Arabidopsis thaliana thermomemory of prolonged cold stress and its ecophysiological consequences with respect to the plastidic antioxidant system

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Jelena Cvetkovic
from Belgrade (Serbia)

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LIST OF ABBREVIATIONS

A	ampere/absorption
A_{530}	absorbance at λ = 530 nm
ABA	abscisic acid
Act2	Actin 2
ANOVA	analysis of variance
APS	ammonium persulfate
APXs	ascorbate peroxidases
Asc	ascorbate
A. thaliana	*Arabidopsis thaliana*
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	control plants
°C	Celsius degree
CAT	catalase
2CPA	2-Cys peroxiredoxin A
2CPB	2-Cys peroxiredoxin B
cDNA	complementary DNA
CO_2	carbon dioxide
Col-0	Columbia-0
Chl	chlorophyll
Chl a	chlorophyll a
Chl b	chlorophyll b
CBF	c-repeat binding protein
COR15A	cold-regulated 15A gene
CSD2	Cu/Zn-superoxide dismutase
Cu/Zn-SOD	copper-zinc superoxide dismutase
DD_{50}	flowering day difference
DHA	dehydroascorbate
DHAR	DHA reductase
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides
DTT	dithiothreitol
EDTA	ethylenediamintetra-acetic acid
Eff qY	efficient quantum yield
Fig.	figure
FD	ferredoxin
FNR	ferredoxin NADPH reductase
GLR	glutaredoxin
GOX	glycolate oxidase
GPXs	glutathione peroxidases
GR	glutathione reductase
GSH	glutathione
GSSG	oxidized glutathione
HCl	hydrochloric acid
H_2O	water
H_2O_2	hydrogen peroxide
Heynh.	Gustav Heynhold
KCl	potassium chloride
KCN	potassium cyanide
kDa	kilo dalton
KH_2PO_4	monopotassium phosphate
K_2HPO_4	dipotassium phosphate
Ini qY	initial quantum yield

IM	inner membrane
L.	Carl von Linné
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
MDHAR	monodehydroascorbate reductase
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate
N	number
NA	non-acclimated plants
NBT	nitro blue tetrazolium
NP	non-primed plants
O_2	molecular oxygen
$^1O_2$	singlet oxygen
O_2•	superoxide radical anion
OH•	hydroxyl radical
P	primed plants
p	Spearman correlation coefficient
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGP	phosphoglycolate phosphatase
PM	plasma membrane
PMSF	phenylmethylsulfonyl fluoride
PT	primed and triggered plants
PSI	photosystem I
PSII	photosystem II
PRXs	peroxiredoxins
PrxIIE	peroxiredoxins type IIE
PrxQ	peroxiredoxin Q
qRT-PCR	quantitative real-time PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
RuBisCO	ribulose-1,5-bisphosphate carboxylase oxygenase
sAPX	stromal ascorbate peroxidase
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
T	triggered plants
tAPx	thylakoid-bound ascorbate peroxidase
TBS	Tris-buffered saline
TEMED	tetramethylethylene-diamine
TF	transcription factor
Tyl	thylakoid
UV	ultraviolet
V	volt
v/v	volume per volume
w/v	weight per volume

1 INTRODUCTION

1.1 Priming concept

Initial research on acquired physiological immunity in plants and their sensitization mechanisms was conducted in early 1933 by Chester (1933). Since then, scientists have conducted many experiments in order to discover and understand phenomenon of defense priming. Terms such as adaptation, acclimation, induction, acquired resistance and cross protection have an overlapping meaning with the term 'priming'. These terms are discussed and separated from each other in Hilker et al. (2015). A transient environmental stress experience can prepare an organism for improved defense responses when the next stress occurs (Hilker *et al.* 2015; Balmer *et al.* 2015; Conrath *et al.* 2015; Crisp *et al*. 2016; Li *et al.* 2014). Memorizing the previous (priming) experience during the lag-phase is necessary preparation for future (triggering) stress responses. According to Hilker et al. (2015), primability is the plant's ability to prepare itself for improved inducible stress responses.

In the context of biotic stress resistance, the priming concept was exhibited via stronger pathogen response mechanisms (Conrath *et al.* 2002; Shah and Zeier, 2013; Gamir *et al.* 2014) and herbivory attacks (Hilker and Fatouros, 2015; Kim and Felton, 2013). Primed organisms behaved differently to future stress when are primed with abiotic stressors such as temperature, osmolality, illumination and drought (Hincha and Zuther, 2014; Saidi *et al*. 2011; Sani *et al.* 2013; Li *et al.* 2014; Balmer *et al.* 2015). Stress response has not only been studied at the plant level but also on bacteria and fungi. Rilling et al. (2015) have recently highlighted the role of priming and memory of antimicrobial response. Induced priming defense is also possible by the application of chemical stimuli such as including the beta-amino-butyric acid (BABA) (Zimmerli *et al.* 2000) and phytohormone abscisic acid (ABA) (Goh *et al.* 2003).

The storage of the information on the priming effect was not clearly explained until now. It is still not clear whether chromatin marks, micro RNA levels, transcriptional factors or phytohormons and secondary metabolites accumulation are key elements contributing to the information storage. According to Crisp et al. (2016),

during recovery, RNA metabolism, post-transcriptional gene silencing, and RNA-directed DNA methylation have a potential to play key roles in resetting the epigenome and transcriptome and in altering the memory. The questions about the second stress response and its speed, timing, strength and sensitivity and the expected priming costs and/or benefits (Figure 1-1) were discussed frequently. The response to a triggering stress stimulus is linked with the organism's experience of a previous priming stimulus and can be different from the first one (Hilker *et al.* 2015; Bruce *et al.* 2007). Faster kinetics, earlier response, higher sensibility and reaction than a non-treated organism are just some of the possible response patterns to the triggering stress. Exposing pre-treated plants to a second (triggering) stress is expected to benefit them. It is assumed that an investment into priming causes modest costs (Figure 1-1, Hilker *et al.* 2015). The costs and benefits are usually studied by quantifying the fitness and morphological parameters.

Investigation of the importance of antioxidative system priming is gaining a greater attention. Karpinski and Szechynska-Hebda (2010) highlighted the importance of chloroplast networks in performing a biological quantum computation and memorizing light training with the help of non-photochemical quenching and photo-electrophysiological signaling (PEPS) plants to optimize their Darwinian fitness. The results from Li et al. (2014) indicate that cold training activated the subcellular antioxidant systems in winter wheat plants by depressing the oxidative burst in photosynthetic apparatus and, therefore, enhanced the tolerance to subsequent low temperature stress.

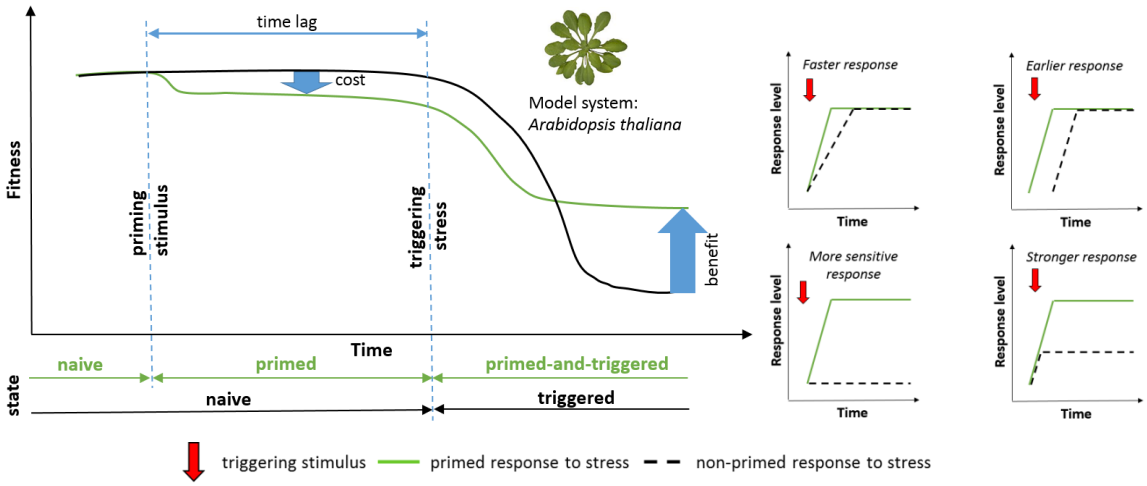


Figure 1-1. Priming scheme with possible cost and benefits consequences (left) and response patterns of pre-treated and non-treated plants upon triggering stimulus (right). Modified after Hilker et al. (2015).

1.2 Environmental stress

Any kind of environmental change can disturb the highly integrated and regulated process of photosynthesis and their role in balancing the light energy absorbed by the photosystems with the energy consumed by metabolic sinks of the plant (Ensminger *et al.* 2006). As a result of the changed environment, the plants respond by regulating the gene expression (Lopez-Maury *et al.* 2008) and the modulation of metabolites (Hurry *et al*. 2000) and antioxidants (Asada, 1999; Foyer and Noctor, 2003; del Rio *et al.* 2006; Navrot *et al.* 2007). The temporal (acclimation) or permanent (adaptation) activation of the regulatory systems is dependent on the duration of stress (Chinnusamy *et al.* 2004). Environmental stress cannot be studied separately. The presence of an abiotic stress can have the effect of reducing or enhancing the susceptibility to a biotic pest or pathogen, and *vice versa* (Atkinson and Urwin, 2012).

1.2.1 Abiotic stress

Different environmental perturbations as a result of one or a combination of several factors such as high light intensity, salinity, drought, heat/chilling, ozone, herbicides and heavy metals induce the elevated ROS production (Mittler, 2002; Mittler *et al.* 2004) and are reflected on the plant growth and development. For instance, exposure to certain heavy metal ions (Cu^{2+}, Fe^{2+}, Hg^{2+}) shifts the ROS metabolism balance towards the accumulation of hydrogen peroxide and, in the end, converts to high reactive radicals that can cause cell death (Karuppanapandian *et al.* 2006). Ozone (O_3), one of the well-known air pollutants, is becoming more important as deforestation continues. The oxidizing potential of O_3 and the consequent formation of radicals are the reasons for high toxicity of this pollutant (Li *et al.* 2009). Drought stress causes complex reactions (turgor lost, membrane damage) and involves several defense steps. By minimalizing the water loss by ABA stimulating stomatal cell, the plants stimulate the central hormone (Pei *et al*. 2000).

Temperature requirements for optimal growth are plant specific. Frequently, temperature is a crucial stress factor. In nature, acclimation to low temperature is

usually initiated by short-term fluctuations in temperature, which perturbs the metabolic homeostasis and induces a stress response (Ensminger *et al.* 2006). Together with elevated light, these stressors are known to increase the ROS production inside chloroplasts (Foyer *et al.* 1994). Redox status, even after short cold stress, is harshly imbalanced. Adjustments in photosynthesis to maintain the balance of energy flow occur when low temperatures cause an imbalance between the source of energy and the metabolic sink (Ensminger *et al.* 2006). Cell membrane is component where the first impact occurs (Quinn, 1988). The reduced leaf expansion and chlorosis are some of the phenotypic symptoms of long stress exposure (Yadav, 2010).

The length of the cold stress period plays a crucial role for the photosystem. Long-term (months) cold hardening of winter rye at 5 °C was found to increase the *in situ* photosynthetic capacity (Hurry *et al.* 1994). In contrast to long cold stress, hours of cold resulted in a half maximal Calvin Cycle inhibition. According to Thomashow (1999), reductions or cessations in plant growth, increased level of plant hormones and antioxidants as well as modifications in cell wall occurred during cold acclimation. The changes in membrane lipid composition, accumulation of osmolites and sugars as well as increased transcript abundance are responses to longer exposure (days, weeks) to low positive temperatures (Thomashow, 1999; Cook *et al.* 2004; Hannah *et al.* 2006, Knight and Knight, 2012). When temperature tolerance is insufficient, chilling injury results in cellular dysfunctions, loss of vigor, wilting, chlorosis, sterility and, in the worst scenario, plant death (Knight and Knight, 2012).

The C-repeat binding factors, CBFs or DREB1, are a family of transcriptional activators which play a huge role in the cold-acclimation process (Thomashow, 1999). Within this transcription factor family, there are three transcriptional activators such as CBF1 (DREB1b), CBF2 (DREB1c) and CBF3 (DREB1a). After a few minutes of cold stress exposure, plants induce a transcript of these genes and reach the maximum after two hours (Cook *et al.* 2004). The CBFs, as a group of APETALA 2/ethylene-responsive element binding factor (AP2/ERF), contribute not only to the cold acclimation process but also towards an increase in the freezing tolerance through the regulation of genes COR (cold regulated), KIN (cold inducible) and ZAT (zinc-finger transcription factor) (Sakuma *et al.* 2002; Gorsuch *et al.* 2010). Induction of freezing tolerance and activation of CBFs, ZAT and COR

genes as well accumulation of proline and sugars were discussed frequently (Zuther *et al.* 2015, Juszczak *et al.* 2016, Thomashow, 1999). *COR15A* (At2g42540) is a cold-regulated gene of *Arabidopsis thaliana* that encodes a chloroplast-targeted polypeptide (Lin and Thomashow, 1992), enhances the *in vivo* freezing tolerance of chloroplasts in non-acclimated plants by almost 2°C (Artus *et al.* 1996) and increases the cryostability of cellular membranes (Steponkus *et al.* 1998). *ZAT10* (At1g27730) is a zinc finger transcription factor that enhances the tolerance of plants to salinity, heat and osmotic stress when it is constitutively expressed (Mittler *et al.* 2006). *BAP1* (At3g61190) encodes a membrane-associated protein with a calcium-dependent phospholipid-binding activity (Hua *et al.* 2001) and is induced by various stimuli such as temperature variations, mechanical stress, and biotic stresses (op den Camp *et al.* 2003; Yang *et al.* 2007) with negatively regulating defense responses and cell death in *Arabidopsis thaliana* (Yang *et al.* 2006).

1.2.2 Biotic stress

Plants interact within the environmental communities and that can result in plant injure by other organisms (bacteria, viruses, fungi, parasites, insects, etc.). The ROS production is one of the first cellular responses following successful pathogen recognition (Torres *et al.* 2006). Pathogen attacks stimulate the production and the accumulation of ROS as a defense response (Levine *et al.* 1994). Insect feeding causes wounding and, therefore, results in ROS production in the damaged tissue. Harvey et al. (2007) proposed mollusks, and less likely insects, as the major herbivores consuming *Arabidopsis thaliana* in spring due to the temperature climate conditions. Herbivores such as arthropods can cause significant damage in agricultural plants and have an influence on the plant biodiversity structure (Allan and Crawley, 2011). Specificity in the defense induction is mediated by the perception of herbivore-associated molecular pattern and varies across herbivore species (Falk *et al.* 2014). Recognition of herbivory-associated molecular pattern and induction of low molecular weight compounds via insect oral secretion during feeding, allow plants to respond in a wound-induced manner (Mithofer and Boland, 2008). According to Schäfer *et al.* (2011), perception of herbivore-associated molecular pattern in *Arabidopsis thaliana* plant species specifically triggered the biosynthesis of oxylipins such as jasmonate acid

and isoleucine conjugate biosynthesis as defense metabolites. The synchronized behavior of defense molecule biosynthesis and signaling with insect feeding behavior is already known (Goodspeed *et al.* 2012). In addition to all of these plant defense strategies against herbivory and microbial pathogens, the role of small RNAs and genes involved in their biogenesis is important (Ruiz-Ferrer and Voinnet, 2009).

According to the priming definition, preparations by an occasional and discontinuous (a)biotic stress are resulting on better organisms performance when next stress occurs (Hilker *et al.* 2015; Conrath *et al.* 2001).

1.3 Reactive oxygen species (ROS) and antioxidative defense systems

Environmental stresses (abiotic and biotic) lead to an enhanced generation of reactive oxygen species (ROS) in plants due to disruption of cellular homeostasis (Foyer *et al.* 1994; Hurry *et al.* 1994; Mittler, 2002; Ensminger *et al.* 2006).
The molecular oxygen on the Earth is important as its usage allows all aerobic organisms to achieve the goal of a higher energy yield as compared to fermentation (Dismukes *et al.* 2001). As a di-radical and being rather unreactive, O_2 univalent reduction leads to the formation of chemically more reactive oxygen species (D'Autreaux and Toledano, 2007). ROS are by-products of the normal cell metabolism in plants where the balance between production and elimination is disturbed under stress conditions (Karuppanapandian *et al.* 2011). As a result of the high accumulation, ROS can quickly inactivate enzymes, destroy membranes of vital cell organelles, degrade nucleic acids, lipids and proteins and lead to irreparable metabolic dysfunction and death. Imbalances in photosynthesis result in a stronger generation of ROS. Distinct biological properties such as chemical reactivity, half-life and lipid solubility are characterizations of various ROS (Halliwell and Gutteridge, 2000).

1.3.1 ROS production

ROS is generated from the O_2 by energy/electron transfer reactions and result in generation of the superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), hydroperoxyl radical ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2), alkoxy radical ($RO^{\cdot}$), peroxy radical ($ROO^{\cdot}$), singlet oxygen (O_2^1) and excited carbonyl (RO*) (Halliwell, 1977; Dismukes *et al.* 2001; Karuppanapandian *et al.* 2011; Klotz, 2002; Noctor and Queval, 2007).

Singlet oxygen is a highly reactive molecule in comparison to O_2 and can interact with various biological molecules, such as proteins, DNA and unsaturated fatty acids (Mittler, 2002; Wagner *et al.* 2004). Preferential targets for chemical reactions are double bands (Karuppanapandian *et al.* 2011). The production of superoxide radical is a result of oxygen reduction (Halliwell, 1977; Fridovich, 1986). Due to a short life time (2-4 μs) and restricted movement, this radical has oxidizing and reducing properties (Halliwell, 1977). It is converted to hydrogen peroxide spontaneously or by reacting with superoxide dismutase or can alter other ROS such as hydroxyl radical and singlet oxygen (Noctor and Queval, 2007; Haber and Weiss, 1932; Fridovich, 1986). The O_2^- and H_2O_2 have preferred biological targets and HO• has indiscriminate reactivity towards biological molecules (D'Autreaux and Toledano, 2007). Hydroperoxyl radicals are formed from superoxide radicals and can cross membranes and initialize lipid autoxidation (Halliwell and Gutterigde, 2000). Hydrogen peroxide is a long-living molecule (approx. 1ms) with the ability to diffuse and generate damages and inactivate enzymes away from the generation site by acting as a messenger (Moller *et al.* 2007; Halliwell, 1977; Halliwell, 2006). Depending on the concentration, hydrogen peroxide arriving from chloroplasts and mitochondria electron transport chains, photorespiration and oxidation of fatty acids, can have a dual role: signaling and descrutive (Asada, 1999; Foyer and Noctor; 2000; Moller, 2001, Dat *et al.* 2000). Hydroxyl radical, as a most reactive oxidant from ROS, can be produced in the presence of hydrogen peroxide and transitional metals via Fenton reactions and it is responsible for the irreversible chemical modifications of various cellular components (Haber and Weiss, 1932; Halliwell and Gutteridge, 2000; Mittler *et al.* 2004). This non-scavenger radical does not have a direct signaling function but can elicit signaling role via products of its reactions and its excess results in

programmed cell death (Halliwell, 2006; Moller *et al.* 2007; Karuppanapandian *et al.* 2011).

As a byproduct of various cell pathways under stressful and non-stressful conditions, ROS are produced in chloroplasts, mitochondria, peroxisomes, the endoplasmic reticulum, apoplast, cell wall and plasma membrane (Mittler *et al*. 2002). Due to cell rhythm, more ROS are produced from chloroplasts and peroxisomes during the day, while at night they are produced mainly in mitochondria (Asada, 2006; Moller, 2001).

Chloroplasts are places where different varieties of ROS are formed. Miyake et al. (1991) highlighted that 30% of the electrons transferred through photosynthesis reactions at PSI and PSII can flow into ROS metabolism. According to Asada (2006), chloroplasts are the primary source of O_2^1 production due to the association with the electron transport chain sites such as ferredoxin at PSII and Fe-S clusters at PSI (Takahashi and Asada, 1988). Singlet oxygen can be produced in chloroplasts as a result of photosensitization of chlorophyll molecules and under UV stress (Chalapathi Rao and Reddy, 2008). Superoxide anions (O_2^-) are mainly generated together with O_2^1 at the thylakoid membrane by the process Mehler reaction (Foyer and Noctor, 2000).

Mitochondria can generate cytotoxic ROS such as hydrogen peroxide and superoxide radical via its own electro transport chains reactions where anions can be further reduced by Mn-superoxide dismutase to hydrogen peroxide (Moller, 2001; Navrot *et al.* 2007). The same author highlights that hydrogen peroxide can then react with iron and copper anions and produces highly toxic hydroxyl radical in Fenton reaction that penetrates membranes.

Peroxisomes are sites where the majority of hydrogen peroxide is generated via its own photorespiratory pathway by different flavin oxidases (del Rio *et al.* 2006; Mano and Nishimura, 2005). Superoxide anions are also produced here (Sandalio and del Rio, 1988). Due to a short half-life (1µs), it is subsequently converted into hydrogen peroxide and oxygen by dismutases (del Rio *et a*l. 2006).

Other sources of ROS generation in plants are the endoplasmic reticulum, apoplast, the plasma membrane and the cell wall. Endoplasmic reticulum is the site of hydrogen peroxide production (Delaunay-Moisan and Appenzeller, 2015). Apoplastic hydrogen peroxide production is connected with the presence of cell

wall oxalate oxidase (Lane, 2002). Plasma membrane NADP-dependent oxidase transfers electrons from cytoplasmic NADPH to oxygen and resulting in superoxide radical formation, which can be converted to hydrogen peroxide by dismutation (Heyno *et al.* 2011). Kim et al. (2010) stressed that cell wall peroxidases site are a site of hydrogen peroxide production.

1.3.2 Cellular damages and signaling role by ROS

Indicators and signals of environment come from ROS formation and their conducted response program ability (Foyer *et al.* 1994; Hurry *et al.* 1994; Mittler, 2002). Their size and diffusion characteristics determine the potential of information to signal translation (Mittler *et al.* 2004).

Accumulation of ROS initiates redox disequilibrium and can lead to a cell collapse via damaging the proteins, nucleic acids and lipids as well as lead to a quick (genetically or not orchestrated) cell death namely, programed cell death and necrosis (Gadjev *et al.* 2008; Moller *et al.* 2007). Through high concentrations of antioxidants or translational and transcriptional signaling inhibitors, cell death can be stopped and damages on cellular components can be prevented (Halliwell, 2006; Moller *et al.* 2007; Hiltscher *et al.* 2014). According to Mittler et al. (2004), tightly conducted redox balance between enzymatic and non-enzymatic antioxidants is crucial for cell death avoidance. Singlet oxygen from chloroplasts affects membrane proteins and lipids near the site of production (Asada, 2000). The attack of ROS on crucial proteins and lipids, which undergo oxidation and subsequent inactivation by ROS, causes many modifications in their structure and properties (Moller *et al.* 2007). The superoxide anions accumulation and conversion to hydrogen peroxide occurs spontaneously or with the help of superoxide dismutases (McCord and Fridovich, 1969; Marklund, 1976; Kurepa *et al.* 1997). In addition to that, hydrogen peroxide produced by glyoxysome and the mitochondrion influences the cytosolic concentration of ROS (Mittova *et al.* 2004; Noctor *et al.* 2002). The destructive characteristic of hydrogen peroxide and superoxide anions is primarily due to their contribution to the hydroxyl radical production rather than the direct damage (Karuppanapandian *et al.* 2011). This radical causes random changes of DNA observed for many genes (Moller *et al.* 2007).

Apart from the degradation role upon relatively high sub-lethal concentrations, the different types of ROS trigger various signaling cascades and control gene expression inside and outside of chloroplasts (Maruta *et al.* 2012, Karuppanapandian *et al.* 2011; Neil *et al.* 2002; Foreman *et al.* 2003; Mullineaux and Karpinski, 2002; op den Camp *et al.* 2003). Mittler *et al*. (2004) defined more than 152 genes in Arabidopsis that are tightly regulated in the ROS gene network and that affect plant growth and cell metabolism.

Changes in gene transcription and Ca^{2+} signaling pathways in plant cells occur in response to altered temperatures (Dai *et al.* 2007, Ma *et al.* 2009). *COLD1* encodes a regulator of G-protein signaling that is localized in the plasma membrane and endoplasmic reticulum in rice during chilling temperatures (Ma *et al.* 2015). According to these authors, the sensing low temperature is perceived via Ca^{2+} channel and G-protein activity.

Plant stress hormones such as salicylic acid (SA), jasmonate (JA) and ethylene (ET) are located downstream of the ROS signal and as a secondary messenger, hydrogen peroxide, influences the induction of hormone signaling pathways interactions (Orozco-Cardenas *et al.* 2001; Mittler *et al.* 2004; Yan *et al*. 2007).

The plasma membrane NADP oxidase can trigger calcium chanels and mitogen-activated protein kinase signaling pathways and stop the hormone signaling transfer of JA, SA and ET (Evans *et al*. 2005; Overmyer *et al.* 2003).
Intracellular ROS signaling in *Arabidopsis thaliana* is also induced in response to ozone exposure (Kangasjärvi and Kangasjärvi, 2014). ROS and chemical control of cell death after ozone exposure include bimodal damages with similarities to pathogen-induced oxidative damage (Overmyer *et al.* 2003).

One of the best-studied plant signaling molecules is hydrogen peroxide (H_2O_2). H_2O_2 is relatively stable (steady state levels ~10^{-7} M) and is able to diffuse due to modulated changes in membrane permeability or by transport through aquaporins (Bienert *et al.* 2006). Due to the selective reactivity and diffusibility, H_2O_2 has a perfect role in signaling. However, it does not act alone. Hydroxyl radical, HO•, despite very short half-life and high toxicity, seems to operate in H_2O_2 sensing (D'Autreaux and Toledano, 2007). Hydrogen peroxide derived from the chloroplast has a clear retrograde signaling function (Maruta *et al*. 2012). In response to APx silencing, CBF1 transcript level was reduced due to ROS-signaling. Control of the

antioxidant capacity depends on the chloroplast-to-nucleus redox signaling, considering that all enzymatic components are nuclear encoded (Heiber *et al.* 2007). Generation of the same signaling molecule does not imply that the plant transfer the same message. For instance, H_2O_2 produced in peroxisomes induces transcripts involved in protein repair responses, while H_2O_2 produced in chloroplasts induces early signaling responses, including transcription factors and biosynthetic genes involved in production of secondary signaling messengers (Sewelam *et al.* 2014). According to Mullineaux et al. (2006), compartment-specific H_2O_2-mediated signaling is an important factor limiting the accumulation of H_2O_2 to its sites of production where the possible breakdown in the integrity of this spatial component and H_2O_2 diffusion into other subcellular compartments would promote oxidative stress and trigger signaling associated with cell death.

1.4 ROS scavenging and detoxification systems

According to oxford dictionary, the term ‘antioxidant’ is defined as a substance that, in small amounts, inhibits the oxidation of other compounds. Through evolution, plants have established a complex crosstalk between antioxidative defense systems with the common goal; to protect cells from oxidative damage and scavenge harmful ROS (Karuppanapandian *et al.* 2011). Strong detoxification systems are present in cellular compartments where ROS are produced, such as chloroplasts, mitochondria, peroxisomes and cytosol (Noctor and Foyer, 1998; Asada, 1999; del Rio *et al.* 2006).

Photosynthesis, as a sensor of imbalances, regulates the photophysical, photochemical and metabolic processes in the chloroplast (Ensminger *et al.* 2006). In a fluctuating environment, it is necessary to have high photoxidative protection to ensure plant survival. In intact chloroplasts, ROS levels are controlled on a site of superoxide and the hydrogen peroxide generation by the chloroplast antioxidant system (Asada, 2000). Stress response by modulating and regulating concentrations of non-enzymatic and enzymatic protection components is unique for the plant antioxidant protection system (Figure 1-2).

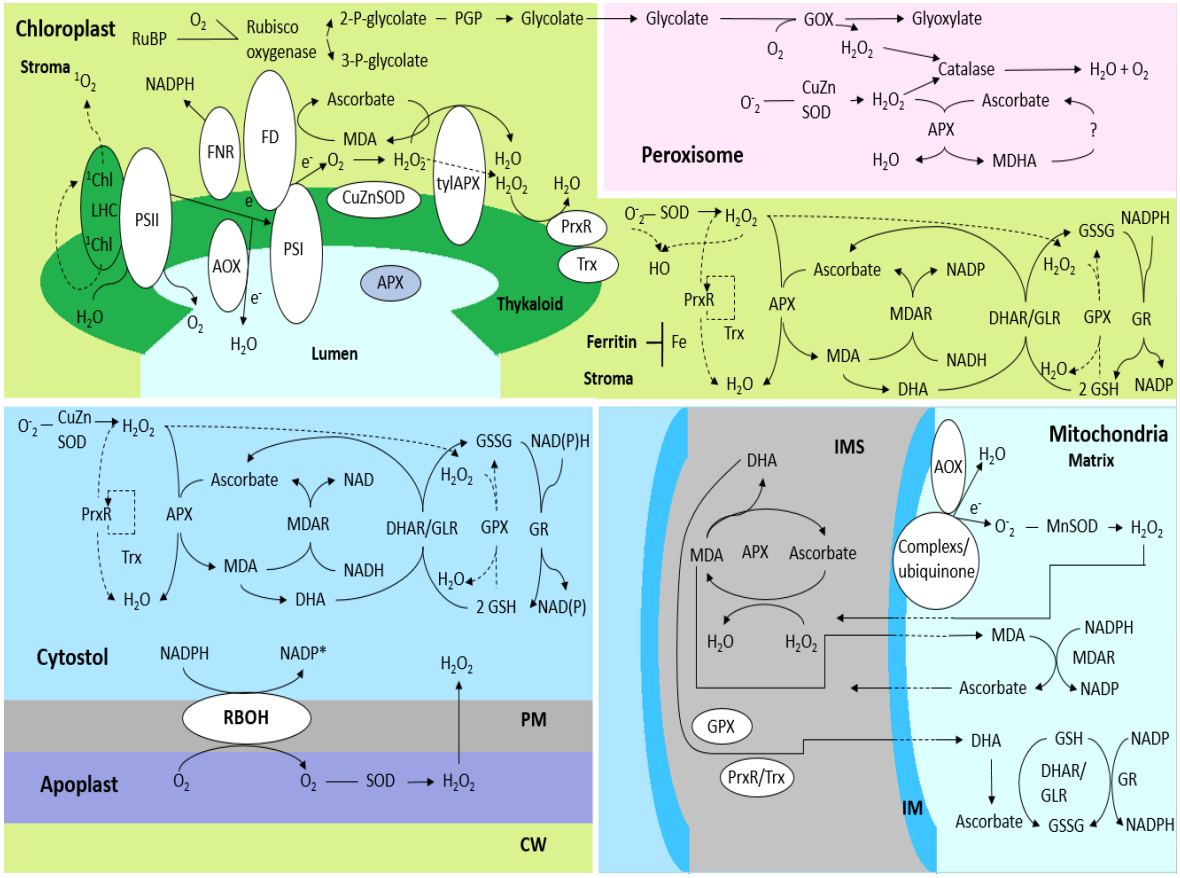


Figure 1-2. ROS generation, scavenging pathways, detoxification systems, and their localization in plant cells. Modified after Miller et al. (2010)

DHA, dehydroascrobate; DHAR, DHA reductase; FD, ferredoxin; FNR, ferredoxin NADPH reductase; GLR, glutaredoxin; GR, glutathione reductase; GOX, glycolate oxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; IM, inner membrane; IMS, IM space; MDA, monodehydroascorbate; MDAR, MDA reductase; PGP, phosphoglycolate phosphatase; PM, plasma membrane; PSI, photosystem I; PSII, photosystem II; RuBP, ribulose-1,5-bisphosphate; Rubisco, RuBP carboxylase oxygenase; Trx, thioredoxin; tyl, thylakoid.

1.4.1 Enzymatic detoxification systems

Enzymatic antioxidant defense includes several enzymes such as CAT (catalase), APx (ascorbate peroxidase), GPx (quaiacol peroxidase), and SOD (superoxyde dismutase). It also includes the enzymes required for the regeneration of the active forms of the antioxidants such as MDAR (monodehydroascorbate reductase), DHR (dehydroascorbate reductase) and GR (glutathione reductase) that are localized in different compartments of the cells. All enzymes actin corcern to neutralize ROS production at side of generation (Asada, 1999; del Rio *et al.* 2006; Mittler *et al.* 2004; Dietz *et al.* 2006; Entsminger *et al.* 2006).

The enzymatic H_2O_2 detoxification systems in chloroplasts include the APx-dependent ascorbate-glutathione (Halliwell-Foyer-Asada) cycle and the PRx-dependant scavenging system (Kangasjärvi *et al.* 2008). Except in chloroplasts, the ascorbate-glutathione cycle is present in the cytosol, mitochondria and peroxisomes (Figure 1-2) (Ishikawa *et al.* 1996; Jimenez *et al.* 1997; Miller *et al.* 2010). According to Knight and Knight (2012), the tolerance of ROS accumulation upon stress is mediated via transcriptional changes. Some transcriptional changes can be buffered synergistically or antagonistically with the other stress induced changes. The highly efficient chloroplast antioxidant system and its components are synchronized through gene expression regulation.

Ascorbate peroxidases (APxs) (EC 1.11.1.11) are haem-binding enzymes localized in cytosol, peroxisoms, chloroplasts and mitochondria (Welinder, 1992; Asada, 1999; Pereira *et al.* 2005). APx has three soluble isoforms (mitAPx, sAPx, cAPx) and two member-bound isoforms (tAPx, mAPx) with defense and scavenging roles, respectively, within the ascorbate-glutathione cycle (Foyer and Noctor, 2005). Ascorbate peroxidase catalyzes the reduction of hydrogen peroxide (H_2O_2) to water and oxidizes ascorbate to dehydroascorbate (DHA) (Welinder, 1992) is:

$$H_2O_2 + \text{ascorbate} \rightarrow 2\ H_2O + \text{DHA}$$

The genes for the chloroplast isoforms (soluble stromal and thylakoid-bound) were differentiated early in plant evolution (Pitsch *et al.* 2010). These genes are nuclear encoded and post-translationally targeted to chloroplasts (Asada, 2000). The chloroplast isoforms are influenced more by the oxidative inactivation than the cytosolic isoforms (Kitajima, 2008). Ascorbate peroxidase function depends on the availability of the reduced ascorbate (Foyer and Noctor, 2005).

Monodehydroascorbate reductase (MDAR) (EC 1.6.5.4) is a dinucleotide enzyme localized in the chloroplast, mitochondria and the cytosol (Miyake *et al.* 1998; Dalton *et al*. 1993; Jimenez *et al.* 1997; Mittler, 2002; del Rio *et al.* 2002). It catalyzes regeneration of reduced ascorbate using electrons derived from the photosynthetic electron transport chain (Hossain and Asada, 1984):

$$2\ \text{MDAR} + \text{NADH} \rightarrow 2\ \text{ascorbate} + \text{NAD}^+$$

Dehydroascorbate reductase (DHA) (EC 1.8.5.1) is a monomeric enzyme similarly located as the monodehydroascorbate reductase (Hossain and Asada, 1984). DHAR catalyzes the regeneration of the ascorbate from its oxidized state (DHA) through the following reaction (Hossain and Asada, 1984):

$$\text{DHA} + 2\ \text{GSH} \rightarrow \text{ascorbate} + \text{oxidized glutathione}$$

Glutathione peroxidases (GPxs) (EC 1.11.1.9) are enzymes localized in the chloroplasts, cytosol, mitochondria and the endoplasmic reticulum (Navrot *et al.* 2006; Rodriguez Milla *et al.* 2003). GPx catalyzes the reduction of peroxide to water and alcohol by thioredoxin and other electron donors (Herbette *et al.* 2002):

$$\text{Trx}_{\text{red}} + H_2O_2 \rightarrow \text{Trx}_{\text{ox}} + 2\ H_2O$$

In chloroplasts, GPx1 and GPx7 have an important regulatory and protective role.

Glutathione reductase (GR) (EC 1.6.4.2), as a flavoenzyme, has a subcellular localization in the cytosol, chloroplasts and the mitochondria (Asada, 1999; Edwards *et al.* 1990). Asada (2000) explained the role of glutathione and GR in hydrogen peroxide scavenging through the Halliwell-Asada pathway. The reaction that catalyzed this antioxidant (Ghisla and Massey, 1989) is:

$$\text{GSSG} + \text{NADPH} \rightarrow 2\ \text{glutathione} + NADP^+$$

Catalase (CAT) (EC 1.11.1.6) is a haem-containing enzyme localized in peroxisomes and glyoxisomes (McClung, 1997; Mittler, 2002). There are three isoforms present in Arabidopsis that are on different chromosomes, which are differentially expressed and independently regulated (Frugoli *et al*. 1996). This enzymatic antioxidant catalyzes the dismutation of hydrogen peroxide (H_2O_2) to water and oxygen (McClung, 1997) through the following reaction:

$$2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$$

Superoxide dismutases (SODs) (EC 1.15.1.1) are enzymes localized in the cytosol, chloroplasts, peroxisomes and the mitochondria (Bowler *et al.* 1992; Halliwell and Gutteridge, 2000; del Rio *et al.* 1996). These enzymes are placed where the first oxidative stress defense occurs in a changed environment (Scandalios, 1993). According to del Rio et al. (1996), SOD has three isoforms classified on the basis of their metal cofactor (Fe^{2+}, Mn^{2+} and Cu-Zn), all encoded by the nucleus and post-translatory targeted to specific cell compartments (Bowler

et al. 1992; del Rio *et al.* 1996). The reaction that catalyzed by these metalloenzymes (Fridovich, 1986) is as follows:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2\ H^+ \rightarrow 2\ H_2O_2 + O_2$$

Chloroplast Cu-Zn SOD (CSD2) in Arabidopsis is known to be miRNA-regulated (Sunkar *et al.* 2006).

Peroxiredoxins (PRxs) (EC 1.11.1.15) are thiol peroxidases localized in the cytosol, nucleus, mitochondria and in chloroplasts (Haslekas *et al.* 1998; Baier and Dietz, 1997; Horling *et al.* 2003). PRx function depends on the small thiol proteins (Trx, GRx) and has a role in reducing the hydrogen peroxide to water (Figure 1-3) (König *et al*. 2003).

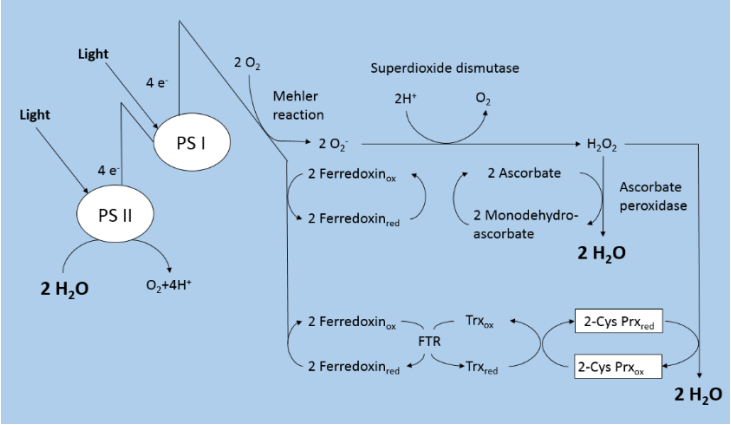


Figure 1-3. PRx-dependant scavenging system as alternative water–water cycle for ROS detoxification in photosynthesis. Modified after Dietz et al. (2006).

PRxs have four isoforms with similar sequences and catalytic mechanisms (Horling *et al.* 2003). Among the nine encoded PRxs in Arabidopsis genome, 2CPs (A,B), PrxIIE and PrxQ are localized to the chloroplasts (Horling *et al.* 2003).

1.4.2 Non-enzymatic detoxification systems

In addition to the scavenging enzymes, ascorbic acid and glutathione are essential mediators in plants which protect against accumulation of toxic amounts of reactive oxygen species (Asada, 1999). The non-enzymatic antioxidants tocopherols, carotenoids and flavonoids also have an important role in protection (Telfer, 2002; Trebst, 2002; Noctor and Foyer, 1998).

