The role of the plastidic antioxidant system during short term cold priming

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy
of Freie Universität Berlin

by

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from Siegen (Germany)

Berlin 2016

The investigations described in the following thesis were performed under supervision of Prof. Dr. Margarete Baier at the Institute of Biology, section Plant Physiology of the Freie-Universität Berlin (01.12.2012 – 30.06.2016).

1st Reviewer: Prof. Dr. Margarete Baier (FU Berlin)

2nd Reviewer: Prof. Dr. Anke Steppuhn (FU Berlin)

Date of defense: 23.09.2016

Acknowledgement

There are a lot of people I would like to thank, for the support over the last couple of years, while I was performing the experiments and was writing this thesis:

First I would like to thank Prof. Dr. Margarete Baier, for her sustained support over the last three and a half years, for providing guidance and support, fruitful discussions and the sharing of knowledge. This gave me the opportunity to develop myself scientifically and as a person: Thank you!

I am very thankful that Prof. Dr. Anke Steppuhn was willing to be my second supervisor and the second reviewer of this work.

Furthermore I would like to thank the CRC973 'Priming and Memory of Organismic Responses to Stress' and connected to this the IRTG for the provided opportunities to advance my knowledge during meetings and training courses. Special thanks are due to Prof. Dr. Monika Hilker and Dr. Tobias Otte for their brilliant organization and tireless work in the CRC shaping the existing stimulating and friendly atmosphere.

I am very grateful for the financial support of the Freie Universität Berlin and the DFG of my project.

I would like to thank the whole research group of plant physiology for their support. Especially, Elena Reifschneider for her experimental support. Christiane, Rainer, Jelena, Elena, Andreas, Aneth, Marina, Andras and Brit for the continuous scientific and private discussions, providing new input and being supportive during the daily life. I thank all the borrowed eyes, which read over the thesis in search for last spelling mistakes and weird grammar, especially Andreas and Rainer.

Special thanks are due to Munya for taking his time proofreading the whole thesis.

Last but not least I would like to thank my family and friend for giving me the support needed during my PhD-time. As in every aspect in life, you supported me at every step I made and enabled me to write this words. My sincere gratitude for this.

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2-Cys peroxiredoxin (A/B)	2CP(A/B)	3’-phosphoadenosine 5’-phosphate	PAP
abscisic acid	ABA	adenosine triphosphate	ATP
ADP-glucose pyrophosphorylase (3)	APL(3)	ascorbate	asc
ascorbate peroxidase	APX	BON association protein 1	BAP1
bonzai 1	BON1	calcium ion	Ca^{2+}
carbon dioxide	CO_2	chalcone synthase	CHS
chilling-tolerance divergence 1	COLD1	chlorophyll	chl
Arabidopsis thaliana var. Col-0	Col-0	cold regulated	COR
cold regulated 15A	COR15A	control	C
copper/zinc superoxide dismutase 2	CSD2	C-repeat/DRE binding factor 1	CBF1
Arabidopsis thaliana var. Cvi-0	Cvi-0	cycle threshold (value)	Ct(-value)
E3 SUMO-protein ligase	SIZ1	executer (1/2)	Ex(1/2)
fluorescent in blue light	FLU	gibberellic acid	GA
glutathione peroxidase (1/7)	GPX(1/7)	heat shock transcriction factor A2	HSFA2
heat shock transcription factor C1	HSFC1	high expression of osmotically responsive gene 1	HOS1
high light	HL	hydrogen peroxide	H_2O_2
hypersensitive response	HR	inducer of CBF expression 1	ICE1
iron dependent superoxide dismutase 2/3	FSD2/3	Arabidopsis thaliana var. Kas-1	Kas-1
Knockout	KO	lethal temperature 50	LT50
light harvesting complex	LHC	low light	LL
methyl viologen	MV	mitogen activated protein kinase kinase kinase	MAPKKK
mitogen activated protein kinases	MAPK	molecular oxygen	O_2
mondehydroascorbate	MDA	mondehydroascorbate reductase	MDAR
Arabidopsis thaliana var. Ms-0	Ms-0	nicotinamide adenine dinucleotide phosphate	$NADP^+$
non-photochemical quenching	NPQ	open stomata 1	OST1
oresara 1	ORE1	pathogen related	PR
peroxiredoxin	PRX	peroxiredoxin IIE	PRXIIE
peroxiredoxin Q	PRXQ	phenylalanine ammonia-lyase (1)	PAL(1)

