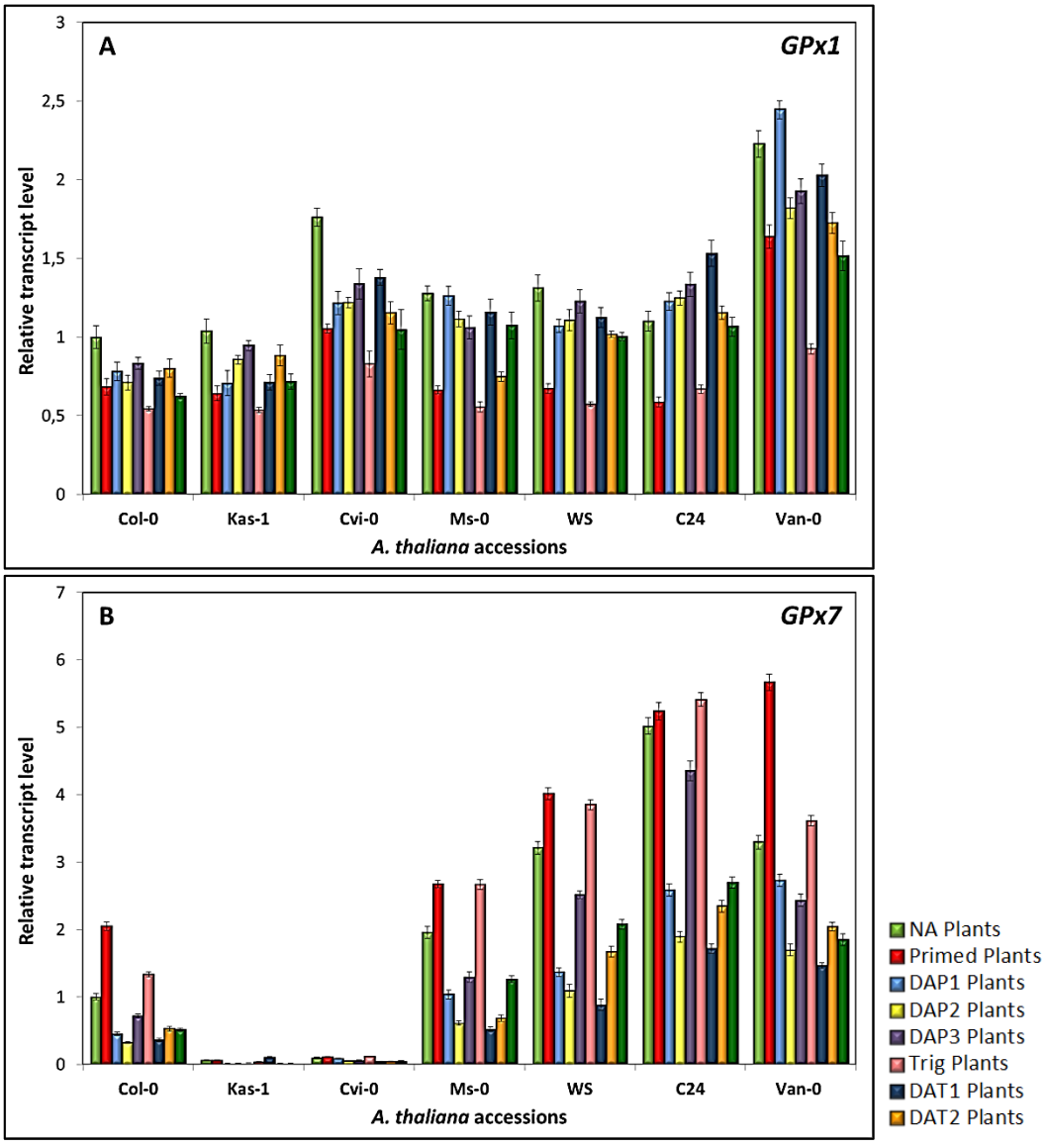

Figure 3-70. Transcript levels of **A.** *GPx1* and **B.** *GPx7* in seven accessions of *A. thaliana* upon short-term cold stress treatment. The mRNA abundances of these genes were measured in NA plants (light green bars), Primed plants (red bars), DAP1 plants (light blue bars), DAP2 plants (yellow bars), DAP3 plants (violet bars), Trig plants (pink bars), DAT1 plants (navy blue bars), DAT2 plants (orange bars) and DAT3 plants (dark green bars) using qRT-PCR. All analyses were performed for one biological replicate representing RNA isolated from pool of five rosettes.

In NA plants the highest levels of *GPx1* transcripts were detected in Cvi-0 and Van-0, while that of *GPx7* in C24 (Figure 3-70). It demonstrated that Cvi-0, Van-0 and C24 activated *GPxs* expression upon non-stressful conditions the strongest among all tested accessions.

Subsequent analyses of plants subjected to short-term cold stress treatment revealed that the transcript levels of *GPx1* after 24 hours at 4 °C (Primed plants) were decreased (in comparison with NA plants) in all tested accessions (Figure 3-70). Among them, the strongest decrease in the level of this transcript was detected for originating from dry and hot habitat Cvi-0, while the weakest reduction was shown for coming from continental climate Col-0. In contrast, *GPx7* mRNA abundance was higher in Primed than NA plants for almost all accessions, except Kas-1 and Cvi-0, in which this mRNA was accumulated at hardly detectable and similar levels at all experimental time-points (Figure 3-70).

Moreover, the analyses of changes in *GPxs* mRNA abundances upon cold treatment (Primed plants) revealed that the expression of *GPx1* and *GPx7* at the transcript level after application of priming stimulus in all almost all tested accessions (except Kas-1 and Cvi-0) was regulated inversely (down-regulation of *GPx1* and up-regulation of *GPx7* expression) (Figure 3-70). It suggested that *GPx1* was generally less essential in acclimation to low temperatures than *GPx7*.

During lag phase (DAP1-3 plants) the transcript levels of both *GPxs* were lower than in Primed plants in almost all tested accessions, except DAP1-3 plants of C24 and DAP1 plants of Van-0 (Figure 3-70), demonstrating that activation of *GPxs* expression by cold stress lasted generally shorter than that of other chloroplast antioxidant enzymes.

After application of triggering stimulus (Trig plants), stronger than in Primed plants activation of *GPxs* mRNAs accumulation was shown only in C24 (Figure 3-70), for which in DAP3 plants (in comparison with Primed plants) the transcript level of *GPx1* was the strongest increased (in comparison with Primed plants) and that of *GPx7* the weakest decreased among all tested accessions.

During de-acclimation phase following application of triggering stimulus (DAT1-3), lower than in NA plants levels of transcripts encoding both chloroplast *GPxs* were observed among all tested accessions (Figure 3-70), indicating that cultivation of *A. thaliana* accessions under short-term cold stress conditions caused the decrease in the expression activity of *GPxs*.

Chapter 4

DISCUSSION

4.1 Chloroplast antioxidant system among *A. thaliana* accessions – developmental control and adaptation vs. acclimation

Accessions of *A. thaliana* evolved by separation and adaptation to specific climatic conditions, such as temperature, light intensity and UV radiation (Koornneef *et al.* 2004). Since, many of these environmental stimuli enhance ROS production, in all accessions a highly efficient antioxidant system developed (Sharma and Dubey, 2007; Gonzáles-Pérez *et al.* 2011; Ma *et al.* 2012; Krasensky and Jonak, 2012). It is a network of antioxidant enzymes and low molecular weight antioxidants (e.g. ascorbate and glutathione), in which the combination of individual components defines the protection potential (Asada, 1999; Mittler *et al.* 2004). Thus, only the overall read-out of the antioxidant system is important for plant survival and, therefore, adaptation and evolution. Moreover, the response pattern of this system depends on the inherited regulation capacity and adaptation to specific habitats (Mittler *et al.* 2004). Consistent with these statements, the comparison of seven accessions of *A. thaliana* originating from various environments revealed that the regulation of chloroplast antioxidant enzymes takes place in accession- and habitat-dependent manner and, furthermore, depends on the developmental stage of the plant.

4.1.1 The expression regulation of chloroplast antioxidant system is age-dependent

The expression of many genes is influenced by the developmental stage of the plants (Kaufmann *et al.* 2010). For chloroplast antioxidant enzymes Peña-Ahumada *et al.* (2006) showed that the transcription of peroxiredoxins (*Prxs*) and thylakoid-bound ascorbate peroxidase (*tAPx*) was activated during the seedling development earlier than that of stromal ascorbate peroxidase (s*APx*), Cu/Zn-SOD (*Csd2*) and monodehydroacrobate reductase (*MDHAR).* At later developmental stages (2-week-old to 6-week-old plants), the transcript levels of genes encoding ascorbate peroxidases

(s*APx* and *tAPx*), glutathione peroxidases (*GPx1* and *GPx7*), monodehydroascorbate reductase (*MDHAR*) and glutathione reductase (*GR*) increased with the age of the plants, while for *2CPs* slightly lower mRNA levels were detected in older than in younger plants (Baier *et al.* 2000; Rodriguez Milla *et al.* 2003).

Present comparison of young (3-week-old) and not fully developed (5-6 leaves of approx. 1 cm in length each) *A. thaliana* accessions with older (6-week-old) and fully developed ones also revealed that the dynamics in the accumulation of transcripts encoding chloroplast antioxidant enzymes strongly depends on the age and developmental stage of plants (see chapter 3.2). An increase in the transcripts levels with the age of the Col-0 plants was observed for almost all analyzed antioxidant enzymes (*Csd2*, *APXs*, *2CPs*, *PrxQ*, *GPXs*, *DHAR* and *GR*), except *PrxIIE*, *MDHAR* and *GR*, which were accumulated at the same level in younger and in older plants. The results were consistent with those obtained by Baier *et al.* (2000) and Rodriguez Milla *et al.* (2003) for almost all tested enzymes (*APXs*, *GPXs* and *GR*) excluding *2CPs*, for which an increase in the transcript accumulation with the age of the plants was observed. Since the level of *2CPs* mRNA was shown to increase in response to higher light intensities (Baier *et al.* 2000; Horling *et al.* 2003), the differences in the results of the present analyses and those obtained for *2CPs* by Baier *et al.* (2000) might be due to the different light regimes in both experimental approaches (twofold higher light intensity in the present studies (120 µmol m^{-2} s^{-1}) than in the previous ones).

Among remaining accessions, the strongest reorganization of the gene expression between 3-week-old and 6-week-old plants was observed for WS and Cvi-0 (see chapter 3.2). In WS four (*PrxQ*, *PrxIIE*, *MDHAR* and *GR*) out of twelve analyzed transcripts were accumulated at significantly higher levels in younger than in older plants, while in Cvi-0 higher levels of mRNA at the earlier developmental stage were detected only for *PrxIIE* (remaining transcripts were accumulated at higher levels in older plants). In combination with high superoxide and hydrogen peroxide levels in Cvi-0 and WS (Figures 3-32 and 3-33) this observation suggests that in chloroplasts of WS are earlier released into redox imbalance and senescence than in Cvi-0.