Ascorbic acid is localized in almost all plant cells and organelles such as the chloroplast, cytosol, mitochondria, peroxisomes, and vacuole as well as in the apoplast (Smirnoff, 2000; Wheeler *et al*. 1998; Noctor and Foyer, 1998). Ascorbate exists mostly in a reduced form in chloroplasts under non-stress conditions (Smirnoff, 2000). As a substrate for APx and hydrogen peroxide detoxification in a broad range of (non)enzymatic reactions, ascorbate is the main ROS detoxifying compound in aqueous phase (Foyer and Lelandais, 1996). Ascorbate can directly scavenge superoxide, hydroxyl radicals and singlet oxygen (Noctor and Foyer, 1998). Ascorbate is not only the main low molecular weight antioxidant but also the most abundant (10-100 mM) one (Noctor and Foyer, 1998). In addition to being an antioxidant, ascorbate is involved in the cell growth and division (Liso *et al.* 1988). Biosynthesis of the low molecular weight antioxidant ascorbate is promoted under high light and cold stress conditions (Streb *et al*. 2003).

Glutathione is an abundant (0.2-10 mM) tripeptide localized in the chloroplasts, mitochondria, endoplasmic reticulum, vacuoles and the cytosol (Noctor and Foyer, 1998). It is a substrate for various peroxidases, glutathione transferases, and glutathione reductase (Noctor and Foyer, 1998; Ball *et al.* 2004). Together with its oxidized form, glutathione serves to maintain the cell redox equilibrium (Noctor and Foyer, 1998). Similar to ascorbate, GSH can scavenge diverse ROS ranges and regenerates through the ascorbate-glutathione cycle ascorbate (Noctor and Foyer, 1998). These non-enzymatic antioxidants have a role in the detoxification of hydrogen peroxide and other toxic compounds (Dixon *et al.* 1998).

Tocopherols are localized in the cell membranes (Kiffin *et al.* 2006; Fryer, 1992). They play their role as antioxidants by protecting the lipids in membranes from peroxidation, detoxification lipid peroxides and specially by quenching singlet oxygen in chloroplasts, thereby playing a role in PSII protection (Igamberdiev *et al*. 2004; Kruk *et al*. 2005).

Carotenoids are localized in (none) photosynthetic tissues such as chloroplasts, chromoplasts, elaioplasts and amyloplasts (Yamamoto and Bassi, 2004). In chloroplasts, they function as harvesting light pigments as they absorb light in the range of 450-570 nm (Young, 1991). They quench singlet oxygen and are of high importance in photosystem assembly, as precursor of ABA synthesis and a component of the light harvesting complex (Young, 1991, Xiao *et al.* 2011).

Flavonoids are localized in the vacuole and have a role in scavenging hydrogen peroxide and hydroxyl radicals (Winkel-Shirley, 2002; Grace and Logan, 2000). They can modify the lipid composition and decrease membrane fluidity, which results in a disrupted diffusion of free radicals and peroxide reactions (Arora *et al.* 2000).

As a result of non-stable environmental conditions, plants modulate the concentrations of low molecular weight antioxidants and the expression of genes encoding antioxidant enzymes, making the chloroplasts the main power station role.

1.5 Model system

According to biogeography, *Arabidopsis thaliana* (L.) Heynh. is native to Europe and central Asia. The Caucasus region and Iberian Peninsula could be potential Arabidopsis ancestral areas (Figure 1-4) (Beck *et al.* 2008; Pico *et al.* 2008). The annual angiosperm, mouse-ear cress, belongs to the Brassicaceae family. These small and rapid grown weedy plant has been sequenced by the Arabidopsis genome initiative 2000 (2n = 10, 157 Mb). The relative short generation time (less than six weeks depending of population and growing conditions) and the large number of seeds per generation are characteristics of this species (Napp-Zinn, 1985, Abbott and Gomes, 1989). *Arabidopsis thaliana* can express either a summer-annual life history with an overwintering characteristic (as seeds) and germinating and flowering in spring and summer next year or a winter-annual life history by overwintering as a vegetative rosette and flowering in spring (Engelmann and Purugganan, 2006). After germination, the next stages of its life cycles are vegetative rosette and reproductive corymb transitions.

Now, sixteen years after sequencing, this plant is a model plant for connecting and bridging the molecular biology, ecology and physiology. The Arabidopsis resource centers possess hundreds of different seed stocks from the natural accessions available worldwide. According to Koornneef et al. (2004), *Arabidopsis thaliana* accessions varied in different aspects such as flowering time, seed size, flower architecture and pathogen resistance. High phenotypic plasticity and adaptive genetic differentiation are two requirements necessary for a broad geographical

distribution in plants (Koornneef *et al.* 2004; Mitchell-Olds and Schmitt, 2006). The accessions such as Kas-1 and Cvi-0 are grown at the altitudes of 1580 m in India and 1100-1200 m in Cape Verde Island, respectively. In contrary to these two accessions, Col-0 (origin Poland), Ms-0 (origin Russia), WS (origin Belarus), Van-0 (origin Canada) and N13 (origin Russia) are grown at altitudes between 100-200 m (Versailles Biological Resource Centre) (Figure 1-4). According to Versailles Biological Resource Centre, the average temperatures under which plants are grown differ among accessions and can be very contrasting (from October to March Cvi-0 grown on average temperature between 22°C and 26°C and at the same time Kas-1 on -12°C to -2°C). In addition to that, average monthly precipitations can also differ. In the same period, from October until March, Van-0 accessions are exposed to very high amounts of precipitation (100-170 mm) while the WS accession is exposed to very low precipitation (30-40 mm).

With all the developed techniques available and species knowledge, *Arabidopsis thaliana* is a potential tool for studying the impact of natural variations in environmental conditions.

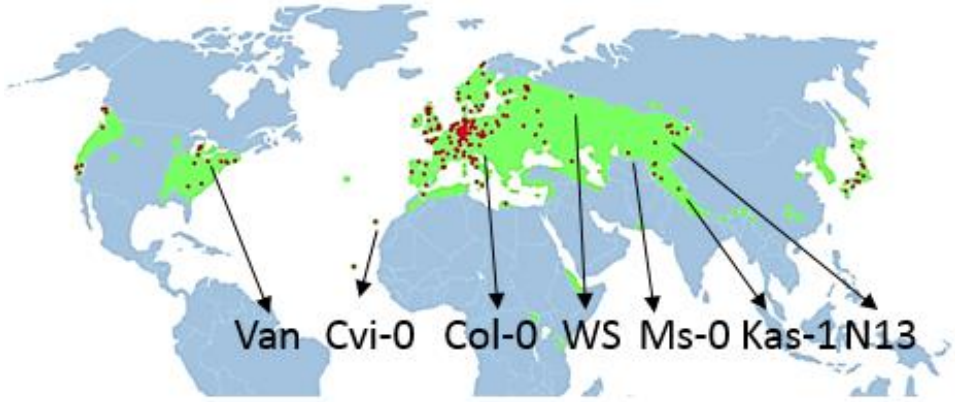


Figure 1-4. Distribution of selected *Arabidopsis thaliana* accessions. Modified after Koornneef et al. (2004)

1.6 Aim of the study

The main goal of this study is to investigate the mechanisms of gene expression regulation and chloroplast-to-nucleus signaling. The hypotheses that the chloroplast antioxidant system serves as a priming hub in plant stress management and controls the activation of the extra-plastidic signaling cascades,

was tested. In literature, priming in the context of oxidant protection was recorded in potato and wheat plants (Bengtsson *et al*. 2014, Li *et al.* 2014). Defining the key elements responsible for memory establishment and the advantage of being primed under changed environment conditions are important traits that could be introduced into crop plants. Avoiding chemical / technical manipulations and the utilization of natural capacity in order to have plants with an increased stress capacity can be future project goals.

2 MATERIAL AND METHODS

2.1 Plant material

Sets of different *Arabidopsis thaliana* seeds were collected from the Versailles nested core collections (INRA) in France. Seven *Arabidopsis thaliana* accessions with broad ecological and physiological differences were chosen for experiments according to the previous experiments performed at the MPI Golm (Germany) (Table 2-1). Gene modified (transfer DNA) lines of *Arabidopsis thaliana*, *sAPX*- and *tAPX*-knock-out lines (*sapx, tapx, tapx sapx*), were used also in this study.

Table 2-1. List and description of different *Arabidopsis thaliana* accessions used in experiments. Modified after Zuther et al. (2012)

Abbreviated name	Name	Country of origin	Latitude of origin	Longitude of origin	LT_{50} NA	LT_{50} ACC	INRA accession ID
N13	Konchezero	RUS	62.12	34.01	-5.52	-11.76	266AV
Ms-0	Moscow	RUS	55.45	37.35	-7.72	-11.87	93AV
Kas-1	Kashmir	IND	34.00	76.00	-4.71	-11.88	434AV
Ws-0	Wassilewskija	RUS	52.22	30.38	-6.02	-10.38	84AV
Col-0	Columbia	POL	52.73	15.15	-5.34	-9.13	186AV
Van-0	Vankuwer	CAN	49.16	23.07	-6.03	-8.79	161AV
Cvi-0	Cape Verde Islands	CPV	16.00	−24.00	-4.98	-7.78	166AV

2.1.1 Growth conditions

Seeds of seven different *Arabidopsis thaliana* accessions were vernalized in darkness for three days at 4 °C on Arabidopsis soil, which consisted of 70 volumes “Topferde” (Einheitserde, Germany), 70 volumes “Pikiererde” (Einheitserde, Germany), 25 volumes Perligran Classic (Knauf, Germany) supplemented with 0.5 g l^{-1} dolomite lime (Deutsche Raiffeisen-Warenzentrale, Germany) and 0,5 g l^{-1} Axoris Insekten-frei (COMPO, Germany). After the stratification period, seeds were transferred to a climate-controlled chamber with a day / night temperature of 20 ± 2 °C / 18 ± 2 °C at 120 µmol photons $*m^{-2}*s^{-1}$ light (L36W/840 Lumilux Cool White fluorescent stripes) with a 10 h light / 14 h dark photoperiod. One week old seedlings were transferred individually to 6 cm diameter pots and were grown

under controlled conditions in total 28 days. The relative humidity in the growth chamber was 60 ± 5 % during the day and night periods.

2.2 Stress treatments

Four week old plants were transferred to cold and high light stress conditions for a required experimental time. Stress treatments were applied for duration of 2.5 h after onset of light due to physiological reasons.

2.2.1 Cold stress conditions

In the cold chamber, plants were kept under 4 °C in a 10 h light / 14 h dark photoperiod for two weeks with the same illumination, aeration setting and humidity as in the growth chambers. This treatment was followed by a five day deaclimation phase in a 20 °C growing chamber for genetic studies. This phase lasted for 60 days in a greenhouse for ecological studies. For experimental cold triggering requirements, plants were transferred to a 4 °C cold chamber for 24 hours. After triggering stress, plants were cultivated at a day / night temperature of 20 ± 2 °C and 18 ± 2 °C until harvesting.

2.2.2 High light stress conditions

The light intensity was increased up to 220–250 µmol photons $*m^{-2}*s^{-1}$ in a climate controlled growth cabinet (Percival-CU-41L4X, Percival Scientific Inc. Perry, USA). All the other parameters such as the day / night length, illumination, aeration setting and humidity were controlled and coordinated within standard growth conditions. Light stress was performed for 24 h duration. Afterwards, the plants were transferred back to the standard 120 µmol photons $*m^{-2}*s^{-1}$ light condition for deaclimation and until harvesting.

2.3 Outdoor and indoor ecological set up

Four-week-old *Arabidopsis thaliana* plant sets, which consisted of two half sets of two week long cold primed and non-primed plants, were transferred to the field on the first Monday of March and May. In parallel with the field planting, half of the plant material was transferred to standard temperature and light controlled greenhouse conditions. In total, 90 plants per accession were planted in an open basket growth regime and split into the trays for both field and greenhouse plantings. Garden boxes with size 56 x 30 x 15 cm were filled with the soil obtained from the field and were installed in the experimental area. Sixteen randomly organized primed and non-primed plants of seven different *Arabidopsis thaliana* accessions were arranged in each box. In the greenhouse, primed and non-primed plants were cultivated in different trays in a random pattern. During the May experiments, due to temperature increase and stable weather conditions, the plants in the field were attacked by slugs. For that reason, in the first week of May, metaldehide pellets 'Mesurol Schneckenkorn' (Bayer, Germany) were used as pesticide against slugs.

2.4 Measurements of chlorophyll-a fluorescence

MINI-PAM fluorimeter (Walz, Germany) was used for the determination of initial (F_M / F_V) and efficient (ΔF / F_m') quantum yield of photosystem II. Firstly, the photosynthetic yield was measured in the light acclimated plants. The duration and intensity of the light pulse was 0.8 s and >3000 µmol quanta m^{*-2} s^{*-1}. Dark leaf clip DLC-8 was used for single point measurements. Closing the clips for 20 min resulted in the dark adaptation of plants. Secondly, the maximum (initial) quantum yield after 20 min of dark acclimation was measured. The parameter 'Measuring intensity' was set to the sensor unit 4 and the parameter 'Sensitivity range' was set to the sensor unit 2. For field studies, chlorophyll-a fluorescence measurements were made in the garden areas and greenhouse every day in the first experimental week and then weekly for the next four weeks. For the non-field studies, measurement dynamics were according to the experiment requirements.

2.5 Plant habitus analysis

For morphological parameter determination, digital images of plant tops were taken in the field and greenhouse and were subsequently analyzed by the ImageJ software package. The images were captured at a distance of 50 cm. For field studies, morphological measurements in the field and greenhouse were performed weekly for a period of four weeks.

2.5.1 Rosette diameter and leaf area

Rosette diameter was measured in mm by analyzing digital images (using ImageJ software) of the single rosette taken at the time points defined by the experiment. For this measurement, the ‘Segmented line’ tool was used.

The leaf areas (mm^2) were determined via 8-bit images (ImageJ software package). ‘Threshold’ and ‘Measurement setting’ parameters were set up manually with a rationale of easier leaf and background differentiation.

2.5.2 Fresh weight

After harvesting the entire rosette, fresh weight was immediately determined on the ABT320-4B-Balance (Kern & Sohn GmbH, Germany).

2.5.3 Leaf number

Leaf number was determined by counting the number of leaves from the digital images taken for defined experimental time points.

2.6 Fitness measurements

The seeds from the outdoor and greenhouse experiments were collected and analyzed after a few months of drying period. Plants in the field and the greenhouse were bagged in cellophane before the first siliques opened. Seeds were collected and air-dried for a minimum of 4 weeks in these bags before

cleaning. Collected seeds were additionally kept for a couple of weeks in the dark and at room temperature before taking the measurements. Equipments used for seeds measurement were digital balance (ABT320-4B, Kern & Sohn GmbH, Germany) and Nikon camera with special lens (AF Micro Nikkor 60mm, Germany).

2.6.1 Average seed weight

Software package ImageJ and its 'Analyze particles' function was used for average seed weight determination. Seed weight analysis involved taking the digital images of seeds spread in a petri dish, followed by weight measurements. The parameters in the software such as 'Selection the area of interest', 'Threshold' and 'Size pixel' were set up manually to the sensor unit 6-150 and 'Circularity' to the sensor units 0.7-1.0 in order to count number of particles. The weight of the individual seed was calculated by dividing the total weight of the seeds by the number of seeds.

2.6.2 Total amount of seeds and total number of seeds per plant

The total number of seeds is presented as the weight of all seeds produced from a single plant. This number was measured from a simple weight scale.

Total number of seeds per plant was calculated as the total amount of seeds divided by the average seed weight measured previously.

2.7 Weather data

The meteorological data for the experimental time were obtained from the meteorological station 'Dahlem' located in Berlin (Institut für Meteorologie der Freien Universität Berlin, Germany). Measurements of air temperature, humidity and precipitation were obtained at one-minute intervals from beginning of March to the time of the last harvest.

2.8 Transcript analyses

The plant material was collected and pooled before, immediately after and one to five days after cold pre-treatment for transcript analyses. Five plants were immediately frozen in liquid nitrogen and stored at -80 °C. All samples were taken at 2.5 hours after the onset of light.

2.8.1 Total RNA isolation

Total RNA was extracted from the 100 mg rosette material. Collected plant material was homogenized using liquid nitrogen and the Retsch Mixer Mill MM10 (Retsch, Germany). RNA was isolated with the GeneMATRIX Universal RNA Purification Kit (EURx, Poland) according to the manufacturer's instructions. In order to avoid sample contamination with genomic DNA, 1 U RNase-free DNase I (Fermentas GmbH, Germany) was applied on-column digestion.

2.8.2 RNA quantification

After RNA extraction, the samples were analyzed for quantity and purity with the NanoPhotometer P300 (Implen, USA). This spectrophotometer measured the absorbance at 230 nm, 260 nm and 280 nm. Nucleic acid concentration was determined according to measured absorbance at 260 nm. Calculated A_{260} / A_{230} and A_{260} / A_{280} ratios were used for detection of undesired sugar / phenol compounds and protein contaminations. RNA samples with ratios between 1.8 and 2.0 were used for analysis.

2.8.3 Electrophoretic separation of RNA

RNA integrity was analyzed by RNA electrophoresis. Electrophoretic separation was performed in a 1% (w/v) agarose gel in 1x MOPS buffer (20 mM MOPS pH 7.0, 5 mM sodium acetate, 1 mM EDTA). After the melted agarose gel was cooled down to approximately 60 °C, it was supplemented with formaldehyde to a final concentration of 0.9% (v/v). Gels were cast into horizontal gel trays and left for solidification at room temperature. RNA sample of 3 µl was mixed 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 ethidium bromide). After RNA denaturation at 65 °C for 15 minutes, the sample was cooled down on ice and then loaded into the gel pockets. Electrophoresis was performed for approximately 20 min at constant voltage of 90 V in 1x MOPS buffer. Ethidium bromide stained RNA bands were visualized at UV light and using Gel Imager (Intas, Germany).

2.8.4 First strand cDNA synthesis and test of DNA contamination

The high quality RNA samples were directly used for first strand cDNA synthesis with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Two micrograms of Arabidopsis RNA were reverse transcribed in 20 µl total volume of mixture containing 1 x RT Buffer, 10 mM dNTP Mix, 20 U Multiscribe Reverse Transcriptase, and 10 µM Oligo(dT) Primers (Sigma-Aldrich Chemie GmbH, Germany). The sample mixture was incubated at 25 °C for 10 minutes (annealing the primers), followed by cDNA synthesis for 120 minutes at 37 °C and reverse transcription for 5 minutes at 65 °C. cDNAs were diluted in a 1:2 ratio with RNase-free water prior to qRT-PCR. Afterwards, the samples were tested for DNA contamination with primers flanking two short introns and an exon of the 2CPA gene At3g11630 (ATbas-O1H GACTTTACTTTCGTCTGC; ATbas-O4H ATCACTCCTTCCTTGTCG) by DNA gel electrophoresis after 40 cycles.

2.8.5 Quantitative real-time PCR analyses

Transcript abundance rates were analyzed by qRT-PCRs in the CFX96 thermocycler (BioRAD, Germany) according to the MIQE standards (Bustin *et al.* 2009). Primers used for qRT-PCR analysis are presented in Table 2-2. In order to prevent the amplification of genomic DNA, all primers were designed to span exon–intron border using the QUANTPRIME software (Arvidsson *et al.* 2008). Due to the ability of SYBR Green to intercalate into double-stranded DNA and emission of green light, fluorescence signal increased after each polymerization cycle. Each PCR reaction mixture contained 50 ng tested cDNA, 2 µl 10X buffer (160 mM ammonium sulfate, 1 M Tris-HCl pH 8.3, 0.1 % v/v Tween-20), 0.8 µl 50 mM $MgCl_2$, 0.4 µl 5 mM dNTP, 0.2 µl 10X SYBR Green (Sigma-Aldrich, Germany),

0.04 µl 5U/µl OptiTaq Polymerase (EURx, Poland) and 0.12 µl of 50 µM gene-specific primers (300 nM final concentration) in a total volume of 20 µl. All reactions were performed in triplicates per biological sample. Within 3 min at 95 °C, the activation of the polymerase started and the PCR was initialized. Amplification was performed in 40 cycles.

Each cycle included DNA denaturation at 95 °C for 15 s, primer annealing at 60 °C for 30 s, and an extension step at 72 °C for 30 s. During the temperature increase, fluorescence was detected continuously. The analyses of transcript abundance were performed in minimum of three biological replicates to ensure accuracy.

Table 2-2. Primers used for gene expression analyses by qRT-PCR.

Gene	***AGI code***	**Forward primer**	**Reverse primer**
2CPA	At3g11630	CCCAACAGAGATTACTGCCT	ATAGTTCAGATCACCAAGCCC
2CPB	At5g06290	TCATACCCTCTTCCTCGGCATC	ACCGACCAGTGGTAAATCATCAGC
ACT2	At3g18780	AATCACAGCACTTGCACCAAGC	CCTTGGAGATCCACATCTGCTG
BAP1	At3g61190	ATCGGATCCCACCAGAGATTACGG	AATCTCGGCCTCCACAAACCAG
CHS	At5g13930	TTCCGCATCACCAACAGTGAAC	CGCACATGCGCTTGAACTTCTC
COR15A	At2g42540	AACGAGGCCACAAAGAAAGC	CAGCTTCTTTACCCAATGTATCTGC
CSD2	At2G28190	CTCAACAGGACCATTTCAACC	ATTGTTGTTTCTGCCAACGCCA
GPX7	At4g31870	CGTTAACGTTGCGTCAAGATGTGG	TGACCTCCAAATTGATTGCAAGGG
GR	At3g54660	GAAATTCCGCAAAGACTCCTC	CAGACACAATGTTCTCCTTATCAG
MDAR	At1g63940	TGGGAGAAACAGTGGAGGTTGG	TGGTAGAAGCTGGAACTCCTCAG
PAL1	At2g37040	GCAGTGCTACCGAAAGAAGTGG	TGTTCGGGATAGCCGATGTTCC
sAPX	At4g08390	AGAATGGGATTAGATGACAAGGAC	TCCTTCTTTCGTGTACTTCGT
tAPX	At1g77490	GCTAGTGCCACAGCAATAGAGGAG	TGATCAGCTGGTGAAGGAGGTC
YLS8	At5g08290	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT
ZAT10	At1g27730	TCACAAGGCAAGCCACCGTAAG	TTGTCGCCGACGAGGTTGAATG

2.8.6 Quantification of qRT-PCR results and standardization

The CFX Manager 3.0 software was used for calculation of the Cq values. Regression testing and further analyses of primer amplification efficiency were conducted via this software. Transcript levels of all analyzed genes were normalized to the transcript levels of *ACT2* (At3g18780) and *YLS8* (At5g08290) according to Czechowski et al. (2005).

2.9 Protein analyses

2.9.1 Protein isolation and quantification

Total protein content was isolated according to Kangasjärvi et al. (2008). Hundred milligrams of plant material, frozen immediately in liquid N_2 after harvesting, was ground in 300 µl buffer (330 mM sucrose, 25 mM Hepes pH 7.4, 10 mM $MgCl_2$, 100 mM NaF, 0.1% Triton and 0.1% (w/v) SDS) by plastic pestles until homogeneity was reached. During preparation, the tubes with the samples were stored on ice. Afterwards, centrifugation was performed for 30 minutes at 16 000 x g at 10 °C. The supernatant was collected and stored in three 1.5 ml plastic reaction tubes. One tube was used for protein quantification directly after isolation and the two others were stored at -80 °C until SDS-PAGE separation.

The protein level was determined using the BIO-RAD DC Protein Assay (Bio-Rad, Germany). According to manufacturer's recommendations, 10 µl of protein sample or standard BSA sample (0-15 µg) was mixed with 990 µl of diluted Protein Assay Dry Reagent Concentrate (1:5). After 15 minutes of incubation in dark at room temperature, the absorbance was measured spectrophotometrically at 595 nm (Ultrospec 2100 pro, England). The standard BSA curve was used for protein concentration determination.

2.9.2 Electrophoretic separation

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) is a technique for protein separation according to their molecular weight in an electric field (Laemmli, 1970). According to Laemmli's discontinuous gel method, proteins are first concentrated at the border of stacking gel in the sharp narrow band and then separated in the resolving gel according to their molecular weight. The polyacrylamide gels are casted between two glass plates divided by spacers. Five milliliters of 12 % resolving solution (375 mM Tris-HCl, pH 8.8; Rotiphorese Gel solution (12 % (w/v) acrylamide, 0.32 % (w/v) bis-acrylamide); 0.1 % (w/v) SDS, 0.1 % (w/v) APS; 0.04 % (w/v) TEMED) was polymerized between glasses and pre-overlaid with 1 ml of isopropanol to avoid contact with molecular oxygen. Afterwards, 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 the resolving gel. A comb with sample pockets was inserted before polymerization. Total protein extract was diluted (15 µg/15 µl) according to previous quantification and mixed with 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) to the final 0.83 µg/µl protein concentration. Proteins were denaturalized for 5 minutes at 95 °C and cooled down on ice before loading. At the end, 6 µl of the sample mix (5 µg of protein) were separated on gels in 1x SDS-PAGE running buffer (25 mM Tris-HCl, pH 8.3; 200 mM glycine; 1 % (w/v) SDS) for approximately 1.5 hours at constant 30 mA. PageRuler Prestained Protein Ladder (Fermentas, Germany) was separated on a gel in parallel with the samples.

2.9.3 Transfer of proteins and immunodetection with specific antibodies

Western blot method was used for detection of proteins that are bound to the membrane. According to the Kyhse-Andersen (1984) protocol, proteins were transferred from the polyacrylamide gel onto the nitrocellulose membrane (Whatman, Germany) using a semi-dry blotting method in FastBlot B44 chamber (Biometra, Germany). Membrane and gel were incubated for 5 minutes in the transfer buffer (25 mM Tris-HCl, pH 8.3; 150 mM glycine; 10 % (v/v) methanol) were assembled between six layers of wet filter paper (Schleicher & Schuell GmbH, Germany). The transfer was performed for 45 minutes at constant current of 2 mA per cm^2 of the membrane. After the transfer, membrane staining was performed with 0.2 % (w/v) Ponceau S (Sigma Aldrich, Germany) in 3 % (v/v) acetic acid as efficiency control.

Immunological detection of the proteins bound to the nitrocellulose membrane was the next step after semi-dry transfer. The membrane was washed for 15 minutes with TBS buffer (20 mM Tris-HCl, pH 7.6; 0.8 % (w/v) NaCl) and blocked with 5 % (w/v) fat-free milk powder overnight at 4°C. Twenty-four hours later, the membrane was washed three times for 15 minutes with TBST buffer (TBS buffer supplemented with 0.1 % (v/v) Tween-20) and incubated for 1 hour at room temperature with polyclonal anti-APXs (AS08 368, Agrisera-Sweden) primary antibodies in TBS buffer (1:1000). Afterwards the membrane was washed 3 times for 5 minutes with TBST buffer in order to remove unbound antibodies. Horseradish peroxide-conjugated anti-rabbit secondary antibodies (Sigma-Aldrich,

Germany) diluted in TBS buffer (1:10 000) recognizes primary antibodies. The membrane was incubated in a 1:10 000 dilution for 45 minutes at room temperature. The membrane was washed again three times for 5 minutes with TBST buffer.

2.9.4 Chemiluminescent detection of proteins

Chemiluminescence imager (ImageQuantTM LAS 4000 Mini Biomolecular Imager, GE Healthcare, United States) was used together with Western Blotting substrate (Pierce ECL, Thermo Scientific, USA) for protein detection. According to the manufacturer's protocol, horseradish peroxidase which is conjugated to secondary antibodies catalyzes the oxidation of luminol to 3-aminophthalate from the detection solution (Detection reagent 1-Peroxide Solution in Detection reagent 2-Luminol Enhancer, 1:1). Low-intensity light at 428 nm was then emitted and detected by chemiluminescence imagers. The amount of emitted light is correlated with the amount of protein on the membrane and that provided the necessary information for the quantification.

2.9.5 Quantification of protein levels

The band intensity detected by the imager was quantified by ImageJ software with the help of 'Analyze Gels' tool. Digital pictures of the Ponceau S stained membrane were used also for normalization. The large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) detected on the membrane was used for the normalization of the measured activity of primary antibodies.

2.10 Pigment analysis

2.10.1 Determination of chlorophyll content

Chlorophylls were extracted from 15 mg of frozen plant material homogenized in 1 ml 80% (v/v) acetone supplemented with a trace of $CaCO_3$ (Porra *et al.* 1989). After 3 h of incubation in the dark at - 20°C, the samples were centrifuged for 5 min at the maximal speed (Microcentrifuge 5424, Eppendorf, Germany). The supernatant after centrifugation was analysed spectrophotometrically (Ultrospec

2100 pro, Biochrom, England) at 647 nm and 664 nm. Chlorophyll contents, chlorophyll a and chlorophyll b, were calculated from the absorbance by the following equations (Porra *et al.* 1989):

$$\text{Chl a } (\mu g\ ml^{-1}) = 12.25 \times A_{663.6} - 2.55 \times A_{646.6}$$

$$\text{Chl b } (\mu g\ ml^{-1}) = 20.31 \times A_{646.6} - 4.91 \times A_{663.6}$$

$$\text{Chl a+b } (\mu g\ ml^{-1}) = 17.76 \times A_{646.6} - 7.34 \times A_{663.6}$$

The results were standardized on gram fresh weight.

2.10.2 Determination of anthocyanin content

Anthocyanins were extracted and analyzed according to Mancinelli et al. (1991). Fifty milligrams of frozen plant material was extracted in 1 ml 1% (w/v) HCl in methanol. After 30 minutes of incubation in the darkness on a shaker, the samples were centrifugated for 5 minutes at the maximal speed (Microcentrifuge 5424, Eppendorf, Germany). The supernatant was analyzed spectrophotometrically (Ultrospec 2100 pro, Biochrom, England) at 530 nm and 657 nm. The $A_{530corr}$ was calculated according to the following equation (Mancinelli *et al.* 1991):

$$A_{530corr} = A_{530} - 0.25 \times A_{657}$$

The results were standardized on gram fresh weight.

2.11 Ascorbate measurements

For ascorbate determination, the plant material was immediately frozen in liquid nitrogen and directly analyzed. All samples were taken at 2.5 hours after the onset of light.

The ascorbate content was quantified according to Noctor and Queval (2007). Forty milligrams of frozen plant material was extracted in 400 µl 0.2 M HCl. After 5 min centrifugation at 13000 x g at 4° C, 250 µl of the supernatant was transferred into 85 µl 0.2 M NaH_2PO_4 pH 5.6 and adjusted to pH 5-6 with 220 µl 0.2 M NaOH. The supernatant was divided into two tubes in order to quantify the reduced and total ascorbates spectrophotometrically. Reduced ascorbate content was analyzed

with 100 µl of the supernatant added to 520 µl 0.2 M NaH_2PO_4 buffer pH 5.6 and 375 µl distillated water. The decrease in A_{265} in the presence of 1 U ascorbate oxidase (Applichem, Germany) was monitored spectrophotometrically. For the determination of total ascorbate content, 200 µl of supernatant in 200 µl 0.2 M NaH_2PO_4 buffer pH 5.6 and 0.12 M NaH_2PO_4 buffer pH 7.5 was incubated with 40 µl 25 mM DTT for 30 min at room temperature. Similar to the reduced ascorbate measurements, the decrease in A_{265} in the presence of 1 U ascorbate oxidase (Applichem, Germany) was monitored spectrophotometrically at 265 nm. Total ascorbate pool was standardized on gram fresh weight.

2.12 Hydrogen peroxide measurements

Hydrogen peroxide oxidizes an acidic environment in the presence of Fe^{2+}, sorbitol and xylenol orange (o-cresolsulfonephthalein-3,3-bis-methyliminodiacetic acid sodium salt) to a purple product that can be measured spectrophotometrically at 560 nm (Gay *et al.* 1999). Quantification of the H_2O_2 level in the plant tissue started with the homogenization of 100 mg frozen plant material in 300 µl 5 mM KCN buffer. The extract was centrifuged at 16 000 x g (Microcentrifuge 5424, Eppendorf, Germany) for 15 minutes at 4 °C. Ten volumes of freshly prepared working reagent (1 volume of Reagent A (25mM ammonium ferrous (II) sulfate, 2.5 M H_2SO_4) with 100 volumes of Reagent B (100 mM sorbitol, 125 µM xylenol orange) was added to 1 volume of a sample and incubated for 20 min in the dark at room temperature. Following incubation, the sample was analysed spectrophotometrically (Ultrospec 2100 pro, Biochrom, England) at 560 nm. The standard H_2O_2 curve (concentration range of 1-1000 µM) was used for calibration.

2.13 Statistical analyses

The Student t-Test, Turkey Post-hoc tests and Pearson correlation analyses were carried out at $\alpha<0.05$ and were conducted using the R software (ver 3.2.2, Vienna, Austria).

3 RESULTS

The study focused on the analysis of priming effects on the plant growth and fitness and the underlying molecular regulatory mechanisms. Ecological approaches to understand the interactions between plants and their environment were combined with molecular approaches to study the links between genetic and phenotypic variation (Trontin *et al*. 2011).

The initial research, which provided the experimental proof that all the tested *Arabidopsis thaliana* accessions (Table 2-1) can germinate without a cold stratification period, opened up a new chapter in which different factors and strategies influencing natural variation and thermomemory development, were investigated.

3.1 Ecophysiological evaluation of priming

Due to their broad ecological distribution, *Arabidopsis thaliana* plants can germinate in autumn or spring and live as winter or summer annual plants. Winter annual Arabidopsis plants face a long cold period before they go into reproductive development. Throughout the spring season, when the temperature slowly increases, short cold periods are very common and can restrict plant growth. *Arabidopsis thaliana* accessions require more than one week at 4°C to be stably cold acclimated (Zuther *et al.* 2012).

To test how the cold pre-treatment influences the tolerance to fluctuating spring temperature conditions, experiments were set up in March and May of 2014 and 2015. Two weeks of cold priming provided winter-like temperatures to the plants. Short direct exposure to harsh winter temperature variations in March and mild temperatures in May have been expected to yield more information about the different memory strategies of the individual accessions. As a control, parallel treated plants were transferred to temperature controlled greenhouse chambers.

The outdoor experiment not only showed general and specific ecological adaptations in plants, but also provided a chance to observe the eventual ecophysiological costs and benefits of priming. Experiments with cold primed and

non-primed plants were performed in parallel under the same conditions and using the same equipment (Figure 3-1).

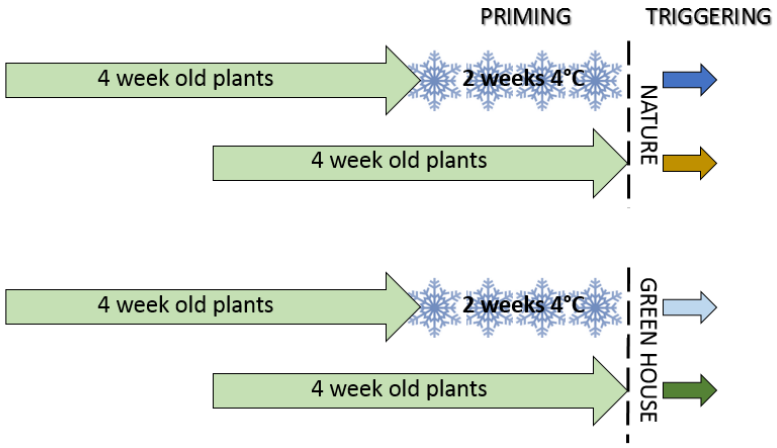


Figure 3-1. Outdoor and indoor experimental design. Four week old plants were primed for two weeks at 4 °C. In parallel to cold priming, another plant set was grown under stable growth conditions in growth chambers. Afterwards these plants were exposed to the triggering stressors in the nature (field) or transferred to the stable greenhouse conditions. Presented color code is used in graphs. Dark blue: primed and 'nature triggered', Yellow: non-primed and 'nature triggered', Light blue: primed and transferred in the greenhouse, Green: non-primed and transferred to the greenhouse.

Cold acclimation is a process that occurs during a low temperature exposure and results in elevated freezing tolerance. The experiments were performed with 7 *Arabidopsis thaliana* accessions previously characterized for acquired freezing tolerance (Zuther *et al*. 2015). The accessions N13, Ms-0 and Kas-1 had the lowest LT_{50} (temperature at which 50 % damage occurred) values after two weeks at 4°C (Table 2-1). Different from these three accessions, accession Cvi-0, had the highest LT_{50} value, mainly due to its ecological background. This accession originates from a very hot and dry island in the Atlantic Ocean. In its natural habitat, it is usually not exposed to freezing temperatures. Freezing tolerance in Cvi-0 after cold acclimation was not elevated compared to the other tested accessions. The remaining accessions, Col-0, WS and Van-0, originating from more temperate climates, displayed LT_{50} values within the two extremes. The LT_{50} values declined during the deacclimation period for all the tested accessions, as described in Zuther et al. (2015). While the accessions with higher LT_{50} had a stronger and quicker response to warm temperature after cold acclimation, the accessions with low LT_{50} showed almost no response over the investigated

deacclimation period, indicating natural genetic variability in the deacclimation response.

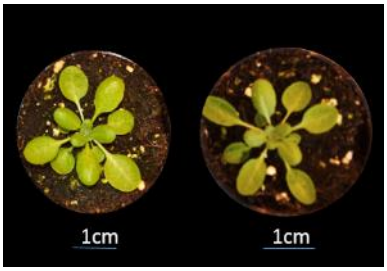


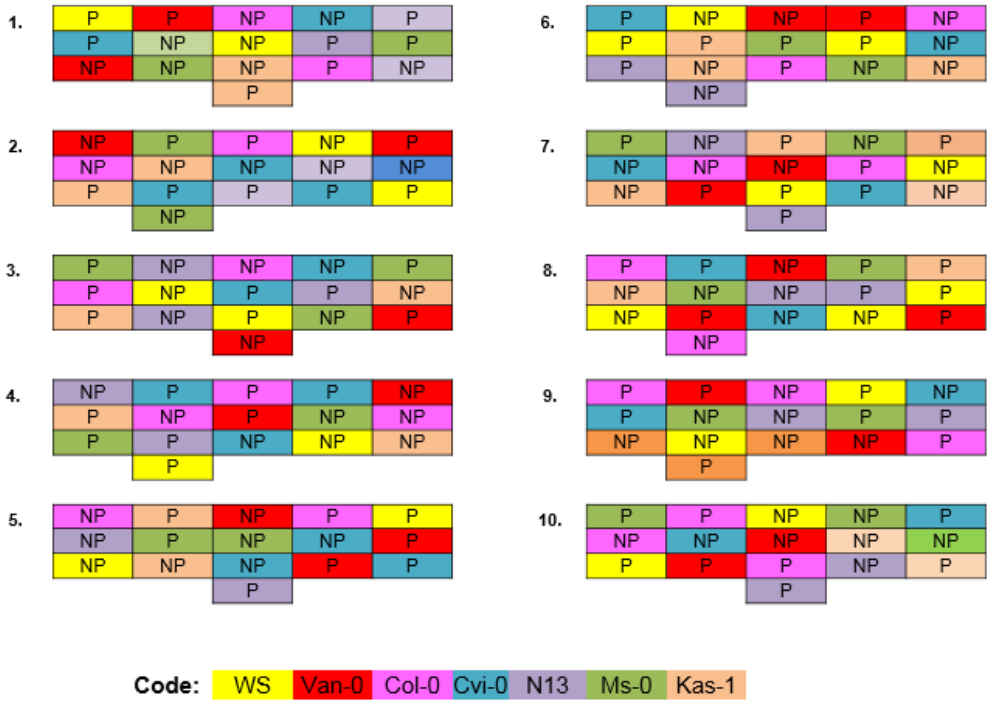
Figure 3-2. Phenotype of *Arabidopsis thaliana* accession Van-0, one of the seven *Arabidopsis thaliana* accessions used in field and greenhouse experiment, just before planting in field and transfer to greenhouse Four and six week old plants before (left) and after (right) two weeks at 4°C.

Among various abiotic stresses, cold is a major stressor that limits plant growth and development in continental climates (Shibasaki *et al.* 2009). After two weeks of cold priming, primed and non-primed plants (i.e. with and without cold stress experience) were exposed to stressors in the field and to stable greenhouse conditions. Rosette diameter, leaf area and number of leaves were similar between the treatments (Figure 3-2). Morphological differences that appeared during the time of the experiment present a picture of the ecological consequences of priming on morphology.