photosynthetic electron transport	PET	photosystem (I/II)	PS(I/II)
pinformed protein	PIN	pipecolic acid	Pip
plastidic antioxidant system	PAS	plastoquinone	PQ
primed and triggered	PT	primed only	P
programmed cell death	PCD	reactive oxygen species	ROS
related to apetala 2A	RAP2.4A	respiratory burst homologue	RBOH
ribulose-1,5-bisphosphate-carboxylase/oxygenase	RuBisCO	RuBisCO large subunit	rbcL
salicylic acid	SA	salt tolerance zinc finger	ZAT10
ß-glucuronidase	GUS	state transition 7/8	STN7/8
superoxide	O_2^-	superoxide dismutase	SOD
systemic acquired acclimation	SAA	systemic acquired resistance	SAR
thioredoxin	TRX	thylakoid bound/stromal ascorbate peroxidase	tAPX/sAPX
tonoplast intrinsic protein 1/2	TIP1/2	triggered only	T

1 Introduction

Adaptation enables plant species to optimize their phenotype for their current habitat, maximising their fitness (Orr, 2005). This includes the ability to endure biotic and abiotic stresses. But evolutionary adaption to stresses is not only necessary to survive and reproduce in the habitat, it is also essential for the colonization of new habitats (Preston and Sandve, 2013). Even if plants are adapted to certain habitats, climatic changes occur on seasonal and geological time-scales. Changes on the geological time scale happened, both in the northern and southern hemisphere, leading to the contraction of the tropics (Zachos et al., 2001; Stickley et al., 2009). Continuous adaptation to altering environmental conditions is necessary to ensure survival and evolutionary success.

In the twentieth century the average temperature increased by 0.6 °C (Jones et al., 2001). This changes forces plants to adapt in a short time to new environmental conditions. Trends for a migration toward higher elevations and latitudes are reported to escape the steadily increasing temperatures (Walther et al., 2002; Parmesan and Yohe, 2003). Due to the increase in the rate of global warming it is believed that changes in the natural environment of plants are changing faster than the species are able to migrate. This puts more pressure on adaptation and the ability to react to stress on very short time scales (Jump and Peñuelas, 2005).

To launch appropriate responses against stress, the stressors need to be identified and signalling must be initiated and decoded correctly. Often the same signalling molecules that operate in a signalling cascade are used for various kinds of stress (Tuteja and Sopory, 2008). Mitogen activated protein kinases (MAPK) are stimulated by a range of different stimuli (Rodriguez et al., 2010). McClean et al. (2007) could show by computational modelling that in the case of MAPKs specificity can be achieved by mutual inactivation of the adjacent pathways, enabling yeast to respond only to one stress, even if two stresses were applied activating the same mitogen activated protein kinase kinase kinase (MAPKKK) to launch the initial response.

Calcium ions are involved in the responses to cold (Knight et al., 1996), drought and salinity (Knight et al., 1997). In 2000 in an Arabidopsis root model it was found that for those three stresses a Ca^{2+} influx into the cytosol is triggered, but the intensity of this influx was unique for

each stress (Kiegle et al., 2000), indicating how specificity could be introduced into the signalling of the universal second messenger Ca^{2+}.

Reactive oxygen species (ROS) also belong to this group of unspecific signalling molecules that are induced by a range of environmental stresses and can be used as signal molecules (Apel and Hirt, 2004).