4.1.2 The expression regulation of chloroplast antioxidant system is challenged by the growth temperature

Under low and high temperatures ROS accumulates in plant cells influencing the regulation of their antioxidative defence system (Thomashow, 1999; Iba, 2002; Heidarvand and Amiri, 2010). Among the chloroplast antioxidant enzymes heat stress resulted in the down-regulation of *Csd2* transcript levels (Guan *et al.* 2013), while chilling led to a stronger expression of *GPx1* (Soitamo *et al.* 2008) and *sAPx* (Kangasjarvi *et al.* 2008) and down-regulation of *tAPx* and chloroplast *Prxs* (*2CPs*, *PrxQ* and *PrxIIE*) (Kangasjarvi *et al.* 2008). However, all of these expression analyses were performed so far only in the Col-0 background. Here, such studies are presented for seven accessions of *A. thaliana* originating from habitats with contrasting average temperatures. It was proposed that such exposure to diverse temperatures in the natural environments might differentially challenge the expression regulation of the chloroplast antioxidant enzymes. Indeed, the present studies revealed that various accessions differentially responded to three different growth temperatures (10, 20 and 30 °C) spanning the temperature range at their natural habitats (see chapter 3.3). Among all of them, the strongest deprivation from the expression regulation of the chloroplast antioxidant system by growth temperature in a habitat-dependent manner was observed for *Csd2* in Cvi-0 and Kas-1 (Figure 3-39). Thus, at 30 °C the transcript level of *Csd2* was higher in originating from an alpine habitat with lower temperatures Kas-1 than in coming from dry and hot climate Cvi-0. In contrast, at 10 °C a higher accumulation of *Csd2* mRNA was detected for Cvi-0 than for Kas-1. Moreover, at 10 °C also the levels of *2CPA*, *2CPB*, *PrxIIE*, *sAPx* and *GR* transcripts were higher in Cvi-0 than in Kas-1, indicating that in Cvi-0 the detoxification of ROS is activated in response to cold. In contrast, Kas-1, originating from cold climate, is well adapted to low temperatures and, therefore, does not perceive low temperatures as a stress conditions and does not activate the ROS scavenging system.

4.1.3 Various accessions have a different acclimation capacity to cold stress

The survey of nine geographically diverse accessions of *A. thaliana* (Hannah *et al.* 2006) revealed a significant correlation between freezing tolerance of these accessions and the temperature at their natural habitats, subsequently suggesting that temperature is a highly probable selective pressure limiting the geographical distribution of *A. thaliana*

accessions. Consistent with these results, present data demonstrate a link between the complexity of the transcriptional response of cold-responsive genes and the acclimation capacity (see chapters 3.5 and 3.6). Thus, in non-acclimated (NA) plants of accessions characterized by the highest freezing tolerance (lower LT_{50}; Figure 3-55), such as N14, N13 and Ms-0, the amounts of transcripts encoding cold-induced transcription factors (*CBF1*, *CBF2* and *CBF3*) and CBF regulated genes (*COR6.6*, *COR15A*, *COR15b*, *COR47* and *COR78*) (Thomashow, 1999) were higher than in the NA plants of less freezing tolerant accessions (WS, Col-0, Cvi-0, Kas-1, Van-0, C24, Can-0, Sah-0). However, after cold acclimation a stronger induction of cold-responsive transcripts were detected in less freezing tolerant accessions than in the most tolerant ones, indicating a higher resistance to cold stress and better acclimation capacity to low temperatures for N14, N13 and Ms-0 than for the other tested accessions (data kindly provided by E. Zuther and D. Hincha, MPIMP, Golm, Germany).

Besides the activation of cold-responsive genes, cold stress is also known to induce the production of reactive oxygen species (ROS), such as superoxide radical anion and hydrogen peroxide. When accumulated at high levels, ROS have a potential to cause cellular damages, therefore plants have evolved a wide range of defence mechanisms including the ROS-mediated activation of antioxidant enzymes (Thomashow, 1999; Iba, 2002; Heidarvand and Amiri, 2010). The capacity of these enzymes for ROS detoxification was shown to be balanced with regard to ROS production (Foyer and Shigeoka, 2011). Present analyses revealed that a two-week-long cold treatment (4 °C) generally resulted in the stronger enhancement of ROS accumulation (compared to NA plants) in the less freezing tolerant accessions (WS, Col-0, Cvi-0, Kas-1, Van-0, C24, Can-0, Sah-0) than in the most freezing tolerant ones (N14, N13 and Ms-0) (Figures 3-61 and 3-62). Consistent with Foyer and Shigeoka (2011), in N14, N13 and Ms-0, accumulating upon cold treatment low amounts of ROS, the expression of almost all chloroplast antioxidant enzymes was either down-regulated or similar to NA plants. In contrast, less freezing tolerant accessions, in which higher amounts of ROS were detected, accumulated the transcripts encoding almost all chloroplast antioxidant enzymes (except *GPxs*) at higher levels than NA plants (see chapter 3.5). Taken together, low levels of ROS and, in response, a weak activation of chloroplast antioxidant enzymes upon cold treatment in the most freezing tolerant accessions

demonstrate that these accessions have generally better acclimation capacity to low temperatures than the accumulating high amounts of ROS less freezing tolerant ones.

In addition to enhanced ROS production, cold stress leads also to the inhibition of sucrose synthesis. In turn, lower sucrose synthesis causes decreased P_i cycling between the cytosol and the chloroplasts. Thus, at low temperatures the chloroplasts become P_i limited impending the synthesis of ATP which is needed for regeneration of RuBisCO. This might cause the feedback inhibition of photosynthesis (Hurry *et al.* 2000) and subsequently lead to the lower chlorophyll synthesis. Therefore the sucrose and chlorophyll contents might be good indicators of the acclimation capacity to low temperatures. Present comparison of seven accessions of *A. thaliana* revealed that upon cold treatment the most freezing tolerant accessions (N14, N13 and Ms-0) accumulated generally higher amounts of sucrose than the less freezing tolerant ones (data kindly provided by E. Zuther and D. Hincha, MPIMP, Golm, Germany). So, if the photosynthetic activity and the chlorophyll content depends on the sucrose availability, cold treatment should result in the accumulation of higher chlorophyll amounts in N14, N13 and Ms-0 than in the remaining accessions. Indeed, the analyses of chlorophyll accumulation revealed that after 14 days of cold treatment all accessions accumulated lower levels of chlorophyll than NA plants, but stronger decrease was generally observed in the less (WS, Kas-1, Van-0 and Sah-0) than in the most freezing tolerant ones (N14, N13 and Ms-0) (Figure 3-65). It indicates that at low temperatures photosynthesis is affected the weakest in N14, N13 and Ms-0, and, thus, these accessions have better acclimation capacity to cold.

Similar to the long-term cold stress, upon short-term cold stress treatment (Primed plants compared to NA plants) a stronger induction of one of the cold-responsive gene (*COR15A*) expression was also observed for the accessions originating from warmer climates (Col-0, WS and Van-0) than in those coming from cold habitats (Kas-1 and Ms-0) (Figure 3-65). Also the accumulation of almost all transcripts encoding chloroplast antioxidant enzymes (except *GPxs)* in response to 24 h of cold treatment was stronger in Col-0, WS and Van-0 than in Ms-0 and Kas-1 (see chapter 3.6.1). Since the capacity of antioxidant enzymes for ROS detoxification is balanced with regard to ROS production (Foyer and Shigeoka, 2011), it indicates that upon cold treatment Col-0, WS and Van-0 accumulate higher amounts of ROS than Ms-0 and Kas-1. Furthermore, higher ROS levels in Ms-0 and Kas-1 than in Col-0, WS and Van-0 might

indicate that the photosynthesis, which activity was shown to be inhibited under excess ROS production (Scarpeci *et al.* 2008), under cold stress is the least affected in the accessions originating from cold habitats and the most in those from warm environments. Indeed, the photosynthetic activity measurements (own group data) revealed that the photosynthesis was most affected by cold-stress treatment in Cvi-0, being the accession coming from the hottest and driest climate.

Taking into account the expression data and the ROS and chlorophyll accumulation patterns (see chapters 3.5 and 3.6), it is concluded that the acclimation capacity of *A. thaliana* accessions is determined by the average temperature at their natural habitats.

4.1.4 Do accessions of A. thaliana have a thermomemory?

The analyses of *A. thaliana* accessions subjected to short-term cold stress revealed significant differences in the responses of the chloroplasts antioxidant system at the transcript level after application of the first (priming stimulus) and the second (triggering stimulus) cold stress stimulus (see chapter 3.6). Since the exposure of plants to the first stressful stimulus (e.g. cold stress) can be memorized and subsequently affect the response of plants to the future exposure to the same or another stress stimulus (Conrath *et al.* 2006; Bruce *et al.* 2007), it was postulated that the analyzed accessions of *A. thaliana* are able to generate a thermomemory in the expression regulation of the chloroplast antioxidant enzymes. Memory of plants was described by (Trewavas, 2003) as: "… ability to access past experience so that new responses incorporate relevant information from the past" and "…information storage of previous signalling, with the ability to retrieve the information at a much later time". Such an ability to store the information from the previous exposure to the stressful conditions was proposed to make plants more resistant to future exposure to the same or different stimulus (Conrath *et al.* 2006; Bruce *et al.* 2007). Thus, primed plants (plants exposed the first time to the stress conditions) should display either faster and/or stronger, activation of the various defence responses that are induced after the application of the second (triggering) stress stimulus (Bruce *et al.* 2007).

Since the induction of the chloroplast antioxidant system is one of the defence mechanisms activated under stressful conditions, for plants able to generate thermomemory stronger increase in the levels of transcripts encoding enzymatic components of this system was expected after application of triggering stimulus than

after that of priming. However, such response pattern was detected only for *Csd2* in Kas-1, *2CPB* in Ms-0, *GPx1* and *GPx7* in C24 and for *tAPx* in Kas-1, Ms-0 and WS (see chapter 3.6), indicating that only these accessions are able to generate thermomemory in the expression regulation of the chloroplast antioxidant enzymes. Since almost all of these accessions (except C24) origin from cold climates, it indicates that, similar to acclimation capacity, also the generation of thermomeory might be determined in regard to the temperature at the natural habitats of accessions.

However, Foyer and Shigeoka (2011) postulated that the capacity of antioxidant enzymes for ROS detoxification is balanced with regard to ROS production. If so, a stronger activation of analyzed chloroplast antioxidant enzymes in Kas-1, Ms-0, WS and C24 after the application of triggering stimulus than after the priming one, may indicate that triggering with the short-term cold stress enhanced the production of ROS even stronger than the priming. Furthermore, it might demonstrate that Kas-1, Ms-0, WS and C24 are more stressed (produce higher amounts of ROS) after the application of triggering stimulus than after the priming one. Thus, in this case priming of plants with short-cold stress would not result in the stronger activation of the chloroplast antioxidant enzymes after the application of triggering stimulus, but via down-regulation of chloroplast antioxidant system in response to triggering stimulus would rather lead to the decrease in the "costs" of such an activation.

Both hypotheses described above are highly probable, but the data obtained for the photosynthetic activity of the selected accessions (own group data) rather support the first one, which indicated that priming resulted in a stronger activation of the chloroplast antioxidant system in triggered than in primed plants. Since photosynthesis is slowed down or even inhibited under cold stress conditions (Hurry *et al.* 2000), it is postulated that less stressed plants should be more photosynthetically active. Thus, the present results demonstrated that the photosynthesis was less affected (higher photosynthetic activity in triggered than in primed plants) after the application of triggering stimulus than after the priming one only in Kas-1 and Ms-0. Since, the accumulation of transcripts encoding the highest number of analyzed antioxidant enzymes in these accessions was higher in triggered than in primed plants, it indicates that higher activity of the chloroplast antioxidant enzymes is positively correlated with the photosynthetic activity. Thus, the accessions able to generate the thermomemory in

the expression regulation of the chloroplast antioxidant system are rather those, in which the activity of this system is higher in trigerred than in primed plants.