3.1.1 Testing the thermomemory on ecological level

Randomization between the treatments (primed / non-primed) and among the accessions is an important factor in the experimental setting. The planting scheme for the seven accessions (labeled with different color codes) is illustrated in Table 3-1.

Table 3-1. Experimental set up and coding system used in March and May 2014 and 2015 for the ten differently arranged tray / boxes. Randomized pattern of 2 week primed (P) and non-primed (NP) plants have been used for field planting as well as for transfer in the greenhouse chamber. Accessions used in experiments are N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0. In total 624 plants, arranged in 26 boxes and 26 trays have been used per month per season for conducting experiments.



The outdoor experiments started on the first Monday of March and May for both years (2014 and 2015). An overview of the experimental design is given in Figure 3-3. In parallel with the field experiments, accessions were grown in the greenhouse. While the outdoor plants were exposed to the local weather fluctuations, the greenhouse plants were kept under a constant day / night temperature of 20 ± 2 °C / 18 ± 2 °C at 120 µmol photons $*m^{-2}*s^{-1}$ light.

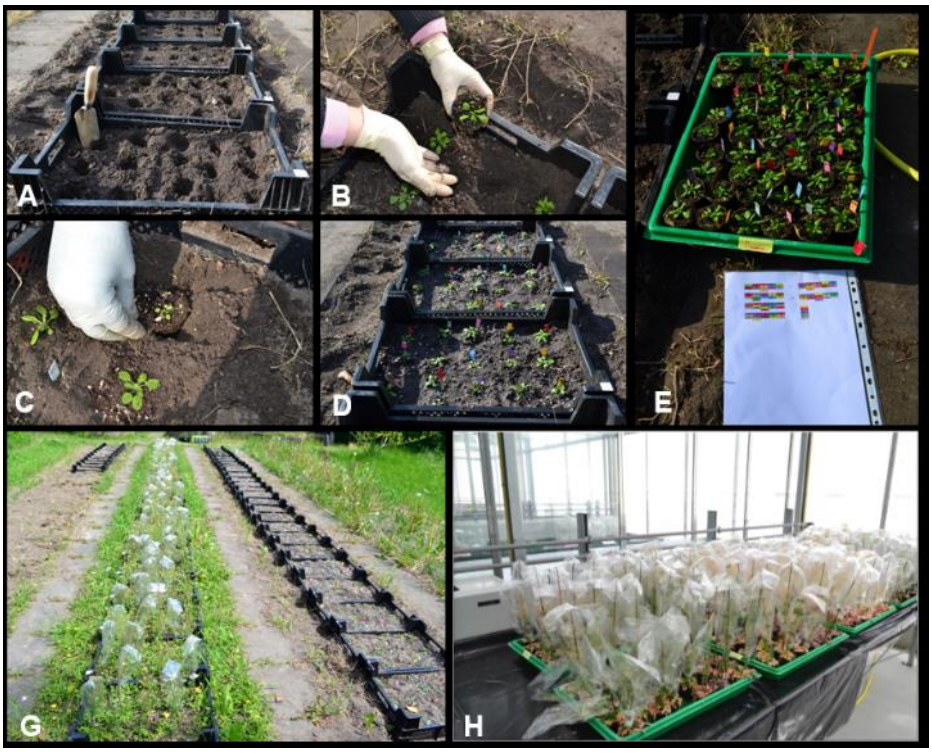


Figure 3-3 Experiments in March and May 2014 and 2015 in garden area and greenhouse of the institute. On Monday, in the first week in March and in the first week in May, two week long cold primed and non-primed plants of *Arabidopsis thaliana* were planted in the field and transferred to the greenhouse. In June and July the seeds were collected. A: soil and boxes preparation for planting, B: the plastic pots were removed and the plants transferred into soil, C-E: plants were randomly planted and labeled in 26 boxes (16 plants per box) and transferred in 26 trays to the greenhouse, G: parallel running March and May experiments, H: plants were bagged into cellophane bags and the seeds were collected in the greenhouse and in the field.

3.1.2 Natural temperature and light regimes during the experimental period

Data on temperature, light intensity and humidity were obtained from the weather stations (Fichtenberg and Botanical garden) of the Meteorology Department of Freie Universitaet, situated approximately 300 m (linear distance) from the field area. *Arabidopsis thaliana* plants were exposed to these natural temperature and light variations in the field throughout the experimental time (March-July).

In March, the mean temperature for both years was approximately 10 °C, while the maximum temperatures were 20 °C in 2014 and 17 °C in 2015. Temperatures below 0°C were recorded twice in March 2014 and six times in March 2015 during

nighttime. The minimum day time temperatures were around 4 °C (Figure 3-4, Figure 3-5).

The light intensity varied strongly in March. Midday light intensities were between 20 and 70 kLux. In the second and third week of March 2015, the illumination intensities were below 5 kLux. The light intensities on the sunniest day in March were 10 times higher than in the growth chamber. It rained frequently in March as expected for this time of the year.

In April, temperature, illumination and precipitation were still highly variable. On average, both day and night temperatures increased (Appendix Figure A1; Figure A2).

In the beginning of May (2014 and 2015), the night-time temperatures ranged from 5 to 10 °C. During May, in both years, 2014 and 2015, the average day temperature was approximately 20 °C, which is optimal for Arabidopsis growth (Rivero *et al.* 2013) (Figure 3-4, Figure 3-5). Throughout late spring within May, the temperature was continuously above 5°C. Concomitantly, the daily light intensities were more similar in May as compared to March. The mid-day, light intensity was more than 25% higher than those observed during the sunny days in March. There were major differences between light intensities measured in growing chambers and in the field, during full sunlight. Plants in the field were exposed to almost 20 times more sun energy compared to plants in the growth chambers. Humidity during May was elevated, thereby, excluding drought effects.
April, June and July weather data for 2014 and 2015 are presented in Appendix (Figure A1 and Figure A2).

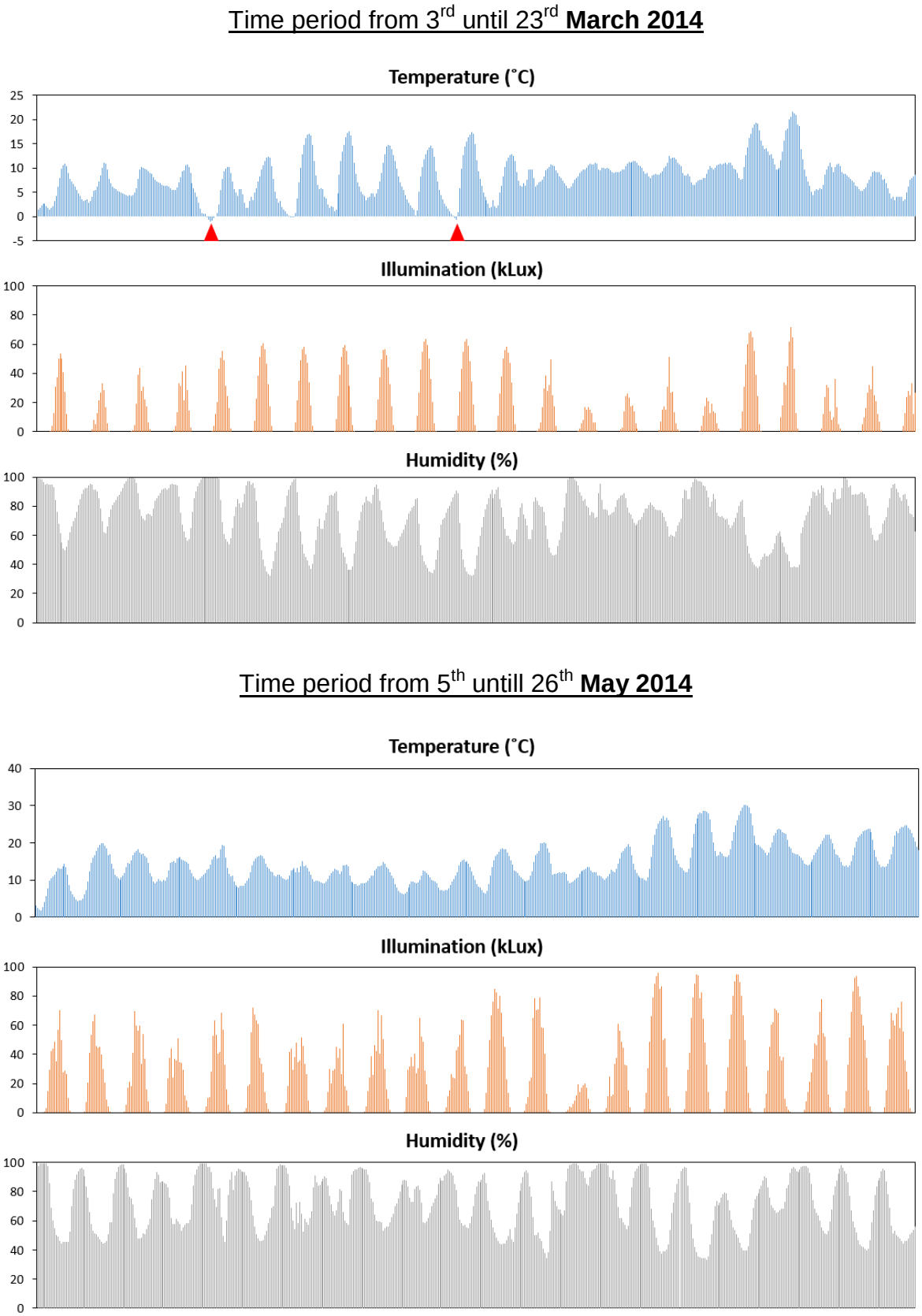


Figure 3-4. Meteorological data for the months of March and May 2014 provided by Meteorology station of the FU Berlin, Dahlem (Germany). Temperature (°C), illumination (kLux) and humidity (%) were recorded every hour during the experimental periods. Red triangles represent days when temperature values were below 0 °C during the nights.

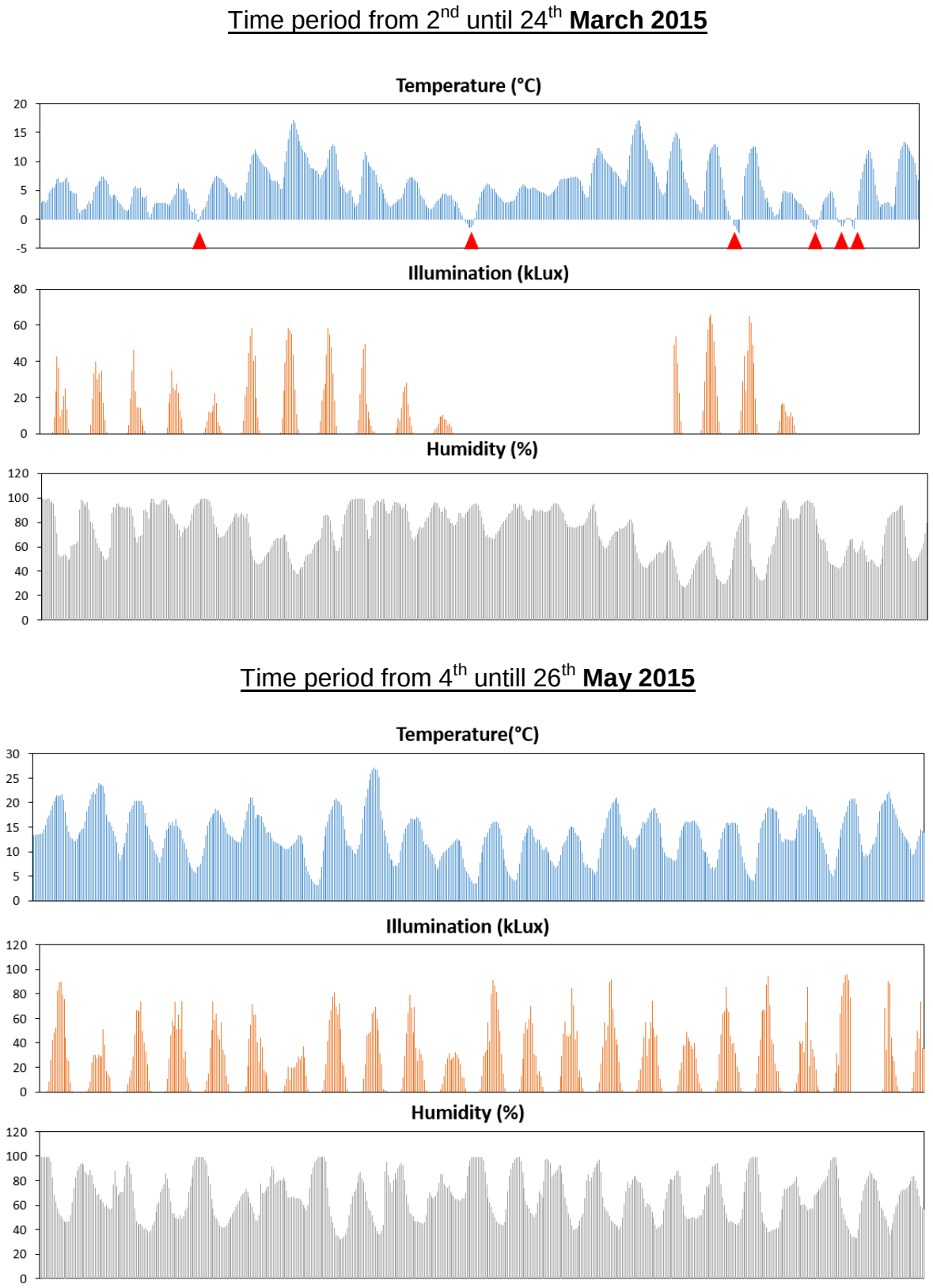


Figure 3-5. Meteorological data for the months of March and May 2015 provided by Meteorology station of the FU Berlin, Dahlem (Germany). Temperature (°C), illumination (kLux) and humidity (%) were recorded every hour during experimental periods. Red triangles represent days when temperature values were below 0 °C during the nights.

3.1.3 Impact of natural variation in the environmental parameters

To evaluate plant growth and fitness, parameters namely, seed yield, seed mass, rosette diameter, leaf area and flowering time, were measured in the seven *Arabidopsis thaliana* accessions after cold pre-treatment or without cold pre-treatment.

3.1.3.1 Priming effect on the morphological level

Morphological differences were recorded for the first four weeks in the field and under greenhouse conditions for the experimental seasons 2014 and 2015 (Figure 3-6, 3-7 and 3-8).

Differences in rosette diameter

The rosette diameter provides information about the rate of plant growth. Temperature had a strong effect on plant growth and development. During May, the plants in the experiment had bigger rosettes after four weeks of growing outside than those planted in March. The rosette diameters were 2 to 5 fold higher than their counterparts planted in March (Figure 3-6).

Two week of cold pre-treatment had positive effect in the field among all accessions, but negatively affected the plant growth under greenhouse conditions. This priming effect was confirmed by significant rosette diameter changes in two experimental seasons 2014 and 2015. There were stronger differences between primed and non-primed plants in the field in March than in May.

Stable and non-stable conditions in the greenhouse and in the field had a strong influence on the plant growth. The plant growth rate under the greenhouse conditions was higher compared to the growth rate in the field. It was not possible to see any specific trend in the rosette diameter among accessions in the field and greenhouse.

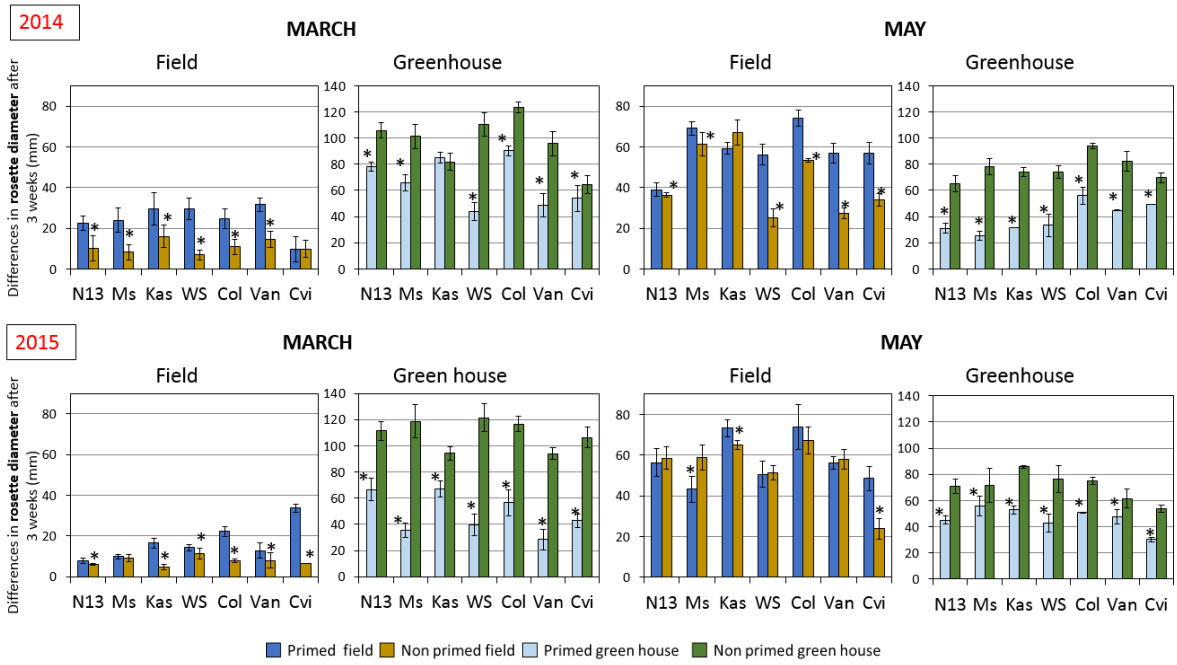


Figure 3-6. Differences in rosette diameter (mm) recorded after 3 weeks under field and greenhouse conditions in March and May of 2014 and 2015, respectively. The accessions used in the experiments were N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0. They are ordered from the lowest LT_{50} value after cold acclimation on the left to the highest on the right. The values of two week long primed plants values are compared with the values of non-primed ones. Statistically significant changes (Student T-Test *p < 0.05) between two bars of the same accession are labeled with an asterisk. Bars represent arithmetic means ± standard deviation from 10 independently grown plants.

Ratio between primed and non-primed plant rosette diameters

To further compare the priming effects, the ratio between the rosette diameters of primed (P) and non-primed (NP) plants was calculated during the first, second, third and fourth week. If the ratio P / NP >1 at any given time point this means that primed plants have an advantage over non-primed ones. Values smaller than one represent the extent of a negative priming effect. In general, primed plants had bigger rosettes than the non-primed ones already after the first week of growth in the field as well as in the greenhouse during both, March and May 2015, irrespective of the accession type.

Throughout the March experimental period, the P / NP ratio remained elevated (>1) in all accessions in the field. In the meantime, this ratio decreased below one in the greenhouse in all accessions. The fastest decrease was recorded for accessions N13, Ms-0 and Kas-1. The decline in the rosette diameters under

greenhouse conditions could be explained by the transition of these plants from a vegetative phase of growth to a reproductive one (i.e. bolting).

The trend in P / NP ratio during the late spring period within May was the same as in March for all accessions. In general, the differences from outdoor grown plants were not as pronounced as they were in March (Figure 3-7).

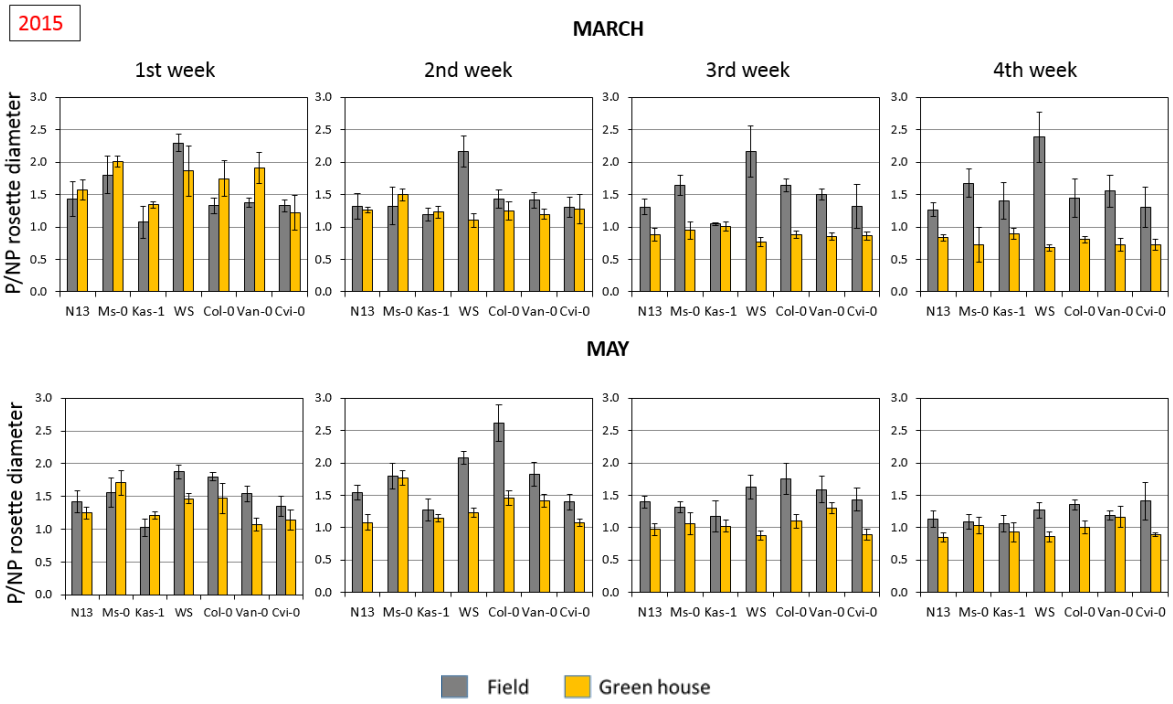


Figure 3-7. P / NP rosette diameter ratio monitored over a period of 4 weeks under field and greenhouse conditions in March and May 2015. Accessions used in experiments: N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0 are arranged from the lowest LT_{50} value after cold acclimation on the left to the highest on the right. Statistically significant changes (Student T-Test *p<0.05) between two bars of the same accession are labeled with an asterisk. Bars represent arithmetic means ± standard deviation from 10 independently grown plants.

Leaf area

The leaf area defines the photosynthetic area, which controls the energy uptake of the plants. It is strongly influenced by the temperature, as well as other factors during the plants growth.

Primed plants displayed larger leaf areas than non-primed plants studied under field conditions. In the greenhouse, non-primed plants had larger leaf areas and started flowering earlier than the plants grown in the field (Figure 3-8).

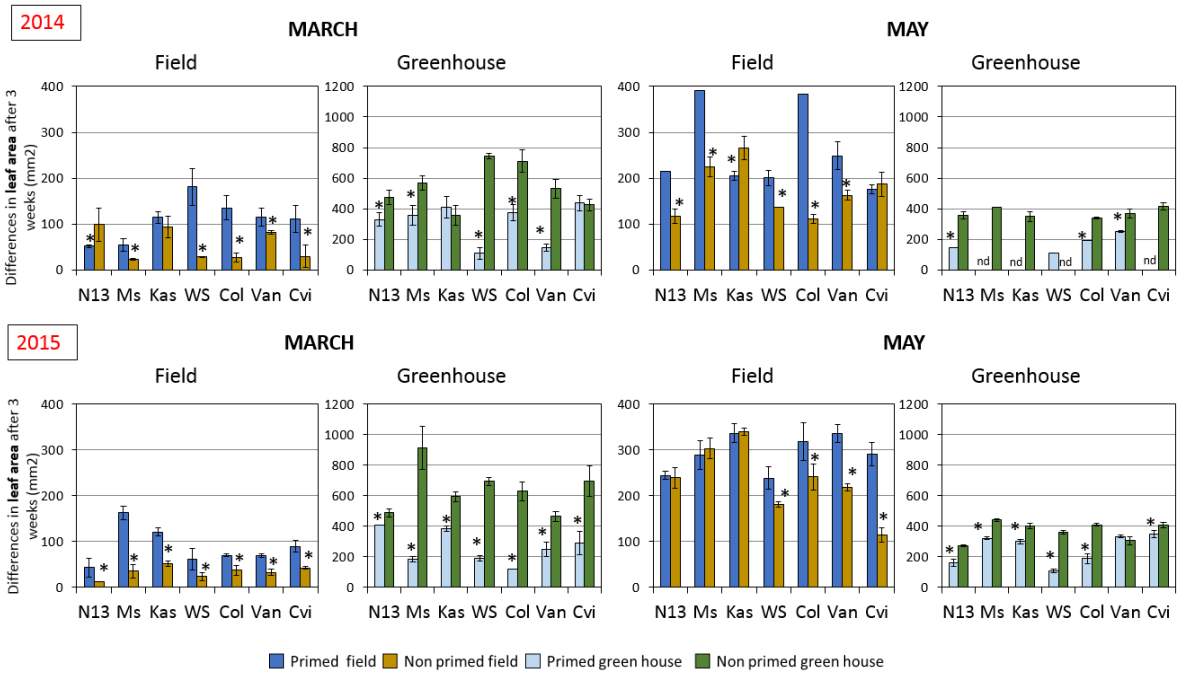


Figure 3-8. Differences in the leaf areas (mm^2) after 3 weeks of growth under field and greenhouse conditions in the March and May of 2014 and 2015 respectively. The accessions used in experiments were N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0. They are arranged from the lowest LT_{50} value after cold acclimation on the left to the highest on the right. The values of two week long primed plants are compared with the values of non-primed ones. Statistically significant changes (Student T-Test *p<0.05) between two bars of same accession are labeled with an asterisk. Bars represent arithmetic means ± standard deviation from 10 independently grown plants.

3.1.3.2 Seed mass and seed number

The ability to adjust metabolic processes to the variations in the environment is crucial for the Darwinian fitness of plants and other sessile organisms, which cannot move away from unfavorable conditions (Külheim *et al.* 2002). A critical factor in determining plant fitness is seed mass. Total seed mass is negatively correlated with the number of seeds produced and positively correlated with seedling survival (Coomes and Grubb, 2003). For this study, the average seed weight and total number of seeds per plant were determined after the harvest in July 2014 and 2015.

Average seed weight

Average seed weight number was obtained as described previously in Material and methods section. Variations in seed weight reflect the differences in acclimation strategies that exist among the seven *Arabidopsis thaliana* accessions (Figure 3-9). Accessions such as Kas-1 and Cvi-0 had, in general, slightly heavier seeds (approximately 30 µg per seed) as compared to the other tested accessions (average seed weight ≈ 20 µg). Seed weight did not significantly change between 2014 and 2015 nor between March and May experiments.

Small significant differences were presented between the primed and non-primed plant treatments, but without a common trend throughout the accessions.

Variations in seed weights among accessions did not change this trend under temperature fluctuations. Same average seed weight values were also observed under the control conditions in the greenhouse.

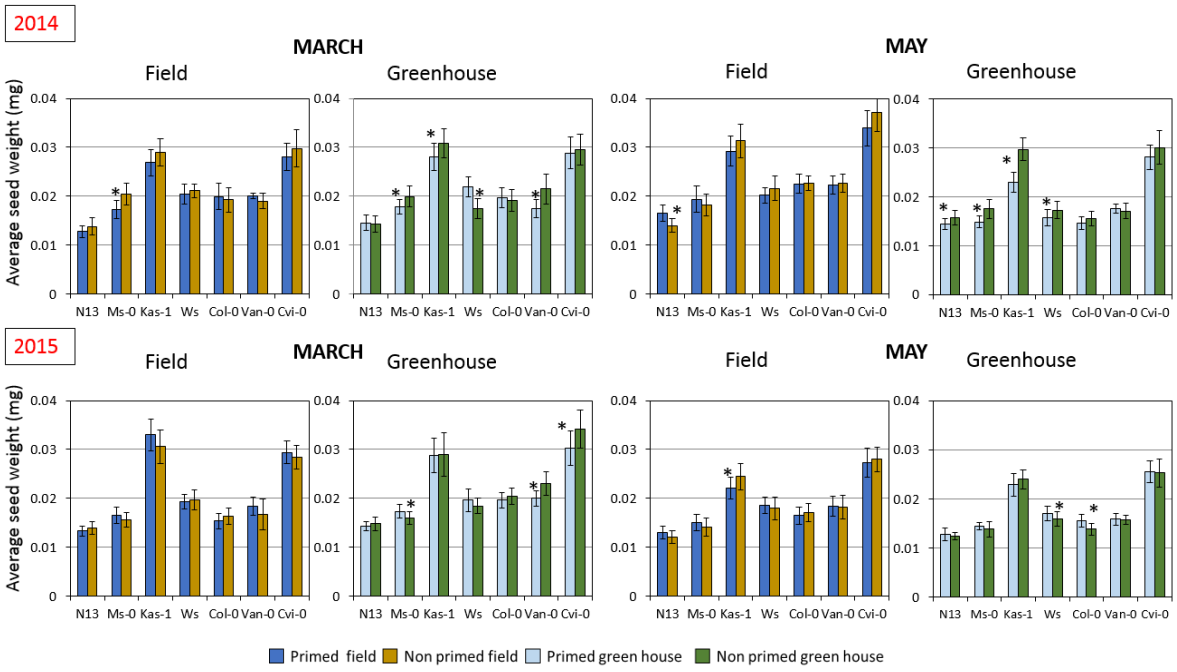


Figure 3-9. Average seed weights in grams of the plant sets grown in March and May of 2014 and 2015 respectively. Accessions used in experiments: N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0 are arranged from the lowest LT_{50} value after cold acclimation on the left to the highest on the right. Plants that were primed for two weeks are compared with non-primed ones. Statistically significant changes (Student T-Test *p<0.05) between two bars (primed and non-primed) of same accession are labeled with an asterisk. N = 25 ± SD.

Total number of seeds per plant

Total number of seeds per plant was calculated from the previously determined parameters (average seed number, seed number for defined seed mass and total seed production), as described in the Material and Methods section (Figure 3-10). This number represents the accession-spreading strategy for the next generation under temperature fluctuations. The accessions such as Kas-1 and Cvi-0, produced a lower amount of seeds per plants than accessions N13 and Ms-0. Their respective seed yield was lower than that of the accessions WS, Col-0 and Van-0. The number of seeds per plant varied between experimental years and, in some accessions, the variation was up to ± 10 %.

All the cold pre-treated accessions had elevated total seed numbers when compared to non-treated plants under field conditions in March and May (both years), demonstrating a positive priming effect on the seed yield. Significant differences were present between the primed and non-primed treatments in the greenhouse for accessions N13, Ms-0 and Kas-1. On the contrary, the accessions WS, Col-0 and Van-0 produced the same amount of seeds in the greenhouse, irrespective of whether they were cold pre-treated or not.

The number of seeds per plant varied between the experimental years and also between the seasons. Despite these variations, cold pre-treatment increased the seed number in all accessions in the field.

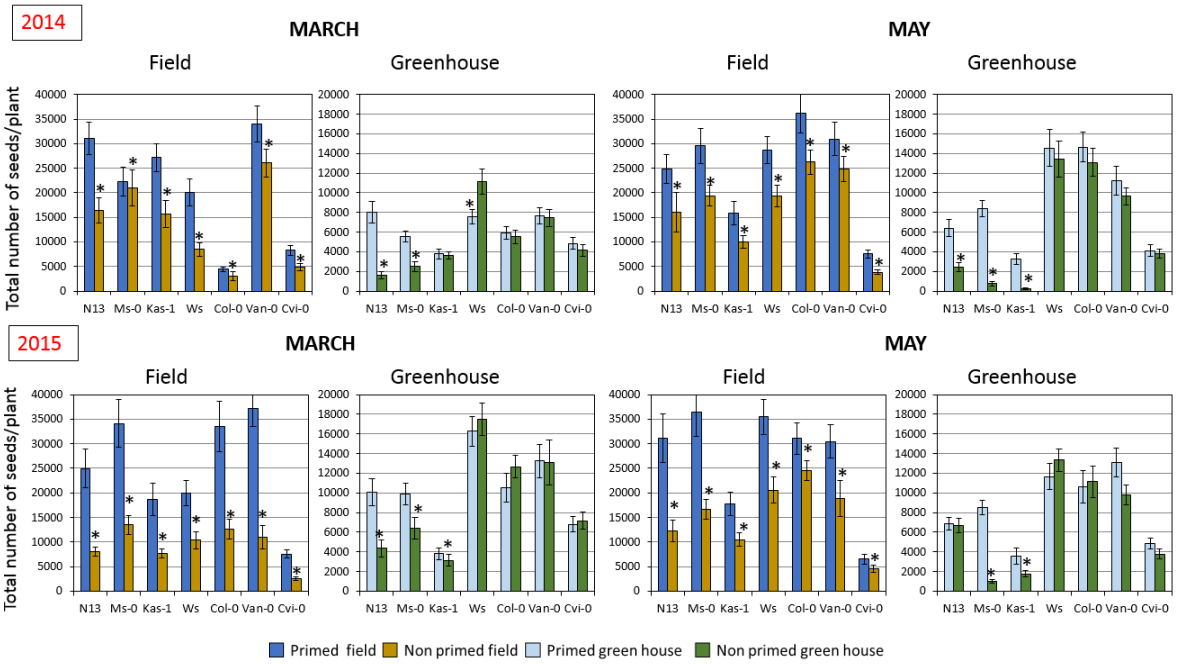


Figure 3-10. Total number of seeds per plant of the plant sets grown in March and May of 2014 and 2015 respectively. Accessions used in experiments were N13, Ms-0, Kas-1, WS,

Col-0, Van-0 and Cvi-0. They are arranged from the lowest LT_{50} value after cold acclimation on the left to the highest on the right. Plants that were primed for two weeks are compared with non-primed ones. Statistically significant changes (Student T-Test *p<0.05) between two bars of same accession are labeled with an asterisk. N = 25 ± SD

3.1.3.3 Impact of priming on the flowering time

Flowering is a critical stage of development in the life cycle of most crops. It affects the chances of pollination and other crop processes, such as leaf expansion, root growth, and nutrient uptake (Fitter and Fitter, 2002), and determines seed number and ripening (Craufurd and Wheeler, 2009). The average time between two consecutive generations differs in the lineages of *Arabidopsis thaliana* population (Yu *et al.* 2009). Accessions show differential generation times, caused by divergent day / night length, temperature fluctuations, UV radiation, and precipitation rate as well as pre-treatment. In order to determine the generational time, parameters such as age at the onset of flowering and number of flowering plants at the certain date, were used as assessment criteria. The percentage of flowering plants during flowering time in the field and the greenhouse are presented in Figure 3-11 and Figure 3-12.

In March, the air temperatures dropped below 0 °C (Figure 3-11), which challenged the outdoor plants. In May, weather conditions outside had mild imbalances which might have interrupted optimum plant growth. By the end of May, the plants planted in March, started bolting in the field. Plants planted in May flowered in the first weeks of June. By the end of June, all plants had flowered in the field from both experiments. Weather conditions in June and July were stable and in the last week of July, plants were harvested from the field. In the greenhouse, due to the stable temperature conditions, plants flowered faster and finished flowering earlier than the plants planted in the field. Appendix (Figure A1; Figure A2) shows the weather conditions for April, June and July for both years (2014 and 2015).

In general, the accessions N13, Kas-1 and Ms-0 flowered the latest compared to the other accessions under the field as well as under greenhouse conditions (Figure 3-11). The accession Cvi-0 flowered the earliest, irrespective of the growth

conditions. The accessions WS, Col-0 and Van-0 all flowered in the same week, after Cvi-0 and before N13, Kas-1 and Ms-0.

Cold pre-treatment resulted in earlier flowering in all accessions under greenhouse and field conditions. When the accessions N13, Ms-0 and Kas-1 were planted in March, they displayed later flowering than other plants. This flowering trend was recorded not only in the field, but also in the greenhouse. Primed accessions, WS, Col-0, Van-0 and Cvi-1 grown under field and greenhouse conditions in March, flowered in parallel with non-primed plants but with a different flowering rate. In May 2014, primed and non-primed accessions N13, Ms-0 and Kas-1 planted in the field, flowered in the same week and with the same number. Non-primed plants planted in May flowered in the same weeks across the two year period. The accessions WS, Col-0, Van-0 and Cvi-1 planted in May, showed different tendencies and time delays between the two treatments. In the greenhouse, the different treatments resulted in different flowering times. Information on the time management and priming influence on flowering are given in the explanation of Table 3-2.