1.1 Reactive oxygen species

Reactive oxygen species can be produced actively, for example during pathogen infections (Torres et al., 2006). Additionally they can be produced as a by-product of regular cell metabolism (Giorgio et al., 2007). One of the main contributors to ROS production is photosynthesis. The photosynthetic electron transport (PET) does not only produce molecular oxygen (O_2) (Hoganson and Babcock, 1997), but can also transfer energy or electrons to oxygen. This transfer to oxygen produces ROS. As the name suggests ROS are very reactive, mostly very unstable intermediates of aerobic metabolism. Apart from ROS directly produced by photosynthetic electron transport, the process of photorespiration produces ROS in peroxisomes e.g. by the glycolate oxidase (Foyer et al., 2009). Due to their unstable nature, ROS are able to damage DNA, proteins and lipids (Halliwell and Gutteridge, 2015).

I. *Singlet oxygen*

Singlet oxygen is mainly produced at photosystem II (PSII) (Telfer et al., 1994; Krieger-Liszkay, 2005) and in the antenna complexes (Krieger-Liszkay, 2005). The input of additional energy to O_2 from PSII ($P680^+$) allows electrons to overcome the spin restriction (Krieger-Liszkay, 2005). The half-life of singlet oxygen is roughly 200 ns (Gorman and Rodgers, 1992). It is thought to react only with targets in its close neighbourhood, as its maximal diffusion distance was calculated to be 10 nm (Sies and Menck, 1992). However, more recent literature suggests that singlet oxygen can reach the nucleus by shuttling through the cytosol (Fischer et al., 2007). One of the main effects of singlet oxygen is damage of the D1 protein in chloroplasts, which decreases the net photosynthesis (Trebst et al., 2002).

II. *Superoxide*

In the Mehler reaction (Mehler, 1951) electrons are transferred to molecular oxygen. The generated superoxide (O_2^-) can be subsequently detoxified to water via hydrogen peroxide (H_2O_2) (Heber, 2002). Superoxide can be produced at PSI (Asada et al., 1974). However photosystem II (PSII) seems to be more sensitive to superoxide than photosystem I (PSI) (Krieger-Liszkay et al., 2011). For long, it is discussed that the toxic effect of superoxide is not generated by the anion itself, but by hydrogen peroxide and the Fenton reaction (Benov, 2001). Various iron-sulphur cluster containing enzymes can be strongly inhibited by superoxide (Kuo et al., 1987; Flint et al., 1993), e.g. that of ferredoxins (Fisher et al., 2016). Ferredoxins are very important electron carriers (Fukuyama, 2004), mediating the last step of linear electron transfer of the PET chain by reducing nicotinamide adenine dinucleotide phosphate ($NADP^+$) to $NADPH/H^+$ (Hanke and Mulo, 2013).

III. *Hydrogen peroxide*

Hydrogen peroxide is produced spontaneously in aqueous solution. Alternatively, it can be produced by detoxification of superoxide (Asada, 1999, 2006). Within the group of ROS, H_2O_2 has the highest half-life (>1 ms) (Petrov and Van Breusegem, 2012) and, even though its passive diffusion over membranes is limited, it can be actively transported across membranes via aquaporins (Bienert et al., 2006). As other ROS hydrogen peroxide can oxidize proteins (Groen et al., 2005), lipids (Siddique et al., 2012) and DNA (Imlay et al., 1988).

IV. *Hydroxyl radical*

If hydrogen peroxide comes in contact with iron or copper, the Haber-Weiss (or more general Fenton) reaction takes place (Haber and Weiss, 1932; Lloyd et al., 1997). Hydroxyl radicals are among the most reactive oxygens species known (Jakob and Heber, 1996) and are able to cause DNA damage (Cadet et al., 1999). High stress levels lead to induced levels of superoxide, which ultimately cause the release of iron and sulfur. This free metal can be used to generate hydroxyl radicals and by that cause DNA damage (Keyer and Imlay, 1996).