4.2 Transcription vs. translation in the expression regulation of the chloroplast antioxidant enzymes

Previous analyses revealed that the accumulation of transcripts encoding most of the chloroplast antioxidant enzymes increased with the age of the plants (Baier *et al.* 2000; Rodriguez Milla *et al.* 2003). These results were confirmed by the present studies (see chapter 3.2), indicating that the chloroplast antioxidant enzymes play a more important role at the later developmental stages. Therefore, older accessions of *A. thaliana* (6-week-old) were chosen for further investigations of mechanism regulating the expression of genes encoding the chloroplasts antioxidant enzymes. In general, the levels of the transcripts encoding these enzymes in 6-week-old accessions, cultivated under the same, optimized for Col-0 (reference accession) growth conditions (120 µmol m^{-2} s^{-1} light and a day / night temperature of 20 °C / 18 °C), differed stronger than ever observed for Col-0 between control and stress treatments (Baier *et al.* 1997; Baier *et al.* 2000; Panchuk *et al.* 2002; Rodriguez Milla *et al.* 2003; Sunkar *et al.* 2006; Kangasjarvi *et al.* 2008; Chang *et al.* 2009). Several transcripts, e.g. *2CPB*, *sAPx*, *tAPx*, *MDHAR* and *DHAR*, as well as *2CPA* and *GPx7* in C24 were accumulated in twofold higher abundances (compared to Col-0), while the levels of the other ones e.g. *Csd2*, *PrxIIE*, *GPx1*, *sAPx*, *MDHAR* and *DHAR* in WS, as well as *2CPA*, *2CPB*, *GPx7*, *sAPx*, *tAPx* and *GR* in Ms-0 were more than twofold lower than in Col-0 (see chapter 3.1.1). In contrast to the highly variable patterns of transcript accumulation, less variation was observed at the protein level (see chapter 3.1.2), indicating that the expression of genes encoding the chloroplast antioxidant enzymes might have been also regulated at the post-transcriptional and/or translational level. Indeed, the comparison of mRNA and polysomal RNA levels revealed that, for most of the analyzed genes and accessions, differences in the mRNA accumulation were balanced at the level of translation initiation (see chapter 3.1.3). These observations indicated that the expression of genes encoding the chloroplast antioxidant enzymes among selected accessions of *A. thaliana* was controlled at the transcript and translation initiation levels in gene- and accession-specific manner:

4.2.1 Accessions of A. thaliana strongly differ in the accumulation of transcripts encoding chloroplast antioxidant enzymes – transcripts abundance patterning

A huge collection of microarray data (AtGenExpress and Arabidopsis eFP Browser) demonstrates that the accumulation of transcripts encoding the chloroplasts antioxidant enzymes in *Arabidopsis thaliana* is regulated in an accession-specific manner and depends on the kind, duration and intensity of stress stimuli (Kilian *et al.* 2007; Winter *et al.* 2007). However, the mechanisms underlying this regulation have been studied in details only exemplarily for a limited number of genes and accessions (e.g. *2CPA* in Col-0, *APxs* in Col-0 and *Csd2* in Col-0 and WS). Nevertheless, already these single gene studies show extreme differences in the relevance of the transcriptional and post-transcriptional regulation. For example, they indicated that the expression of *2CPA* and *APxs* is predominantly transcriptionally regulated (Baier *et al.* 2000; Baier *et al.* 2004; Klein *et al.* 2012), while that of *Csd2* rather post-transcriptionally in micro-RNA dependent manner (Sunkar *et al.* 2006).

Here, the mechanisms of transcript accumulation regulation will be discussed for genes encoding all chloroplast antioxidant enzymes in seven accessions of *A. thaliana*. Accession- and gene-specific control of mRNA abundances will be indicated.

4.2.1.1 Regulation of Csd2 transcript abundance

Regulation of the *Csd2* transcript abundance has been studied in detail. These studies revealed that in Col-0 the accumulation of mRNAs encoding *Csd2* and its copper chaperone *CCS1* is controlled by miR398 (Sunkar *et al.* 2006; Beauclair *et al.* 2010). This micro-RNA itself is responsive to diverse abiotic and biotic stresses, such as oxidative stress, ultraviolet (UV-B) stress, heat stress and drought stress (Sunkar *et al.* 2006; Jagadeeswaran *et al.* 2009; Jia *et al.* 2009). Among them, oxidative stress promotes a decrease in the *miR398* level, permitting post-transcriptional induction of *Csd2* expression, and thereby helping plants to cope with stressful conditions (Sunkar *et al.* 2006). In contrast, the expression of *miR398* is up-regulated in response to high temperature, UV-B and drought, leading to decrease in the accumulation of *Csd2* transcripts (Zhou *et al.* 2007; Trindade *et al.* 2010; Guan *et al.* 2013).

In the present studies, the highest levels of *Csd2* mRNA were detected in Cvi-0 and Ms-0 (Figure 3-7). Furthermore, the strong accumulation of *Csd2* mRNA in these accessions was correlated with the high levels of superoxide in their most

photosynthetically active medium-age leaves (Figure 3-32), indicating conservation or mimicking of ROS-mediated down-regulation of *miR398* expression (Sunkar *et al.* 2006) and, thus increase in the *Csd2* transcript accumulation. However, high levels of superoxide detected in Cvi-0 did not support the statement of Abarca *et al.* (2001), who showed that Cvi-0-specific SOD provides increased protection against oxidative stress and thus, leads to accumulation of lower amounts of $O_2^{\bullet-}$. Therefore, it is suggested that the overexpression of *Csd2* is not sufficient to protect plants from oxidative stress and other mechanisms (e.g. overexpression of other antioxidant enzymes) must be also involved in this process.

In contrast to Cvi-0 and Ms-0, the *Csd2* transcripts were accumulated at low levels in Kas-1, WS, C24 and Van-0 (Figure 3-7), suggesting either, up-regulation of *miR398* expression or destabilization of *Csd2* mRNA by other mechanisms. Among these accessions, the expression of *miR398* was studied only in WS (Beauclair *et al.* 2010). The analyses revealed that in this accession *miR398* was stronger accumulated than in Col-0, leading to the lower levels of the *Csd2* transcript in WS than in Col-0. For Kas-1, it is suggested that weak *Csd2* mRNA accumulation was due to the up-regulation of *miR398* expression by environmental stimuli at the natural habitat of this accession. Kas-1 originates from Kashmir in India, where it grows at high altitude (1580 m) and is probably subjected to strong UV-B radiation. Since, UV-B leads to the up-regulation of *miR398* expression (Zhou *et al.* 2007), the weak accumulation of *Csd2* transcript in Kas-1 might be a result of increased *miR398* accumulation in response to UV-B.

Taken together, all results obtained for the *Csd2* transcript accumulation indicate that the post-transcriptional regulation of the *Csd2* expression by *miR398* is conserved among *A. thaliana a*ccessions (conservation of this regulatory mechanism will be discussed in details later).

4.2.1.2 2CPs-APxs cluster

Compared to the reference accession Col-0, the strongest differences in the accumulation of the transcripts encoding 2-Cys peroxiredoxins (*2CPA* and *2CPB*) and ascorbate peroxidases (s*APx a*nd *tAPx*) were detected for Cvi-0 and Ms-0 (Figures 3-9 and 3-14). In 6-week-old Cvi-0 and Ms-0, the transcript levels of both, *2CPs* and *APxs*, were respectively higher and lower than in Col-0, indicating a positive correlation between the accumulation of *2CPs* and *APxs* mRNAs. Such coupling of the *2CPs* and

APxs expression at the transcript level was observed already before in the screened for lower *2CPA* promoter activity *rimb* mutants. Thus, in these transgenic lines the levels of *2CPA* transcript were co-decreased (compared to Col-0) together with those of *sAPx a*nd *tAPx* (Heiber *et al.* 2007). It indicated a common transcriptional regulation of the *2CPA* and *APxs* expression. This conclusion was supported later by the identification of transcription factor Rap2.4a, being the activator of the *2CPA* transcription (Shaikhali *et al.* 2008). In *Rap2.4a* T-DNA insertion lines of *A. thaliana* var. Col-0, beside *2CPA*, the levels of the *sAPx* and *tAPx* transcripts were also decreased (Shaikhali *et al.* 2008).

The inverse transcriptional regulation of the *2CPs-APxs* cluster in Cvi-0 and Ms-0 prompted for more detailed analyses and discussion of *2CPs* and *APxs* expression regulation in these accessions. Since it was shown that the *2CPA* expression in Col-0 background is regulated by Rap2.4a in a ROS-dependent manner (Baier *et al.* 2004; Shaikhali *et al.* 2008), Cvi-0 and Ms-0 were compared for ROS accumulation. However, the comparison revealed that the levels of $O_2^{\cdot-}$ and H_2O_2 were similar in both of these accessions (Figures 3-32 and 3-33), indicating that differences in the *2CPA* levels between them were not due to the contrasting accumulation of ROS and thus, different activation of Rap2.4a. This conclusion was supported by analyses of the *Rap2.4a* transcript levels in Ms-0 and Cvi-0 (own group data). They demonstrated that, in comparison with Col-0, Cvi-0 accumulated *Rap2.4a* mRNA in a 3.3-fold lower abundance, while in Ms-0 the level of this transcript was 8.8-fold higher than in Col-0, further showing that the differences in the *2CPA* amounts observed between Ms-0 and Cvi-0 are independent from the Rap2.4a-mediated regulation. However, Rap2.4a belongs to the bigger family of AP2-type transcription factors, which highly diversified during evolution (Magnani *et al.* 2004). So far, the investigation of these transcription factors in Col-0 background showed that various members of the Rap2.4 gene family can form a combinatorial gene expression control system, in which individual transcription factor (TF), either compete or is redundant with another TF from the same family (own group data). Therefore, it is suggested that Cvi-0 and Ms-0 by loss/reduction or activity modification of one component of the Rap2.4 gene network, could completely or slightly shift the read-outs of the whole network and, thus change the regulator of *2CPA* expression from Rap2.4a into another member of the Rap2.4 family. Indeed, the analyses of *Rap2.4a-h* expression regulation in Cvi-0 and Ms-0 (data obtained for non-treated plants in cold stress studies; I. Juszczak and E.Zuther; SFB973,

project C4) revealed that other than Rap2.4a members of the Rap2.4 family might be responsible for the discrepancies in the *2CPA* transcript accumulation between these accessions. Thus, Cvi-0 expressed *Rap2.4d, Rap2.4e* and *Rap2.4h* at two-, five- and elevenfold higher levels than Ms-0, indicating that these three Rap2.4 transcription factor might be responsible for the regulation of *2CPA* expression in Cvi-0 and Ms-0. Moreover, Rap2.4d was recently identified as a regulator of the *sAPx* and *tAPx* expression (own group data), suggesting that this transcription factor might be also responsible for the differential patterning of the *APxs* transcripts accumulation in Ms-0 and Cvi-0. The sequence comparison of the Rap2.4d-binding motif between tested accessions of *A. thaliana* did not reveal any differences, indicating that the binding affinity of Rap2.4d to the promoters of chloroplast *APxs* is the same in all accessions and the regulatory force of this transcription factor might only depend on its level and activity. Indeed, the analyses of *Rap2.4d* transcript accumulation revealed that this TF is expressed in Cvi-0 at a fivefold higher level than in Ms-0. Thus, it is concluded that the inverse regulation of *2CPA* and *APxs* at the transcript level in Ms-0 and Cvi-0 is due the differential (inverse) regulation of *Rap2.4d, Rap2.4e* and *Rap2.4h* expression.

Moreover, it is proposed that the inverse regulation of the *2CPA* and *APxs* expression in Ms-0 and Cvi-0 could have been evolved in response to specific environmental parameters at the natural habitats of these accessions. Thus, Cvi-0 originates from a hot and dry climate with long vegetation periods, while Ms-0 comes from a cold, arid habitat with short summers. It shows that the natural habitats of these accessions are extremely different in the most important environmental parameters (e.g. temperature, light intensity), which might differentially strain regulation of *2CPA* and *APXs* expression. Therefore, the inverse expression regulation of *2CPs-APxs* could be observed for Ms-0 and Cvi-0.