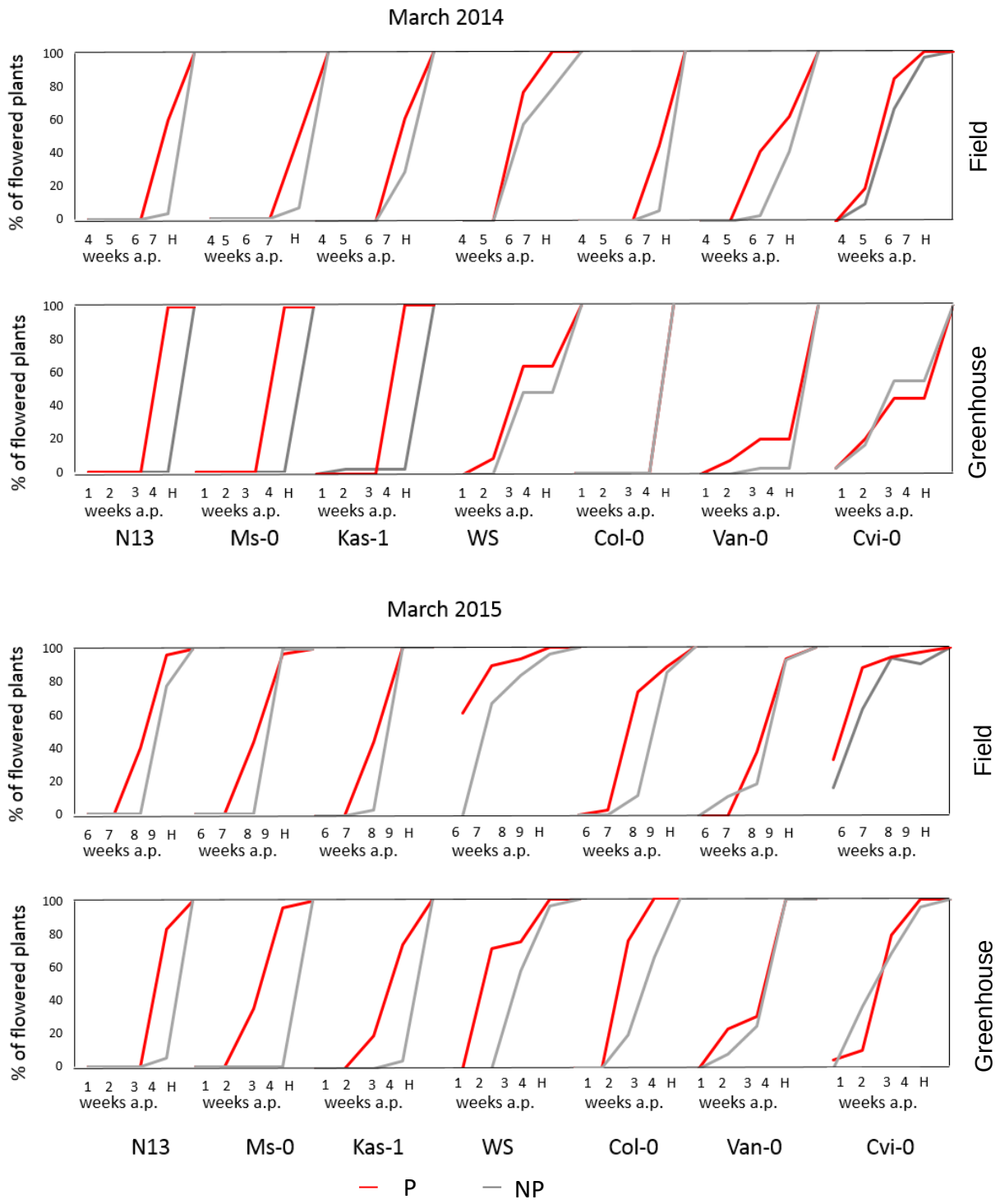


Figure 3-11. Percentage of flowered plants during the experiments in March of 2014 and 2015. Numbers 1, 2, 3, 4, 5, 6, 7, 8 and 9 represent weeks after the start of experiment (March) when the first flower opened. The letter H represents the harvesting day when all of the plants have been collected from, both, the field and the greenhouse area and were then stored for additional drying. Accessions used in the experiments are N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0. They are ordered from the lowest LT_{50} value after cold acclimation on the left to the highest on the right. The data of plants that were primed for two weeks are compared with values of non-primed ones. weeks a.p.(weeks after planting) N = 30

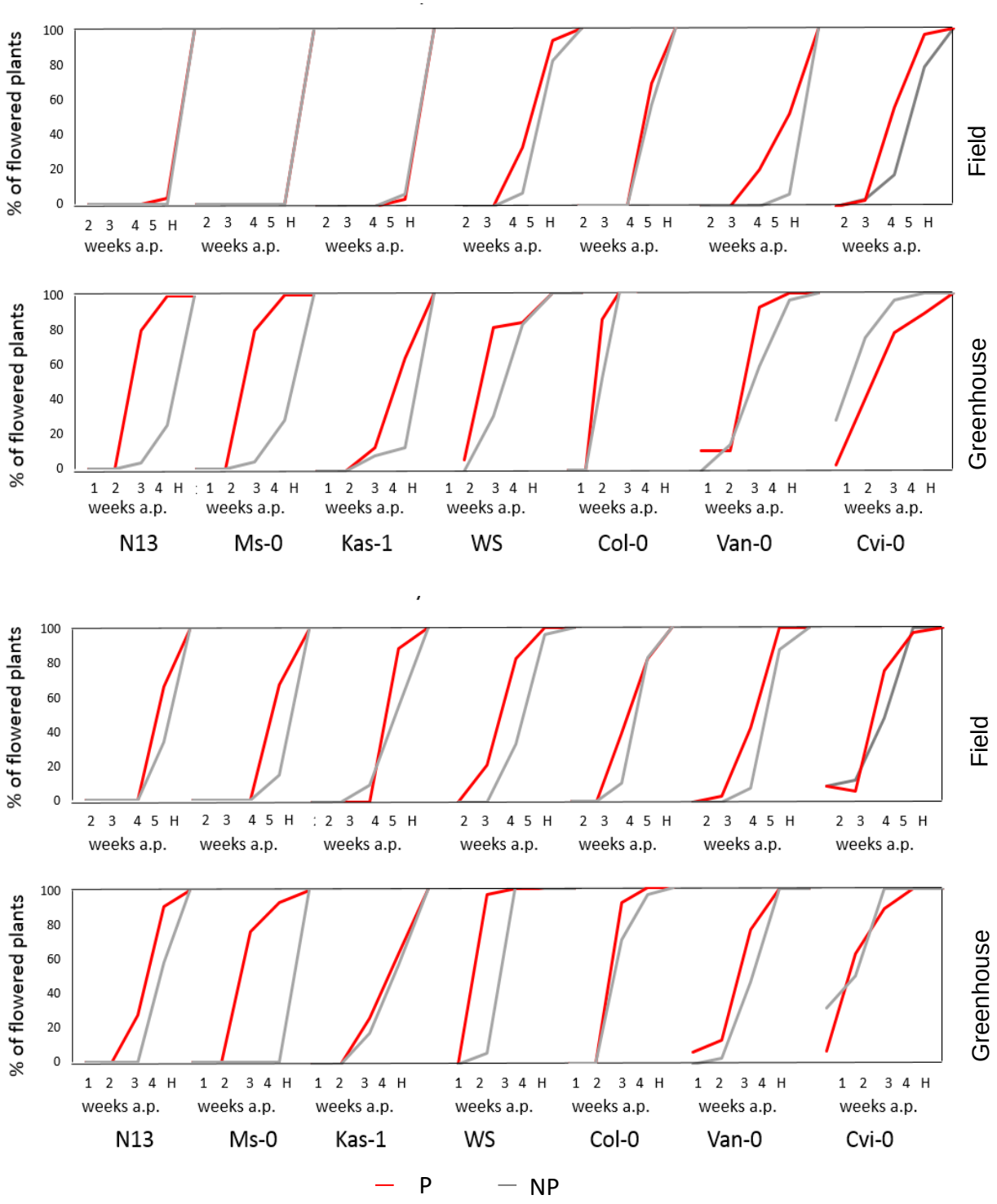


Figure 3-12. Percentage of flowered plants during the experiments in May of 2014 and 2015. Numbers 1, 2, 3, 4, 5, 6, 7, 8 and 9 represent weeks after the start of experiment (May) when the first flower opened. The letter H represents the harvesting day when all of the plants have been collected from, both, the field and the greenhouse area and were then stored for additional drying. Accessions used in the experiments are N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0. They are ordered from the lowest LT_{50} value after cold acclimation on the left to the highest on the right. The data of plants that were primed for two weeks are compared with values of non-primed ones. weeks a.p.(weeks after planting) N = 30

Flowering day difference

Flowering day difference (DD_{50}) is defined as the difference in the number of days between primed and non-primed plants when 50% from the total plant set flowers.

In general, in the field, flowering differences are much higher between the two treatments for accession N13, Ms-0 and Kas-1 when compared to the accessions WS, Col-0, Van-0 and Cvi-0 in March (Table 3-2). For obtaining 50 % of flowering status, in the March sets, non-primed plants had almost 50 days delay of flowering, compared to the primed ones. Primed accession WS, Col-0, Van-0 and Cvi-0 flowered earlier than the remaining accessions. The non-primed plants did not display an extended flowering delay. Depending on the accession, the differences between the treatments were not more than 13 days. For that reason, DD_{50} value was not high for accession WS, Col-0, Van-0 and Cvi-0. In the absence of a cold-acclimation treatment, accessions N13, Ms-0 and Kas-1, planted in May, all flowered at the same time and with the same frequency as their primed counterparts. The DD_{50} values for these last three accessions were 0 and 3.5 (approx.) for the years 2014 and 2015 respectively. The other accessions did not show drastic differences between years.

Under greenhouse conditions, between the years, DD_{50} values (approx. 8) remained constant for N13, Ms-0 and Kas-1 accessions. The four remaining accessions (WS, Col-0, Van-0 and Cvi-0) planted in May and grown under greenhouse conditions showed similar DD_{50} values when compared to them.

Table 3-2. DD_{50} (day's differences between flowering time of primed and non-primed plants when 50% of plants flowered) values from the field and greenhouse in March and May experiments (2014, 2015). Accessions used in experiments: N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0 are ordered from the lowest LT_{50} value after cold acclimation on top to the highest on the bottom. Accessions coming from the colder habitats and with the highest LT_{50} value are marked in red.

Field	Flowering differences in days	
	March 2014	March 2015
N13	50.4	52.5
Ms-0	49.7	44.8
Kas-1	52.5	2.8
WS	4.9	5.6
Col-0	13.3	2.1
Van-0	4.9	2.1
Cvi-0	1.0	3.5

Field	Flowering differences in days	
	May 2014	May 2015
N13	0.0	4.2
Ms-0	0.0	4.2
Kas-1	0.0	2.1
WS	1.4	4.9
Col-0	1.0	2.1
Van-0	14.0	2.1
Cvi-0	2.1	1.4

Green house	Flowering differences in days	
	March 2014	May 2015
N13	7.0	8.4
Ms-0	7.0	8.4
Kas-1	7.0	8.4
WS	8.4	9.1
Col-0	0.0	5.6
Van-0	1.4	0.0
Cvi-0	9.8	1.4

3.1.4 Photosynthesis damages caused by temperature variation

Chlorophyll-a fluorescence can give insights into the plant´s ability to tolerate environmental stress and the extent to which the photosynthetic apparatus has been damaged due to such stress (Maxwell and Johnson, 2000). The effective (indicator of the amount of energy used in photochemistry by PSII under steady-state photosynthetic lighting conditions) and initial (maximum potential quantum efficiency of PSII if all capable reaction centers were open) quantum yield parameters can be quantified with a Mini-PAM (Waltz, Effeltrich, Germany), in order to assess the consequences of priming under varying conditions. For the purpose of studying the impact of these environmental influences, a comparison was made between field and greenhouse grown plants.

3.1.4.1 Photosystem II in March under unstable field conditions

The first week in the field was challenging for all accessions and plant treatments. The plants were exposed to drastic temperature and light fluctuations as well as myriads of other abiotic and biotic stressors.

The first 24 hours after planting in the field were decisive. As a result of the previous two-week low temperature treatment, the primed plants displayed 2-3 times higher values of effective and initial quantum yield compared to the non-primed ones (Figure 3-13). This signifies the high amount of stress for the photosystem due to this unstable environment. Because of the changes in PSII, the maximum quantum yield (F_v / F_m) values were low, not only in non-primed plants but also in primed plants. After a day in outdoor conditions, the maximum quantum yield values were around 0.25 for non-treated plants and around 0.58 for the treated ones. These significant differences between treatments among all accessions under field conditions persist up to seven days after planting. Significant differences were not observed among the various accessions.

After transferring the plants to the greenhouse conditions for day, primed plants showed 5% lower values of the maximum quantum yield when compared to the non-primed ones. This value decreased within the next days under controlled

greenhouse conditions and continued until the end of experiment. Under stable greenhouse conditions, accession specific changes were not observed.

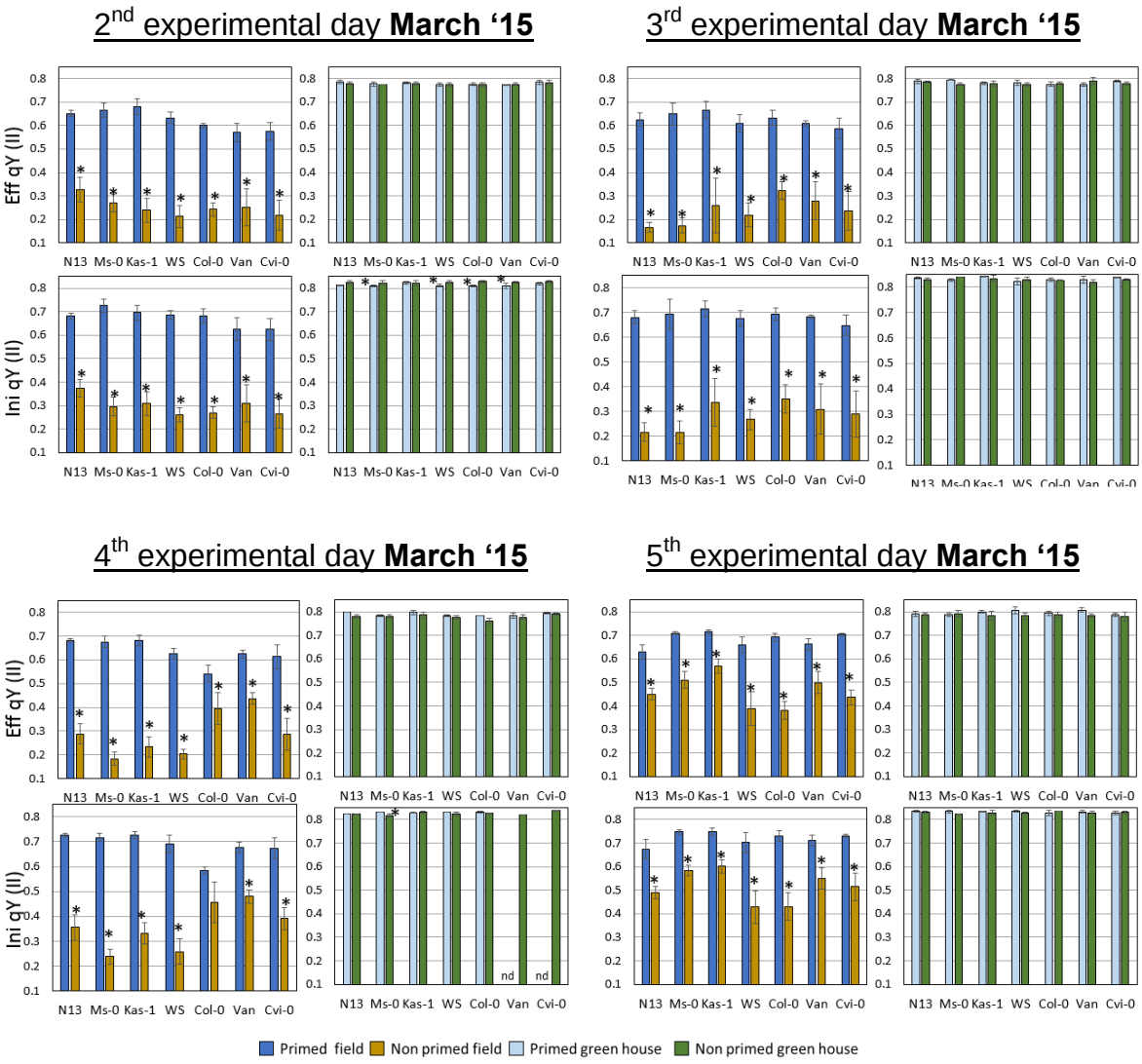


Figure 3-13. Effective (eff) and initial (ini) quantum yield of photosystem II after 1st, 2nd, 3rd and 4th day after planting in the field and transferring to the greenhouse in March 2015. Accessions used in these experiments are N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0. They are arranged from the lowest LT_{50} value after cold acclimation on the left to the highest on the right. The values of the two week primed plants have been compared with the non-primed ones. The data were analyzed by Student T-Test and significant differences (*p<0.05) between two bars of same accession are labeled with an asterisk. n.d (not detected due to technical reasons). N = 10 ± SD.

The acclimation process continued up to three weeks after the experiment started. As was expected, photosynthetic parameters changed in the direction of increment.

After one week under field conditions, the effective quantum yield values for primed and for the non-primed plants were around 0.77. Considering the temperature fluctuations and the several sub-zero events in March 2015, plants were triggered few times by the low temperature during the experiment. Depending on the duration and the magnitude of temperature stress, significant differences were observed between treatments even after three weeks of acclimation. Differential patterns existed under low temperature stress conditions among accessions (Table 3-14).

Photosynthetically measured parameters did not change for plants in the greenhouse in the second, third and fourth week after transfer.

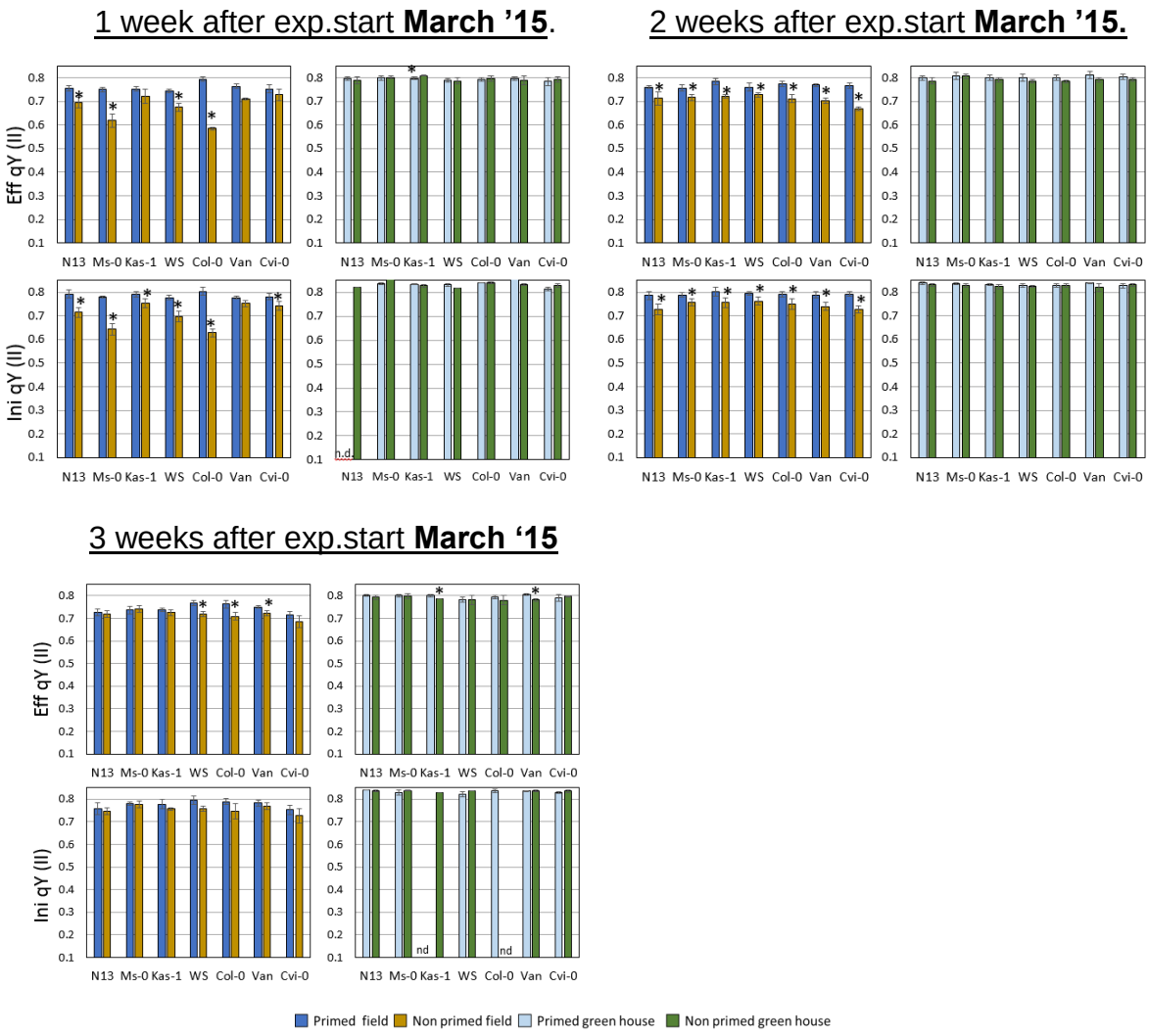


Figure 3-14. Effective (eff) and initial (ini) quantum yield of photosystem II after 2nd, 3rd and 4th weeks after planting in the field and transferring to the greenhouse in March 2015. Accessions used in these experiments are N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0. They are arranged from the lowest LT_{50} value after cold acclimation on the left to the highest on the right. The values of the two week primed plants are compared with the non-primed ones. The

data were analyzed by Student T-Test and significant differences (*p<0.05) between two bars of same accession are labeled with an asterisk. n.d (not detected due to technical reasons). N = 10 ± SD

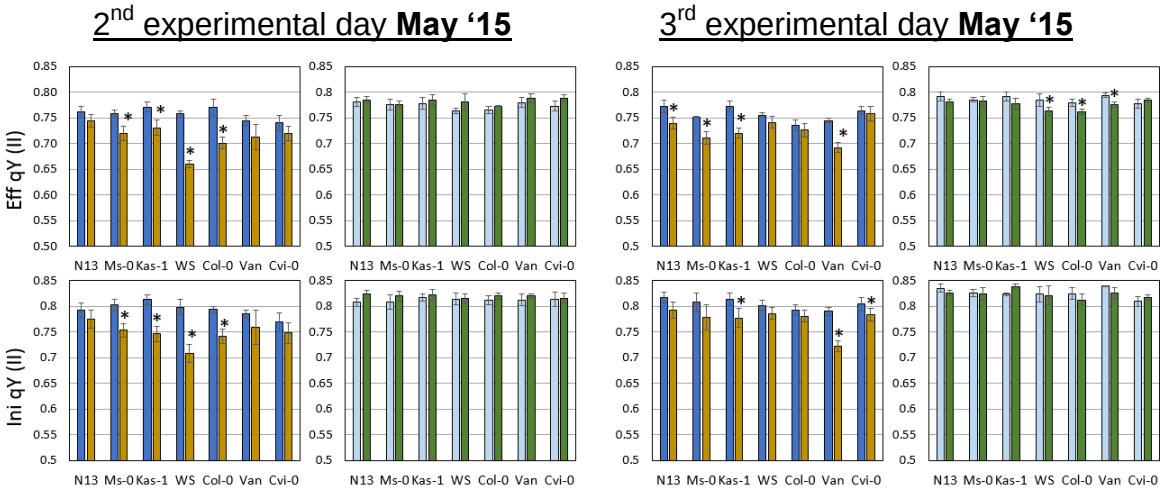
3.1.4.2 Photosynthesis in May under stable temperature conditions

Any kind of a stress on a plant can be manifested directly on PSII as damage. Under more stable temperature conditions, like in the late spring (May), huge and irreversible damages in PSII should not be overly pronounced.

In general, photosynthetic parameters, such as effective and initial quantum yield, did not show very strong significant differences among the accessions and between the treatments, as was the case in March.

After 24 hours under field conditions, non-primed accessions Ms-0, Kas-1, WS and Col-0 displayed significantly reduced quantum yield values compared to the primed plants. Effective and initial quantum yield values of primed plant were around 0.75 and 0.81, as expected. Similar values were measured in the greenhouse grown plants (Figure 3-15).

During acclimation time in the field, these chlorophyll-a parameters showed only slight changes between treatments. In the greenhouse, from the beginning of the experiment, quantum yield quickly attained the appropriate optimal value (0.84) in both treatments and in all accessions.



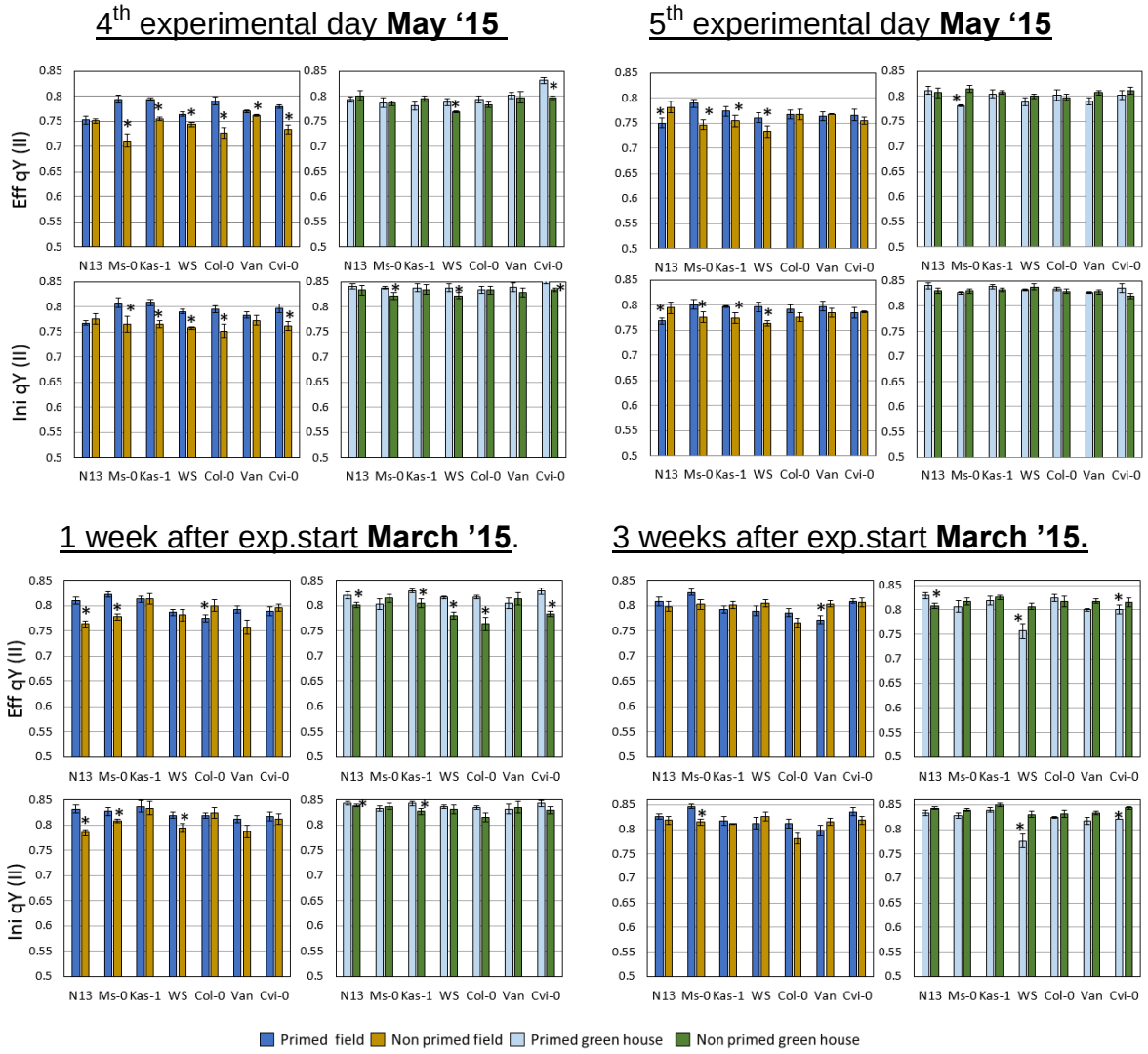


Figure 3-15. Effective (eff) and initial (ini) quantum yield of photosystem II after 1st, 2nd, 3rd and 4th day and 2nd, 3rd and 4th weeks after planting in the field or transferring to the greenhouse in May 2015. Accessions used in these experiments are N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0. They are ordered from the lowest LT_{50} value after cold acclimation on the left to the highest on the right. The values of the two week primed plants are arranged with the non-primed ones. The data were analyzed by Student T-Test and significant differences (*p<0.05) between two bars of same accession are labeled with an asterisk. N = 10 ± SD.

Correlational analysis between LT_{50} values and the ratio of eff / ini qY in plants that were primed two weeks can give hints about the acclimation in the field on the first and third day. LT_{50} data of the first and third deacclimation day (DEACC 1 and DEACC 3) were provided by Dr. Hincha, MPI Golm- Potsdam (data published in Zuther *et al*. 2015). The effective / initial quantum yield ratios measured in the first experimental days in March and May 2015 (Figure 3-13; Figure 3-14; Figure 3-15)

were used to determine the p (statistical significance) value and Spearman correlation coefficient.

After 24 hours in the field, in March 2014 and 2015, strong negative correlations were recorded for the overall plant set; consisting of accessions N13, Ms-0, Kas-1, WS, Col-0, Van and Cvi-0. In 2014, this correlation was significant. Accessions with the lowest LT_{50} values had higher eff / ini qY ratio implying relatively small damages on PSII. On the other hand, plants with high LT_{50} values showed the opposite trend. In contrast to March values, in May 2014 and 2015, after one day, correlation was positive and weak in the field conditions.

After 72 hours in the field, in March as well in May (for both years), primed plant sets did not show significant correlation between LT_{50} values and eff / ini qY values. Spearman correlation data are presented in Table 3-3.

Table 3-3. Spearman correlational analyses with the numerical values of correlation coefficients p and r^2 between the LT_{50} values and eff / ini qY for 2 week primed plants. Upper table: 24h after planting primed plants in the field in March and May 2014 and 2015. Table down: 3 days after planting primed plants in the field in March and May 2014 and 2015. Correlational analysis were performed with a data set consisting of the *Arabidopsis thaliana* accessions: N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0. Significate negative correlations are marked in red.

1 day after priming	March '14	May '14	March '15	May '15
p	0.0217	0.4444	0.1016	0.4440
r^2	-0.8101	0.3571	-0.6671	0.3571

3 days after priming	March '14	May '14	March '15	May '15
p	0.7853	0.2667	1.0000	0.8397
r^2	-0.1429	-0.5000	0.0000	-0.1071

3.1.4.3 Unpredictable temperature changes tested accession capacity

The response to extremely low winter temperatures, even if these occur only infrequently, may be highly species- and location-specific (Pederson *et al*. 2004). Unpredictable sub-zero temperatures tested accession acclimation capacity and recall of thermomemory after several weeks in the field. The triggering stress resulted in distinct changes of eff / ini qY ratio in treated and non-treated plants.

As shown in Figure 3-16 (up), 12 hours of freezing temperature resulted in differences in the quantum yield ratio between treatments, among seven different accessions on 23rd March 2015. Primed and non-primed N13, Ms-0 and Kas-1 plants, that were four week under field conditions responded differently to 12 h cold stress. Compared to the ratio values between the two treatments in WS, Col-0, Van-0 and Cvi-0 plants, these three accessions displayed the opposite trend. In the absence of a cold pre-treatment, N13, Ms-0 and Kas-1 plants showed higher values of eff / ini qY ratio than primed plants. Meteorological data for these two years are given in the Figure 3-4 and Figure 3-5.

In the absence of freezing stress (such as on 24th March 2014), N13, Ms-0 and Kas-1 plants which were acclimated for four weeks in the field, both primed and non-primed, showed eff / ini qY ratios between treatments opposite to the WS, Col-0, Van-0 and Cvi-0 plants. Non-primed WS, Col-0, Van-0 and Cvi-0 plants displayed higher eff / ini qY ratios than primed plants after four weeks of acclimation in the field and under mild weather conditions.

Very small temperature variations during a few hours at the night (on 7th March 2014 and 6th March 2015; Figure 3-16 center) did not show accession specific trends in plants that were acclimated for one week in the field. In most of the tested accessions, primed plants displayed higher eff / ini qY ratio than the non-primed ones.

The plants that were acclimated for one day, on 4th March 2014 and 3rd March 2015 (Figure 3-16 bottom), showed typical priming responses for treated and non-treated plants, with a higher eff / ini qY ratio in all primed plants.

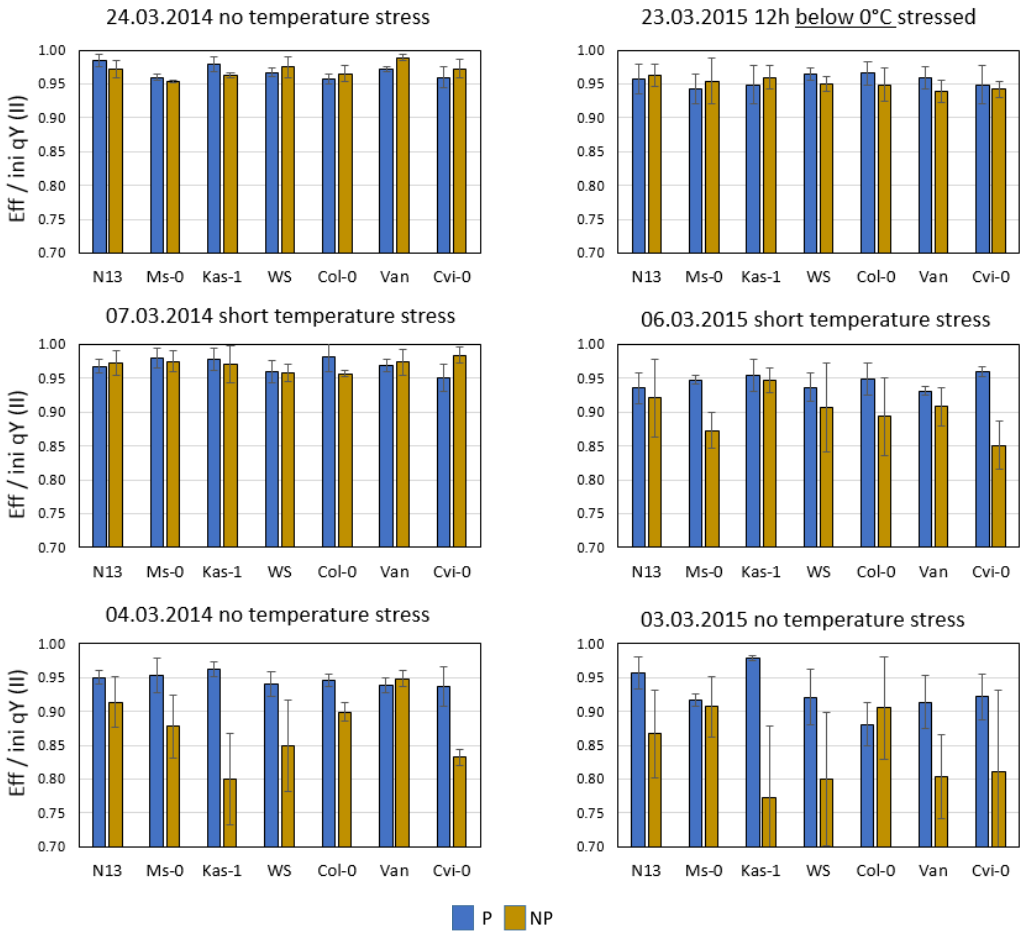


Figure 3-16. Ratio of quantum yield of photosystem II (eff / ini qY) in primed and non-primed plant sets in March 2014 and 2015. Top: eff / ini qY values of 4 week acclimated plants in the field in 2014 and 2015, without temperature stress (left) and 12 hours of temperature stress (right) conditions. Center: eff / ini qY values of 4 days acclimated plants in the field in 2014 and 2015 under very short (2 hours) temperature stress conditions. Bottom: eff / ini qY values of 24 hours acclimated plants in the field in 2014 and 2015 under non freezing temperature stress conditions. Accessions used in experiments: N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0 are ordered from the lowest LT_{50} value after cold acclimation on the left to the highest on the right. N = 10 ± SD.

Figure 3-17 is a simple and clear representation of the accession capacity for unpredictable future stress situations and acclimation speed. The relative effect of priming on the eff / ini qY ratio shows an accession-specific role during longer exposures to freezing temperatures.

Under stable weather conditions and the absence of freezing stress, accessions such as WS, Col-0, Van-0 and Cvi-0, had a quantum yield ratio of NP / P greater than 1. This ratio can change to less than 1 after 12 hours of temperature stress exposure. Regardless of the four week acclimation length, accessions N13, Ms-0

and Kas-1 had contrariwise management. These three accessions displayed eff / ini qY NP / P ratio greater than 1 under freezing temperatures.

Short temperature stress occurred during the first weeks of deacclimation, causing a decline in the quantum yield ratio of non-primed and primed plants to less than 1 in the all accessions in the field.

First exposure to the environmental stress in March 2014 and 2015 generated the same ratio outcomes as for the short stress after one week of acclimation.

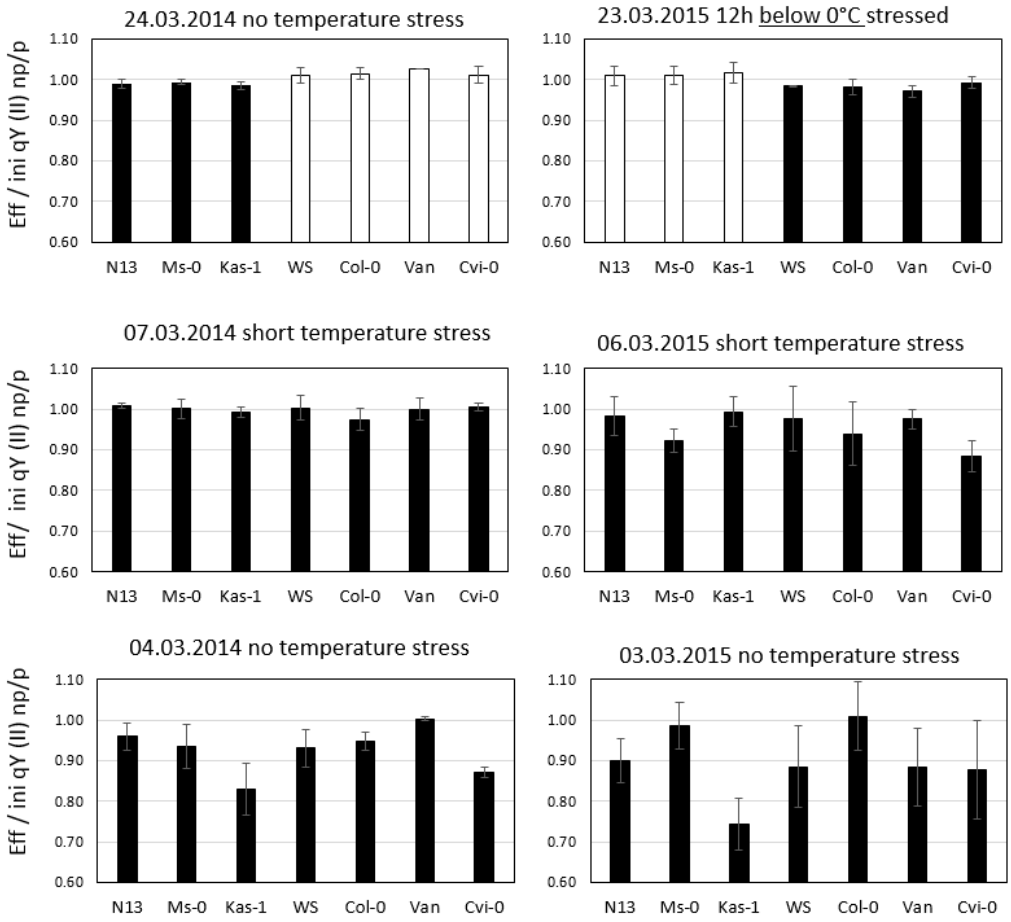


Figure 3-17. Relative effect of priming on eff / ini quantum yield in March 2014 and 2015. Non-primed / primed (NP / P) ratio of quantum yield of photosystem II (eff / ini qY) in seven different *Arabidopsis thaliana* accessions. Top: 4 week acclimated plants without temperature stress (left) and 12 hours of temperature stress (right) conditions. Center: 4 days acclimated plants under very short (2 hours) temperature stress conditions. Bottom 24 hours acclimated plants under non temperature stress conditions. Accessions used in experiments are N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0 are ordered from the lowest LT_{50} value after cold acclimation on the left to the highest on the right. Black: mean values are below 1. White: mean values are above 1. N = 10 ± SD.

3.1.5 Plant attractiveness towards herbivores

The role of herbivores in controlling plant species richness is a critical issue in the conservation and management of grassland biodiversity (Olff and Ritchie, 1998). During the experiment in May 2015, due to favorable weather conditions, the Arabidopsis accessions were immediately attacked after planting by terrestrial gastropod mollusks. As most slug species are generalists, they were attracted by freshly planted (four week old) plants.

The special affinity of slugs to Arabidopsis plants was recorded in the first days of the experiment. Slugs had full freedom to move and eat plants within the experimental area. No physical barriers influenced their nutritional choice.

After only two days in the field almost all of the primed WS and Kas-1 plants were eaten. Primed Van-0, Ms-0 and non-primed Col-0 plants had a lower percentage of appeal to the slugs. In 50% of the cases, primed Col-0 and non-primed Ms-0 and Kas-1 were eaten by slugs as compared to the accessions Cvi-0 and N13, where no attacks were recorded at all.

Three days after planting Arabidopsis plants in the field, plants shoots of both primed accession WS and Kas-1 as well as primed Van-0 and Col-0 were completely eaten. Non-primed accessions Col-0, Ms-0 and Kas-1 were also a feeding choice but not preferred as the Cvi-0 and N13 accessions were still not affected.

Differences in the plants attractiveness towards herbivores vanished after 12 days of planting between treatments and among accession in the field. This was probably due to the lack of fresh plant material in the experimental boxes. In the next months, most of the accessions recovered from this biotic stress. The slug's lack of interest for Arabidopsis plants decreased in the next months due to the temperature rise and low precipitation (Table 3-4).

Table 3-4. % of the slugs attack on *Arabidopsis thaliana* plants in the field during experiment in May 2015. Different attractiveness have been recorded among the accessions and between the treatments (P-2 week primed plants, NP-non-primed plants). Accessions used in experiments are N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0. Accessions that are bold show less than 10% slugs attractiveness for feeding.