1.2 ROS production in different plant compartments

Reactive oxygen species are produced in various plant compartments (Foyer and Noctor, 2003). The respiratory burst homologue (RBOH) proteins produce superoxide at the plasma membrane, releasing superoxide in the apoplast (Torres and Dangl, 2005). The Arabidopsis genome encodes for ten RBOH proteins (Camejo et al., 2016). ROS in the apoplast are important for plant development: if apoplastic ROS levels are reduced, root hair growth is decreased (Foreman et al., 2003). Stomatal closure and seed germination are also affected by knockouts (KO) in RBOH D/F by an impairment of abscisic acid (ABA) accumulation (Kwak et al., 2003). RBOHs are activated in a calcium dependent manner after phosphorylated (Kimura et al., 2012). With contribution of RBOH D, long-distance signalling of ROS can be realized in plants by a variety of stresses (Miller et al., 2009) propagating a ROS wave that is able to travel through the plant (Torres et al., 2006; Mittler et al., 2011) (see chapter 1.8 II. spatial priming). Additionally, apoplastic ROS are important to counter pathogenic infections (Doke, 1983; Auh and Murphy, 1995).

The peroxisomes are the largest contributor to cellular ROS (Foyer and Noctor, 2003; del Río and López-Huertas, 2016). Synthesis takes place by xanthine dehydrogenase and aldehyde oxidase (Yesbergenova et al., 2005), the acyl-CoA oxidases (Bonekamp et al., 2009) and the glycolate oxidase (Foyer et al., 2009). Peroxisomal ROS are discussed to be an important factor for cellular ROS signalling and involved in pathogen infection and senescence leading to organized cell death (del Río et al., 2006).

Round about 1% of the electrons from the mitochondrial electron transport chain are transferred to oxygen (Raha and Robinson, 2000). Programmed cell death (PCD) is accompanied by the release of cryptochrome c from mitochondria and a decrease in cellular adenosine triphosphate (ATP). Both processes are correlated with a rise in the levels of mitochondrial ROS (Rhoads et al., 2006). Fungal infection in oak coincides with a burst of ROS at rupture zones of mitochondria, playing an important role to induce apoptosis (Yao et al., 2002).

In chloroplasts ROS are mainly produced via the Mehler reaction (Mehler, 1951). Chloroplasts generate more than 20 times more H_2O_2 than mitochondria (Foyer and Noctor, 2003). Estimations are that 10% of the total electrons transported through the photosynthetic electron transport

chain under non-stressed conditions are transferred to oxygen (Ivanov and Khorobrykh, 2003). This makes the chloroplast a potent producer of reactive oxygen species in plant cells. Even ROS produced via the glycolate oxidase in peroxisome are connected to the chloroplast, where glycolate is produced during photorespiration.

1.3 Environmental stimuli inducing ROS

ROS are produced under all kinds of environmental stresses (Sewelam et al., 2016). Especially under laboratory conditions the morphology, metabolism and regulatory circuits are adjusted to the stable control conditions, in which the plants are grown. If the familiar environmental conditions change or plants are infected with pathogens higher amounts of ROS are produced (Kaushik and Aryadeep, 2014).

ROS can be produced actively upon pathogen attack to encounter a threat to the plant (Torres et al., 2006) or to adjust plant morphology to the new stress (Gapper and Dolan, 2006). Under most other stresses ROS are mainly produced as a sheer by-product of aerobic metabolism or by the PET chain. Even though, in these cases unwanted, the elevated ROS production can transduce information for signalling (Torres et al., 2006; Mittler et al., 2011), development (Kwak et al., 2003) and act as defensive molecules (Doke, 1983; Auh and Murphy, 1995).