4.2.1.3 The expression of PrxQ, PrxIIE and GPxs at the transcript level is regulated independently from that of the 2CPs-APxs cluster

Among the selected accessions of *A. thaliana* different trends in the accumulation of transcripts than those detected for *2CPs* and *APxs* were observed for *PrxQ*, *PrxIIE* and *GPxs* (see chapters 3.1.1.2 and 3.1.13). This indicated that the expression of *PrxQ*, *PrxIIE* and *GPxs* at the transcript level is regulated independently from that of the *2CPs-APxs* cluster.

Among the chloroplast antioxidant enzymes, PrxQ is the only enzyme localized to the chloroplast lumen (Petersson *et al.* 2006). Thus, it was assumed that, due to the different subcellular localization, diverse function of PrxQ and expression regulation of encoding this protein gene than those identified for 2CPs and APXs might have evolved. The analyses of the *PrxQ* expression at the transcript level in Col-0 revealed that the accumulation of mRNA encoding *PrxQ* is induced in response to ROS and high light intensities (Horling *et al.* 2003). Consistent with ROS regulation, in the present comparison of *A. thaliana* accessions, the abundance of *PrxQ* transcript was high in in the superoxide-accumulating accessions (Cvi-0 and Ms-0) and low in Kas-1, WS, C24 and Van-0, in which superoxide detoxification efficiently prevented its accumulation in the most photosynthetically active middle-age leaves (Figures 3-10 and 3-32). Moreover, the studies of the *PrxQ*-knockout lines (*PrxQ*-KO) revealed that the expression of *PrxQ* is negatively correlated with the photosynthetic activity (compared to wild-type plants improved photosynthetic performance in *PrxQ*-KO) (Petersson *et al.* 2006). Therefore, the accessions were compared for their photosynthetic performance (own group data). The comparison demonstrated that accumulating the lowest amounts of *PrxQ* transcripts Kas-1 was the most photosynthetically active. In contrast, Col-0, Ms-0 and Cvi-0, in which the level of the *PrxQ* mRNA was significantly higher than in Kas-1, were characterized by relatively low photosynthetic performance (Figure 3-10). Thus, it can be concluded that depending on the ROS accumulation accessions of *A. thaliana* differentially regulate the expression of *PrxQ* in order to adjust the photosynthetic activity to environmental stimuli at the natural habitats of these accessions.

The transcripts encoding the last chloroplast peroxiredoxin, PrxIIE, were accumulated in all analyzed *A. thaliana* accessions at the lowest levels among all *Prxs (*Figure 3-8). The results were consistent with those obtained by Bréhélin *et al.* (2003), who showed that *PrxIIE* is expressed at low levels in leaves and high in floral buds and siliques. However, despite the low expression of *PrxIIE*, significant differences between the tested accessions in the accumulation of this mRNA were observed (Figure 3-10). Furthermore, this variability was due to the different environmental stimuli at the natural habitats of selected accessions. Thus, the accumulation of *PrxIIE* transcript was shown to be reduced in response to low temperatures, drought stress, oxidative stress and UV-B stress (AtGenExpress). Consistently, low levels of *PrxQ* mRNA were

detected in Cvi-0 and Kas-1, which at their natural habitats might face the combination of high UV radiation with either low temperatures (Kas-1) or water deficit (Cvi-0). In contrast, the strongest accumulation of *PrxIIE* transcript was observed in Ms-0 growing in its natural environment at low temperatures and under low UV radiation Ms-0, demonstrating accession-specific, UV-dependent and temperature-independent regulation of *PrxIIE* expression.

Similar to *PrxIIE*, the expression of chloroplasts *GPxs* at the transcript level was also regulated in an accession- and habitat dependent manner (Figure 3-12). The conclusion was based on the previous analyses, which showed that both chloroplast *GPxs* play an important role in the acclimation to photooxidative stress (Chang *et al.* 2009) and that the accumulation of *GPxs* transcripts is induced by a combination of low temperatures and high light for *GPx1* (Soitamo *et al.* 2008) or high UV-B radiation and cold for *GPx7* (AtGenExpress). Moreover, the regulation of *GPxs* in *Arabidopsis* seems to be redox-dependent. Thus, in the promoter of *GPx6* from *Lotus japonicus*, being a homolog of *GPx1* and *GPx7* from *A. thaliana*, similar to the redox-regulated antioxidant response elements (ARE) of maize catalase 2 (GGTGACCTTGC) were identified (Guan *et al.* 1996; Scandalios, 2005; Ramos *et al.* 2009). Consistently, the highest level of the *GPx1* transcript among the selected accessions was detected in superoxide-accumulating (Figure 3-32) and originating from cold habitat Ms-0 (Figure 3-12), confirming that the expression of *GPx1* is induced by ROS generated in response to low temperatures. In contrast, accumulating similar amounts of ROS to Ms-0 and coming from dry and hot habitat Cvi-0 accumulated *GPx1* transcript at significantly lower level than Ms-0, demonstrating that the transcription of *GPx1* is specifically induced by low and not high temperatures. However, such clear correlation between the natural habitat of the tested accessions and expression regulation of *GPXs* could be indicated only for *GPx1* (Figure 3-12). On the contrary, up-regulated by UV-B *GPx7* (AtGenExpress) was accumulated at the lowest levels in Kas-1 and Cvi-0, which might face high UV radiation in their natural environments, suggesting that Kas-1 and Cvi-0 at applied low conditions are either characterized by low UV perception or the expression of *GPx7* in these accessions is UV-independent.

4.2.1.4 Expression of ascorbate-recycling reductases vs. APxs transcripts accumulation

The analyses of transgenic lines with suppressed expression of 2-Cys peroxiredoxins, revealed that decreased expression of *2CPs* is compensated by enhanced accumulation of *APxs* transcripts. Moreover, the *2CPs*-mutants overexpressed also *MDHAR* and *GR* (Baier *et al.* 2000), indicating that the expression of ascorbate-recycling reductases (*MDHAR*, *DHAR* and *GR*) is linked to that of the chloroplast ascorbate peroxidases (s*APx* and *tAPx*). Indeed, the present comparison of *A. thaliana* accessions revealed that the expression of *MDHAR*, *DHAR* and *GR* was positively correlated with that of chloroplast ascorbate peroxidases (s*APx* and *tAPx*). Thus, the highest accumulation of transcripts encoding ascorbate-recycling reductases was detected in Cvi-0, in which also *sAPx* and *tAPx* were expressed at significantly higher levels than in other tested accessions (Figures 3-27 and 3-29). In contrast, in accumulating low levels of *APxs* mRNAs, Kas-1, transcripts encoding ascorbate-recycling reductases were also accumulated in low amounts. It demonstrates that the expression of *MDHAR*, *DHAR* and *GR* at the transcript level is regulated with regard to the availability of *sAPx* and *tAPx* mRNAs.

Furthermore, the comparison of selected accessions revealed also that expression regulation of *MDHAR*, *DHAR* and *GR* is dependent on the environmental stimuli acting at the natural habitats of the tested accessions. Thus, the levels of transcripts encoding all chloroplast ascorbate-recycling reductases were the highest in accumulating high levels of $O_2^{\bullet-}$ and originating from one of the driest region all over the world Cvi-0, while subjected at its natural habitats to low temperatures and high UV-B radiation Kas-1 accumulated *MDHAR*, *DHAR* and *GR* mRNAs in low amounts (Figure 3-17). The results are consistent with the microarray data (Arabidopsis eFP Browser), which demonstrated that the expression of *MDHAR* was induced by oxidative stress, while water deficit (drought stress) led to increase in the accumulation of *DHAR* and *GR* transcripts. In contrast, *DHAR* mRNA was accumulated at low levels in plants subjected to low temperatures, *GR* in those cultivated under UV-B stress, while combination of cold and UV-B led to decrease in the accumulation of *MDHAR* (Arabidopsis eFP Browser).

4.2.2 Translational control

Comparison of mRNA and pRNA accumulation patterns obtained for genes encoding chloroplast antioxidant enzymes in the tested accessions of *A. thaliana* revealed that, the expression of these genes is regulated not only at transcript level but also during the translation initiation (see chapter 3.1.3).

So far, modulation of translation activity during its initiation has been described for the genes encoding general control nonrepressed 4 protein (*GCN4*) in yeast (reviewed by Hinnebusch, (1990)), ferritin in mammals (Melefors and Hentze, 1993) and, more interestingly in the regulatory context of chloroplast proteins expression, for the small subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) in amaranth (Berry *et al.* 1988). Moreover, it was shown that in *A. thaliana* var. Col-0 drought stress resulted in the reduction of mRNA association with ribosomes (Kawaguchi *et al.* 2004), while exogenous application of hydrogen peroxide promoted the translation initiation (Shenton *et al.* 2006). However, the increase in the activity of translation initiation in response to H_2O_2 seemed to be concentration-dependent. Thus, lower concentration of H_2O_2 (0.2 mM) induced the translation of mRNA encoding antioxidants and cellular transporters, while higher concentration (2 mM) promoted translation of proteins involved in the ribosome biogenesis (Shenton *et al.* 2006). From these experiments it can be concluded that the cytosolic initiation of translation is redox-regulated. Such regulation of translation by photosynthetic signals was shown already for genes encoding ferredoxin 1 (*Fed1*) in dark-treated tobacco (Petracek *et al.* 1997; Hansen *et al.* 2001), several subunits of photosystem I (*PsaD*, *PsaF* and *PsaL*) in dark-treated spinach (Sherameti *et al.* 2002) and catalase 2 (*Cat2*) in light- and dark-treated leaves of maize (Skadsen and Scandalios, 1987). Since both, dark and light treatments, implicate to ROS production, it indicates that by regulation of ROS levels, chloroplast antioxidant enzymes control the chloroplast redox poise (Baier *et al.* 2000; Heiber *et al.* 2007; Kangasjarvi *et al.* 2008), which later influence the efficiency of translation initiation.

Present analyses revealed that all of the tested accessions strongly differed in the usage of mRNA for translation process (see chapter 3.1.3). To check whether this variability resulted from the different redox status of the selected accessions, ROS levels were compared between all of them (Figures 3-32 and 3-33). The analyses revealed that mRNA binding to ribosomes was generally more efficient in the H_2O_2-accumulating accessions than in the ones, in which lower levels of H_2O_2 were detected

(see Figure 3-33 and chapter 3.1.3), indicating that the translation initiation in the tested accessions was regulated, as shown by Shenton *et al.* (2006), in a H_2O_2-dependent manner. Only for Kas-1 no correlation between accumulation of H_2O_2 and the efficiency of translation initiation (low levels of H_2O_2) could be indicated, demonstrating accession-specific redox-responsiveness of translation.

The analyses of *Fed1* mRNA association with polysomes revealed that the regulation of this process is conferred by the sequences within the 5′UTR of this gene (Petracek *et al.* 1997). Present sequence comparison of 5'UTRs preceding the genes encoding chloroplast antioxidant enzymes in selected accessions of *A. thaliana* (see chapter 3.1.3.6) also indicated that the variability in the efficiency of translation initiation might be due to the differences in the 5'UTRs, more precisely in the sequences of regions flanking translation initiation site (TIS) (Joshi *et al.* 1997). It was proposed that the proteins operating in the cell at low abundances have a less optimal context sequence of TIS than those synthesized in high amounts (Joshi and Nguyen, 1995). Therefore, the sequences of regions flanking TIS of *Csd2*, *MDHAR* and *tAPx*, which differed between accessions (Figure 3-31), were compared with the optimal TIS sequence identified for *A. thaliana* by Rangan *et al.* (2008) as aaaaaaa(A/G)(A/C)aATGGcgaataata (Figure 4-1).