% of slug attack	After 2 days outside		After 3 days ouside		After 12 days outside	
100-80%	WS	P	WS	P	WS	P
	Kas-1	P	Kas-1	P	Kas-1	P
70-60%	Van-0	P	Van-0	P	Van-0	P
	Ms-0	P	Col-0	P	Col-0	P
	Col-0	NP	Ms-0	P	Ms-0	P
50%	Col-0	P	Col-0	NP	Col-0	NP
	Ms-0	NP	Ms-0	NP	Ms-0	NP
	Kas-1	NP	Kas-1	NP	Kas-1	NP
40-0%	WS	NP	WS	NP	WS	NP
	Van-0	NP	Van-0	NP	Van-0	NP
	Cvi-0	**P**	**Cvi-0**	**P**	N13	NP
	Cvi-0	**NP**	**Cvi-0**	**NP**	N13	P
	N13	**NP**	**N13**	**NP**	Cvi-0	P
	N13	**P**	**N13**	**P**	Cvi-0	NP

3.1.6 *Arabidopsis thaliana* life strategies and priming influence on r-K-continuum

The model plant *Arabidopsis thaliana* is widely distributed throughout Western and Central Europe, South-East Asia, as well as in the Mid-Atlantic and the Pacific North-Western of the United States into South-Western Canada (Hoffmann, 2002) and their intra-specific variation is often pronounced along latitudinal or altitudinal gradient. Almost 25 years ago, Aarssen and Clauss (Aarssen and Clauss, 1992; Clauss and Aarssen, 1994b; Clauss and Aarssen, 1994a) gave first hints of Arabidopsis ecological differentiation of flowering ecotypes and suggested *Arabidopsis thaliana* as a representative of an r / K type selection continuum. According to Pianka (1970) concept, *Arabidopsis thaliana* is the r-selected organism due to its short lifetime, high reproductive rate and broad geographical habitus.

Good primability of stress resistance in both r- and K-selected organisms allowed detailed comparison. *Arabidopsis thaliana* accessions have been sorted along the r / K continuum after comparison and determination of priming-sensitive and priming-tolerant lines. Parameters such as early flowering, high seed number per

plant, low seed weight per seed and low germination rate were indicators for r-selected accessions (Pianka, 1970). These characteristics grouped the WS, Col-0 and Van-0 accessions. The opposite of these r-selected accessions is the accession Kas-1 with characteristics of slower and smaller offspring production and higher weight of each seed. Some accessions, such as Cvi-0, Ms-0 and N13 have some features from both groups. The accession Cvi-0, just like the accession Kas-1, is among those that produce larger seeds in smaller amounts. However, at the same time, in our experimental conditions, Cvi-0 flowered first, compared to all the tested accessions. Accessions such as Ms-0 and N13 flowered the latest, as Kas-1, which is a characteristic of K-selected accessions. However, at the same time, slightly higher seed numbers per plant are comparable to the accessions in r-selected group. The various reproductive strategies of the tested Arabidopsis accessions and the influence of cold priming on their position in this continuum are given in Figure 3-18.

A cold-acclimation pre-treatment did not change the suggested accession continuum for some parameters namely, seed weight, number of the seeds and time until flowering, but made this suggested continuum clearer and tougher. Inducible defense is not necessary costly for plant growth and reproduction (Boyle and Walters, 2005). No significant effects on plant growth and yield were associated in response to pathogen and insect herbivory due to possible increment in photosynthesis capacity and efficiency after attract as composition for the cost (Murray and Walters, 1992; Thomson *et al.* 2003).

Exposure to the different stable and unstable conditions, in field and in greenhouse, did not influence the suggested *Arabidopsis thaliana* continuum for the parameters seed weight, number of the seeds and time until flowering. Stable greenhouse conditions showed clearer accession trends for the total number of seeds per plant. At the same time, under field conditions, flowering time was more noticeable. Not only r-K life strategy traits but also some other ecological factors could affect the persistence of stress memory (Hilker *et al.* 2015).

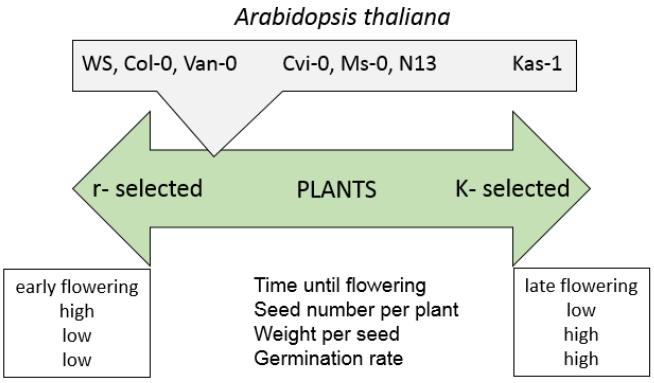


Figure 3-18. Reproductive strategies of *Arabidopsis thaliana* accessions along r-K-continuum. Differences in time flowering, seed number, seed weight and germination rate have been used for positioning along the continuum. Accessions used in ranging are N13, Ms-0, Kas-1, WS, Col-0, Van-0 and Cvi-0.

3.2 Molecular evaluation of priming

In plant stress biology, the term priming is used to describe a phenomenon which differentially and positively affects future plant performance when exposed repeatedly to certain stress conditions (Hilker *et al.* 2015). Temperature below 10°C is usually considered as a chilling stress for plants from tropical or sub-tropical zones. Cold acclimated plants show an elevated tolerance against subsequent cold stress where such an adaptation requires alternations in gene expression (Byun *et al.* 2014). We hypothesized that the chloroplast antioxidant system acts as a memorizing priming hub, where the response to the triggering signal is defined by the changes in signals during priming and lag-phase. Defining the transcriptomic, proteomic and metabolomic elements responsible for the development of thermomemory and consequently the usage of this memory in the necessary moments, was a crucial part of the hypothesis. Different strategies of stress responses and the existence of differential antioxidant machinery among the different Arabidopsis accessions were observed under unfavorable conditions.

Similar to the ecological experiments (Chapter 3.1.), four weeks old plants were cold-treated at 4 °C for 14 days at a light intensity of 120 µmol quanta $m^{-2}s^{-1}$ (Figure 3-19). After five days at optimal growth temperatures (lag-phase), the primed and naïve plants were kept for 24 h at 4 °C or 24 h under elevated light conditions at 250 µmol quanta $m^{-2}s^{-1}$ (triggering). This kind of experimental set up was necessary to test the plants memory of the previously experienced cold stress and to discover different responses to a subsequent stress stimulus after cold pre-treatment.

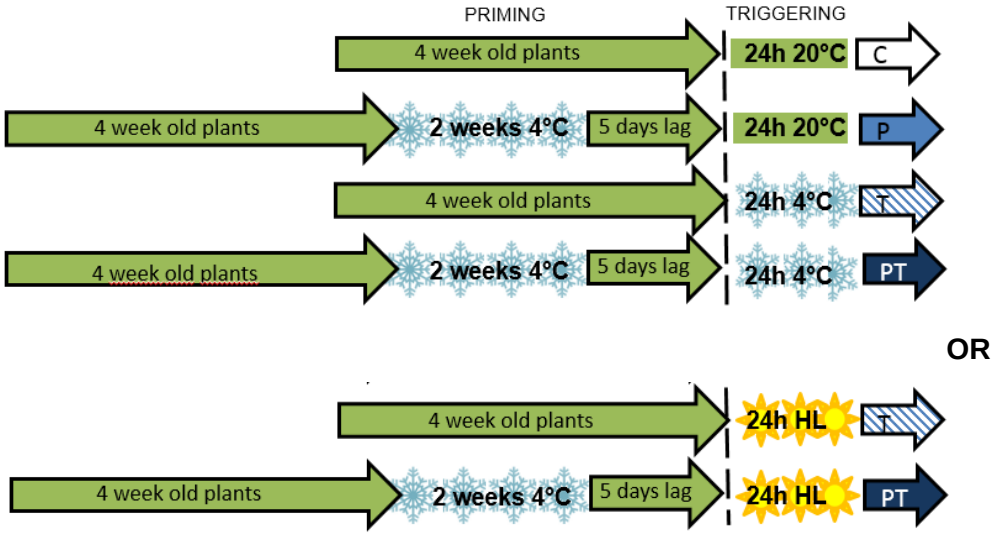


Figure 3-19. Experimental design used in study. Four week old plants were used for studying molecular mechanisms of priming. Two plant sets were primed for two weeks at 4 °C. In parallel to cold priming, another two plant sets were grown under optimum conditions in the growth chambers. Afterwards, cold primed plants were transferred for five days to the stable growth conditions at 20 °C. The triggering stimulus (cold / high light) was applied for 24 hours to naïve and to cold primed plants. The same color code as in the figure is used in graphs in this chapter. White (C): control, light blue (P): only primed, white with blue lines (T): only triggered, dark blue (PT): primed and triggered.

To analyze if and to which extend, cold arrest growth, two parameters namely, leaf number and rosette weight, were compared at the same time point, seven days after the 24 h cold triggering stimulus in untreated (C), only two week cold primed (P), only 24 h cold triggered (T) and primed and triggered (PT) plants (Figure 3-20). Before applying triggering stress, untreated plants were at a chronological age of four weeks and five days (lag phase), whereas primed plants were additionally cold stressed (four weeks optimal growth + two weeks cold stress + five days lag phase). This kind of experimental design was established in order to avoid developmental effects between plant sets. Growth arrests in plants have been observed after plants were transferred from optimal growth temperatures to 4 °C (Scott *et al.* 2004; Juszczak *et al.* 2016). Under our experimental conditions, the effect of cold on growth parameters was similar in P, T and PT plants and was not different from the control (C) plants. Insignificant minor changes were observed in the relative leaf number. The rosette weight in control (C) and primed-only (P) plants was marginally, but insignificantly higher than triggered-only (T) and primed and triggered (PT) plants.

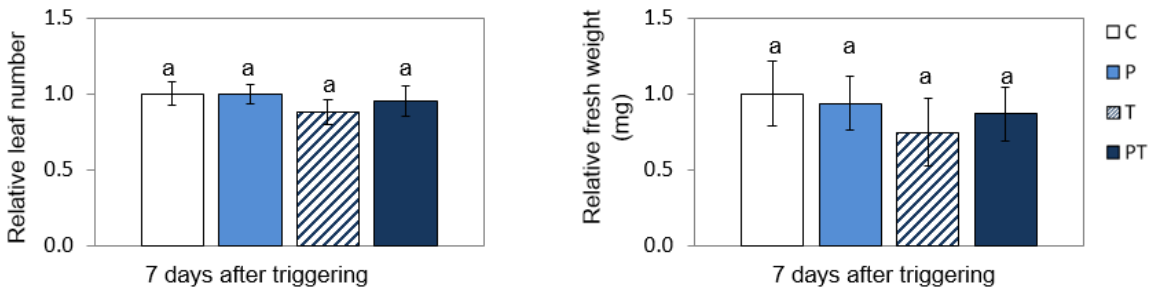


Figure 3-20. Relative leaf number and rosette fresh weight in only-primed (P), only-triggered (T) and primed and triggered (PT) Col-0 plants seven days after cold triggering relative to the leaf number in untreated control plants (C). The significance was tested by ANOVA (Tukey HSD-test; $p < 0.05$). Bars represent arithmetic means ± standard error. N = 15 - 20

3.2.1 Chlorophyll levels

Chloroplasts are one of the first cell compartments where the results of acclimation process due to various stressors, including cold, can be observed (Crosatti *et al.* 2013). Changes in the number of photoreaction centers and antenna size, subsequently affect the total chlorophyll content.
To test the hypothesis of lower availability of photoreaction centers and antenna proteins under cold stress, the total chlorophyll level was calculated from the measured levels of chlorophyll a and b. For testing the chlorophyll composition, the chlorophyll a / b ratio was calculated.

Total chlorophyll content did not significantly change after long cold priming and in the next 24 hours (deacclimation phase) as compared to control plants (Figure 3-21). The result of the cold acclimation process is visible here. Five days after of the initial cold stress and the subsequent return to controlled growth conditions, the total chlorophyll content significantly increased in P plants during the lag phase. During this time, plants over-compensated their chlorophyll levels. One day of triggering at 4 °C was sufficient to provoke significant differences in the total chlorophyll level between T and PT plants. One day triggered plants had the same total chlorophyll level as the control plants meaning that cold treatment was short enough not to disturb regulation of the reaction centers. The second cold stimulus slightly and significantly increased the total chlorophyll level in already primed (PT) plants, which indicates a possible priming effect. No significant changes in the total chlorophyll levels were observed between the treatments after 24 hours of triggering.

Chlorophyll (chl) a and b content did not significantly change immediately and 24 h after cold priming, but a significant increase was observed after five days. The triggering effect on chlorophyll a level was slightly but significantly stronger in PT plants than in T plants immediately after cold triggering stress. This trend was not observed in the measured chlorophyll b level.

The chl a / b ratio slightly but significantly increased during the long cold stress. The two week cold period had a lasting effect on nuclear encoded antenna proteins. This level stayed elevated up to 24 hours after the transfer to the growth chamber. Immediately after 24 h of second cold stress, triggering slightly

increased chl a / b levels in the tested plants due to cold regulation on photoreaction centers. No significant changes in the chl a / b levels were observed one day after triggering.

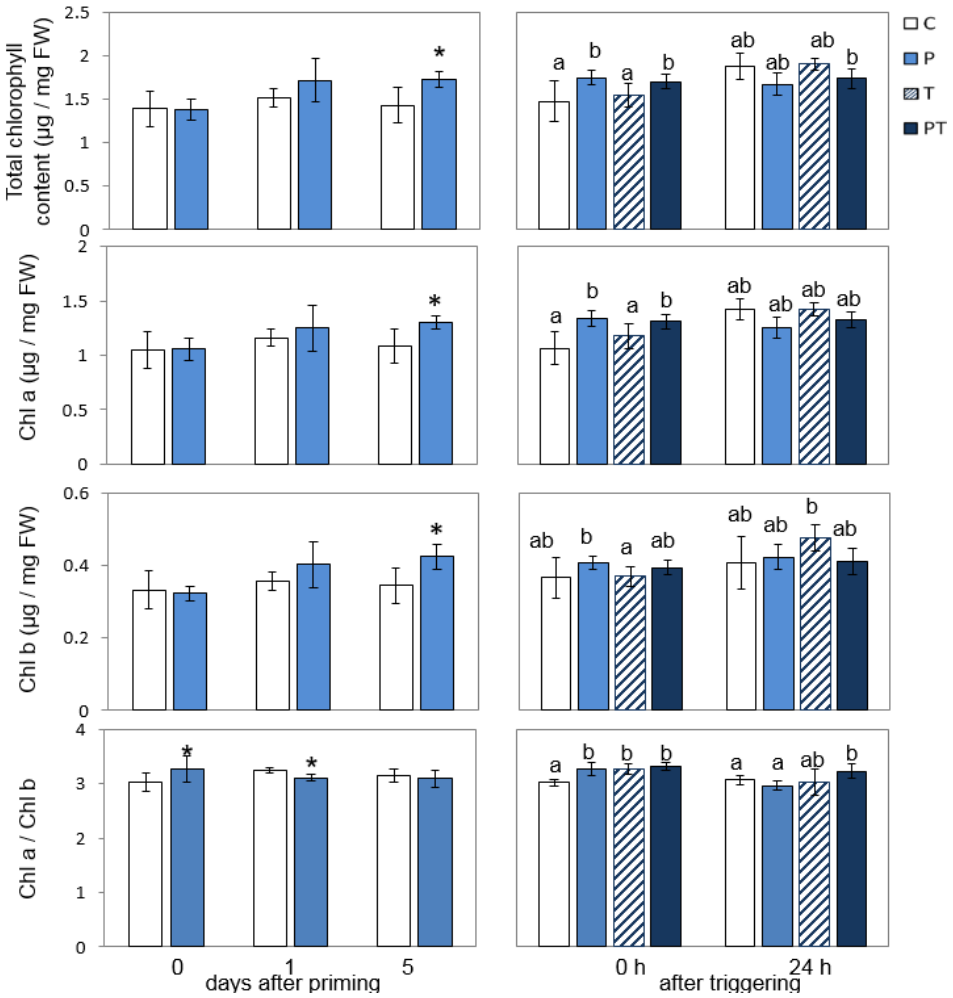


Figure 3-21. Accumulation of total chlorophyll content, chlorophyll a, chlorophyll b and chlorophyll a / b ratio in long cold primed Col-0 plants after priming and cold triggering in only-primed (P), only-triggered (T) and primed and triggered (PT) plants. The crude data are standardized on the values in control plants (C). The data were analyzed by ANOVA (Tukey HSD-test; $p < 0.05$) and Student T-Test (* $p < 0.05$). Bars represent arithmetic means ± standard error. N = 10

3.2.2 H_2O_2 levels

Under stressful conditions, reactive oxygen species (ROS) and redox signals are constantly formed in plant cells and can induce / support abiotic and biotic signaling cascades (Foyer and Noctor, 2015). Among the different reactive oxygen species, hydrogen peroxide (H_2O_2) is the most selectively reactive and diffusional molecule (D'Autreaux and Toledano, 2007).The H_2O_2 levels were measured in rosette leaves, in order to determine the amount and duration of the level at which H_2O_2 was produced and accumulated under long cold stress. The differences in priming-specific responses between T and control plants as well as between PT and T plants were evaluated after 24 h of cold triggering stress (Figure 3-22).

After the long-term cold stress treatment, H_2O_2 was strongly accumulating in comparison to the control (C) plants. A slow decrease in hydrogen peroxide level was measured throughout the following five days. Elevated levels of accumulated H_2O_2 were still persistent in P plants even after six days of deacclimation. Differences in the level of accumulated H_2O_2 among treatments harvested at the same time were observed immediately after short cold triggering. Due to the length of the perceived stress, a weak but significant accumulation of H_2O_2 in T plants was expected. For PT plants, a tendency towards a stronger accumulation of H_2O_2 was noticeable. Twenty four hours after the end of triggering stress, the H_2O_2 level decreased in PT plants. The triggering-specific responses gave indications of the importance of pre-treatment.

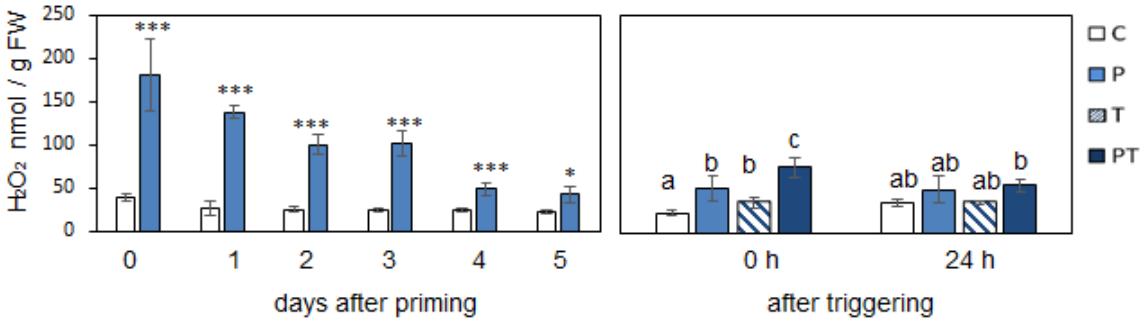


Figure 3-22. Accumulation of hydrogen peroxide immediately after cold priming (day 0), during the lag phase (1 .- 5. day) and after cold triggering (0 and 24 h). The plants were harvested before (C) and after (P) 2 weeks of cold priming at 4 °C and after cold triggering (T, PT). The crude data are standardized on the values in naïve plants prior to priming. The data

were analyzed by ANOVA (Tukey HSD-test; $p < 0.05$) and Student T-Test (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$). Bars represent arithmetic means ± standard error. N = 10

3.2.3 Anthocyanin levels

Anthocyanins play a broad role in the epidermis and mesophyll as plant protective pigments as well as antioxidants (Leyva *et al.* 1995). Similar to H_2O_2, anthocyanins accumulated during the cold period and slowly decreased afterwards (Figure 3-23).

After five days, no significant alterations were observed between the treatments. After the triggering stress, the strongest anthocyanin induction was detected in PT plants. In contrast, a decrease in the anthocyanin level due to the triggering stress treatment was observed in T plants. Twenty four hours later, differences between the treatments were not significant anymore. Differences among treatments due to pre-treatment were correlated with the differences in ROS levels.

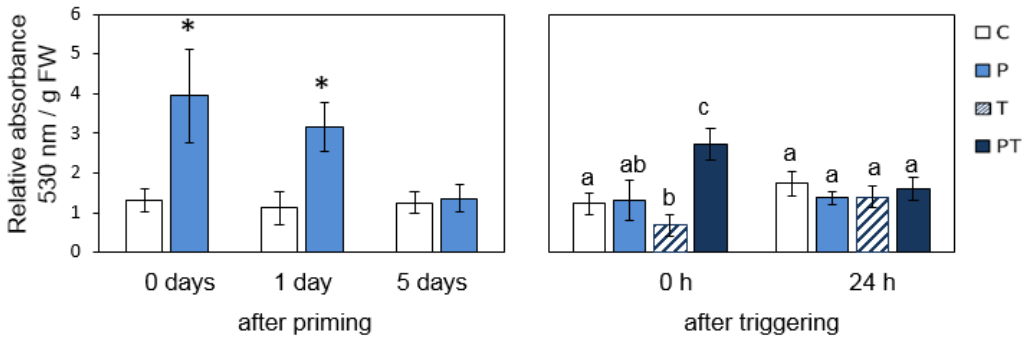


Figure 3-23. Accumulation of anthocyanins before (C) and after cold priming and triggering immediately, 1 and 5 days after priming and triggering (0 h and 24h) in only-primed (P), only-triggered (T) and primed and triggered (PT) Col-0 plants. The data were analyzed by ANOVA (Tukey HSD-test; $p < 0.05$) and Student T-Test (* $p < 0.05$). Bars represent arithmetic means ± standard error. N = 10

3.2.4 Ascorbate levels

Ascorbate is a small molecule that can easily move within the plant cell (Franceschi and Tarlyn, 2002) and with high concentration in the chloroplasts (up to 50 mM in spinach) has a central role in the protection of the photosynthetic

apparatus (Foyer, 1993). Osmoprotectant carbohydrates and proline increased during the two weeks of cold acclimation in Arabidopsis plants (Zuther *et al.* 2015). These findings are consistent with the results found from ascorbate measurements (Figure 3-24).

Total ascorbate content increased during the two weeks at 4 °C in Col-0 plants compared to control plants. Within the first days after the end of the cold period (lag phase), total ascorbate level declined to the control level. The short period of cold triggering stress was not sufficient to change the ascorbate levels in any of the treatments.

Immediately after long cold priming, the percent of the reduced ascorbate had a slight, but an insignificant increase in treated plants in comparison to control plants in which the reduce portion of the ascorbate pool was around 80 %. Reduced ascorbate levels declined slowly during the lag phase and this stayed the same even after the triggering stress in the all treatments.

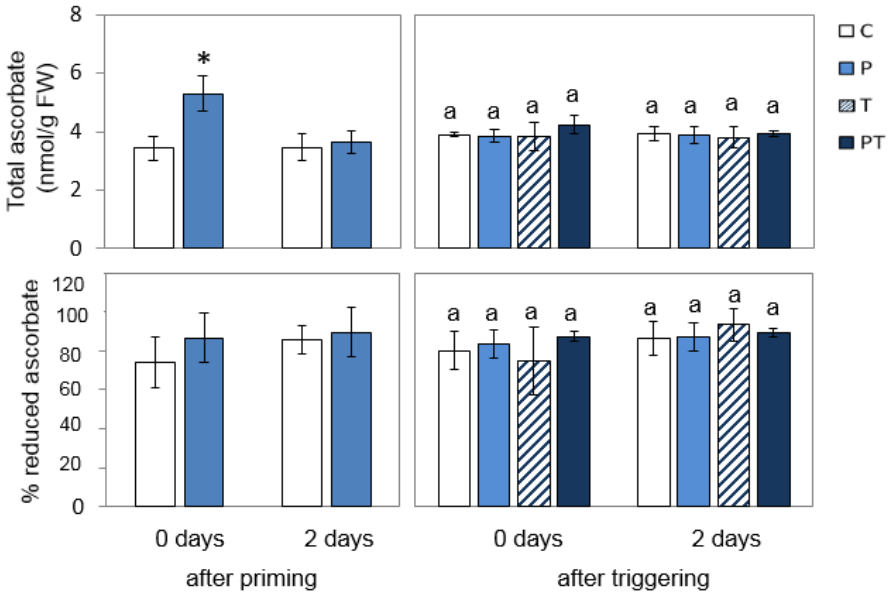


Figure 3-24. Total ascorbate content and the redox state of the ascorbate pool immediately and 2 days after priming and triggering in untreated control (C), only-primed (P), only-triggered (T) and primed and triggered (PT) Col-0 plants. The data were analyzed by ANOVA (Tukey HSD-test; $p < 0.05$) and Student T-Test (* $p < 0.05$). Bars represent arithmetic means ± standard error. N = 10

3.2.5 Effects of cold on chloroplast ROS marker genes after long cold stress and during the deacclimation phase

Chilling injury is associated with complex cellular disfunctions, due to inhibited metabolic activity and impaired electron transfer reactions, and symptoms such as chlorosis, sterility and death (Levitt, 1980). Knight and Knight (2012) suggested that tolerance of ROS accumulation during chilling is mediated via transcriptional changes due to a number of common genes expressed in response to either chilling or the ROS hydrogen peroxide. In my study, the deacclimation process was defined as the time-period between the end of the priming stimulus and the beginning of the triggering stimulus. Information about the priming experience was stored during this phase (Hilker *et al.* 2015). To check whether and to which degree 14 days of cold priming induced destabilization and differential responses, transcript levels of selected cold and ROS marker genes were quantified using the qRT-PCR technique (Figure 3-25).

Cold responsive gene *COR15A* is one of the best studied and functionally characterized cryoprotective proteins. CBFs transcriptional level is upregulated by low temperatures and has effects on *COR15A* regulation (Knight and Knight, 2012). Accumulation of this transcript under cold conditions (Zarka *et al*. 2003) was tested in two week cold primed plants.

The expression of *COR15A* was strongly induced after the two week long cold period. After fourteen days, strong activation of this cold responsive gene was still present (Figure 3-25). Only one day of deacclimation at the standard growth temperature was enough to cause a quick transcript decline to the control level that persisted for the next five days.

ZAT10 was identified as a salt- and cold- response protein (Sakamoto *et al.* 2000) with a control on the mesophyll development under various environmental conditions (Munekage *et al.* 2015). Its role in chloroplast-to-nucleus ROS signaling was previously shown in the context of abiotic stress (Davletova *et al*. 2005).

Two weeks under cold stress caused a drastic increase on the transcript abundance of this gene. This transcript level stayed elevated even after a transfer to the 20 °C growth conditions. After five days, *ZAT10* transcript level decreased and was similar to the level in control plants (Figure 3-25).

The Arabidopsis *BAP1* gene is involved in plant ROS defense (Yang *et al.* 2007) and it is activated in response to the chloroplast singlet oxygen (Gadjev *et al.* 2006). The accumulation in the ROS marker gene *BAP1* was significantly higher after two weeks of cold stress conditions in comparison to the control plants. A strong accumulation of *BAP1* transcripts was observed 24 h after the transfer to the standard growth conditions. Five days after priming, the transcript level of this gene was still elevated but not significantly higher compared to the control plants.

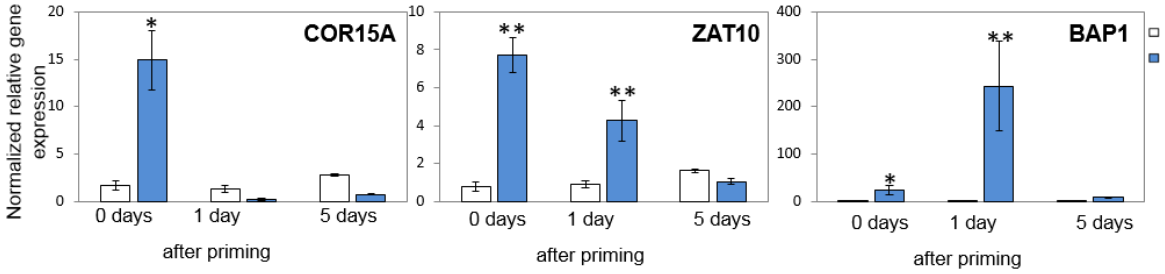


Figure 3-25. Transcript levels of cold- (*COR15A*) and chloroplast-ROS (*ZAT10* and *BAP1*) marker genes immediately after long cold priming (0 days) and 24 h and 5 days later at 4 °C in only-primed (P) Col-0 plants. The transcript levels were normalized on the respective transcript levels of naïve plants immediately before the on-set of priming (C), as well as the mRNA abundance of *ACT2* and *YLS8* genes. Data represent arithmetic means ± SD from three biological replicates where each biological replicate was analyzed in three technical replicates. The data were analyzed by Student T-Test (* $p < 0.05$, ** $p < 0.005$).

3.2.6 The effect of priming after a triggering stimulus

When plants are exposed to repeated and aggressive abiotic agents, they recognize the initial stressor and respond with an appropriate alarm signal (Byun *et al.* 2014). A short stress period just causes an imbalance in the redox status, in contrast to the long cold stress period that induces a freezing tolerance. Increase in H_2O_2, anthocyanin and ascorbate production as well as the expressional regulation of cold and ROS responsive genes (see Results 3.2.2-5) after the long cold stress period, was the basis for further transcript analyses. Transcript accumulation and the activity of various ROS-scavenging enzymes were compared after long stress priming (14 days) and short cold triggering stress (24 h). In order to investigate the priming effect after a triggering stimulus, transcript levels of the selected chloroplast antioxidant genes (*sAPx, tAPx, MDAR, GR,*

2CPA, 2CPB, GPx7) and extra-plastidic genes (*PAL1, APx2, CHS, PAL1*) were compared in P, T and PT plants and normalized on the respective transcript levels of control (C) plants (Figure 3-26; Figure 3-30; Figure 3-31; Figure 3-32).

In general, different transcriptional accumulations and responses were recorded among genes and treatments. Some of the triggering responses were faster, stronger and more sensitive than the other responses and some of them displayed the opposite behavior compared to the response after the first stimulus.

3.2.6.1 Transcript abundance regulation of genes encoding enzymes of the ascorbate-dependent water-water cycle and ascorbate-recycling system

The water-water cycle in chloroplasts plays a dual role in scavenging O_2^- and H_2O_2 at the site of their generation (Yabuta *et al.* 2002). The APx-dependent ascorbate-glutathione cycle, carried out by the stromal and thylakoid APx and the ascorbate regenerating enzymes including monodehydroascorbate reductase and glutathione reductase, occurs in the various chloroplast compartments. According to Kangasjärvi et al. (2008) chloroplast APx isoforms are indispensable during the long term cold acclimation. Here, the transcript levels of the ascorbate-dependent water-water cycle enzymes were compared after two weeks of cold priming and 24 h cold triggering (Figure 3-26).

Immediately after a two weeks period at 4 °C, the transcript level of the *sAPx* significantly increased in the P plants. The transcript level of *tAPx* and the ascorbate regenerative system did not differ significantly (Figure 3-26).

Afterwards, the plants were transferred to optimal growth conditions for five days. During this lag phase, the *sAPx* transcript level in the primed plants remained elevated only during the first day of deaclimation, after that the transcript level decreased. The *MDAR* and *sAPx* transcript levels in P plants on the 5^{th} day decreased to a level that was lower than in the control (C) plants. The *GR* transcript level significantly decreased after the transfer to optimal conditions and stayed low during the lag phase. The *tAPX* transcript level did not increase immediately after the cold-stress period and remained unaltered throughout the next five days of deacclimation in P plants. The *sAPx* and *tAPx* transcript levels in

P plants displayed an opposite behavior. Two weeks of cold stress had caused differences in their regulation after the transfer to optimal conditions.

After five days of lag phase, the triggering (24 h cold) stress was applied to primed and unprimed plants. The general regulation in the *sAPx, tAPx* and *GR* genes observed immediately after two weeks of exposure to cold stress was followed also in T plants after 24 h cold stress. The transcript level of *MDAR* after the 24 h cold stress period was elevated in T plants when compared to the plants immediately after a two week stress period. Significant priming-dependent transcriptional differences were observed only in *sAPx* levels between T and PT plants immediately after the end of the triggering stimulus. No priming effect on the other tested plastid antioxidant genes was observed

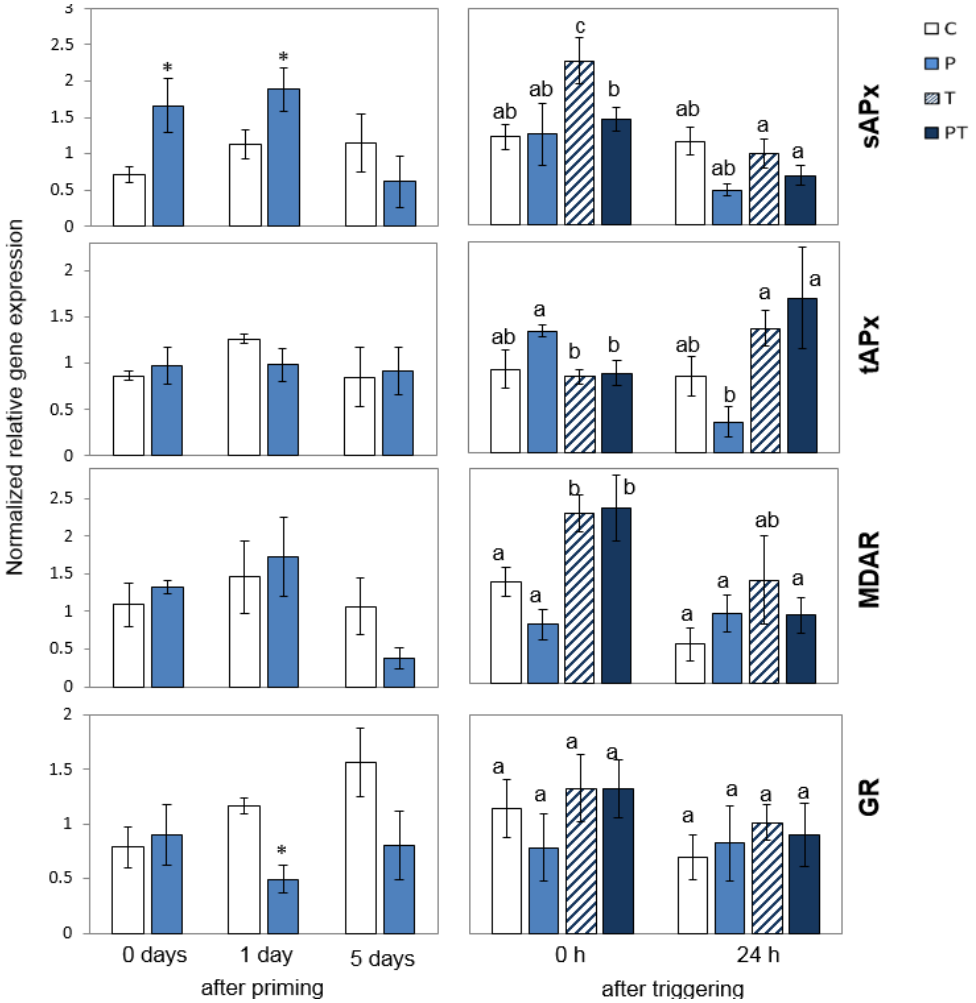


Figure 3-26. The transcript levels of chloroplast ascorbate peroxidases (*sAPx, tAPx*) and reductases (*MDAR* and *GR*) after two weeks of priming (0 days), on day 1. and 5. during the lag phase and immediately after 24 h triggering at 4 °C and 24 h later in only-primed (P), only-triggered (T) and primed and triggered (PT) Col-0 plants. The transcript levels were normalized on the respective transcript levels of naïve plants immediately before the on-set of

priming (C), as well as mRNA abundance of *ACT2* and *YLS8* genes. Data represent arithmetic means ± SD from three biological replicates where each biological replicate was analyzed in three technical replicates. The data were analyzed by ANOVA (Tukey HSD-test; $p < 0.05$) and Student T-Test (* $p < 0.05$).

3.2.6.2 Regulation of enzymes of the ascorbate-dependent water-water cycle on a protein level

Gene expression is regulated at multiple levels with translational regulation being one of the major steps for protein determination under stress conditions in plants (Nakaminami *et al.* 2014). Significant variations in the expression regulation of the chloroplast antioxidant system (Figure 3-26) upon long cold stress treatment were combined with protein analysis. Therefore, the thermomemory development hypothesis was tested utilizing protein studies. For the detection of APxs via Western Blot, the antibodies were raised against a highly conserved region among tAPx, sAPx and the cytoplasmic APxs, corresponding to amino acids 204-215 of tAPx (Kangasjärvi *et al.* 2008). The antibody recognized 38 kDa tAPx and 33 kDa sAPx, which were purified from thylakoid membranes and the chloroplast stroma, respectively. For the comparison and the identification of the chloroplast APx isoforms, samples of tAPx-knock-out lines were analyzed luminometrically at the same time. The steps for protein normalization (protein level of the large subunit of RuBisCO) and detection are described in detail in the Material and Methods (section 2.9).

Priming and lag phase

The protein level regulation, similar to transcript abundance regulation of chloroplast APxs (sAPx and tAPx) indicated relative shifts in thylakoid and stromal ascorbate peroxidase abundance (Figure 3-27). After two weeks at 4 °C, the transcript (Figure 3-26) and protein levels for sAPx significantly increased. At the same time, the tAPx transcript and protein levels both decreased. Significant accumulation of sAPx proteins lasted for three days after the transfer back to optimal growth conditions. The significant decline observed in the tAPx level

persisted for four days during the lag phase. At the end of the lag phase, both APxs protein levels returned to the control level.

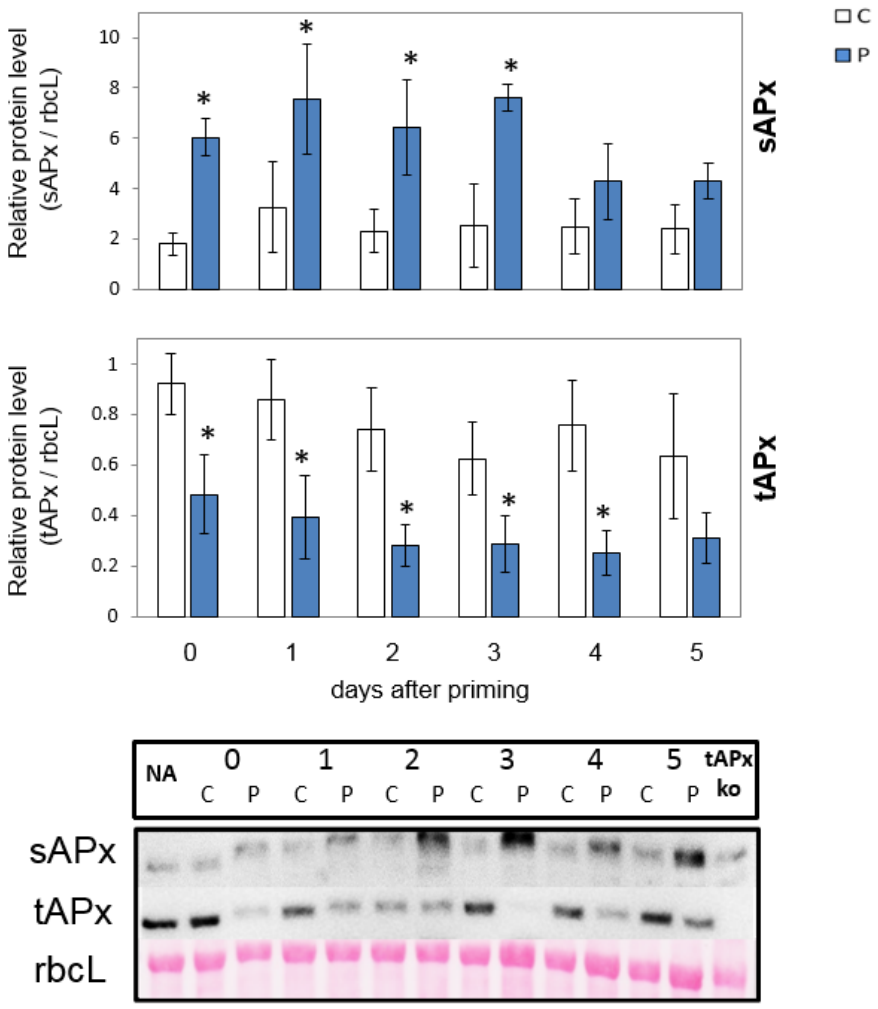


Figure 3-27. Relative protein levels (up) of sAPx and tAPx immediately after two weeks of cold stress (0 days) and during the lag-phase (1.-5. days) in control (C) and only-primed (P) Col-0 plants. The proteins were detected on nitrocellulose membranes (down) with an antibody against the stromal part of Arabidopsis tAPx. The large subunit of RuBisCO (rbcL) was used for standardization as an abundant chloroplast specific protein marker. The band intensities of control (NA) and primed (P) plants were quantified after luminometric detection. Data represent arithmetic means ± SD from three biological replicates. The data were analyzed by Student T-Test (* $p < 0.05$). N =3

The relative sAPx / tAPx and tAPx / total APx ratios were calculated immediately after the two week cold stress period and over the next five days of the lag phase (Figure 3-28). The simultaneous increase in the sAPX protein levels and decrease in the tAPX levels could explain the high regression coefficient of determination of

the sAPx / tAPx ratio obtained from regression (R^2 = 0.94). The result from the linear regression indicates differences in the regulation of analyzed APxs due to the long cold stress treatment. The coefficient of the analyzed tAPx / total APx ratios was very low (R^2 = 0.02), demonstrating that the thylakoid APx proteins did not have a strong impact on the total APxs accumulation. The strength of the quantified relationship between tAPx and the total APx was low. Cold modified the stoichiometry of the *sAPx* and *tAPx* antioxidant genes.