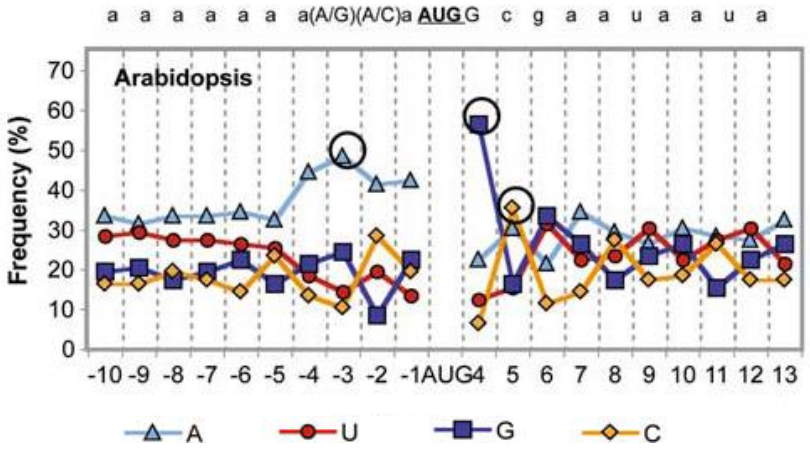

Figure 4-1. Nucleotide frequencies in the TIS sequences of *A. thaliana* (Rangan *et al.* 2008).

The most optimal nucleotides at the position -2 of TIS (A or C) were identified for *Csd2* in Col-0, Cvi-0 and Van-0, as well as for *MDHAR* in C24 (Figure 3-31). In contrast, the remaining accessions contained at this position other, less optimal nucleotides (T or C). Similarly, at the position +4 the most optimal G was identified in the TIS of *tAPx* only in Cvi-0, WS, C24 and Van-0, while other accessions contained at this position T

(Figure 3-31). However, the highest translation efficiencies for almost all tested transcripts (except *MDHAR*) were observed in the accessions, which had rather less optimal sequence of regions flanking TIS, indicating that the specific nucleotide composition of TIS may play a smaller role in translation initiation than assumed by Joshi and Nguyen (1995) and the efficiency of this process might be rather dependent on other structural properties of TIS as proposed later by Rangan *et al.* (2008).

4.2.3 Transcription and translation – antagonizers in expression regulation of chloroplast antioxidant system?

The comparison of the accessions revealed a high variability in the accumulation of transcripts encoding the chloroplast antioxidant enzymes (see chapter 3.1.1). In contrast, less variation was observed at the protein level (see chapter 3.1.2), suggesting that the differences in the mRNA accumulation were antagonized at the translational level. If it was so, the transcription and translation regulation would function like a valves and the combination of both of them would define the dynamics of the responses of plants to environmental factors. Therefore, to study the relative importance of transcriptional and translational regulation, the patterns of mRNA and pRNA accumulation were compared with those obtained for proteins.

Among all analyzed proteins the least variation between tested accessions was observed for 2CPs and GPXs (Figure 3-21), suggesting that the variability in the accumulation of transcripts encoding these genes was almost perfectly antagonized at the level of translation initiation. Indeed, the comparison of mRNA and pRNA levels demonstrated that in almost all tested accessions the high and low transcriptional activity of *2CPs* and *GPXs* was compensated at the translation level by reducing or enhancing the efficiency of mRNA binding to ribosomes, respectively (Figure 3-30). The exceptions from this compensatory regulation was *2CPB* in Ms-0 and *GPx7* in C24 and Van-0, which transcription and translation were regulated in parallel (e.g. low transcripts levels accompanied by low efficiency of mRNA binding to ribosomes), indicating accession-specific regulation of *2CPs* and *GPXs* expression. Moreover, the analyses of the ROS accumulation in the tested accessions (Figures 3-32 and 3-33) suggested that the compensatory regulation of transcription and translation depends on the redox status of plant cells. Thus, the low activity of both, transcription and translation was detected in Ms-0, which among all tested accessions accumulated the highest amount of superoxide

(Figure 3-32) and one of the lowest of H_2O_2 (Figure 3-33). In contrast, highly efficient transcription and translation was observed for *GPx7* in C24 and Van-0, which in comparison with Ms-0 accumulated similar amounts of H_2O_2 and lower of $O_2^{\bullet-}$ (Figures 3-32 and 3-33). It indicates that, in general, low concentrations of superoxide and H_2O_2 promote the transcription and translation and the activities of these processes decrease with the increase of $O_2^{\bullet-}$ levels, demonstrating that transcription and translation in *A. thaliana* depend on the balance between $O_2^{\bullet-}$ and H_2O_2 production, thus on the redox state of the cell. The results are consistent with previously shown for expression regulation of several genes (Baier and Dietz, 1996; Baier and Dietz, 1997; Horling *et al.* 2003; Baier *et al.* 2004, Shenton *et al.* 2006).

Beside 2CPs and GPxs, strong translational compensation was also observed in most of the tested accessions (except Kas-1 and Ms-0) for PrxQ (Figure 3-21). Among them, low transcriptional activity was compensated by efficient translation in WS, C24 and Van-0, while in Cvi-0 high binding efficiency of strongly accumulated *PrxQ* transcript to ribosomes was observed (Figure 3-30). In contrast, much higher level of PrxQ than in Cvi-0, WS, C24 and Van-0, were detected for Kas-1 and Ms-0 (Figure 3-21). In Kas-1 the strong accumulation of PrxQ was correlated with efficient binding of encoding its transcript to ribosomes, while in Ms-0 transcription was supported stronger than translation (Figure 3-30), indicating accession-specific regulation of *PrxQ* expression.

Similar to PrxQ in Kas-1, high protein levels of Csd2 in Kas-1, sAPx in Ms-0 and GR in WS results from with support of translational activity, while strong accumulation of tAPx protein in Cvi-0 correlates with high levels of mRNA encoding this protein (Figure 3-21), further showing the accession specific regulation of gene expression.

Nevertheless, the gene- and accession-specific expression regulation of the chloroplast antioxidant enzymes cannot be explained for every gene in every accession by a general feedback of the chloroplast/cellular redox poise on translation (as described by Shenton *et al.* (2006)). All protein levels were standardized to RuBisCO-LSU levels (see chapter 3.1.2), which also excludes the redox-regulation of protein import into chloroplast (Benz *et al.* 2009) and normalizes general translational redox regulation. Furthermore, the sequence comparison of 5'UTRs preceding genes encoding all chloroplast antioxidant enzymes did not give any evidence for specific RNA structures (Baim and Sherman, 1988; Kochetov *et al.* 2007) or codon usage effects, which could influence the translation process (Kanaya *et al.* 2001). Only the SNPs detected for

several tested accessions in the sequence of regions flanking translation initiation site (TIS) of *Csd2*, *MDHAR* and *tAPx* could impact the efficiency of mRNA binding to ribosomes (see chapter 4.2.2). Thus, it can be concluded that accession- and gene-specific patterning of mRNA, pRNA and proteins accumulation results from coordination of transcription and translation in transcript- and accession-specific manner.

4.3 Main chloroplast copper-containing proteins are linked by a micro-RNA regulon adjusting the copper availability

The results obtained for *Csd2* transcript accumulation indicated that the post-transcriptional regulation of the *Csd2* expression by *miR398* is conserved among *A. thaliana a*ccessions (see chapters 3.1.1.1 and 4.2.1.1). However, miR398, beside the regulation of *Csd2* expression, is also known to have a role in adjusting the copper availability in chloroplasts. In this process this micro-RNA link the expression of the main chloroplast copper-containing proteins, namely PetE, CSD2 and CCS1 (Bouché, 2013). Therefore the selected accessions were also compared for expression regulation of *PetE2* and *CCS1* at the transcript levels (see chapter 3.4). Among these proteins, plastocyanin is an important and often limiting electron carrier in the photosynthetic electron transport chain (Schöttler *et al.* 2004). Consequently, higher plants prioritize the delivery of copper to PetE by down-regulation of other copper-containing proteins (Abdel-Ghany, 2009). Such copper-dependent control of PetE, CSD2 and CCS1 expression by miR398 was shown for *A. thaliana* var. Col-0 cultivated under different copper concentrations (Abdel-Ghany, 2009). Subsequent analyses revealed that the levels of *miR398a* and *miR398b/c* were negatively correlated with *PetE2* expression also in other than Col-0 accessions, demonstrating that the copper regulon has been conserved over a wide range of species diversification and adaptation do distinct habitats (see chapter 3.4). Furthermore, the accumulation of both, *Csd2* and *CCS1* transcripts was controlled by miR398 in accession-specific manner, while the miR398 interaction sites were absolutely conserved (Figure 3-49).

4.3.1 PetE2 affects miR398 regulation

The accumulation of *PetE2* mRNA strongly differed between tested accessions at 20 °C (Figure 3-45). Since, it was proposed that the plastocyanin levels are adjusted to

maintain the photosynthetic performance under all kinds of growth conditions (non-stressful and stressful) (Abdel-Ghany, 2009), it indicates that in all selected accessions *PetE2* expression sensitively responded to the temperature and light conditions optimized for Col-0 to ensure sufficient photosynthetic activity. However, the systematic comparison of the photosynthetic parameters is not available for all tested accessions. Nevertheless, already the data obtained for Col-0, Cvi-0 and WS demonstrated that different accessions of *A. thaliana* cultivated under the same growth conditions (wide range of light intensities, as well as ambient and elevated CO_2) did not differ in the photosynthetic parameters, such as quantum yield of photosystem II and non-photochemical quenching (Li *et al.* 2008; Jung and Niyogi, 2009). Thus, it indicates that the observed accession-specific regulation of *PetE2* expression was not due to the adjustment of the photosynthetic activity to the environmental demands.

However, *PetE2* expression itself is strongly regulated by environmental parameters (e.g. light intensity), carbohydrate availability, abscisic acid and the redox state of plastoquinone pool (Oswald *et al.* 2001). Most of these parameters vary between accessions and are affected by the growth temperature (Cross *et al.* 2006; Davey *et al.* 2009). Moreover, in the regulation of copper availability in chloroplasts the expression of *PetE2* is linked to that of *miR398* (Bouché, 2013). Therefore the levels of *PetE2* transcripts and *miR398* were compared between all accessions acclimated to three different temperatures (see chapters 3.4.1.3 and 3.4.2.3). The comparison revealed that the levels of *PetE2* transcript were generally better correlated with the availability of miR398 in the accessions originating from colder habitats (Kas-1, Ms-0, WS and Van-0) than in Cvi-0 and Col-0 coming from warmer climates (see Table 3-1). Moreover, the negative correlation between *PetE2* transcript levels and both, miR398a and miR398b/c, were the weaker the stronger *PetE2* was expressed at 20 °C (Figure 3-45 and Table 3-1). Consistent with comparison of P*etE2*-deficient lines and Col-0 wild-type plants (Abdel-Ghany, 2009), the data demonstrate that *PetE2* transcripts do not directly act on *miR398* levels, but trigger a non-linear parameter translating plastocyanin availability into miR398 abundance.

4.3.2 miR168a and Ago1 regulation

The expression of *miR168a* and *Ago1* is transcriptionally co-regulated (Vaucheret *et al.* 2006). *miR168* is post-transcriptionally stabilized by *Ago*1 (Vaucheret *et al.* 2006) and,

vice versa, *miR168a* controls *Ago1* homeostasis via a feedback regulatory loop (Li *et al.* 2012). However, in the acclimation response of selected *A. thaliana* accessions to different growth temperatures, the correlation coefficients between the *Ago1* transcript levels and *miR168a* levels were generally low (Table 3-1), demonstrating low co-regulation of this genes and, therefore, different adjustment of the transcriptional and post-transcriptional effects. Nevertheless, calculated Spearman's correlation coefficients (r_s) for the transcript pair *Ago1*/*miR168a* indicate that the co-regulation of *Ago1* and *miR168a* might be habitat-dependent. Thus, the correlation coefficients between *Ago1* transcript levels and *miR168a* levels were higher for accessions originating from cold habitats (Kas-1 and Ms-0) than for those coming from warmer climates, indicating that the expression co-regulation of *Ago1* and *miR168a* is enhanced by cold.

4.3.3 Csd2 and CCS1 expression regulation by miR398

The post-transcriptional regulation of the *Csd2* and *CCS1* expression is conserved in all tested accessions of *A. thaliana* (Table 3-1). However, the *Csd2* transcripts levels were better correlated with the levels of *miR398a* and *miR398b/c* in Van-0, C24, Ms-0 and WS than in Kas-1, Cvi-0 and Col-0. Similarly, the Spearman's correlation coefficients calculated for pairs *CCS1*/*miR398a* and *CCS1*/*miR398bc* in WS, Ms-0, C24 and Van-0 were also lower than in Col-0, Kas-1 and Cvi-0, indicating a better correlation between the levels of *miR398s* and *CCS1* transcript. Thus, these data show that upon temperature acclimation the miR398-dependent regulation of *Csd2* and *CCS1* expression dominates in Van-0, C24, Ms-0 and WS, while it is overlaid (to variable extends) in remaining accessions.

Since, the Ago1 protein forms with miR398 a RISC complex (Vaucheret *et al.* 2006), which enables cleavage of the *Csd2* and *CCS1* target transcripts, the regulatory impact of miR398 on *Csd2* and *CCS1* expression might depend on the availability of Ago1. However, at 20 °C in Van-0, C24, Ms-0 and WS lower levels of the *Ago1* transcript than in Col-0, Kas-1 and Cvi-0 were detected (Figure 3-50). Therefore, a higher than in other tested accessions regulatory impact of miR398 on the *Csd2* and *CCS1* expression in Van-0, C24, Ms-0 and WS cannot be explained by high availability of Ago1. Moreover, the analyses of plants acclimated to lower (10 °C) or higher (30 °C) temperatures than 20 °C gave no indications that the strength of regulatory impact of miR398 on their target genes is modulated with regard to *Ago1* transcript abundance

(Figure 3-45, 3-46 and 3-50). Since, also the miR398 binding sites in *Csd2* and *CCS1* mRNAs were fully conserved (Figure 3-49), it is concluded that the differences in the correlation between the abundances of miR398s and their target transcripts result from accession-specific transcriptional regulation of the *Csd2* and *CCS1* expression.

Among all tested accessions, the most accession-specific variation in the expression regulation of *Csd2* was observed for Cvi-0 at 10 °C (Figure 3-45). Thus, *Csd2* mRNA was accumulated in Cvi-0 in higher amounts at 10 than at 20 °C, while in other selected accessions the level of this transcript increased with the growth temperature. Since, the same trend in the regulation of the *Csd2* expression in response to the temperature fluctuations was not observed in other cold-sensitive accessions, such as Col-0 and WS (Figures 3-45 and 3-55), it demonstrates that induction of *Csd2* is not essential for cold-tolerance, but is specific for Cvi-0. Furthermore, for *CCS1*, which also remains under control of miR398 (Beauclair *et al.* 2010) higher level of encoding its transcript, in contrast to *Csd2*, was detected in Cvi-0 at 20 °C than at 10 °C (Figure 3-46). Thus, it shows that the atypical regulation of gene expression by miR398 in Cvi-0 is specific only for *Csd2*. Furthermore, this Cvi-0-specific regulation of *Csd2* transcript accumulation demonstrates that the post-transcriptional regulation of *Csd2* expression by miR398 can be overwritten up to the effect of inversion.

The escape of Cvi-0 from the conserved regulation of *Csd2* expression by miR398 might be an adaptation to the harsh environmental conditions at its natural habitat. The accession originates from the volcanic mountains of the Cape Verde Islands, where grows on the black, rocky walls covered by mosses. Low amounts of rain are restricted in this place to the second half of the year, which limits the vegetation period of Cvi-0 to the late summer and autumn. Therefore, Cvi-0 has to develop quickly in dry and hot environment with high UV radiation. Probably, due to those climatic factors influencing the growth of Cvi-0 during its vegetation period the accession is less sensitive to drought, heat, UV-B and to paraquat (Abarca *et al.* 2001). The tolerance was linked by Abarca *et al.* (2001) to a Cvi-0-specific and more active *Csd2* gene allele. However, based on the *Csd2* transcript abundance regulation and correlation analyses (Figure 3-45 and Table 3-1) it is proposed that the higher stress tolerance of Cvi-0 results not primarily from the exchange of two amino acids in CSD2 of Cvi-0 (compared to CSD2 from Col-0), but is linked to specific expression regulation and its capability to overwrite the miR398-driven copper regulation.

In contrast to Cvi-0, in accessions from colder and less dry habitats with shorter vegetation periods, such as Kas-1, WS and Van-0, the *Csd2* and *CCS1* expression is much stronger linked to the *PetE2* expression in miR398-dependent way (see chapter 3.4). The most common interpretation of the copper homeostasis regulons proposes them as mechanisms controlling the copper pool in order to maintain expression of essential and indispensable plastocyanin (Bouché, 2013). Thus, limiting *CCS1* and *Csd2* expression for the sake of stabilization of the *PetE2* expression is advantageous if the risk for photooxidative superoxide formation is low. In contrast, under excess light and the low temperatures Mehler reaction activity is supported leading to the superoxide production (Schöner *et al.* 1990). As a consequence of high $O_2^{\bullet-}$, plants face a strain on its detoxification and therefore, activate involved in antioxidative defence CSD2 and CCS1.

Here, the uncoupling of *Csd2* expression from miR398 regulation was specifically observed in Cvi-0 at low temperature and 120 µmol m^{-2} s^{-1} light. The light intensity was much lower than that at the natural habitat of Cvi-0, but low temperature limited regeneration of the electron acceptors of photosystem I, supporting photooxidative stress (Yamasaki *et al.* 2002) and, in response to stress, inducing the *Csd2* expression.

4.4 Conclusion

The comparison of the transcript, polysomal RNA and protein data generated for a population of seven accessions of *A. thaliana* revealed that the expression of genes encoding the chloroplast antioxidant enzymes is regulated at all possible steps of gene expression in a gene- and accession-specific manner (see chapter 3.1). Furthermore, tested accessions also differed in the relative importance of transcriptional and translation regulation of chloroplast antioxidant system (see chapter 4.2.3). In general, Col-0, Cvi-0 and Ms-0 stronger supported transcription, while Van-0, WS, C24 and Kas-1 rather translation (Figure 3-30), demonstrating accession-specific patterning of mRNA, pRNA and proteins accumulation.

Moreover, the analyses of developmental and temperature responses of the tested accessions demonstrated that the regulation of this process depends of the developmental stage of the plant and is responsive to the environmental parameters (e.g. growth temperature). It indicated novel regulatory mechanisms in the gene expression patterning of *A. thaliana* accessions, which sensitively respond to settings

influenced during their evolutionary adaptation processes. Consistent with this assumption, Cvi-0 and Ms-0, the accessions originating from contrasting habitats, evolved contrasting mechanisms to control the *2CPs-APxs* gene cluster in response to ROS accumulation (see chapters 3.1.1 and 4.2.1.2) and in Cvi-0 the conserved in other tested accessions post-transcriptional regulation of *Csd2* expression by miR398 was less prominent (see chapters 3.4 and 4.3). It is concluded that the specific control of *2CPs-APxs* gene cluster and the escape from the conserved copper-homeostasis dominated regulation of *Csd2* and *CCS1* expression observed for Cvi-0 result from adaptation to the extremely harsh conditions at its natural habitat and provide enhanced tolerance of this accession to a wide range of stress conditions.

SUMMARY

Photosynthesis is a predominant source of ROS in plants. In the light, approximately 30 % of electrons which pass the photosynthetic electron transport chain can flow into reactive oxygen species (ROS) metabolism. If insufficiently antagonized, ROS accumulation can pose a threat to plant cells by causing damage of cellular components, such as proteins, nucleic acids and lipids. In order to prevent these destructive effects of ROS on cell functionality, chloroplasts have evolved a highly efficient antioxidant system, consisting of antioxidant enzymes and low molecular weight antioxidants. Here, the expression regulation of enzymatic components of this system was compared among seven accessions of *A. thaliana*, which cover a wide range of northern habitats (from Russia to Cape Verde Islands).

The comparison of temperature and developmental responses in selected accessions demonstrated that the expression of key chloroplast antioxidant enzymes responds to environmental stimuli, however the response dynamics hardly correlated with the climatic parameters (temperature and UV radiation) at the natural habitats of the accessions. Moreover, the analyses of transcript (mRNA), polysomal RNA (pRNA) and protein data revealed that the tested accessions used different strategies to adjust the chloroplast antioxidative defence system to environmental demands. Among them, Col-0, Cvi-0 and Ms-0 invested more into the expression regulation of this system at the transcript level, while Van-0, WS, C24 and Kas-1 supported rather translation. Nevertheless, both strategies of regulation led to the expression of chloroplast antioxidant enzymes at sufficient level to efficiently protect plants from ROS accumulation in Col-0, WS, C24 and Van-0. In contrast, Cvi-0, Ms-0 and Kas-1 accumulated high amounts of ROS. In response to ROS accumulation most of the antioxidant enzymes were expressed in Cvi-0 at significantly higher level than in Kas-1 and Ms-0. Moreover, in Cvi-0, the otherwise conserved post-transcriptional regulation of *Csd2* expression by miR398 was less prominent. It is concluded that these Cvi-0-specific mechanisms result from adaptation to the extremely harsh conditions at its natural habitat and provide enhanced tolerance of this accession to a wide range of stress conditions.

ZUSAMMENFASSUNG

Hauptquelle für reaktive Sauerstoffspezies (*engl.* reactive oxygen species; ROS) in Pflanzen ist die Photosynthese. Annähernd 30 % der Elektronen aus der photosynthetischen Elektronentransportkette führen unter Belichtung zur Produktion von ROS und müssen entsprechend entgiftet werden. Bei unzureichender Entgiftung können zelluläre Komponenten, wie Proteine, Nukleinsäuren und Lipide, durch ROS geschädigt werden. Um den ROS-induzierten Zelltod zu verhindern, haben Chloroplasten ein hocheffizientes antioxidatives Schutzsystem entwickelt. Dies besteht aus Enzymen, die in der Lage sind ROS schrittweise zu Wasser zu reduzieren, und niedermolekularen Antioxidantien. In der vorliegenden Arbeit wurde die Regulation der Expression des antioxidativen Schutzsystems in sieben *Arabidopsis thaliana* Akzessionen vergleichend untersucht. Diese Akzessionen besiedeln unterschiedliche Habitate über die gesamte nördliche Hemisphäre (von Russland bis zu den Kapverdischen Inseln) verteilt.