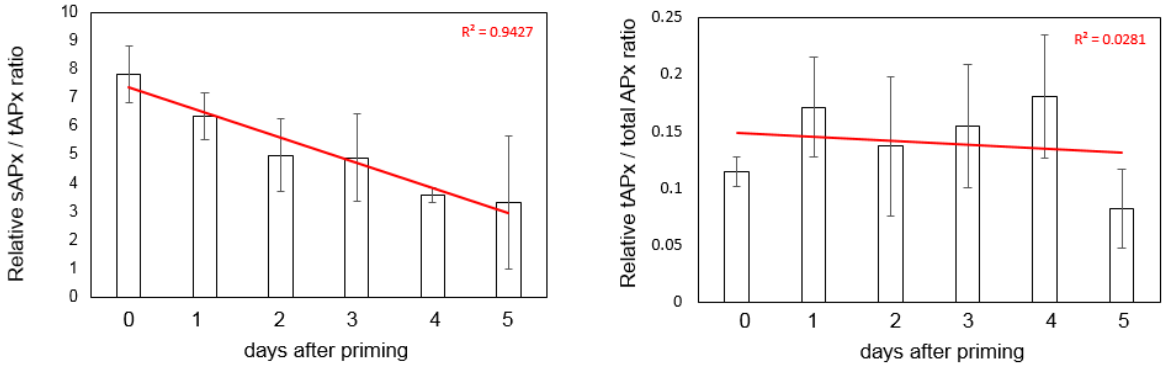


Figure 3-28. Relative sAPx / tAPx (left) and tAPx / total APx protein ratio (right) immediately after two weeks of cold stress (0 days) and during the lag-phase (1.-5. days). Data represent arithmetic means ± SD from three biological replicates. The data were tested for linearity by calculating regression curves. The R^2 was calculated as a measure of the linearity probability.

Triggering and post-stress phase

The protein abundance regulation of sAPx and tAPx was studied immediately after the application of the 24 h cold triggering stress and five days later (Figure 3-29). One day at 4 °C was not long enough to cause translational differences of APxs between the treatments. The long term priming increased the sAPX protein levels, but this could not be observed after the subsequent 24 h cold treatment. Significant priming-dependent translational differences in the sAPx protein level were not observed when comparing T and PT plants. This trend stayed the same throughout the entire time of the experiment.

The general cold regulation effects observed after implementation of the two weeks of cold stress displayed the same trend as after the short cold stress period on the tAPx protein level. The downregulation of the tAPx protein level after the short cold stress period was significant for T and PT plants. Primed plants (P) had accumulated the same amount of protein as the control (C) plants. According to

the results of the X^2 (chi-squared) test, the differences in tAPx protein concentrations between T and PT plants were not due to randomness. Statistically significant (X^2=7.4E-05) differences between treatments due to the priming-dependent pre-treatment, suggested a synergistic effect of priming and triggering in PT plants.

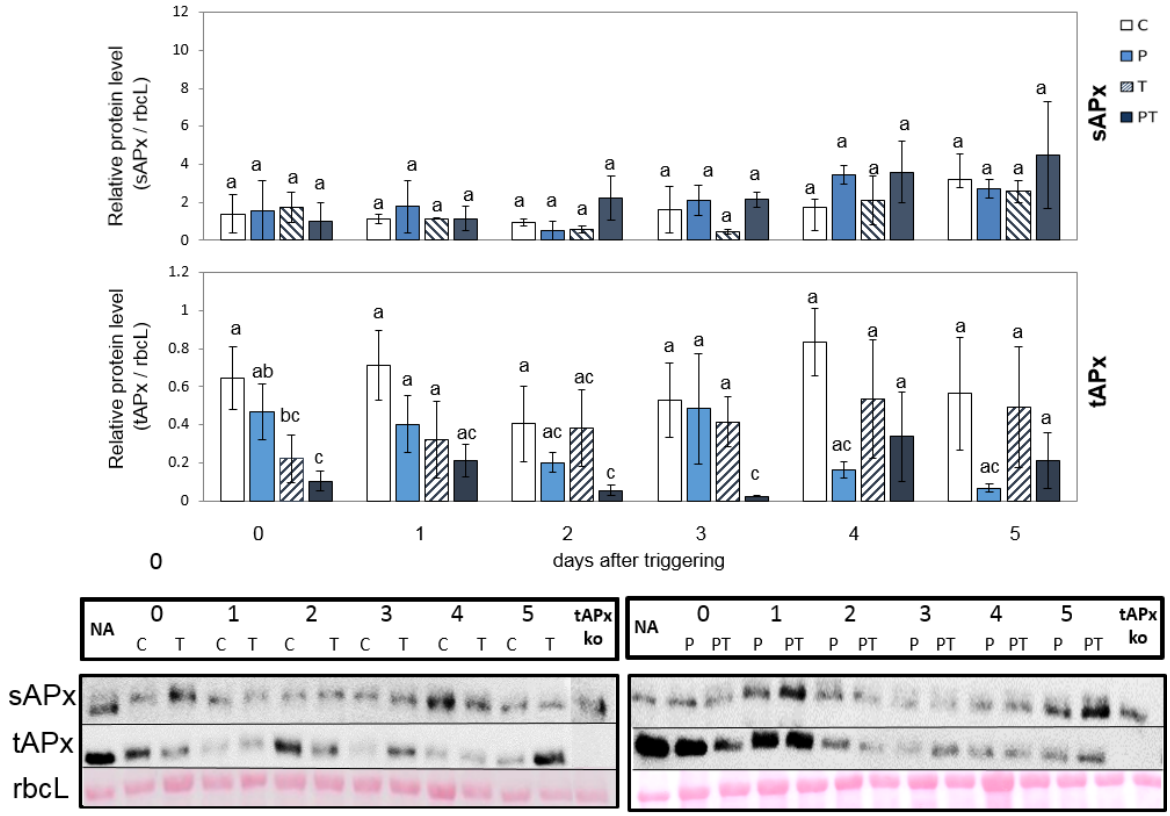


Figure 3-29. Relative protein levels (up) of sAPx and tAPx after 24 h triggering at 4 °C (0 days) and in a next 4 days (1.-5. days) in only-primed (P), only-triggered (T) and primed and triggered (PT) Col-0 plants. The proteins were detected on nitrocellulose membranes (down) with an antibody against the stromal part of Arabidopsis tAPx. The large subunit of RuBisCO (rbcL) was used for standardization as a abundant chloroplast specific protein marker. The band intensities of control (C), only primed (P), only triggered (T) and primed and triggered (PT) plants were quantified after luminometric detection. Data represent arithmetic means ± SD from three biological replicates The data were analyzed by Student T-Test (* $p < 0.05$). N = 3

3.2.6.3 Transcript abundance regulation of genes encoding enzymes of the ascorbate-independent water-water cycle

Thioredoxins acting as regulators of scavenging mechanisms and as components of signaling pathways (Santos and Rey, 2006) have huge relevance in antioxidant protection. Peroxiredoxins (Prx) and glutathione peroxidases (Gpxs) form a potential alternative water-water cycle for ROS detoxification. This cycle acts independently from the ascorbate. The priming hypothesis was also tested by comparing the responses in the expression of genes encoding the enzymes of the ascorbate-independent water-water cycle upon the application of long cold priming and short cold triggering (Figure 3-30).

The mRNA level of *2CPB* was induced after two weeks of cold stress application as opposed to the *2CPA* and *GPx7* transcript levels, which decreased transiently in the treated (P) plants. Twenty four hours after the transfer to optimal conditions, the transcript levels of *2CPA* and *2CPB* decreased. After five days at 20 °C, the transcript levels of three tested enzymes did not differ significantly. Short cold stimulus was applied to the naïve and already primed plants after the lag phase. No priming effect was observed on the ascorbate-independent genes.

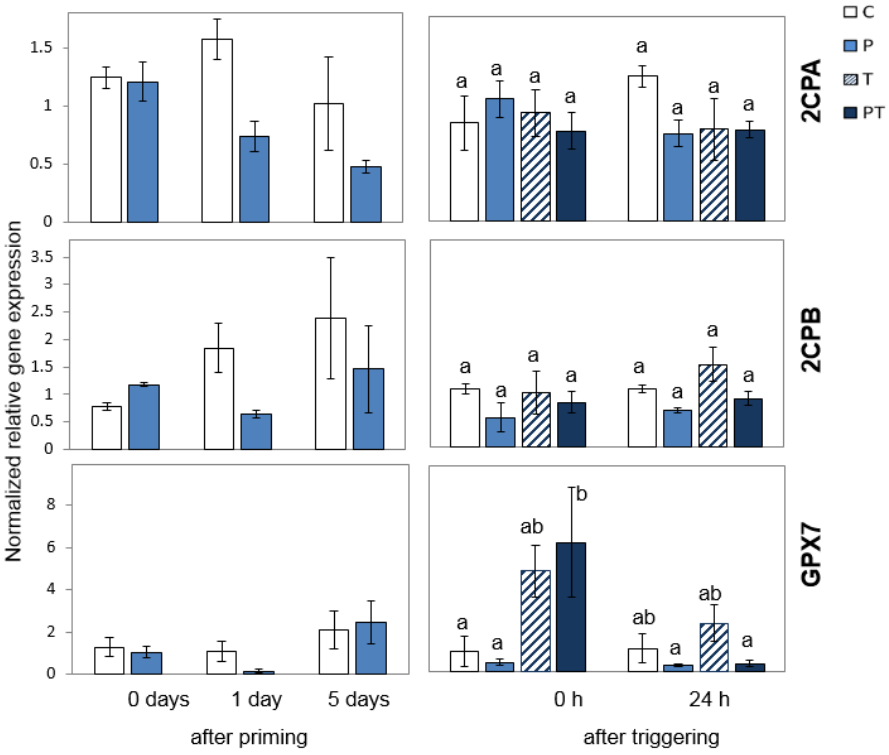


Figure 3-30. Transcript levels of chloroplast peroxiredoxins (*2CPA, 2CPB*) and glutathione peroxidase (*GPX7*) after two weeks of priming (0 days), 1 and 5 days during the lag phase

and immediately after 24 h triggering at 4 °C and 24 h later in only-primed (P), only-triggered (T) and primed and triggered (PT) Col-0 plants. The transcript levels were normalized on the respective transcript levels of naïve plants immediately before the on-set of priming (C) as well as the mRNA abundance of *ACT2* and *YLS8* genes. Data represent arithmetic means ± SD from three biological replicates where each biological replicate was analyzed in three technical replicates. The data were analyzed by ANOVA (Tukey HSD-test; $p < 0.05$) and Student T-Test (* $p < 0.05$).

3.2.6.4 Transcript abundance regulation of genes encoding enzymes of the plastidic and extra-plastidic antioxidant system

The strong induction of the cold- and ROS- marker genes (*COR15A, ZAT10* and *BAP1*), as well as the significant changes in the ascorbate-dependent water-water cycle regulation after two weeks of cold priming and one day of cold triggering were indications for changes of the buffering in the induction of chloroplast ROS signaling. Here, it was compared the regulation of the copper-zinc superoxide dismutase (*CDS2*) gene (the first gene of chloroplast antioxidant ROS defense), the *FER1* gene (known to be specifically activated after paraquat or H_2O_2 treatment) (op den Camp *et al.* 2003) and the ascorbate peroxidase *APX2* (one of the three cytosolic H_2O_2 scavenging enzymes) (Figure 3-31).

Prolonged exposure (14 days) to cold stress resulted in a strong significant decline in the *CSD2* gene and the up-regulation of the *FER1* transcripts in treated (P) plants. The transcript level of cytosolic ascorbate peroxidase, *APX2*, did not significantly change in P plants when compared to the control (C) plants. During the memory phase, abundance of the three transcripts did not change. The transcript level of *CSD2* increased to the control level in response to the triggering stress in PT and declined in T plants. Additionally, priming response was observed as a reaction to the application of the triggering stimulus in *CSD2* and *APX2* transcript levels. Significant changes were not observed for *FER1*.

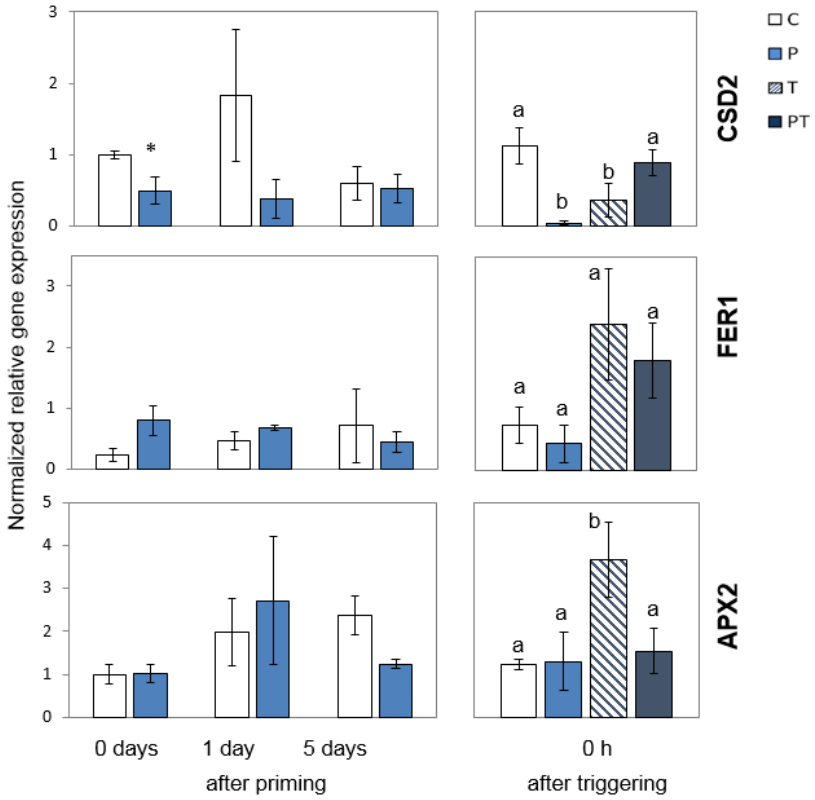


Figure 3-31. Transcript levels of genes for antioxidant enzymes located in chloroplast (*CSD2, FER1*) and cytosol (*APX2*) after two weeks of priming (0 days), 1 and 5 days during the lag phase and immediately after 24 h triggering at 4 °C in only-primed (P), only-triggered (T) and primed and triggered (PT) Col-0 plants. The transcript levels were normalized on the respective transcript levels of naïve plants immediately before the on-set of priming (C), as well as the mRNA abundance of *ACT2* and *YLS8* genes. Data represent arithmetic means ± SD from three biological replicates where each biological replicate was analyzed in three technical replicates. The data were analyzed by ANOVA (Tukey HSD-test; $p < 0.05$) and Student T-Test (* $p < 0.05$).

3.2.6.5 Transcript abundance regulation of cold / stress / ROS marker genes

Simultaneous down-regulation of the plastid antioxidant system and the up-regulation of the extra-plastidic antioxidant system attenuated the ROS levels during cold conditions and shifted the ROS signature, which buffers the post-stress response (Juszczak *et al.* 2016). Two weeks at 4 °C induced high transcript levels of cold and ROS marker genes (Figure 3-25). Subsequently, the same transcript analyses for *COR15A, BAP1* and *ZAT10* were performed after the short triggering stress exposure to the already primed and naive plants (Figure 3-32).

Enzymes such as chalcone synthase (CHS) and phenylalanine ammonia-lyase 1 (PAL1), respond to a broad variety of stressors and catalize crucial steps during the biosynthesis of numerous components of non-enzymatic antioxidants. According to Schulz et al. (2015), anthocyanin and flavonoid biosynthesis is stimulated during cold acclimation due to activation of these two genes. The influence of priming on memory development at crucial moments was determined by analyzing the accumulation of *PAL1* and *CHS* transcripts (Figure 3-32).

The *COR15A* transcript level strongly increased after the short cold stress period in previously primed as well as naive plants. A highly positive priming effect in the *COR15A* transcript accumulation was observed in PT plants sets immediately after the cold stress. The plants memorized the previous cold treatment over a five day long lag phase and displayed a stronger response to the triggering stimulus than T plants. Non-elevated transcript levels were observed one day after the transfer to the control conditions.

After one day of cold stress, the *ZAT10* transcript abundance increased in T and PT plants. The *ZAT10* induction in PT plants was less than half as strong as compared to the T transcript level, suggesting a secondary support of the antioxidant activation.

Immediately after the triggering treatment, the *BAP1* transcript levels were elevated even a week after the priming (P). The T plants showed a significantly elevated transcript level when compared to the control (C) plants and at the same time, PT plants showed a slight but an insignificant priming effect. The relatively noisy transcript regulation persisted up to one day after triggering.

The transcript levels of *PAL1* and *CHS* after triggering in PT plants were strongly induced in comparison to T plants. The transcript level in PT plants was, in general, higher for both genes than the combined value of P and T plants. The positive priming effect led to a stronger induction of these two genes.
One day of cold triggering was not sufficient to induce significant *PAL1* transcriptional changes on T plants, but was strong enough for ‘prime-prepared’ plants. The induction of *CHS* transcript level due to short cold triggering in T plants was half as strong when compared to ‘prime-prepared’ (PT) plants.

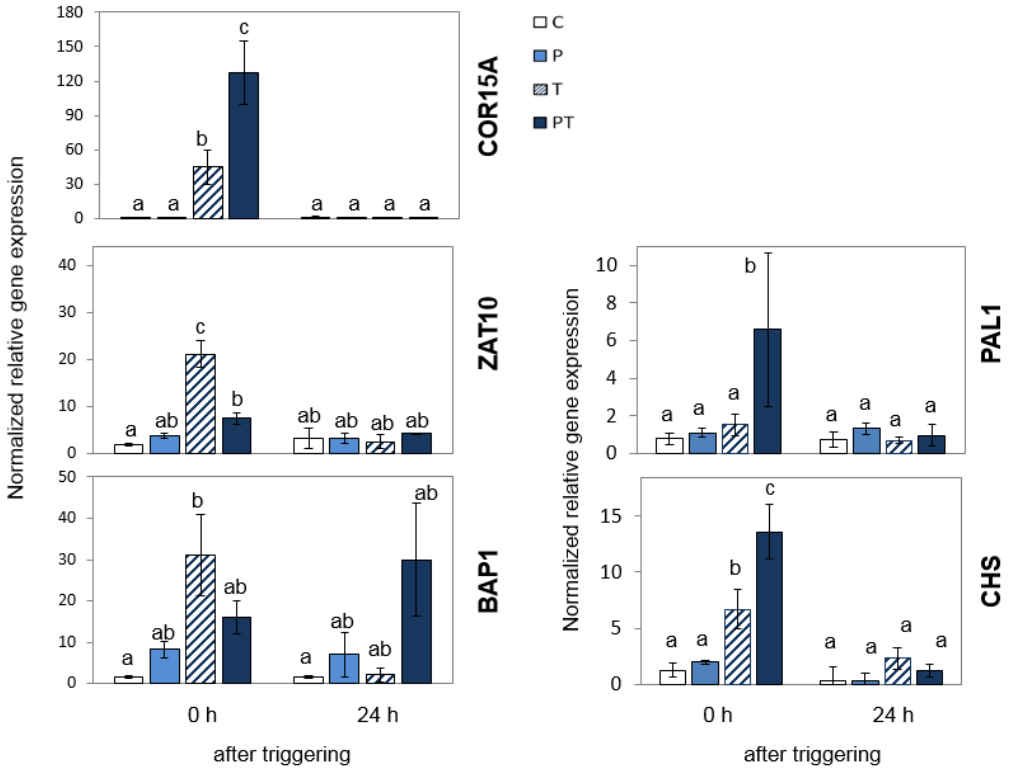


Figure 3-32. Transcript levels of cold- (*COR15A*), stress- (*PAL1* and *CHS*) and chloroplast-ROS (*ZAT10* and *BAP1*) marker genes immediately after 24 h triggering at 4 °C and 24 h later in only-primed (P), only-triggered (T) and primed and triggered (PT) Col-0 plants. The transcript levels were normalized on the respective transcript levels of naïve plants immediately before the on-set of priming (C), as well as the mRNA abundance of *ACT2* and *YLS8* genes. Data represent arithmetic means ± SD from three biological replicates where each biological replicate was analyzed in three technical replicates. The data were analyzed by ANOVA (Tukey HSD-test; $p < 0.05$).

3.2.7 Testing priming candidate regulators

Van Hulten et al. (2006) suggested that priming could provide a better protection without the costs associated with the constitutive stress related to gene expression. In order to determine the possible priming regulators and costs of priming, the effective and initial quantum yields (same method as for Results 3.1.4) were measured in Col-0, *tapx, sapx* and *tapx sapx* plants that were treated as follows: control (C), P, T and PT. Since there were significant changes in the transcript and protein abundancies of the chloroplast stromal and thylakoid ascorbate peroxidases in response to the long cold stress, these genes were

chosen as possible candidates for the regulation of priming and hence analyzed further via qRT-PCR.

According to Fryer et al. (1998), elevated levels of the chloroplasts scavenging enzymes (SOD, GTR, DHAR, MDHAR and APX) may play a role in limiting the degree of photodamage experienced by maize at chilling temperatures (Jahnke *et al.* 1991; Massacci *et al.* 1995; Hodges *et al.* 1997). The effects of the possible APXs regulators on photodamage was tested by chlorophyll-a fluorescence measurements after the two week cold priming and one day cold triggering stress (Figure 3-33).

Chlorophyll-a fluorescence gives information about the photosynthetic regulatory processes occurring when some of the possible scavengers are blocked (Baker, 2008). The effective (Eff-qY) and initial (Ini-qY) quantum yields after long cold priming in Col-0 and knock-out lines *tapx, sapx* and *tapx sapx* showed significant changes between the two plants sets. The maximum quantum yield (Ini-qY) of the photosystem II measurements gave a picture of the possible damages in the photosynthetic electron transport chain caused by the cold (i.e. chronic photoinhibition). The cold treated (P) plants displayed significantly lower both qY values when compared to control (C) plants. The eff / ini qY (II) ratio was similar between plants, except for the *tapx* knock-out plants where the difference between primed (P) and non-primed (C) plants was significantly expressed (Figure 3-33). To visualize the possible restrictions and / or profits in the photosynthetic dynamics after cold long priming, the plants were triggered with a 24 h cold stress period. In general, eff-qY values for Col-0, *sapx* and *tapx sapx* knock-out plants declined after the application of the second stress and were not significantly different between T and PT plants. The *tapx* knock-out line showed a significant decline in the eff-qY (II) parameter after the short cold stress period in PT plants as opposed to the T plants. One day of cold caused small damages of the photosynthetic electron transport chain (which is reflected by a decrease in Ini-qY) in T when compared to the damages after the long cold stress period (P). The PT plants showed no chronic photoinhibition in contrast to the control (C) and T plants. The eff / ini qY ratio overall, decreased in the T and PT plants after the short stress.

The triggering pulse slightly damaged the photoreaction centers (except in *tapx* T plants) and the significant positive priming effect was observed only in Col-0

plants, demonstrating the supported activation of the electron flux through the photosystem II by priming. Lack of stromal and / or thylakoid function resulted in damages of the photosynthetic electron transport chain due to PT plants had a slight higher eff / ini qY ratio.

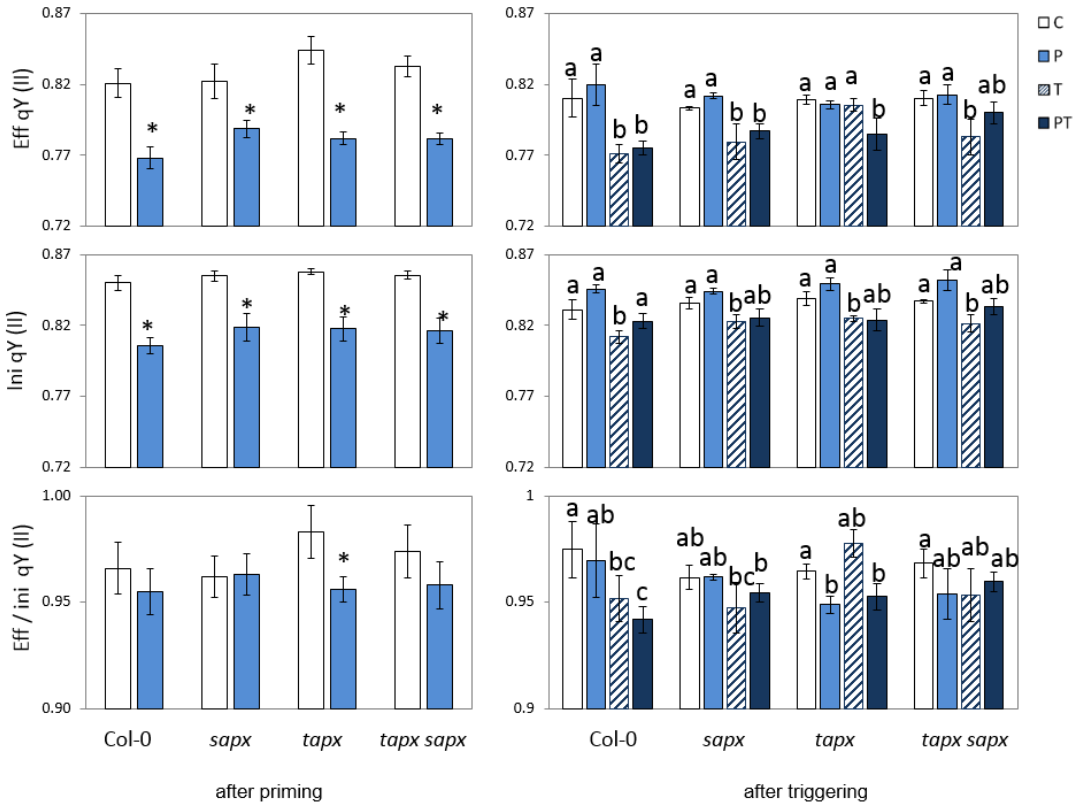


Figure 3-33. Effective (ΔF / F_M') and initial (F_V / F_M) quantum yield and their ratio of photosystem II after two weeks of cold priming and after 24 h triggering at 4 °C in only-primed (P), only-triggered (T) and primed and triggered (PT) Col-0 plants and knock-out lines *tapx, sapx* and *tapx sapx*. Data represent arithmetic means ± SD. The data were analyzed by ANOVA (Tukey HSD-test; $p < 0.05$) and Student T-Test (* $p < 0.05$). N = 5

According to the previous transcript analysis (Figure 3-26; Figure 3-31) and chlorophyll-a fluorescence data (Figure 3-33) the long cold stress regulated the expression of APxs in a treatment specific way. In the same APx knock out lines gene expression of *ZAT10, BAP1* and *COR15A* was quantified via qRT-PCR to assess the APxs relevance during priming. According to Kangasjärvi et al. (2008), the absence of tAPx will promote the *sAPx* expression at 10 °C but not *vice versa*, suggesting there is at least some degree of functional and regulatory redundancy. The response of *tapx*, *sapx* and *tapx sapx* to the second cold stimulus was tested in C, P, T and PT plants (Figure 3-34) and compared with the Col-0 plants.

The cold marker gene, *COR15A*, showed an immediate increase in the transcript abundance values after the 24 h cold stress in the tested plants, except in *tapx sapx* T plants. The transcript differences between T and PT plants were significant in Col-0, *tapx*, *sapx* plants. The transcript levels in T and PT *tapx sapx* plants were slightly elevated. This implies that the previous priming experience altered the transcript level upon the application of a new stressor. The lack of sAPx and tAPx in *tapx sapx* plants resulted in a smaller transcriptional change due to the pleiotropic effects.

The ROS marker gene BAP1 had a very noisy transcript regulation upon short cold triggering. The lack of sAPx in *sapx* and *tapx sapx* plants caused no significant differences between T and PT plants, but led to strong accumulation differences between these two mutants. In contrast, the lack of a thylakoid bound APx in *tapx* plants resulted in a significant priming effect between T and PT plants.

Among all the tested lines, the *ZAT10* induction after triggering stress was significant in treated plant sets. Similar to the *BAP1 sapx* and *tapx sapx* plant transcripts, the relative *ZAT10* expression was high, but the priming was not significant. In contrast, differences were observed between the transcript levels in Col-0 and *tapx* after the triggering treatments, but they differ in priming specific manner (significant down- and up-regulation in PT transcript levels respectively).

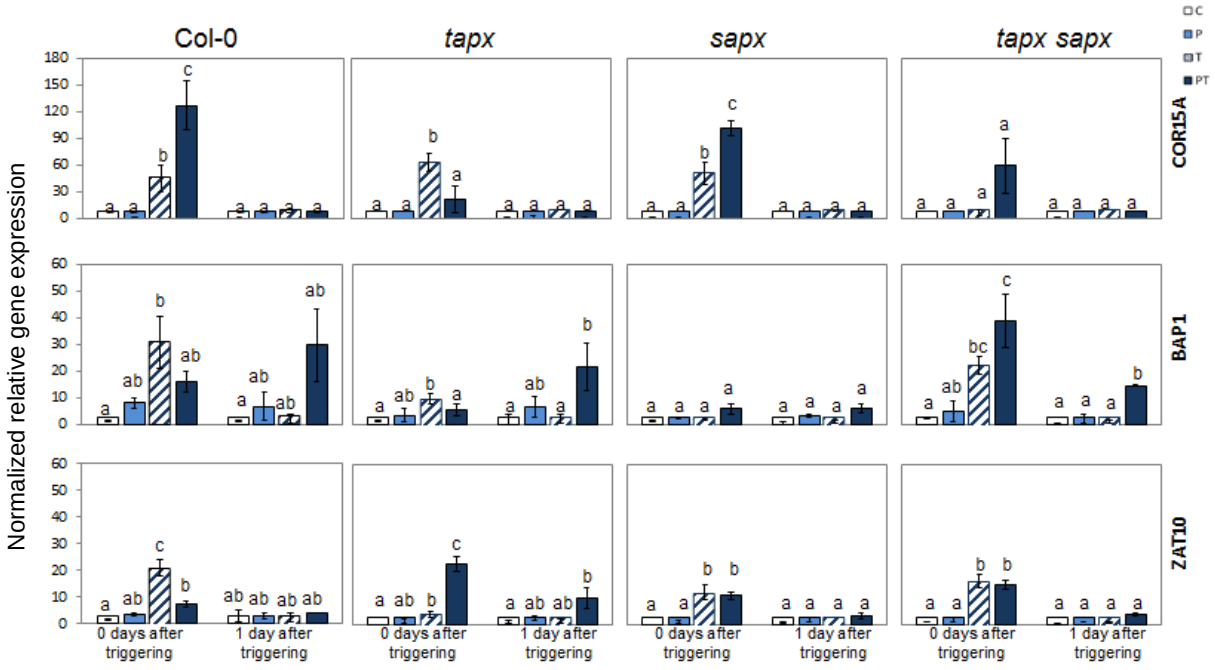


Figure 3-34. Transcript levels of cold (*COR15A*) and chloroplast-ROS marker genes (*BAP1* and *ZAT10*) immediately after 24 h triggering at 4 °C and 24 h later in only-primed (P), only-triggered (T) and primed and triggered (PT) Col-0 plants and knock-out lines *tapx*, *sapx* and

tapx sapx. The transcript levels were normalized on the respective transcript levels of naïve plants immediately before the on-set of priming (C), as well as the mRNA abundance of *ACT2* and *YLS8* genes. Data represent arithmetic means ± SD from three biological replicates where each biological replicate was analyzed in three technical replicates. The data were analyzed by ANOVA (Tukey HSD-test; $p < 0.05$).

In order to compare the amplitude of the observed priming effects in the mutant lines to the response in Col-0 plants, the relevant transcript ratio of PT / T was calculated (Figure 3-35).

The priming dependent decline of *ZAT10* induction after triggering was observed in Col-0 plants and, to a smaller extent, in the *sapx* and *tapx sapx* plants. The positive priming effect was inversed in *tapx* plants, resulting from a lack of strong induction in T plants.

Long cold priming resulted in a stronger induction of *BAP1* in the PT plants than in the T *sapx* and *tapx sapx* plants, demonstrating that priming had the opposite effect on *tapx* and wildtype Col-0 plants.

The relevant priming effect was also observed in *COR15A*. The primed and triggered Col-0, *sapx* and *tapx sapx* plants had a higher PT / T ratio than the *tapx* plants. Similar to the *ZAT10* transcript, the *tapx* plants showed an opposite regulation of *COR15A* after priming when compared to the other lines, demonstrating a regulatory effect of tAPX on the cold and two ROS-marker genes.

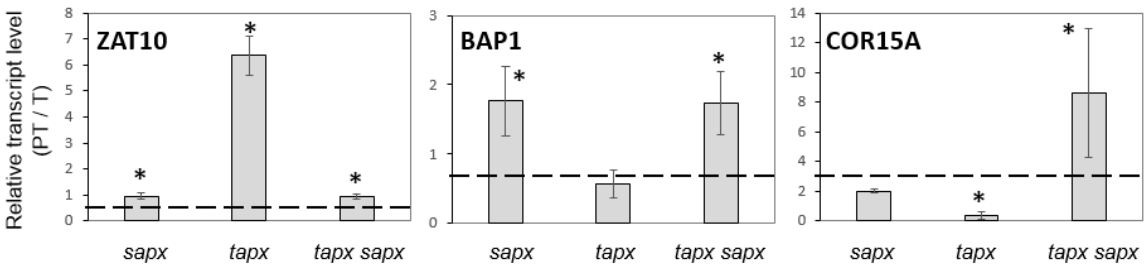


Figure 3-35. Relative transcript level (PT / T) of chloroplast-ROS- (*ZAT10* and *BAP1*) and cold- (*COR15A*) marker genes immediately after 24 h triggering at 4 °C in *tapx*, *sapx* and *tapx sapx* knock-out lines. Significant increases and decreases relative to the response in Col-0 (dotted line) are labeled with an asterisk. Data represent arithmetic means ± SD from three biological replicates where each biological replicate was analyzed in three technical replicates. The data were analyzed by Student T-Test (* $p < 0.05$).

3.2.8 Test for priming specificity

The advantage of being primed for a particular stress is important for plants and results in a faster and / or stronger activation of the various defense responses when the stress recurs (Conrath *et al.* 2006). The importance of testing the responses to different second stressors (not cold) was huge. The priming specificity, with respect to the antioxidant system, was tested with two different abiotic stressors, cold and moderate excess light. The expression regulation of the cold regulated genes (*PAL1, CHS, FER1, APx2, COR15A, ZAT10* and *BAP1*) was analyzed on log 2 scale immediately after the application of a short excess light triggering stress in T and PT plants (Figure 3-36).

Among the several different functional classes induced under high light are genes *PAL1, CHS, APx2* (Rossel *et al.* 2002) and *FER1* (Long *et al.* 2008). Just doubling the light intensity (250 µmol quanta $m^{-2}s^{-1}$) was enough for different transcriptional changes between plant treatments. However, this light intensity was not enough to provoke significant transcriptional changes in the genes that are regulated upon high light among light triggered (LT) and cold primed-light triggered (PLT) plants. In addition to the transcript response to cold priming, they also displayed a specific priming response upon cold triggering. Specificity to the second stress is important for memory establishment.

According to Kimura et al. (2003) *COR15A* is one of the 10 genes that showed upregulation by high light, drought, cold and salt stress. Under 24 h of cold triggering, the transcript level significantly increased in PT plants. Twenty four hours of moderately elevated light triggering was not sufficient to provoke huge changes in the transcript level in either one (LT, PLT) of plants treatments. According to Mittler et al. (2006), ZAT10 plays a key role both as a positive and negative regulator of plant defenses and can modulate the activation of defense responses during different stress combinations. In this study, the combination of cold priming and light triggering significantly blocked ZAT10 induction, as also observed after cold triggering. Priming did not affect the type of the response to light triggering but led to a stronger reaction. Contrary to that, the singlet oxygen marker gene *BAP1*, displayed a priming response upon excessive light triggering. The *BAP1* transcript can alternatively be induced by high light (Laloi *et al.* 2006), which was the case in LT plants. In PLT plants, as a result of stress type, moderate light triggering blocked the stronger *BAP1* expression.

Overall, the effects of cold triggering were stronger than after moderated light triggering in respect to the same pre-treatment. The cold priming and ROS-dependent effects on the tested genes could not be fully distinguished after changing the type of stress that was applied.

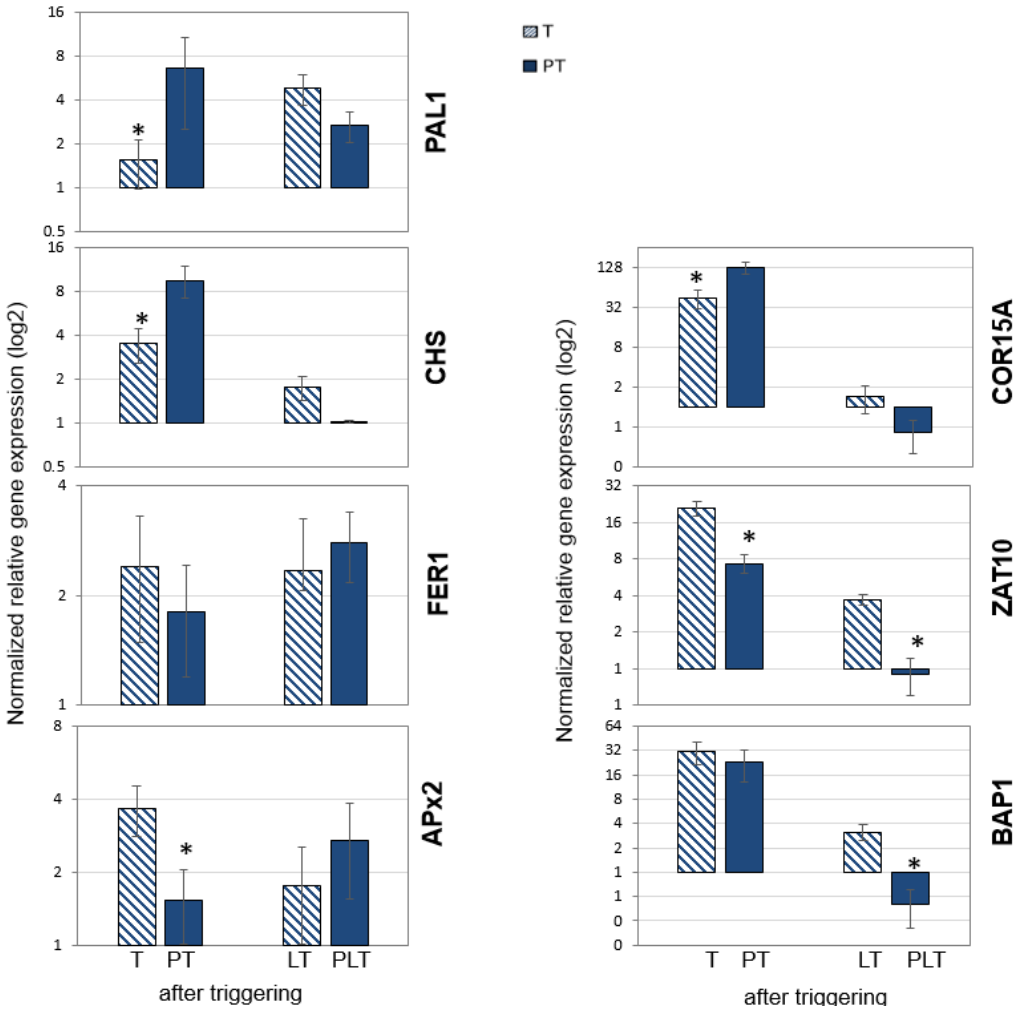


Figure 3-36. Log2 scale of normalized relative gene expression levels of stress- (*PAL1, CHS, FER1 and APx2*), cold- (*COR15A*), and chloroplast-ROS marker genes (*BAP1* and *ZAT10*) marker genes immediately after 24 h triggering at 4 °C or moderate excess light (250 µmol quanta $m^{-2}s^{-1}$) in only cold triggered (T), only light triggered (LT), cold primed and cold triggered (PT) and cold primed and light triggered (PLT) Col-0 plants. The transcript levels were normalized on the respective transcript levels of naïve plants immediately before the onset of priming as well as the mRNA abundance of *ACT2* and *YLS8* genes. Data represent arithmetic means ± SD from three biological replicates where each biological replicate was analyzed in three technical replicates. The data were analyzed by Student T-Test (* $p < 0.05$).