Vergleiche der Temperatur- und Entwicklungsabhängigkeit zeigten, dass die Expression der plastidären antioxidativen Enzyme durch interne Stimuli und solche aus der Umgebung gesteuert wird. Es konnten nur selten Korrelationen zwischen der Expression der plastidären antioxidativen Enzyme mit klimatischen Parametern der natürlichen Lebensräume (Temperatur und UV-Strahlung) festgestellt werden. Der Vergleich von Transkriptspiegeln (mRNA), polysomaler RNA (pRNA) und Proteinspiegeln zeigte, dass die analysierten Akzessionen unterschiedliche Strategien verwenden, um ihr plastidäres antioxidatives Schutzsystem an die Umweltanforderungen anzupassen: Beispielsweise, investieren Col-0, Cvi-0 und Ms-0 mehr in die Regulation der Transkriptspiegel, während Van-0, WS, C24 und Kas-1 eher die Translationsaktivität steuern. Ungeachtet der Unterschiede in beiden Strategien sind Col-0, WS, C24 und Van-0 in der Lage sich effektiv vor ROS zu schützen. In Cvi-0, Ms-0 und Kas-1 dagegen konnten hohe Mengen an ROS akkumulieren. In Folge dessen wurden in Cvi-0 antioxidative Enzyme deutlich stärker exprimiert als in Kas-1 und Ms-0. In Cvi-0 kommt hinzu, dass die sonst in allen anderen Akzessionen konservierte post-transkriptionellen Regulation der Csd2 Transkription durch miR398 weniger stark ausgeprägt war. Dieser für Cvi-0 spezifische Mechanismus lässt vermuten, dass diese

Akzession individuelle Anpassungs- und Toleranzmechanismen an ihre extremen natürlichen Bedingungen entwickelt hat.

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Aus dateschutzrechtlichen Gründen ist der Lebenslauf in der Online-Version der Dissertation nicht enthalten.

Due to the data protection reasons, the resumé is not included in the online version of the thesis.

LIST OF PUBLICATIONS

Juszczak I., Baier M. (2012) The strength of the miR398-Csd2-CCS1 regulon is subject to natural variation in *Arabidopsis thaliana*. *FEBS Lett.* **586:** 3385-3390

Juszczak I., Rudnik R., Pietzeniuk B., Baier M. (2012) Natural genetic variation in the expression regulation of the chloroplast antioxidant system among *Arabidopsis thaliana* accessions. *Physiol. Plant.* **146:** 53-70

MEETING ABSTRACTS

Juszczak I., Pietzenuk B., Kier L., Baier M. (2010) Natural genetic variation in the chloroplastic water-water cycle among *Arabidopsis thaliana* accessions. SEB Annual Main Meeting, Prague, Czech Republic (poster presentation)

Juszczak I. (2010) Natural genetic variation in the chloroplastic water-water cycle among *Arabidopsis thaliana* accessions. 19. Photosynthese Workshop, Frankfurt am Main, Germany (oral presentation)

Juszczak I., Pietzenuk B., Baier M. (2010) Natural variation in the chloroplast antioxidant system among *Arabidopsis thaliana* accessions. 4th SFB 429 International Symposium: "Signals, sensing and plant primary metabolism". Potsdam, Germany (poster presentation)

Juszczak I., Baier M. (2011) From transcript to protein: Natural genetic variation in the chloroplast antioxidant system among *Arabidopsis thaliana* accessions. Botanikertagung 2011, Berlin, Germany (poster presentation)

Juszczak I., Baier M. (2011) From transcript to protein: Natural genetic variation in the chloroplast antioxidant system among *Arabidopsis thaliana* accessions. Plant Biology 2011 Meeting, Minneapolis, United States (poster presentation)

Juszczak I. (2012) Natural genetic variation in the expression regulation of the chloroplast antioxidant system among *Arabidopsis thaliana* accessions. Havel-Spree Colloquium, Potsdam-Golm, Germany (oral presentation)

Juszczak I., Baier M. (2012) Natural variation in the complexity of chloroplast copper-containing proteins expression regulation among *Arabidopsis thaliana* accessions. 23rd International Conference on Arabidopsis Research, Vienna, Austria (poster presentation)

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APPENDIX

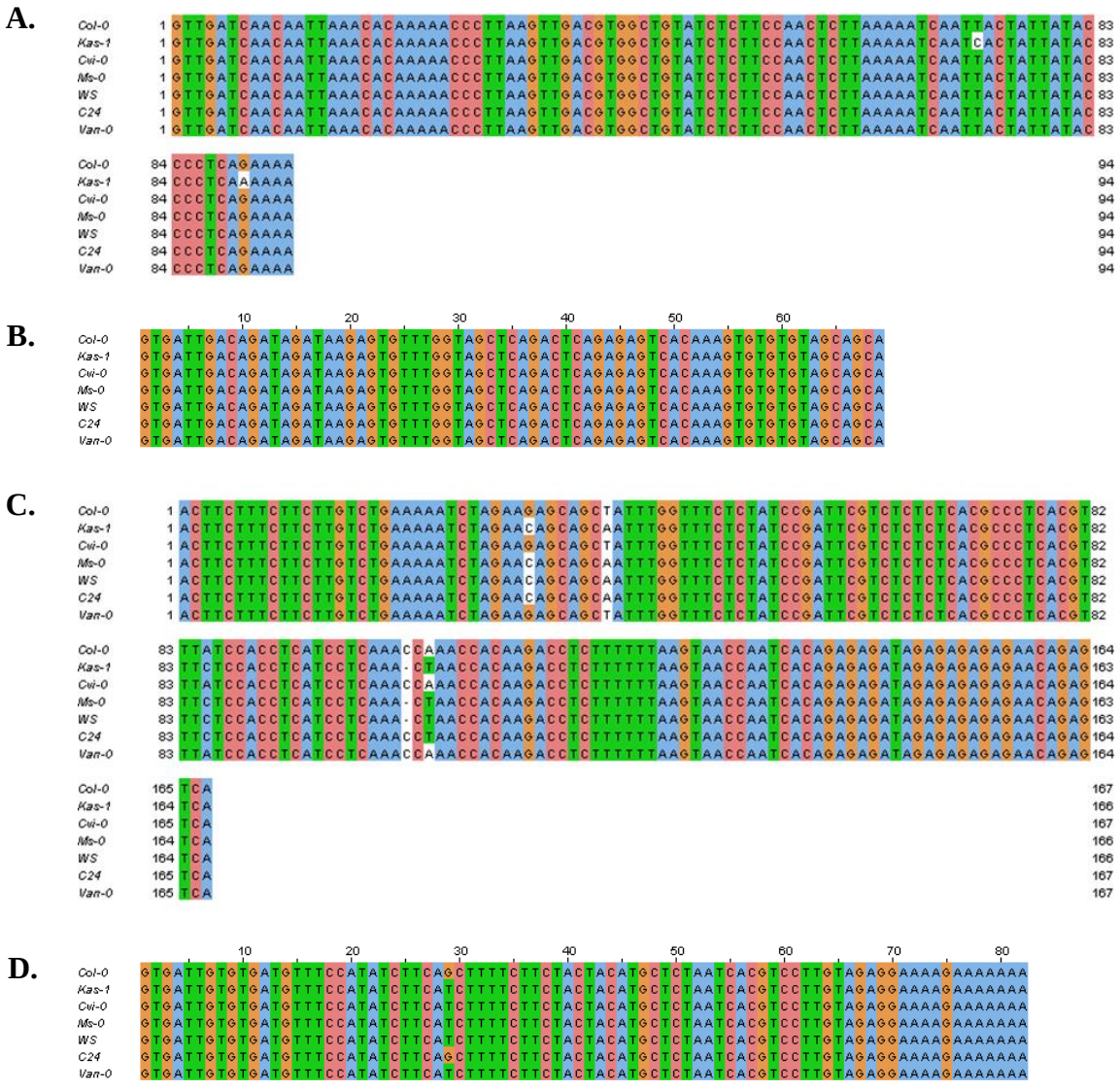

Figure A1. Comparison of 5'UTRs preceding **A.** *sAPx*, **B.** *2CPA*, **C.** *tAPx*, **D.** *PrxQ*, **E.** *PrxIIE*, **F.** *GPx1* and **G.** *GR* in tested accessions of *A. thaliana*.

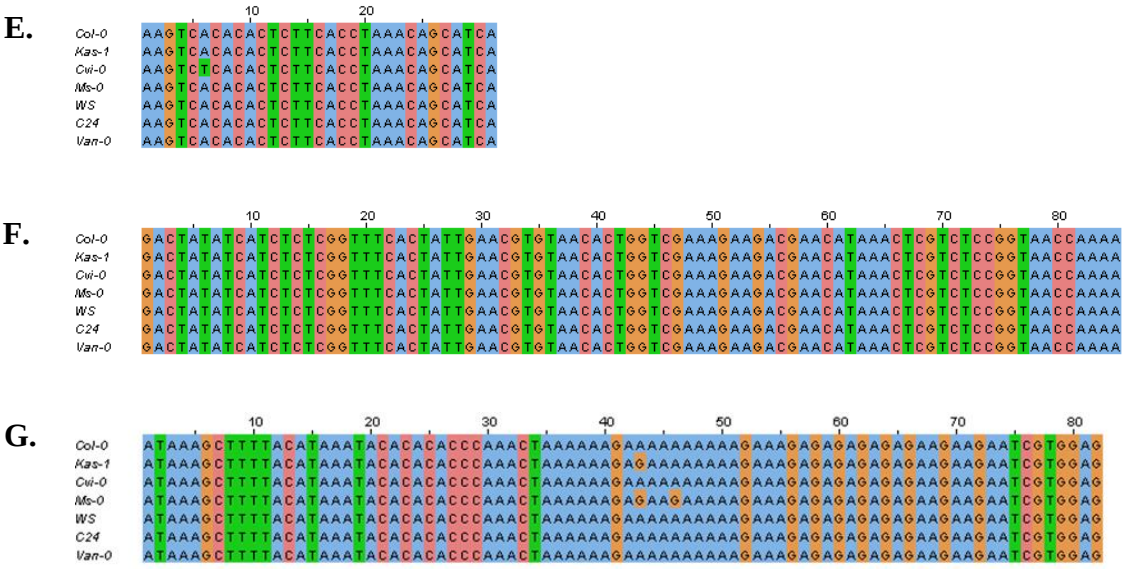

Figure A1 (continued). Comparison of 5'UTRs preceding **A.** *sAPx*, **B.** *2CPA*, **C.** *tAPx*, **D.** *PrxQ*, **E.** *PrxIIE*, **F.** *GPx1* and **G.** *GR* in tested accessions of *A. thaliana*.

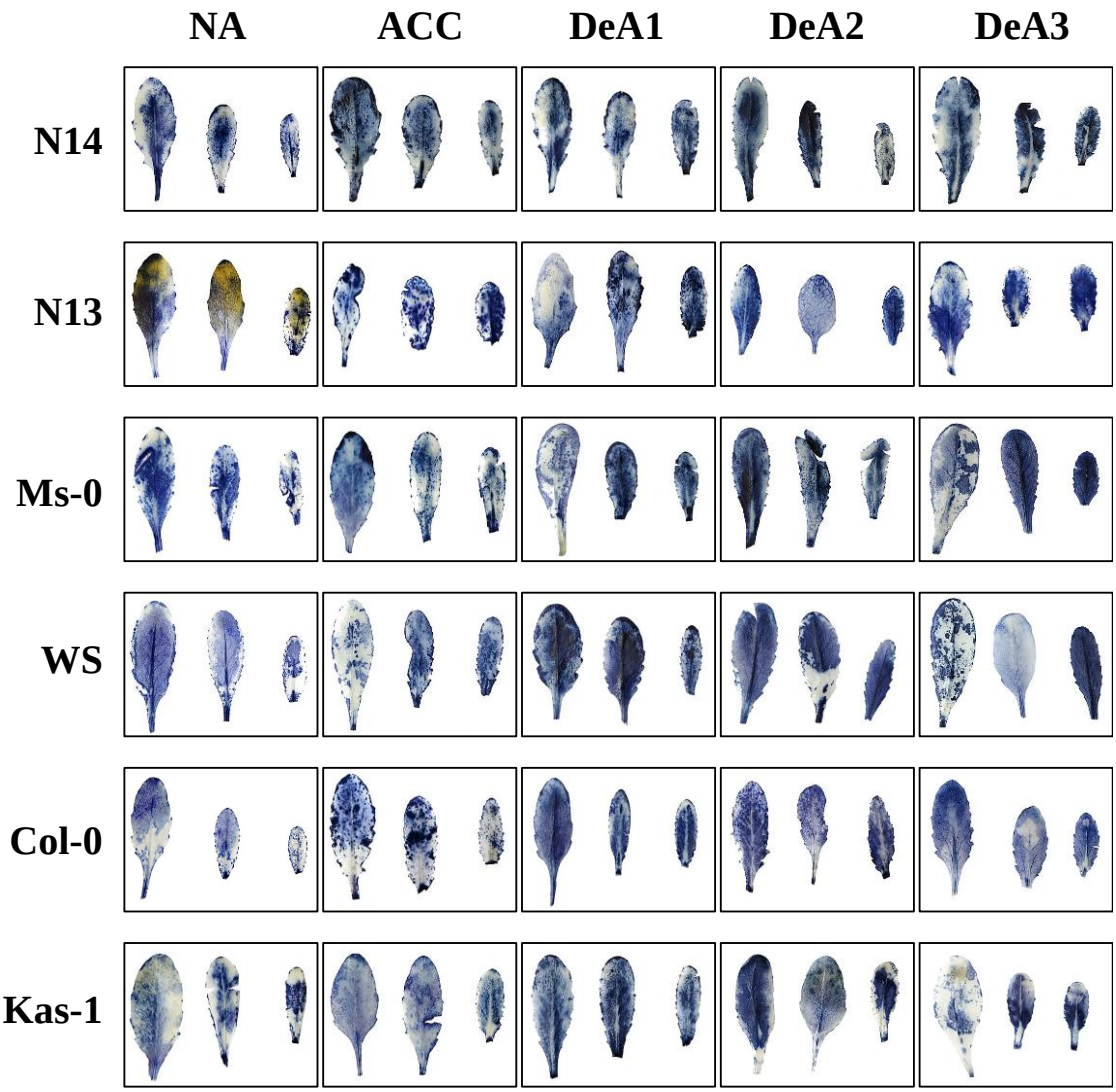

Figure A2. Endogenous production of superoxide radical anion ($O_2^{\bullet-}$) in ten *A. thaliana* accessions upon long-term cold stress treatment. Superoxide was detected by NBT staining in the mature, middle-old and young leaves of these accessions before (NA plants) and after (ACC plants) 14 days of cold acclimation, as well as on the first three days of de-acclimation phase following cold treatment (DeA1-3 plants).

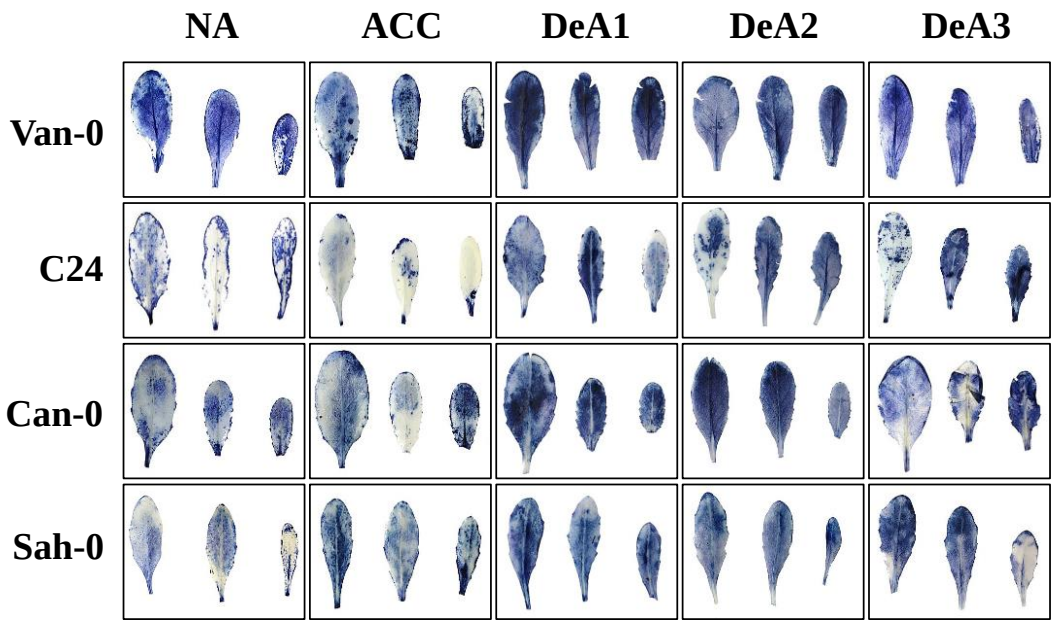

Figure A2 (continued). Endogenous production of superoxide radical anion ($O_2^{\bullet-}$) in ten *A. thaliana* accessions upon long-term cold stress treatment. Superoxide was detected by NBT staining in the mature, middle-old and young leaves of these accessions before (NA plants) and after (ACC plants) 14 days of cold acclimation, as well as on the first three days of de-acclimation phase following cold treatment (DeA1-3 plants).

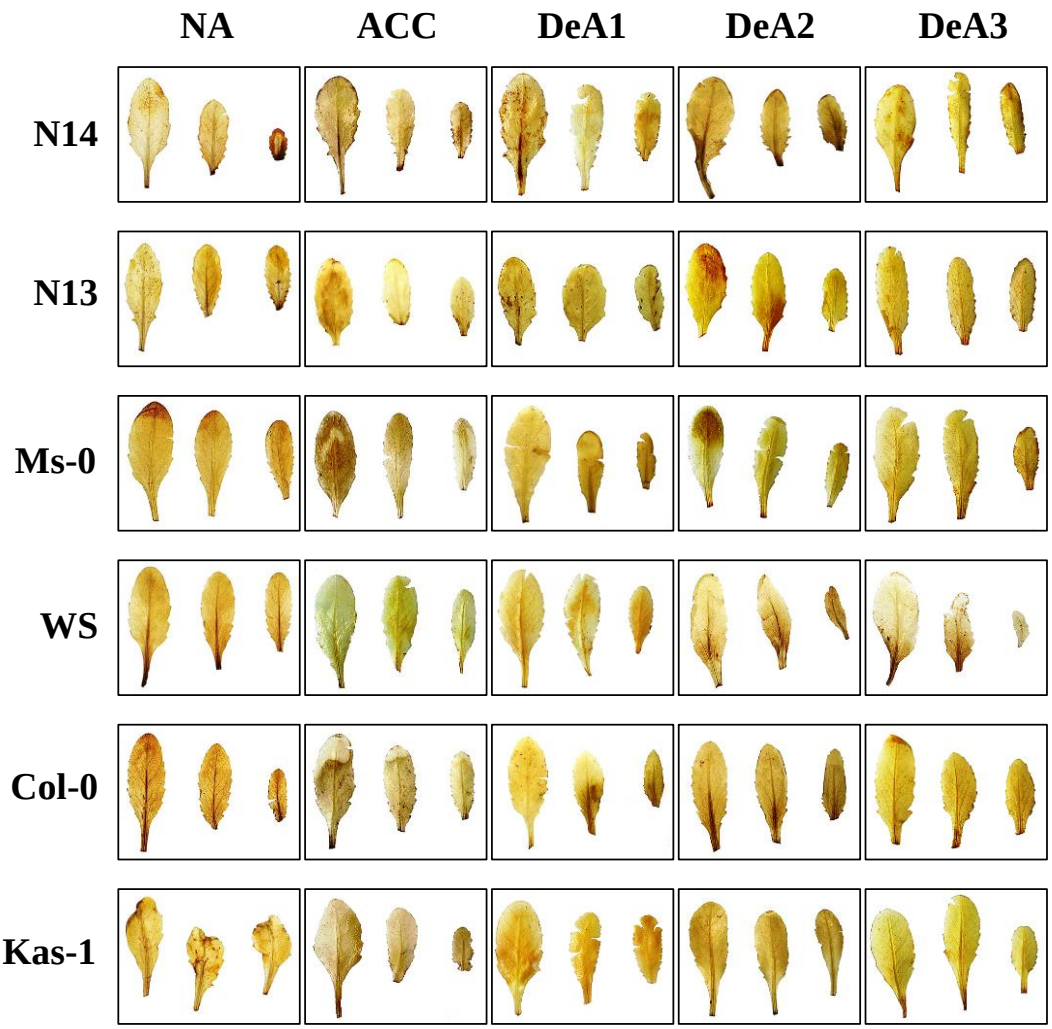

Figure A3. Endogenous production of hydrogen peroxide (H_2O_2) in ten *A. thaliana* accessions upon long-term cold stress treatment. Hydrogen peroxide was detected by DAB staining in the mature, middle-old and young leaves of these accessions before (NA plants) and after (ACC plants) 14 days of cold acclimation, as well as on the first three days of de-acclimation phase following cold treatment (DeA1-3 plants).

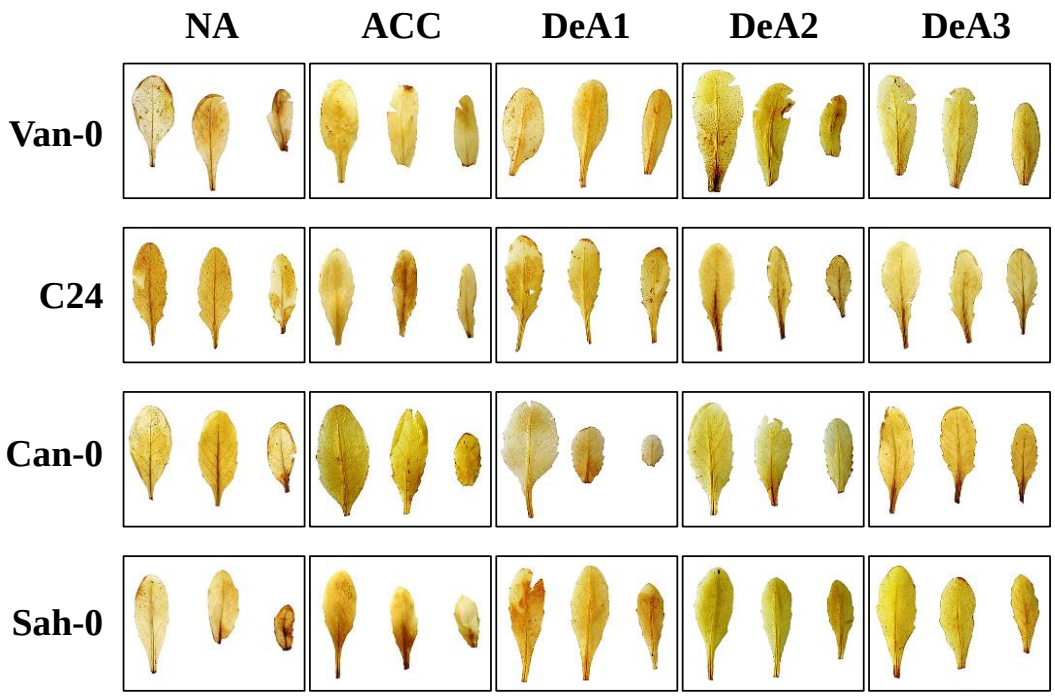

Figure A3 (continued). Endogenous production of hydrogen peroxide (H_2O_2) in ten *A. thaliana* accessions upon long-term cold stress treatment. Hydrogen peroxide was detected by DAB staining in the mature, middle-old and young leaves of these accessions before (NA plants) and after (ACC plants) 14 days of cold acclimation, as well as on the first three days of de-acclimation phase following cold treatment (DeA1-3 plants).