3.2.9 Comparison of *Arabidopsis thaliana* accessions for post-cold regulation

Arabidopsis thaliana accessions, Col-0, Ms-0, Kas-1 and Cvi-0, originate from distinct habitats and have the accession specific transcript regulation of genes for antioxidant enzymes. The priming hypothesis was tested on a response of various plastidic antioxidant enzymes by comparing the transcriptional changes immediately after triggering (T plants) stress and transcriptional abundance as a result of pre-treatment (PT plants). The priming-dependent differences in their responses are shown in Figure 3-37.

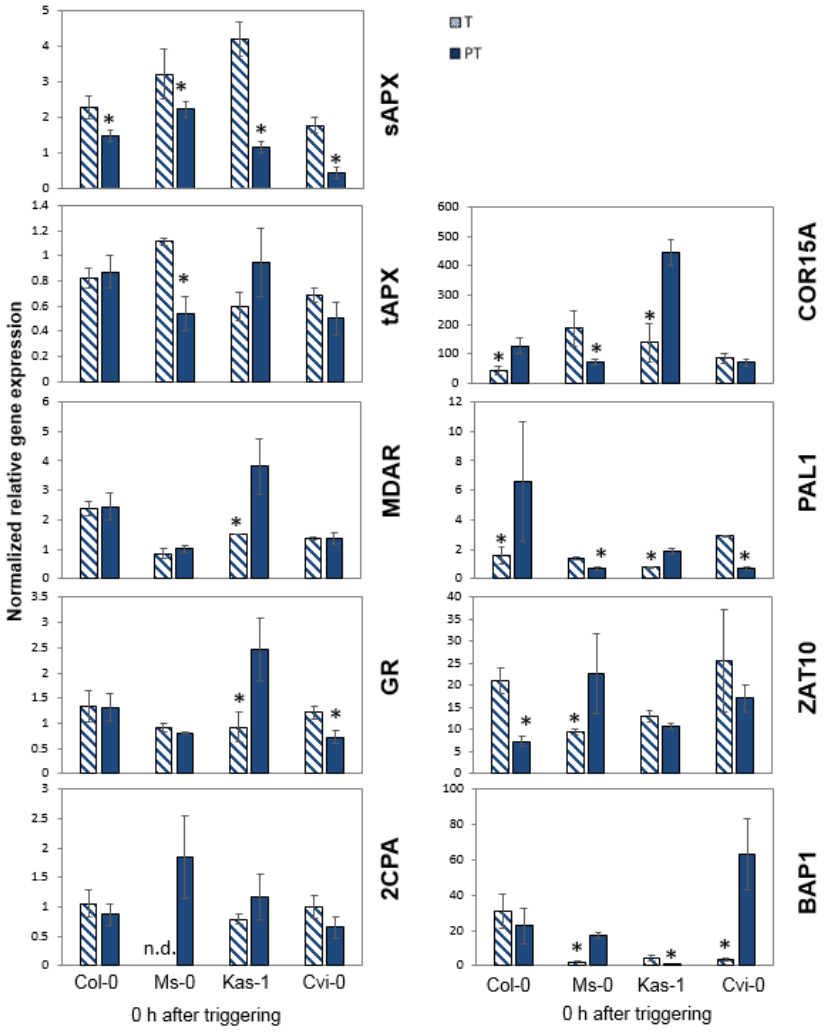


Figure 3-37. The transcript levels of chloroplast ascorbate-dependent and independent cycle genes (*sAPx, tAPx, MDAR, GR* and *2CPA*) and cold- (*COR15A*), stress- (*PAL1* and *CHS*) and chloroplast-ROS marker genes (*ZAT10* and *BAP1*) immediately after 24 h triggering at 4 °C in only-triggered (T) and primed and triggered (PT) Col-0, Ms-0, Kas-1 and Cvi-0 plants. The transcript levels were normalized on the respective transcript levels of naïve plants immediately before the on-set of priming as well as the mRNA abundance of *ACT2* and *YLS8*

genes. Data represent arithmetic means ± SD from three biological replicates where each biological replicate was analyzed in three technical replicates. The data were analyzed by Student T-Test (* $p < 0.05$).

Upon long cold priming and short cold triggering, a habitat dependent variation in the regulation of genes encoding enzymes of the ascorbate-dependent water-water cycle (*sAPX, tAPx, MDAR, GR*) was observed. The highest *sAPX* transcriptional effect after the application of the second cold stress was detected in the Kas-1 accession. Reduced transcript levels of the same gene were also detected in PT plants of the accessions Ms-0, Col-0 and Cvi-0 when compared to T plants, indicating similar effects of priming throughout all tested accessions. In contrast to *sAPX*, among the tested accessions, no effects were observed in *tAPX* transcript levels in the accessions Col-0, Kas-1 and Cvi-0 upon triggering stress. The relative transcript level declined in the pre-treated (PT) and non-pre-treated (T) plants in these three lines, while the accession Ms-0 displayed a priming dependent transcriptional decline. Moreover, significant differences in the transcript levels of genes encoding enzymes of ascorbate-recycling system (MDAR, GR) were observed between T and PT plants. The characteristic priming response that was observed for *MDAR* and *GR* genes appeared to be habitat-dependent. The tendency of a higher transcript accumulation of these two genes in PT plants was observed for the freezing tolerant accession Kas-1. The accession that originated from warm climate background, Cvi-0, displayed a stronger accumulation of *GR* transcript in the previously naïve plants, when compared to PT plants.

The 2-Cys peroxiredoxin A was analyzed in a same way as the ascorbate-dependent enzymes. The accumulation of *2CPA* transcript level for both plant treatments (T and PT) was not regulated in an accession or a gene specific manner.

In general, a high accumulation of *COR15A* mRNA was observed after second cold stress. A significantly high accumulation was observed in PT plants in Kas-1 and Col-0 plants. A low accumulation of transcript in PT plants for this cold responsive gene was specific to the accessions Ms-0 (significant) and Cvi-0. The decreased ability of cold acclimation in Cvi-0 is probably an evolutional adaptation to exposure to consistently high temperatures.

The two most freezing tolerant accessions, Kas-1 and Ms-0, had contrasting priming patterns for the *PAL1.* It was shown that the priming dependent *PAL1* transcriptional regulation took part in all accessions.

The tendency of lower *ZAT10* transcript induction in PT plants after short triggering was observed not only in Col-0 (significant), but also in the Kas-1 and Cvi-0 accessions. Contrary to the all accessions, a significant transcript accumulation of this gene was observed in the Ms-0 accession. The *ZAT10* priming dependent tendency for accessions Ms-0 and Col-0 was entirely opposite to the *tAPx* transcript expression pattern. A simultaneous and significant induction of the *ZAT10* transcript and a significant decrease in the tAPx transcript level was observed for the Ms-0 accession. An opposite trend was observed in the accession Col-0. The two other accessions, Kas-1 and Cvi-0, did not display a significant priming behavior, but had the same tendency for *ZAT10* and *tAPX* transcript abundance regulation.

The Col-0 was the only tested accession where the BAP1 transcript levels did not display significant priming specific response after short cold triggering. The accessions Kas-1 and Ms-0 showed the same priming-dependent transcriptional trend in *BAP1* and *ZAT10* and an opposite trend in *COR15A*.

Arabidopsis thaliana accessions showed differential amplitudes in their response to low temperature as well as strategies for the activation and regulation of chloroplast APX and cytosolic ROS-signalling at the same time. Only two genes, *sAPx* and *PAL1*, displayed a tendency to be primeable throughout all of the tested accessions. The cold and ROS marker genes showed contrasting transcript regulation after the stress treatment of two cold tolerant accessions, Kas-1 and Ms-0. The Kas-1 was the only accession with elevated genes encoding enzymes of ascorbate-recycling system after the second cold stress suggesting at a ROS avoidance strategy and a fast induction of a cold marker gene (*COR15A*). Habitat-dependent priming mechanisms of the individual accessions could not verified clearly for the tested genes. After the short triggering stress, an up-regulation of the chloroplast antioxidant system a down-regulation of extra-plastidic ROS-signaling molecules was characteristic of the cold tolerant Kas-1 and Ms-0 accessions when compared to the more cold sensitive Col-0 and Cvi-0. The thermomemory was maintained during the lag phase and subsequently enabled the plants to perform differential response to the short triggering stimulus.

4 DISCUSSION

It is discussed in the scientific community that the response and adaptation to periodically repeated internal and external signals are characteristics of intelligent behavior. Trewavas (2003) defined memory of living organisms as an ability to access past experience in a way that the later response can incorporate the relevant information from the past. In this study, plant memory was examined through a molecular based process of storing and recalling information. The use of a memorized experience resulted in an increased variety of possible responses throughout the evolution of plants.

The number of studies involving priming research has vastly increased in the past years. However, only little attention has been given to the development and storage of plant memory in context of cell specific compartmentation until now. The present study focused on the regulation chloroplast antioxidant system, its genes and the discovery of putative transcriptional regulators correlated to long cold temperature disturbance as well as the ability to recall information from past key moments. The accumulation of cold and ROS-responsive transcripts in combination with metabolites and reactive oxygen species gave an insight in the generation of thermomemory. Priming attendance was important in plant life not only as a controlling ROS-signaling mechanism upon future stress but also as a reproductive determinate. The persistence of a memory, the importance of the chloroplast redox control as well as costs and benefits of ‘stress preparation’ have not been in a mutual relationship addressed in the literature.

4.1 Costs and benefits of cold priming in plants

The term ‘priming’ or ‘habituation’ by Thellier (2011), was mentioned and discussed previously mainly in the plant phytopathology and herbivory literature. Priming and acclimation are two terms used to describe the effects of external stimuli on the phenotype of individuals (Hilker *et al.* 2015). The acclimation process refers to the plants acclimation in a continuously changing environment or a new habitat whereas priming refers to the overcoming strategies for occasional

but discontinuous stress occurrences. Within the project, priming is defined as a plants survival strategy, which is developed during a two week period of cold where non-freezing temperatures are preparing the plants for future stress exposure. Upon the subsequent implementation of a shorter triggering stress phase (during which the chloroplast redox status is imbalanced), the plant benefits from the previous priming event. In spite of the fact that efficient priming has its benefits, an investment into priming is costly. These observations have not experimentially proven until now.

Various accessions of *Arabidopsis thaliana*, with diverse selection pressure for low temperature tolerance, were grown for four weeks under short-day growth conditions and used for all the present experiments. At this age, the plants strongly regulate their chloroplast gene expression and, for the plants that require vernalization, four weeks of vegetative growth were sufficient. The chloroplast antioxidant system in priming stress responses hypothesis was tested under highly controlled growth conditions before stress treatments and to take measurements and harvest always at the same time of the day to exclude circadian effects.

4.1.1 Variations in plant growth and reproductive strategy are important for an unpredictable environment

In general, field studies on reproductive timing, fitness-related quantitative traits and flowering time are rare because the photoperiod, temperature, and light intensity are variable (Mishra *et al*. 2012). It has been shown in many studies that diverse abiotic and biotic stress factors were able to induce stress responses. However, the effects of cold priming on morphological and fitness parameters as well as the on-set of flowering have not been studied in detail.

Different tradeoffs exist between growth, reproduction and stress defense

The aims of this study were to investigate the priming costs and benefits over a three year experimental time period under controlled as well as fluctuating environment conditions. The results from this study show different tradeoffs between growth, reproduction and stress defense, among the different examined accessions. The priming and memory function were analyzed at plant architecture level where was expected that transfer duration from optimal growth temperatures

to 4°C would have an effect on growth extent (Gorsuch *et al.* 2010). After the two week cold priming and 24 h cold triggering there was no modifications of the plant architecture between treatments (Figure 3-2; Figure 3-20). The plant phenotype, meristem changes, the relative leaf number as well as relative fresh weight remained unchanged (Figure 3-20).

Constant preparation for cold stress is waste of energy and resources

The thermomemory in plants involves comprehensive physiological adjustments that reflected after (three weeks under stable conditions) on plant growth (Figure 3-6; Figure 3-8). Taking into consideration that cold is a major stressor that limits plant growth and development in continental climates (Shibasaki *et al.* 2009), the present data (Figure 3-6) suggest certain priming costs under controlled conditions (up to three times smaller primed WS and Van-0 rosettes in March 2014 and 2015 compared to non-primed ones). It is certain to be costly as it involves changes in gene expression patterns and metabolite profiles. In addition, the data confirm the hypothesis that constant preparation for cold stress utilized energy and resources, which could be otherwise used for growth and reproduction when freezing stress is not present. The growth conditions the plants experienced during the more stable month May, when midday temperatures were not falling below than 15°C during both years (2014, 2015) (Figure 3-4; Figure 3-5), and comparable to the green house where the temperature was set at 20° C. The already low P/NP ratios of rosette diameters declined to values below one (Figure 3-7) for plants grown under greenhouse conditions, which could be explained by the faster arrival of the bolting stage compared to field conditions. This study concerned also the fact that the high light conditions in laboratory studies (typically 600-800 µmol quanta m^{-2} s^{-1}) are equivalent to the rather low light in the field where light intensities on sunny days can exceed 2000 µmol quanta m^{-2} s^{-1} (Mishra *et al.* 2012). All accessions grew better in the field (Figure 3-6; Figure 3-8) and this is an example of thermomemory benefit in a fluctuating temperature environment. Expanding the leaf area to harvest more sunlight was another example of plant priming strategy in a non-stable environment. In some accessions such as WS (March, 2014) and Ms-0 (March, 2015) primed plants displayed almost four times bigger leaf areas than non-primed ones after three weeks of growth under field conditions (Figure 3-8). All accessions developed a P/NP rosette diameter ratio >1 in the field (Figure

3-7) during the cold temperature fluctuations in March from the first week of the experiment until more than four weeks later and it is linked to the priming edge. In May, due to the more stable weather conditions (absence of freezing) (Figure 3-5) and the earlier onset of flowering in primed plants (Figure 3-12), these trends in ratio did not persist longer than three weeks (Figure 3-7). Additionally, it can be concluded that differences in leaf size and shape were due to the changes in light levels and stable temperatures.

Morphological parameters such as rosette diameter as well as the leaf architecture differed among accessions (Figure 3-6; Figure 3-8). The reason for this is that these parameters are strongly influenced by the growth environment. Consistent with Tisne et al. (2008), morphological parameters are additionally under the control of plant developmental processes. The pre-treatment and thermomemory development make these morphological differences more visible according to study data.

Reduction in fitness results from the indirect effects of stress inside or outside the chloroplasts and ecological variation

In this study, priming and memory was linked to size traits and fitness. In order to address the potential fitness benefits by seed number, the differences in seed weight were compared between treatments and among the accessions (see Results 3.1.3.2). Herridge et al. (2011) identified a number of quantitative trait loci of 91 different *Arabidopsis thaliana* accessions which are responsible for generating differences in the average seed sizes. Plant organs are known to be under the direct influence of several abiotic factors such as temperature, light and day length (Orozco-Arroyo *et al.* 2015). It is assumed that among the accessions in this study, plants will have generally numerous seed size strategies and be sorted along r-K-continuum. It is observed that the species which originate from high altitudes (Kas-1 and Cvi-0 at the altitudes of about 1580 and 1200 m above sea level respectively) produce heavier but fewer seeds than the species from low altitudes. The primed and non-primed Kas-1 and Cvi-0 accessions have slightly heavier seeds compared to the other tested accessions (Figure 3-9). The other tested accessions displayed a trend to produce more rather than bigger seeds as a result of selection pressure (Figure 3-9; Figure 3-10). The weights of the individual seeds did not differ significantly between treatments in the greenhouse

or in the field (Figure 3-9). The accessions Kas-1 and Cvi-0 may benefit from their competitive superiority, while accessions as N13, Ms-0, WS, Col-0 and Van-0 may benefit from elevated numbers of seeds, as an adaptation strategy. This conclusion is consistent with the seed size-number trade-off model from Leishman (2001) where is highlighted the negative correlation between the seed size and number across a range of species from a range of habitats.

The pre-treatment dependent differences were only observed in the greenhouse for the seed numbers in the accessions N13, Ms-0 and Kas-1 (Figure 3-10). The primed Ms-0 plants produced eight times more seeds than non-primed plants under greenhouse conditions (May, in both years). On the contrary, the accessions WS, Col-0 and Van-0 produced the same amount of seeds in the greenhouse, irrespective of pre-treatment (primed and non-primed differences not significant, $p > 0.05$) (Figure 3-10). The largest difference in the total number of seeds per plant was observed between the accessions Kas-1 and WS (in both years) with the WS plants producing a five times higher amount of seeds compared to Kas-1 accession. The reduction in fitness can result from the indirect effects of stress inside or outside the chloroplasts and/or ecological variation among tested accessions. These conclusions are supported by the work of Zhen and Ungerer (2008) which showed that natural selection prefers the mutations compromising cold-acclimation in accessions coming from warmer habitats which is the case in this study with WS, Col-0 and Van (the average temperature from April to September goes up to 18°C). The resources required for cold-acclimation in *Arabidopsis thaliana* can then be used more efficiently for growth and reproduction. Community composition and seedling-seedling competition in an annual-dominated society can also be linked to such effects. Here, a positive priming effect on the seed yield was observed in all the cold pre-treated accessions as opposed to the non-treated plants under field conditions (Figure 3-10). It can be concluded that cold pre-treatment resulted in a higher seed number of similar mass (Figure 3-9). In several other studies, similar trends were observed. For example, the seed numbers in *Lactuca sativa* L., *Brassica rapa* L. and *Allium cepa* L. increased after a treatment at low temperature (Toledo *et al.* 1981, Linwattana *et al.* 1997, Reghin *et al.* 2005).

The link between rosette diameters, leaf areas and plant fitness was made in case of primed plants which were transferred to the field. Nevertheless, the strength of

this positive relationship (bigger rosettes, more expanded leaf area, and higher total number of seeds) varied in extent among accessions and depends on their position along the r-K-continuum (Figure 3-18).

Environmental fluctuations cause a delay in the flowering and, therefore, reflect on fitness

The induction of flowering is a crucial event in a plants life cycle. It was also affected by the two week period of cold priming. It was previously demonstrated in Heyer et al. (2004) that flowering time control is strongly influenced by modifying sugar balances in the apex and that all accessions monitored in this study displayed increased levels of sugar and prolin after two weeks of cold acclimation (Zuther *et al.* 2015). For the accessions originating from cold habitats such as N13, Kas-1 and Ms-0, cold priming mimics the environmental conditions that are required for meristem transition. Accessions, which germinate in autumn and require vernalization during winter for flowering in spring, required important sugar-induced senescence inhibition in order to allow the plants to resume growth in the spring (Masclaux-Daubresse *et al.* 2007). The onset of flowering in the accessions N13, Kas-1 and Ms-0 occurred several weeks later compared to Cvi-0, which started to flower within the first week after planting in May (Figure 3-11; Figure 3-12).

For the plants which were planted in March, the difference between the latest and the earliest flowering accessions was more than six weeks (Figure 3-11). Fifty percent of the primed plants coming from colder habitats such as N13, Ms-0 and Kas-1 flowered 50 days earlier than the non-treated ones in fluctuating field conditions in March (Table 3-2). Earlier flowering of pre-treated plants can result of continual sugar accumulation under cold temperatures in March as well as an earlier stimulated induction of senescence. Under the more stable May and greenhouse conditions, the flowering day differences (DD_{50}) were less than three days on average. The variation in DD_{50} numbers between the treatments was not more than 10 days in the greenhouse. Due to the more stable May and greenhouse conditions, it is assumed that deaccimation resulted in faster decline of the sugar content of pre-treated plants and a weaker induction of senescence. Sucrose levels declined back to the non-acclimated level during the first day of deacclimation (Zuther *et al.* 2015). The observed effects on flowering time show

that environmental fluctuations caused a delay in the onset of flowering and, therefore, reflected an increase in fitness (up to four times in both years) compared to the greenhouse conditions (Figure 3-10).

Cold priming influenced several ROS-sensitive signal transduction pathways, which were reflected as a terrestrial mollusk control response

So far, it is not clear how the cold-primed plants react to herbivore attacks and whether priming induced metabolic changes are a loss or a gain in that situation. The results from my research revealed differences in plants attractiveness to terrestrial *Arion sp.* under field conditions (Table 3-4). Damage caused by these herbivores was observed on both the primed and non-primed plants in the first feeding days. Up to 80% of the primed WS and Kas-1 plants were consumed within two days post-planting (Table 3-4). After all of the WS and Kas-1 accessions were consumed, slugs moved on to the primed Van-0 and Col-0 accessions. More than 50% of accession Ms-0 (both treatments) as well as Col-0 and Kas-1 plants (both non-primed) was attacked on the third day of the experiment. The attraction of slugs to the primed and non-primed accessions Cvi-0 and N13 was minimal within the first three days (0% attack). Differences in the plant's attractiveness towards herbivores disappeared after 12 days of planting where almost all accessions were eaten by the slugs (Table 3-4). A possible reason for the differences lies in the ecological background of each accession. Being the ``preferred nutritional choice`` might also serve as an accession specific strategy explained in some studies as a ``importance of damage overcomposition`` by increasing the fitness after herbivore attack (Paige and Whitham, 1987; Tiffin, 2000). Increased sugar levels in pre-treated plants attracted herbivores. It can be also assumed that the induction of repellents, as one of the plant defense mechanisms against herbivores, causes differential behavior among accessions. The role of cyanogenic glucosidase as a possible component against gastropod mollusks could be excluded for the acyanogenic species *Arabidopsis thaliana* (Tattersall *et al.* 2001). However jasmonate biosynthesis and glucosinolates are linked to freezing tolerance. Hu et al. (2013) suggesting that jasmonate may act as an upstream signal of the ICE-CBF/DREB1 transcriptional pathway which regulates the freezing stress responses of Arabidopsis. Here, plants did not have the need to activate this signaling branch due to more stable

weather conditions and non-chilling temperatures in May (Figure 3-5). Among the accessions, there was no significant correlation between latitude of origin and % of the slug attack. The study showed that the two weeks of cold priming influenced several ROS-sensitive signal transduction pathways, which were reflected as a terrestrial mollusk control response (Table 3-4). The considerable pre-treatment variations documented in the first days of slug exposure opened a new research chapter on the inextricable linkages of biotic and abiotic stress pathway to a broad network of molecular interactions.

4.1.2 Chlorophyll-a fluorescence indicates the ecological relevance of priming under (un)controlled stress situation

Chloroplasts are one of the main sites involved in the acclimation process to various environmental conditions (Crosatti *et al.* 2013). The process of photosynthesis plays an essential role, not only as a primary energy producer but also as a highly sensitive cellular energy sensor which can react to myriads of stresses, temperature fluctuations being one of them (Ensminger *et al.* 2006). Here, the drastic decrease in the value for Fv/Fm (Ini-qY) (less than 0.3 in non-treated March plants in the field) indicated chronic inhibition of photosystem II. During cold acclimation, photosynthesis interacts with other processes involving crosstalk between photosynthetic redox, as well as ROS and sugar-signaling pathways (Ensminger *et al.* 2006). Low temperature exposure might result in the collapse in the carbon export and interruption of plant transient starch synthesis (Figure 3-13; Figure 3-14). During the memory phase, plants would have time to repair the damages in the photosynthetic membrane and protein in order to recover from the first stress stimulus. It was suggested by Rapacz and Hura (2002) that during deacclimation, the photosynthetic capacity is being improved due to the acceleration of plant metabolism including repair systems for potential photodamages. This also decreases the assimilate content in leaves which formerly resulted in feedback inhibition of photosynthesis.

In the field experiments, the length and the speed of the recovery phase was dictated by natural fluctuations in the environment. The two week cold treated and non-treated plants were exposed directly to the field or to the greenhouse conditions without a lag phase. The ecological relevance of priming on the plants

reaction to a triggering stress was tested from the first day in March where the midday temperature did not exceed more than 5°C (Figure 3-13; Figure 3-4). The Ini-qY values, representing the level of PSII damages, showed up to three times higher values in the pre-treated plants as compared to the non-treated plants (Figure 3-13). Strand et al. (1997) presented the role of long-term cold temperatures as a signal in developing leaves to suppress photosynthetic gene expression. Here, two week cold treated plants, with already increased soluble sugars in leaves, displayed no high photosynthetic perturbations at low temperatures in March. Presumably the reason for this is that the photosynthetic apparatus is already acclimated, due to adjustments in photosynthetic gene expression during the pre-treatment. It can be concluded from these observations that primed plants had an advantage and are naturally dominant in unstable environmental conditions over non-primed plants. A strong negative correlation ($p= 0.0217$; $r^2 = -0.8101$) was found between the LT_{50} values and the eff / ini qY ratio in March (Table 3-3), manifesting the importance of ecological imprinting in a changing environment. These benefits, reflected in chlorophyll-a fluorescence, were present up to three weeks after the transfer to the field (Figure 3-14). A habitat dependent specificity was observed with the adjustments of the photosynthetic apparatus (Figure 3-16; Figure 3-17). My conclusion from this experiment is that primed plants were able to avoid photoinhibition when they experienced the low temperature stress again later in their life.

Plant photosynthetic stability and recovery potential upon freezing temperatures

The physiological costs of priming were quantified through the reduction in the maximum quantum yield (Fv/Fm) measured after the application of chilling stress. This parameter is sensitive to any environmental stress induced chronic photoinhibition of PSII (Maxwell and Johnson, 2000) and therefore measurable physiological cost of pre-treatment will be shown as a reduction on this parameter. One day under plant transfer to controlled greenhouse conditions was long enough to entirely recover the PSII (Ini-qY = 0.8) (Figure 3-13). In the absence of a second cold stress, the cold damage to PSII was visible through a 5 % decline in the Ini-qY parameter (Figure 3-13).

Plants were exposed to the chilling temperatures in March and were challenged several times by the sub-zero temperatures (Figure 3-4; Figure 3-5). The higher growth light intensities (approx. 10 times) on the sunniest days in March as well as the exposure to higher light levels (i.e. sun light; approx. 20 times as intense) of plants in field conditions as compared to the plants in the growth chambers influenced their photosynthetic performance drastically. During the experiment, all accessions were affected to a varying degree by the fluctuating environmental conditions, especially on the first day of the experiment (March both years) (Figure 3-16; Figure 3-17).

The chlorophyll-a fluorescence measurements showed distinct changes in the eff / ini qY ratio in treated and non-treated plants under freezing temperatures (12 hours of temperatures below 0°C in the last week of March) (Figure 3-17). Four weeks in the field were long enough to see a differential behavior among the accessions when the temperature declined to sub-zero levels. The less cold tolerant accessions such as WS, Col-0, Van-0 and Cvi-0 displayed lower eff / ini qY np/p ratios as compared to the N13, Ms-0 and Kas-1 accessions (Figure 3-17, up). This implies that the cold tolerant accessions showed different ecological program than cold sensitive accessions. Correlation between the latitude of the geographical origin of the accessions and their LT_{50} values was observed in several studies (Zuther *et al.* 2012; Hannah *et al.* 2006; Zhen and Ungerer, 2008). Such accession specific results were not detected in the one week acclimated and the few hours cold triggered plants (Figure 3-17, center). This means that the length of conducted period in the field and intensity of the second cold stress played together against photoinhition and PSII damages.

4.2 Thermomemory establishment and its utilization

The term ‘memory of cold stress’ was proposed by Byun et al. (2014). They compared the transcript abundance of 788 differentially expressed genes (encoding for components of the photosynthetic electron transport chain, chlorophyll, ascorbate and starch biosynthesis and signal transduction elements) upon cold acclimation (24 h at 0°C), deacclimation (72 h at 23°C) and re-acclimation (24 h at 0°C) in three week old Arabidopsis plants. These authors

observed the link between stronger activation of tested gene sets and improved freezing tolerance by the second cold stimulus, following the proposed priming model by Bruce et al. (2007). At the same time Li et al. (2014) performed six nights cold training experiments with two serials of stress applications (first 5°C below outside temperature and then 8.4°C below outside temperature) and highlighted the role of effective scavenging systems of chloroplasts and mitochondria observed as higher total plastid SOD and APxs activities in treated wheat plants. The analyses showed differential transcript regulation related to photosynthetic activity, lipid metabolism and calcium signaling on cold specific gene regulation. Markovskaya et al. (2008) observed that repetitive short cold pulses (6 days, 2 h at 12° C and 22 h at 23° C) caused longer lasting cold tolerance in treated than in non-treated cucumber plants.

The majority of the studies have not investigated the changes induced after the long cold exposure (more than one week) followed by a short second stress period. Aspects of priming and triggering have been poorly understood. The experiments in my study were set up to cover a broad physiological range. The effects of cold priming on the plants response to a triggering stimulus were studied at a transcriptional level, looking at cold- and ROS- marker genes, genes responsible for antioxidative protection and unspecific stress responsive genes. In addition to that, metabolite levels were quantified, by measuring ascorbate content, chlorophyll levels and the known stress indicator anthocyanin. Even in the absence of cold stress, the genes encoding plastid antioxidant enzymes are strongly expressed due to their protection role in cell life, and hence any kind of plastidic transcriptional variation will represent strong absolute change. The abundance of transcripts, metabolites and proteins were shifted by two week cold priming and readjusted during the post stress (memory) phase in Arabidopsis plants (Figure 3-2).

4.2.1 Plants exhibit two types of priming dependent transcript regulation when facing a triggering stress

The present research showed that cold-priming effects on marker genes for selected cold-, ROS- and general stress- responses (Gadjev *et al.* 2006; Rossel *et al*. 2002; Kimura *et al.* 2003; Byun *et al.* 2014; Reymond *et al.* 2000). We

hypothesize that there is a weak and a strong response behavior that together lead to the better antioxidant protection after the application of the second triggering stimulus. Priming-dependent regulation of ROS signaling is a crucial protection strategy under unstable environment.

4.2.1.1 Genes for plastidic antioxidant enzymes have weak responding behavior

In this study, link between temperature and variation of gene expression intensity was proposed. Shifts in the peroxidase patterns can be of great importance for the chloroplast antioxidant function. The hydrogen peroxide level was elevated almost five times after the initial exposure to the two week cold period (Figure 3-22). Jusczcak et al. (2016) showed the down-regulation of thylakoid bound APx along with the up-regulation of stromal APx enzymes after the transfer of various *Arabidopsis thaliana* accessions to 10°C while the peroxiredoxins and thioredoxins were not regulated in a specific manner. Environmental stress indicators namely, anthocyanins showed a typical cold response (four times up-regulation) under the cold stress treatment (Figure 3-23). According to these results, it is assumed that plant protection metabolite pattern affected the strong peroxide signature of the chloroplasts. The antioxidant defense is supported by accumulation of ascorbate (1.4 times total ascorbate induced) (Figure 3-24) and the modulation of cold responsive gene transcription (5 times cold marker gene COR15A induction) (Figure 3-25). Modulation of cold responsive gene transcription and ascorbate accumulation are consistent with the literature where an increase in plastidic antioxidant defense has been suggested to be result of an accumulation of free sugars through the induction of ascorbate biosynthesis (Cook *et al*. 2004) and modulated APx expression (Heiber *et al.* 2013).

During the 14 days of cold acclimation, significant changes in the hierarchy of the ROS scavenging system became visible and this trend was monitored via the transcript levels of s- , t- and c- APx (see Results 3.2.6). Transcript and protein abundance decreased for the main plastid superoxide dismutase *CSD2* (half of the control values) and thylakoid *APx* (1.8 times below control level), respectively (Figure 3-31; Figure 3-27). CSD2 protein level showed a decrease under two week chilling conditions in the accession Col-0 (Juszczak *et al*. 2012). At the same time, the strong induction of *sAPX* gene expression (2.7 times transcript increment)

served as a strategy for dealing with prolonged oxidative stress (Figure 3-26). On the protein level, the sAPx increased 3-fold (Figure 3-27; Figure 3-29). The outstandingly high (R^2 = 0.94) regression coefficient of the sAPx / tAPx protein ratio throughout the lag-phase showed sAPx protection dominance (Figure 3-28). The total ascorbate content was slightly elevated (1.4 times changed) and so were the genes encoding the ascorbate recycling enzymes, *GR* and *MDAR* (both approx. 1.2 fold) which suggests a stronger stromal ROS protection (Figure 3-24; Figure 3-26). Transcript abundance regulation of genes encoding ascorbate-independent water-water cycle enzymes did nor appear primeable with the noted exception of a slight increase (1.8 fold) in the transcript level of *2CPB* (Figure 3-30).

After two weeks of cold acclimation, priming shifted the chloroplasts ascorbate dependent antioxidant protection from the thylakoids to the stroma in order to improved resistance of plant towards oxidative stress. According to Shikanai et al. (1998) exactly this gene qualitative modification of the antioxidative machinery is important when stress occurred. The highly flexible antioxidative system of plants is mainly based on functional shifts rather than a costly *de novo* synthesis of antioxidative resources under cold stress (Saleh and Plieth, 2009). The antioxidant protection shifts shown in this study might constitute a benefit for the plants physiology during future stress periods.

The five-day period between priming and triggering served as a memory phase. This period of time was long enough to remove the established cold acclimation but not to delete their memory mark. During this phase, all elevated transcripts (*sAPx, FER1, 2CPB*) as well as sAPx protein levels gradually decreased, while the down-regulated gene *CSD2* and the tAPx protein level transiently accumulated to the control levels (no significant differences between treatments) (Figures 3-26 to 3-31). The chloroplast antioxidant transcripts were regulated slowly and in a gene specific and stress-dependent regulation manner. In comparison, quick decline of ICE1 (inducer of CBF-expression1)-regulated response was observed under temperature increase (Thomashow, 1999; Byun *et al.* 2014). Zuther et al. (2015) highlighted the sharp decline of sugar, proline and transcript levels in two week cold acclimated plants after the transfer back to ambient temperatures. Here, highly significant alterations of transcript and solute levels in cold acclimated plants were lost during deacclimation. During the lag-phase, where both memory

development and recovery occurred, the total ascorbate (Figure 3-24), anthocyanin (Figure 3-23) and chlorophyll a and b levels (Figure 3-21) recovered and reverted back to the naïve state after long cold exposure. The criterion that the response of the read-out parameters to the priming event has to be entirely lost before applied triggering stress (Hilker *et al.* 2015; Conrath *et al.* 2002) was fulfilled.

Twenty four hours of cold stress was too short to observe any large changes in the chlorophyll a and b levels (Figure 3-21), plant growth (no changes on relative leaf number and fresh weight) (Figure 3-20) or ascorbate levels (Figure 3-24) on naïve plants. Short cold stress only provoked a basic regulatory response such as the hydrogen peroxide induction (significant increase) (Figure 3-22), the anthocyanin levels (significant decrease) (Figure 3-23) and a change in the pigment composition of the photosystems (increase in chlorophyll a/b ratio) (Figure 3-21), but it failed to cause severe metabolic shifts, such as the one observed after the long cold treatment.

The priming-dependent inverse regulation for the genes *sAPX* and *CSD2* was observed (Figure 3-26; Figure 3-31) in primed and triggered plants where the transcript level of *sAPX* was approx. 1.6-fold lower and for *CSD2* approx. 2.4-fold higher than in triggered only plants. Two groups of differentially regulated members of the chloroplast ROS scavenging system could be identified: one group that was down-regulated (i.e. *tAPx* and *CSD2*) and another one that was up-regulated (i.e. *sAPx,* total and reduced ascorbate content, *GR, MDAR*). A priming-dependent effect was observed on the down-regulation of tAPx protein levels (X^2=7.4E-05) (Figure 3-29) while there was no significant accumulation of sAPx proteins after triggering in PT plants (Figure 3-29).

The expression of genes encoding peroxiredoxins (*2CPA, 2CPB*) and glutathione peroxidases (*GPx7*), which scavenge peroxides utilizing a disulfide/dithiol system, did not differ significantly ($p > 0.05$) after second stress treatment and was thus not priming dependent (Figure 3-30). The H_2O_2 detoxification was likely under the control of the primeable *APx2* (Figure 3-31).

4.2.1.2 A highly priming-sensitive gene management as a triggering response is a part of plant protection program

Due to the limitations of plastid function during the cold period, excess ROS-levels can induce C-repeat binding factors (CBFs) (Chinnusamy *et al.* 2004), which in turn regulate various nuclear encoded genes. It is known that *COR15A* is a direct target of CBF3 and CBF2, that *BAP1* and *ZAT10* are co-regulated with CBF3-senzitive genes and that *PAL1* and *CHS* are not influenced by CBF2 and CBF3 in response to various stresses (Wang and Hua 2009; Zhou *et al.* 2011; Laloi *et al.* 2007). Different responses among cold-specific and chloroplast-independent gene (*COR15A*), general stress-induced genes (*PAL1* and *CHS*) and ROS-marker genes for chloroplast-to-nucleus signaling (*BAP1* and *ZAT10*) suggest that cold induced priming processes are acting in parallel.

A two week long cold period quickly induced high levels of marker genes such as *COR15A* (approx. 5 times), *ZAT10* (approx. 8 times) and *BAP1* (approx. 7 times) followed by a decrease in transcript abundance after the transfer to optimal growth conditions (Figure 3-25). There was a greater increase in the *BAP1* transcriptional abundance after temperature change after triggering (from 4 °C to 20 °C) as compared to that after the two week cold priming period. A 35-bp fragment within the promoter sequence of BAP1 is suggested to be the reason for the observed increase in the transcript level due to temperature changes (Zhu *et al.* 2011). This cis-acting temperature-sensitive region is able to mediate differences of small temperature changes such as from 28 °C to 22 °C. In my experiment, the temperature changed 16 degrees and can thus be assumed to cause such a high *BAP1* transcript abundance.

The CBF-regulon controlled COR15A induction as one variant of a strong triggering response

Previous exposure to a certain stress makes a plant more resistant (Bruce *et al.* 2007) and more sensitive to future stress experiences (Hilker *et al.* 2015). The present study showed a high transcriptional activation (approx. 5 times higher than in control plants) of the *COR15A* gene after two weeks of cold stress (Figure 3-25) as a result of regulation via the cold sensitive CBF-regulon (Wang and Hua, 2009). Another explanation for such result can be epigenetic modifications as an

important factor of priming. Chromatin alterations via histone demethylation of the COR15A promoter start after exposure to cold stress and increases constantly over the following days (Kwon *et al.* 2009). The level of histone demethylation is correlated with the level of *COR15A* induction. Chromatin modulations observed after two days of cold stress and after the transfer back to optimal conditions (Kwon *et al.* 2009) could explain, at least in part, the mechanism of *Arabidopsis thaliana* thermomemory and its effect during the subsequent (triggering) cold period in this study. Demethylation of the epigenetic mark H3K27me3 (tri-methylation of lysine 27 on histone H3) under the cold stress stops the signal for transcriptional silencing (Zhang *et al.* 2007). According to the same authors, after the transfer to optimal conditions the repressor mark decrease and this lead to unchanged transcript level of *COR15A* until the end of the lag phase observed in my study (Figure 3-25). During the cold triggering, a full two week histone modification imprint caused a stronger reactivation of the cold induced promoter of COR15A and later a significantly higher accumulation of the transcript (Figure 3-32). Sufficient hydrogen peroxide detoxification, stronger *COR15A* induction and higher maximum photosynthetic capacity (Ini-qY) in pre-treated plants compared to non-treated ones are beneficial strategies that provide better protection and cause less severe damage when next stress occurs.

One strategy to cope with the challenges of cold temperatures is the stronger response of the stress induced CHS and PAL1 genes upon re-occurrence of the stress

The non-chloroplast specific, stress responsive genes, *CHS* and *PAL1* are not under ICE1-CBF-regulon and tAPx-mediated chloroplast-to-nucleus control (Lee *et al.* 2005; Laloi *et al.* 2007) and are controlled by different signaling pathways than the *COR15A* gene. Upon exposure to the second cold stress period, these two enzymes display a significantly lower transcript activation in non-primed than in primed plants (Figure 3-32). These genes are regulated by mitogen-activated protein kinases (MAPKs) and present signaling reliable marker genes for an enhanced induction of defense responses (Kohler *et al.* 2002, Sethi *et al.* 2014). The results are indicating the importance of induced transcripts of *CHS* and *PAL1* and the MPKs signaling components in cold priming. These findings are consistent

with the proposed pathogen-attack simulating the role of priming and MPK mediated signaling by Beckers et al. (2009) and Xu and Zhang (2015).

Results highlighted an important role of *PAL1* and *CHS* in response to low temperatures, which are both involved in anthocyanin biosynthesis and antioxidant function after the second cold stress. The significantly increased metabolic activation of anthocyanin (Figure 3-23) in combination with the up-regulated transcript levels of these two genes (Figure 3-32) during the second cold stress period in already cold primed plants lead to the conclusion of the possible signaling mechanisms of *PAL1* and *CHS* in cold priming and pleiotropically inducible priming pathway. Stimulation of MPK6 (Beckers *et al.* 2009), cytosolic calcium signaling (Bose *et al.* 2011) and signals of plastidic redox imbalances (Vainonen and Kangasjärvi, 2015) are common for both, abiotic and biotic, stress responses. Transcriptional and metabolic induction of *PAL1* and *CHS* lead to an induced physiological state in which cells respond to very low levels of stimulus (24 h cold) in a more rapid and robust manner than non-cold primed cells (Figure 3-32), which is consistent with the definition of pathogen priming given by Conrath et al. (2015).

Evidence of strategic BAP1 suppression and ZAT10 activation in ROS-signaling supports the priming hypothesis

Strong activation of the stress induced *PAL1* and *CHS* genes in already cold primed plants (Figure 3-32) was not a unique priming concept among the ROS marker genes. The strong priming-dependent induction of genes (such as *COR15A*) during the second stress period was not observed for chloroplast ROS signaling markers *ZAT10* and *BAP1*, as they underwent a strong decrease (Figure 3-32).

The *ZAT10* and *BAP1* transcript levels showed a drastic increase (Figure 3-25) immediately after the two week cold treatment. Because of the short cellular half-lifetimes and mobility of H_2O_2 (1ms, 1µm), HO^- (1ns, 1nm), O_2^1 (1µs, 30nm), O_2^- (1µs, 30nm) (Karuppanapandian *et al.* 2011), which limits their diffusion (Moller *et al.* 2007), ROS are not able to serve as priming memory molecules, but rather constitute potential primary signals after the five day long deacclimation phase. Transcript levels of *ZAT10* and *BAP1* genes just before triggering were equal to

the level of the control plants (Figure 3-25). Due to the triggering stimulus, these ROS marker genes showed a different behavior as a result of the change in the chloroplast ROS detoxification compartmentation (Figure 3-32). The transcript level of *ZAT10* was slightly induced and the *BAP1* level was suppressed by the chloroplast ROS signals in comparison to the control levels (Figure 3-32). ROS damages inside the pre-treated plants were minor as shown by the Ini-qY values in only triggered plants (Figure 3-33). Long lasting changes in the chloroplast APxs ratio and the ratio of the signaling molecules O_2^- and H_2O_2 resulted on the future plant stress response. According to Gadjev et al. (2006), these two ROS molecules drive particular signal transduction cascades. After short cold triggering, the effects of priming and ROS signaling regulation showed that *ZAT10* is primeable (Figure 3-32). The chloroplast ROS-marker gene *ZAT10,* via regulation of its target genes, controls activation of extra-plastid protection mechanisms, such as ascorbate peroxidases *APx2* and *APX1* (Mittler *et al*. 2006; Rossel *et al.* 2007). The *APx2* transcript levels showed a priming-specific response during the second stress period (Figure 3-31).

4.2.1.3 The response concept of weak and strong priming-sensitive genes is a planning policy

ROS reactivity dictates toxicity while decreasing signaling ability (D'Autreaux and Toledano, 2007). The ROS signature and chloroplast-nucleus signals depend on the antioxidant capacities. Karpinski et al. (1997) gave indications of chloroplast to nucleus signaling via chloroplast ROS activation of the genes for extra-plastidic ascorbate peroxidase. The regulatory function of stromal / thylakoid ratio and Cu-Zn dismutase define the time points and the intensity range of reactive oxygen species generation and hence the signal release from the chloroplast to the cytosol (Figure 3-28; Figure 3-31). In this study, different responses to priming were expected among the tested genes as a result of different governing signaling mechanisms.

The results of the research at hand can be visualized via a model (Figure 4-1) which highlights the memory of the priming induced modifications of the first cold event and the response during the subsequent stress period. The second cold stress period resulted in a stronger transcriptional activation of the extra-plastidic

stress response genes and metabolites (positive priming effects) and at the same time negative priming effects for the chloroplast ROS signaling marker genes *ZAT10* and *BAP1* due to the changes in the chloroplast ROS buffering capacity.

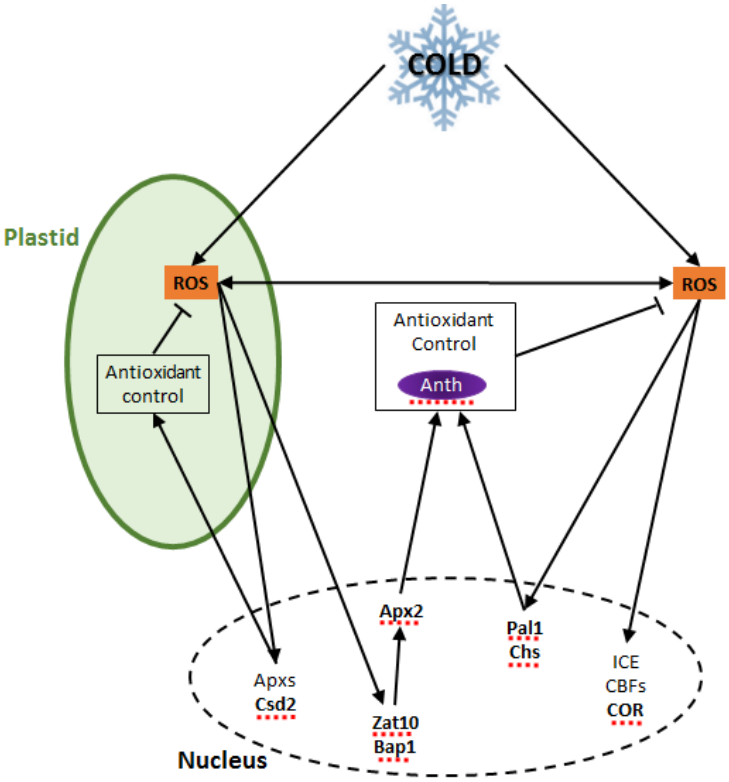


Figure 4-1. Cold priming in *Arabidopsis thaliana*. Two weeks of cold priming results in high ROS generation. Cold and ROS responsive marker genes as well as stromal antioxidative genes are highly up-regulated during cold priming. During the lag phase, transcript levels of these genes were back to control level. By short cold triggering stress, plants respond with a stronger activation of extra plastid specific stress response genes and metabolites and less activation of chloroplast-to-nucleus ROS signaling. Red bolded letters are presenting priming-dependent responses while the arrows an antioxidant regulation in Arabidopsis plants. Abbreviations: APxs: ascorbate peroxidase, CSD2: CuZn SOD isoform 2, ICE: inducer of CBF-expression1, CBFs: regulator of cold responsive genes, Zat10: hydrogen peroxide marker gene, COR: cold responsive gene, Bap1: singlet oxygen marker gene.

The priming specific regulation of *ZAT10* (Figure 3-32) was linked to the priming-induced regulation of stromal and thylakoid bound ascorbate peroxidase expression (Figure 3-28). *ZAT10* transcript level was half-maximal by the triggering stimulus in the pre-treated plants. Stronger induction of this transcript after second cold pulse observed by Byun et al. (2014) was not confirmed as a probably reason of shorter lag phase length (3 days). The gain- and loss-of

function *ZAT10* mutants with decreased transcript abundance showed enhanced stress tolerance (Mittler *et al.* 2006). This lead to conclusion that not only modulated transcript abundance of *ZAT10* but also lower expression of *BAP1* (Hua *et al.* 2001; Yang *et al.* 2007) enhanced stress tolerance through stronger activation of immunity responses in *Arabidopsis thaliana*. According to chi-squared test, decreased *BAP1* transcript abundance in pre-treated plants was result of priming and triggering synergism (X^2 = 1.7E-36) upon second cold stress period. The hypothesis of priming-dependent *BAP1* response upon triggering stress was confirmed. Similar to the pathogen-induced priming, a stronger and faster second stress response of each, *COR15A, PAL1* and *CHS* was observed (Figure 3-32).

Modulated activation (suppression) of the priming-dependent stress mediator genes for chloroplast-to-nucleus ROS signaling (*ZAT10* and *BAP1*) is a strategy to decrease activation of specific stress-responses and to activate extra-plastidic specific ROS responsive genes with pleiotropic and secondary stress mediating protecting function upon triggering. Antioxidant protection is initially provided by the fast-activated cold responsive gene and the low inducible chloroplast enzymes, followed by the modulation of the photosynthetic apparatus (Figure 3-33), ascorbate stability (Figure 3-24), protective metabolites (Figure 3-23) and chlorophyll enhancement (Figure 3-21). This important protection from ROS is necessary to cope with unpredictable temperature changes. Priming has a dual role as an activator of basic stress responses and as a suppressor of chloroplast-nucleus signaling.

4.2.2 Physiological aspects of priming specificity on a triggering stress type are important for memory establishment

Similar to the cold stress response, excess light in Arabidopsis can induce systemic signaling and acclimation (Karpinski *et al.* 1999; Bode *et al.* 2016). Moderate excess light was 2.5 times higher than regular growth light intensity. Transcriptional differences observed in this study, between the primed / non-primed treatments and between the cold / elevated light triggering stressors, were exclusively a result of the priming effect on the triggering response.

Together, cold and excess light are expected in this study to increase the ROS production by affecting the photosynthetic electron transport. A full recovery of

plastid function after the first stress is essential for the subsequent response to the triggering stimulus in Arabidopsis. It is known that *ZAT10* and *BAP1* are induced by cold and high light stresses (Rossel *et al.* 2007; Laloi *et al.* 2006). The cold priming effect was not specific for the different triggering stressors (cold/elevated light) when the *ZAT10* transcript levels are compared as elevated light and cold similarly blocked its induction (Figure 3-36). The elevated light blocked strongly the ROS marker gene *BAP1* in PT plants (Figure 3-36). Here, different triggering stressors showed same *ZAT10* and *BAP1* regulatory trends.

In cold pre-treated plants, the triggering cold stress increased levels of *PAL1* and *CHS* and strongly decrease *APx2* level while the elevated light triggered an opposite effect (Figure 3-36). Cold priming effect on chloroplast-to-nucleus ROS signaling was cold specific for these three genes.

4.3 Putative cold priming regulators are awaiting further research

The transcript results in this study, suggest that the plastid antioxidant system serves as a priming hub and regulates the generation and the mobility of signals by slow capacity regulation. Intracellular redox stabilization mechanisms after priming and triggering have not been studied until now. As a result of the two week cold priming, transcript abundance levels in APx knock-out plants were significantly different than in the wild-type Col-0 (see Results 3.2.7). During the two week cold period, a lot of pleiotropic effects resulted in different responses in the knock-out plants.

Here, conclusion is that when hydrogen peroxide cannot be oxidized at the thylakoid membrane where it is produced, then the stromal antioxidant part took control over it (see Discussion 4.2.1). The loss of one strong expressed antioxidant component (i.e. tAPx) could be compensated by strong support of remaining ones (i.e. sAPx) in response to light stress and moderate cold (Kangasjärvi *et al.* 2008). At the same time, overexpression of thylakoid ascorbate peroxidase showed an enhanced resistance to chilling stress in tomato and tobacco (Duan *et al.* 2012; Sun *et al.* 2010).

The *ZAT10* and *BAP1* transcript levels appeared inverted in *sapx* and *tapx* lines after cold triggering (Figure 3-35). Eff / ini qY ratio as an indicator of photoinhibition

displayed a different response to the lack of either thylakoid and / or stromal peroxidases (Figure 3-33). A strong increase in the *ZAT10* transcript level (Figure 3-34) as a priming effect on triggering stress was observed in *tapx* lines as an inverted response to Col-0. Lack of both APxs resulted in a decreased inhibition of *ZAT10* induction similar to *sapx* line. The primeability of the signals coming from the chloroplast stromal and thylakoid APx genes and the influence on the nuclear gene expression was not clearly linked here as was by the Laloi et al. (2007). According to the chlorophyll-a fluorescence data (Figure 3-33), combined activation of both APx by priming through the PSII is required for photoinhibition avoidance. The *ZAT10* transcript decrease and the slowdown of signaling in wild type, *sapx* and *tapx sapx* lines during the second stress period could stimulate the activation of the other stress responsive genes such as *PAL1* and *CHS* in wild type Col-0 (Figure 3-32). On the other hand, the absence of thylakoid bound APx in the *tapx* line resulted in a stronger transcript decline and priming dependent response of *BAP1* in PT plants (Figure 3-34). However, *tAPX* is involved in the fine tuning of H_2O_2 signaling upon cold stress and was demonstrated in this study to have a role in the regulation of priming in response to abiotic stress, but also upon biotic stress (Murgia *et al.* 2004).

The increased transcript level of *COR15A* in primed and triggered plants in the tested lines (except the *tapx* line) (Figure 3-34) after the second stress period, demonstrated the regulatory effect of APxs on cold as well as two ROS-marker genes.

4.4 Natural variation of priming mechanisms exists among *Arabidopsis thaliana* accessions

The importance of environmental straining on the transcriptional regulation of chloroplast antioxidant system in *Arabidopsis thaliana* accessions has been recently highlighted in Juszczak et al. (2016). The relevance of the intracellular redox stabilization mechanisms during cold priming has been studied for the first time, using various accessions of *Arabidopsis thaliana* from the Northern hemisphere. Here, the transcript abundance patterns of selected genes for antioxidant enzymes as well of the cold- and ROS- marker genes on the Col-0

accession as well as on contrasting lines Ms-0, Kas-1 and Cvi-0 in parallel were tested (Figure 3-37). Diverse Arabidopsis accessions showed differences in the magnitude of their low temperature response and their ability to survive at freezing temperatures (Table 2-1) due to their geographical origin. In NA plants, LT_{50} values ranged from −4.98°C (Cvi-0) to −7.72°C (Ms-0), while in two week cold acclimated plants the range was from −7.78°C (Cvi-0) to −11.88°C (Kas-1) (Zuther *et al.* 2012). These accessions differ in genetically manifested cold acclimation mechanism strengths (strong Ms-0 and Kas-1, medium Col-0 and weak Cvi-0) (Schulz *et al.* 2015; Hannah *et al.* 2006). Presumably, these accessions follow diverse priming strategies for concentration of cytosolic ROS-signaling, chloroplast and genes mediating general stress signaling, as it was already shown in correlation between some CBF regulated genes during cold acclimation (Zuther *et al.* 2012; Zhen *et al.* 2011).

Transcriptional primability exists in cold tolerant accessions

The higher degree of chloroplast antioxidant system regulation and a weaker accumulation of extra-plastidic ROS-signaling molecules were observed in cold tolerant accessions such as Kas-1 and Ms-0 with a short vegetation period (Figure 3-37). The highest transcriptional differences in *sAPX* between the treatments after the second cold stress period were detected in the accession Kas-1. The ROS signaling effects were buffered by the regulation of the cold and the genes for chloroplast antioxidant enzymes in cold tolerant accessions. The elevated transcript levels of the *sAPx* indicated priming-dependent differences in all the tested accessions. In contrast to stromal *APX*, *tAPX* transcript priming effects were observed only for the Ms-0 accession after application of the triggering stress. The cause for such behavior can be found in the pre-treatment influence on the total ascorbate level (for Col-0, Figure 3-24). The priming dependent accession differences are consistent with the statements from Juszczak et al. (2016) who showed the strongest ascorbate induction in the accession Ms-0 and the weakest induction in Kas-1 after the two week cold period. Over-compensation of the chlorophyll level during the lag phase and the exclusion of CBF-regulation due to the limitation in the thylakoid recovery potential in the Ms-0 accession are suggested as possible reasons for the priming variations. The primability is strongly dependent on the plants ecological background.

After the short triggering stress, the priming-dependent characteristics were confirmed for the ascorbate regenerating genes, *MDAR* and *GR* (Figure 3-37). Here, the highest transcript accumulation was observed in the freezing tolerant accession Kas-1 while lower priming imprints were observed in the more cold sensitive accessions.

Changes during deacclimation positively influenced future stress respond

Specific regulation of *PAL1* among the tested accessions was observed after priming. In the accessions Kas-1 and Col-0 a similar positive priming effect for this gene was observe, similar to the results of Beckers et al. (2009) in response to MPKs mediated pathogen priming. The anthocyanin level was strongly increased during the two week long cold treatment and quickly decreased again during the deacclimation phase in the accession Col-0 (Figure 3-23) as well in Kas-1, Ms-0 and Cvi-0 (Juszczak *et al.* 2016). The two freezing tolerant accessions, Kas-1 and Ms-0, had contrasting priming patterns upon triggering stress (Figure 3-37). It was also reported that these two accessions both accumulate higher levels of sugars and proline (Zuther *et al.* 2015) than the cold sensitive accessions after two weeks of priming. Measured H_2O_2 accumulation indicated an accession specific delay in hydrogen peroxide detoxification during the lag-phase, for the accessions with the lowest LT_{50} values (Kas-1 and Ms-0) (Juszczak *et al.* 2016). However, all of these analyses lead to conclusion that the speed and the magnitude of the changes in cold regulated transcripts, metabolic shifts and chlorophyll levels during the deacclimation phase had a big influence on the future stress reactions.

Different gene selections by environment are decisively under cold stress

Due to the diverse composition of the plastids antioxidant system among the different accessions and experienced patterns of selection on *CBF* genes (Zhen *et al.* 2011), distinct priming-dependent protection mechanisms were observed in this study. Relative to the geographical range, the accessions from the warmer climates exhibit levels of nonsynonymous nucleotide polymorphisms at an approximately 2.8-fold higher rate across CBFs family (Zhen and Ungerer, 2008).

The stronger *ZAT10* transcript accumulation was observed in the accession with a cold adaptation strategy, namely Ms-0 (Figure 3-37). The ROS-signaling responsive gene showed different types of regulation (highly transcript

suppression and accumulation) among the tested accessions under cold triggering. The different types of ROS-signaling cascades among the cold tolerant accessions led to the antioxidant protection. This increased resistance to the next triggering stress is a priming strategy. Reported gain- and loss-of-function mutations in *ZAT10* enhanced the tolerance of plants to abiotic stress (Mittler *et al.* 2006).

A similar priming-dependent transcriptional response was observed with *BAP1* and *ZAT10,* while an opposite response in *COR15A* expression was observed in the accessions Kas-1 and Ms-0 (Figure 3-37). The down-regulation of the chloroplast antioxidant system and decrease of extra-plastidic ROS-signaling molecules were characteristics of the cold tolerant accessions as opposed to the more cold sensitive Col-0 and Cvi-0.

The accession Ms-0, with the highest LT_{50} value and the strongest cold tolerance ability among tested accessions, shows priming strategy visible on the higher transcript accumulations of ROS-marker genes and lower accumulation of cold- and pleiotropic stress- marker genes in the pre-treated plants. Accessions with the lower LT_{50} values than Ms-0, such as Kas-1 and Col-0, had an opposite priming strategy and invested more on better extra-plastid protection system for ROS avoidance. Due to the low acclimation capacities in the Cvi-0 accession compared to the continental accessions, multiple independent mutations in the regulatory and coding regions of CBFs result in reduced rates of induction and maximum levels of expression in the downstream genes that they regulate according to Zhen and Ungerer (2008). Taken together, the control of chloroplast ROS for the activation of stress responses differs among the accessions and reflects the existence of different types of memory.

4.5 Conclusion

Plant development of a thermomemory in response to long-term abiotic stress (cold) increased protection to *Arabidopsis thaliana* cells and brought ecophysiological benefits by accelerating the plants ability to react to a fluctuating environment. It was shown that the first stress stimulus is memorized and stored and this information can be recalled in response to a subsequent stress. Cold has

an effect on the ascorbate signature and the ROS-pattern was crucial for memory formation. The configuration of the antioxidant system controls the priming of the ROS responses. Evidence of the potential mediators of the priming memory within the chloroplast antioxidant system was found in this research and these are APxs. The plastidic APxs ratio and Cu-Zn superoxide dismutase were cold responsive regulators of plastidic ROS levels in a highly specific manner, dependent on the type of stressors. The low expression of marker genes for chloroplast ROS signaling was followed by the activation of the pleiotropic stress marker genes (*PAL1* and *CHS*) expression (Figure 3-32). These genes are key enzymes of the phenylpropanoid and flavonoid biosynthesis pathways (Hu *et al*. 2004; Olsen *et al.* 2008) and probably contributed to the stronger extra-plastidic protection in primed plants via anthocyanins. The accession-specific transcriptional differences of the priming effect after triggering stress were the consequences of the ability to discriminate between the different ROS signaling pathways (Figure 3-37). The natural variation in priming effects did not only result in a differential antioxidant protection, but was also noted in the plant fitness (see Results 3.1.3.3). Thermomemory is an advantage for the plants during short vegetation periods in fluctuating environments. Growing faster, flowering earlier and producing high amount of seeds were some of the priming strategies in the unstable environment (see Results 3.1.3 – 3.1.4). The cold-priming effect on chloroplast-to-nucleus ROS signaling was stressor specific (Figure 3-36).

The major goal of the study was to elucidate the priming process and determine the mechanisms of thermomemory in plants after cold stress. Priming was shown to be conveyed via modulations in the stromal and thylakoid APx ratio as well as Cu-Zn superoxide dismutase, resulting in the alteration of the ROS signature in primed and triggered plants compared to only triggered plants. Regulation of chloroplast-to-nucleus ROS signaling is beneficial strategy upon next stress period by supporting protection outside of plastid in model plant species *Arabidopsis thaliana*.

5 SUMMARY

Survival and acclimation to different environmental changes are two main tasks during evolutional process in plants. Low temperature is a limiting growth factor and has great impact on metabolically processes. Slowing down biochemical reactions and making kinetic differences higher upon low temperature causes photostatic redox-imbalances. Due to these imbalances more reactive oxygen species (ROS) are generated. ROS and the chloroplasts as major sites of their production are important initiators and/or converters of retrograde signals to the nucleus under stress conditions. It is accepted that transient environmental stress experience can prepare an organism for improved defense responses when the next stress occurs. Memorizing the previous (priming) experience during the lag-phase prepares for future (triggering) stress responses. We suggest that the chloroplast antioxidant system behaves as a pro-active priming hub under cold stress.

It was shown that the first stress stimulus is memorized and stored and this information can be recalled in response to a subsequent stress. Cold has an effect on the ascorbate signature and the H_2O_2 pattern was crucial for memory formation. Priming was shown to be conveyed via modulations in the stromal and thylakoid APx ratio as well as Cu-Zn superoxide dismutase, resulting in the alteration of the ROS signature in primed and triggered plants compared to only triggered plants. The low expression of marker genes for chloroplast ROS signaling (*ZAT10* and *BAP1*) was accompanied by the activation of the pleiotropic stress marker genes (*PAL1* and *CHS*) expression and contributed to the stronger extra-plastidic protection in primed plants. Cold tolerance-related primability among Arabidopsis accessions was observed via higher chloroplast antioxidant system regulation and weaker accumulation of extra-plastidic ROS-signaling molecules in cold tolerant accession Ms-0 with a short vegetation period. Growing faster, flowering earlier and producing high amount of seeds were some of the plants priming strategies in the unstable environment. The cold-priming effect on chloroplast-to-nucleus ROS signaling was specific to the type of triggering stress. Regulation of chloroplast-to-nucleus ROS signaling is a beneficial strategy when next stress period occurs by supporting protection outside of the plastid in the model plant species *Arabidopsis thaliana*.

ZUSAMMENFASSUNG

Akklimatisation und das Überleben von unterschiedlichsten Umweltbedingungen sind zwei der evolutionären Herausforderungen an Pflanzen. Niedrige Temperaturen limitieren das Wachstum und haben großen Einfluss auf metabolische Prozesse. Sie verlangsamen biologische Reaktionen und vergrößern kinetische Unterschiede zwischen Enzymen, was zu einer gestörten Redox-Balance führt. Durch diese Missverhältnisse können größere Mengen an reaktiven Sauerstoffspezies (ROS) entstehen. ROS und der Chloroplast als Hauptentstehungsort sind wichtige Initiatoren bzw. Umwandler von retrograden Signalen bei Stress. Es ist allgemein akzeptiert, dass vorübergehende Stresserfahrungen Organismen zu erhöhten Stressantworten während eines zweiten, späteren Stresses führen können. Die Erinnerung an vorherige (Priming-) Erfahrungen während einer Phase unter optimalen Bedingungen (lag-phase) ist notwendig um bei folgenden Stressantworten (Triggern) effizienter an die Bedingungen angepasst zu reagieren. Wir behaupten, dass das plastidäre antioxidative Schutzsystem als ein proaktiver Priming-Knotenpunkt während Kältestress wirkt.

Es wurde gezeigt, dass die Information über den ersten Stress gespeichert wird und dass diese während eines Folgestresses verarbeitet wird. Kälte hat einen Effekt auf den Ascorbathaushalt und die Menge an H_2O_2. Dieser Effekt entscheidend über die Entstehung eines „Gedächtnisses“. Weiterhin wurde aufgedeckt, dass in geprimt und getriggertend Pflanzen über die Modulation des Verhältnisses von stromaler und thylakoidgebundener Ascorbatperoxidase und die Cu-Zn-Superoxiddismutase im Vergleich zu nur getriggerten Pflanzen die ROS-Signatur gezielt verändert wird. Die niedrige Expression von zwei plastidären ROS-Markergenen (*ZAT10* und *BAP1*) ging einher mit der Aktivierung von pleiotropen Stressmarkergenen (*PAL1* und *CHS*). Dies führte zu einem besseren Schutz in geprimten Pflanzen. Die Fähigkeit zum Kältepriming wurde exemplarisch in Akzessionen von Arabidopsis beobachtet. Eine stärkere Regulation des plastidären antioxidativen Schutzsystems und eine schwächere Akkumulation von extraplastidären ROS-Signalen wurde z.B. in Ms-0 gefunden. Diese Akzession hat nur eine kurze Vegetationszeit, wodurch sie gezwungen ist schneller zu wachsen, früher zu blühen und mehr Samen zu produzieren; all dies sind Anpassungen an

nicht-stabile Umweltbedingungen. Der Effekt des Kälteprimings auf Plastid zu Nukleus Signalen ist triggerspezifisch. Die Regulation der ROS-Signale von Chloroplasten zum Zellkern ist eine vorteilhafte Strategie in dem Modelorganismus *Arabidopsis thaliana*, indem sie die nicht-plastidäre Abwehr erhöht.

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7 LIST OF PUBLICATIONS

Journal articles

Juszczak I., Cvetkovic J., Zuther E., Hincha D.K., Baier M. (2016) Natural variation of cold deacclimation correlates with variation of cold-acclimation of the plastid antioxidant system in *Arabidopsis thaliana* accessions. *Front. Plant Sci.* **7:** 305.

Posters

Cvetkovic J., Baier M. (2015) Chloroplast redox signaling and memory development upon long-term cold stress in *Arabidopsis thaliana.* VISCEA International Conference Plant abiotic stress tolerance, Vienna, Austria.

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Cvetkovic J., van Buer J., Juszczak I., Baier M. (2013) The chloroplast antioxidant system in priming stress responses. IRTG Summer School, Berlin, Germany.

8 CURRICULUM VITAE

For reasons of data protection, the curriculum vitae is not published in the electronic version.

9 APPENDIX

Meteorology data (Berlin Dahlem) for period 1st untill 30th **April 2014**

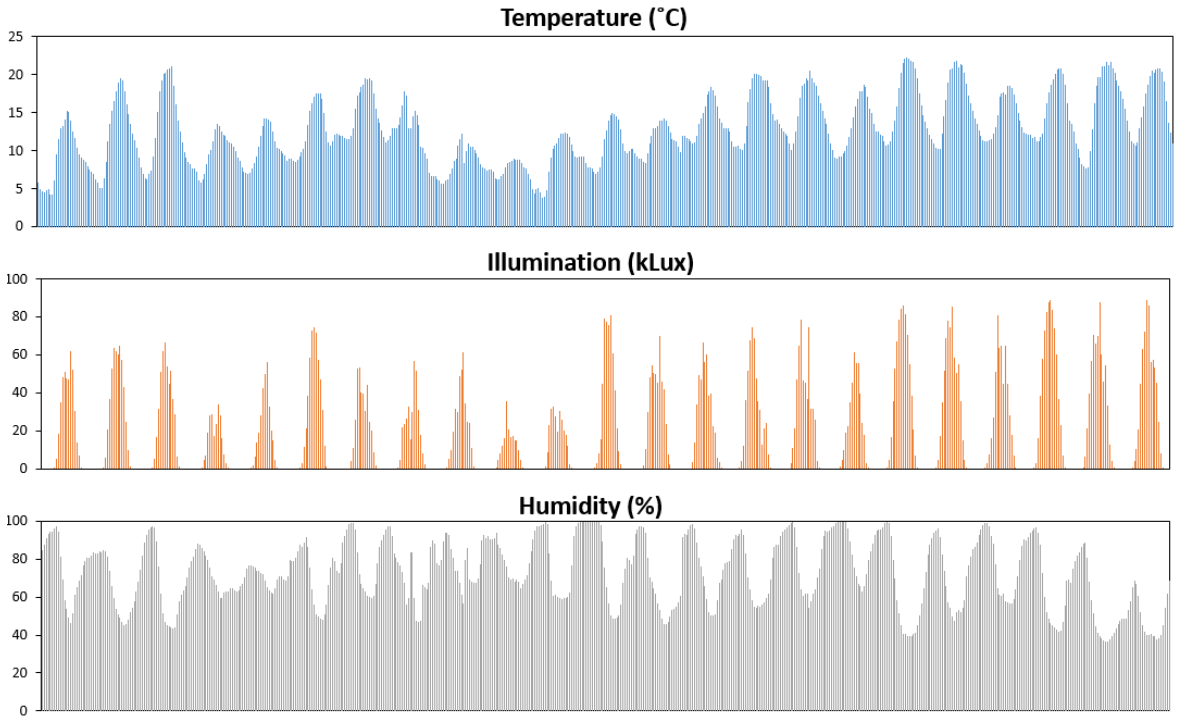


Figure A1. Meteorological data for the months April, May and June 2014 provided by Meteorology station of the FU Berlin, Dahlem (Germany). Temperature (°C), illumination (kLux) and humidity (%) were recorded every hour during the experimental periods.

Meteorology data (Berlin Dahlem) for period 1st **June** untill 11th **July 2014**

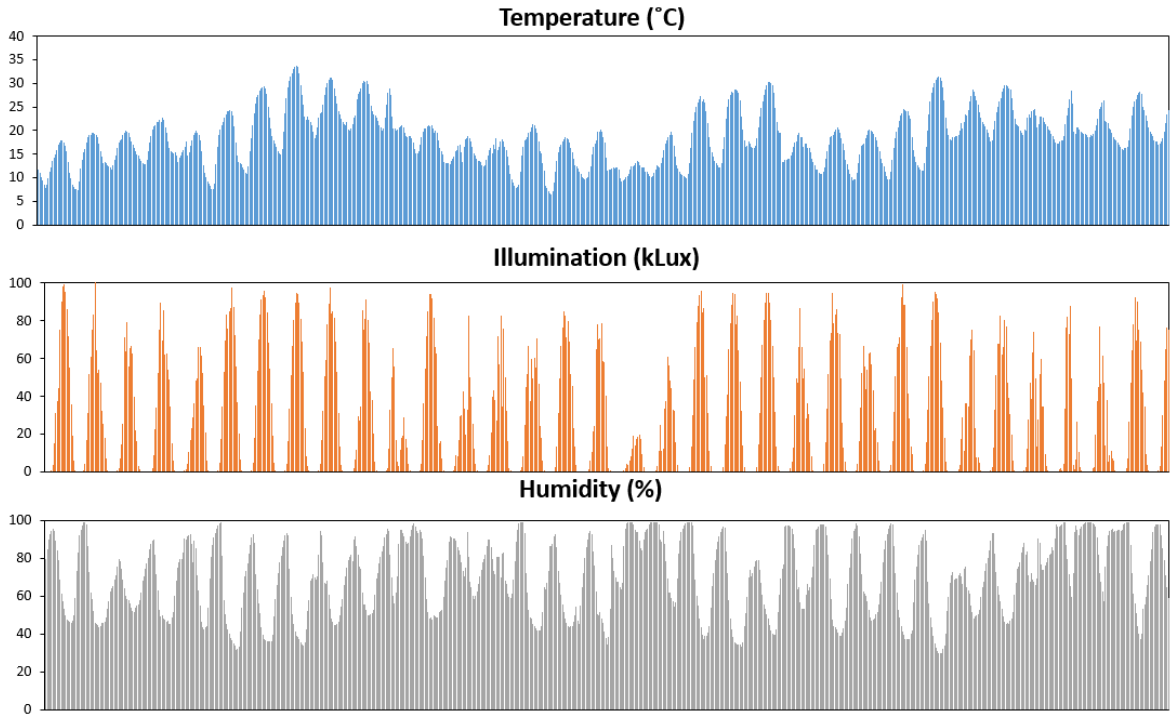
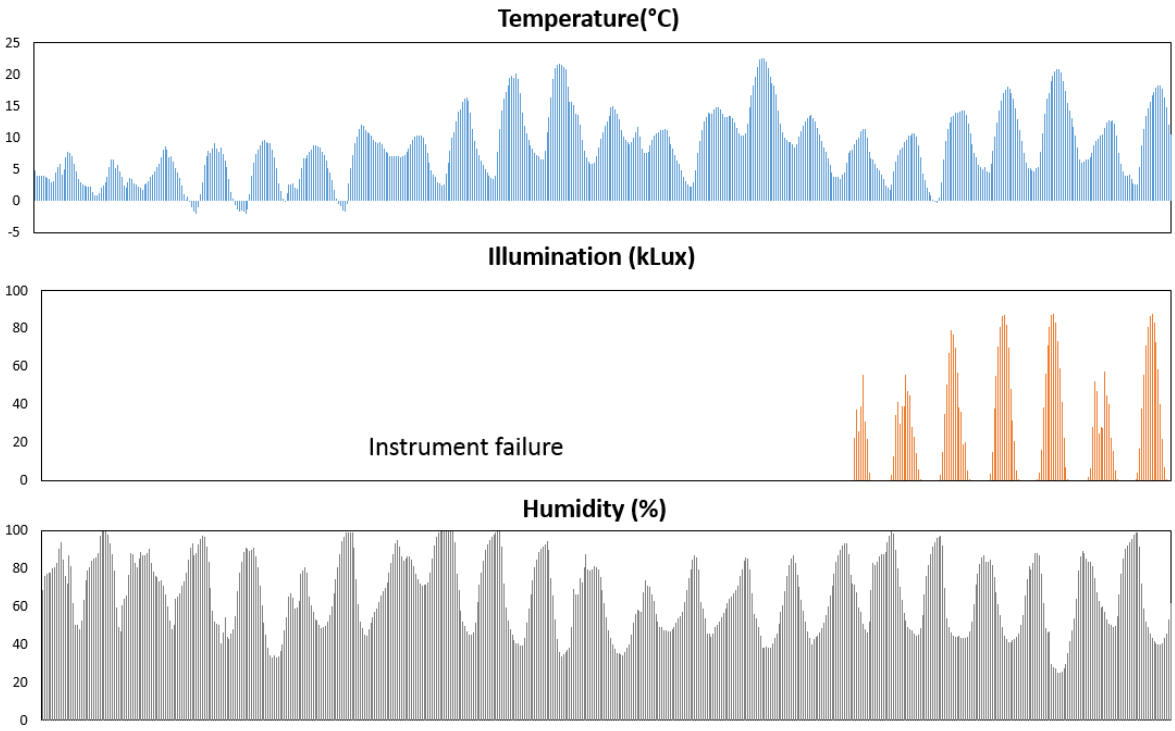


Figure A1 (continued). Meteorological data for the months April, June and July 2014 provided by Meteorology station of the FU Berlin, Dahlem (Germany). Temperature (°C), illumination (kLux) and humidity (%) were recorded every hour during the experimental periods.

Meteorology data (Berlin Dahlem) for period 1st untill 30th **April 2015**



Meteorology data (Berlin Dahlem) for period 1st **June** untill 11th **July 2015**

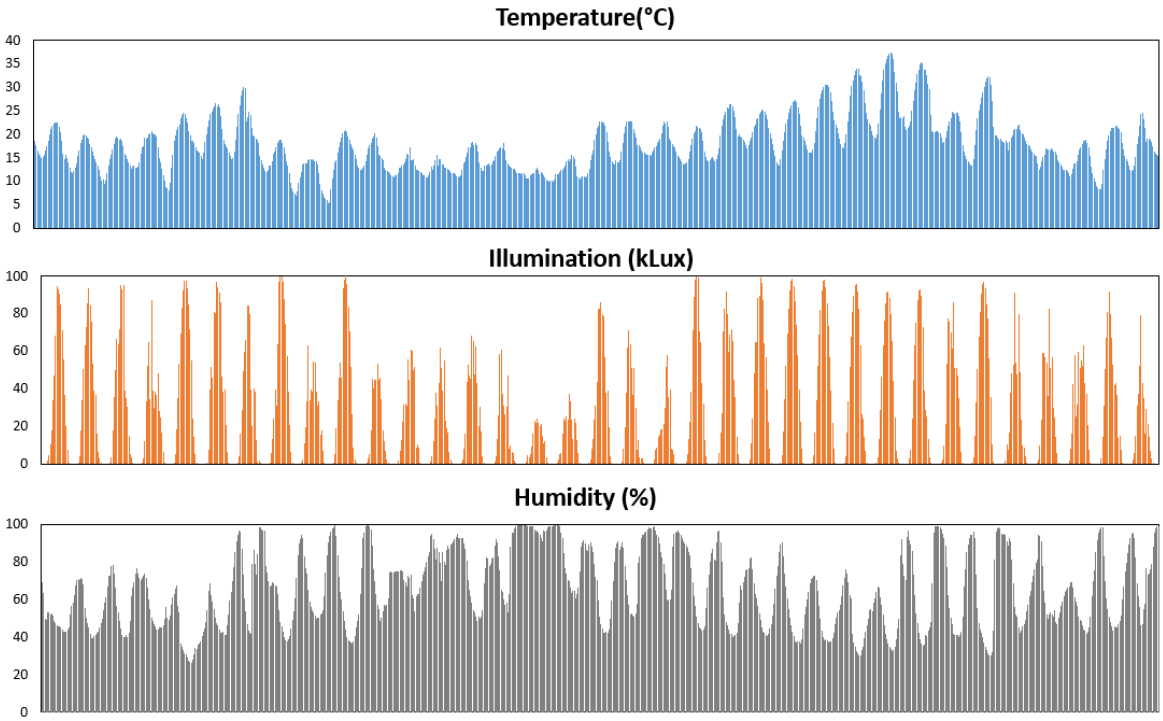


Figure A2. Meteorological data for the months April, June and July 2015 provided by Meteorology station of the FU Berlin, Dahlem (Germany). Temperature (°C), illumination (kLux) and humidity (%) were recorded every hour during the experimental periods.