Under stress conditions the plant metabolism is strongly affected (Shulaev et al., 2008; Obata and Fernie, 2012). During stresses with an osmotic component (e.g. temperature, drought and light stress) the synthesis of compatible osmolytes is induced to retain a positive cell turgor and allow water up-take (Verslues and Juenger, 2011). Osmotic stress initiates stomatal closure (Daszkowska-Golec and Szarejko, 2013). If the stomata are closed, the CO_2 levels decrease in cells and in the intracellular space in the spongy mesophyll cells. With decreasing carbon dioxide (CO_2) levels the net rate of photosynthesis decreases (Farquhar and Sharkey, 1982). The lower the amount of CO_2, the higher is the chance for the ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBisCO) to function as an oxygenase instead of a carboxylase. The emerging P-glycolate must be regenerated by photorespiration to be able to produce hexoses from it (Peterhänsel et al., 2010). The oxygenase function of RuBisCO is also affected by temperature and oxygenation is more likely to take place at higher temperatures (Peterhänsel et al., 2010). Photorespiration

increases the levels of reactive oxygen species in peroxisomes due to the conversion of glycolate to glyoxylate (Voss et al., 2013).

Due to the physical property of membranes, membrane associated proteins are more sensitive to temperature stress, than soluble ones (Suzuki and Mittler, 2006). This partly uncouples the PET chain from the CO_2 fixing pathway in chloroplasts, giving excited electrons the chance to be transferred to molecular oxygen caused by temperature changes (Suzuki and Mittler, 2006). Additionally to the slowdown of the enzymatic processes of photosynthesis by low temperature stress, the absorption of light by chlorophylls is not affected. The excitation energy of the excited electron is normally channelled through the PET chain, under low temperature conditions PET chain related enzymes are slowed down. Thus electrons are relocated to O_2 instead of being used for the Calvin-Benson cycle (Ensminger et al., 2006).

1.4 Cold stress

Cold stress impacts the plant on different biological levels. It strongly limits the maximal distribution of a species (Woodward, 1988) and the agricultural performance (Cramer et al., 2011).

Cold affects the life cycle and development of plants. It does e.g. affect the architecture of leaves (Atkin et al., 2006). It increases leaf thickness, the number of cell layers and decreases the cell size (Atkin et al., 2006). A variety of temperate plant species flower only after they have experienced a longer cold period (Henderson and Dean, 2004). Plants arrest growth under chilling conditions, which is connected with accumulation of salicylic acid (SA) (Scott et al., 2004). This is thought to relocate resources from growth to increase cold tolerance. The primary metabolism is reprogrammed during cold stress and free sugars, mainly sucrose, accumulate (Guy et al., 1992; Stitt and Hurry, 2002; Zuther et al., 2015). In parallel the secondary metabolism is de-regulated. The effects include phytohormone regulation. For example gibberellic acid (GA) levels increase in cold treated seeds and break seed dormancy (Penfield et al., 2005). GA levels are decreased in plants with C-repeat/DRE binding factor 1 (CBF1) -over-expression in rosette stage (Achard et al., 2008). Furthermore *Gossypium hirsutum* (cotton) CBF1, conferring chilling tolerance, was inhibited by GA-treatment (Shan et al., 2007). All major phytohormones can be regulated by cold

stress and help to shape the cold response or adaptation (reviewed in Eremina et al. (2016)). Another example for rearrangements in the plants secondary metabolism is the accumulation of flavonols under cold conditions, probably with the function to stabilize membranes (Schulz et al., 2015).

Besides architectural und metabolic parameters being impaired by cold stress, physical parameters are changed. Temperature below and above optimal conditions affect the kinetic of enzymes. Most enzymes do not have a wide temperature optimum that would allow undisturbed function under cold stress (Peterson et al., 2007). Membranes decrease their fluidity with decreasing temperature (Vigh et al., 1993; Vaultier et al., 2006), if not encountered by desaturation of existing fatty acids (Kodama et al., 1994). By the introduction of a fatty acid desaturase from a chilling-resistant cyanobacterium into a chilling-sensitive cyanobacterium increased the resistance to low temperature stress (Wada et al., 1990). Consistently, the stable induction of a fatty acid desaturase under a 35S promotor from cyanobacteria into tobacco increased freezing tolerance (Ishizaki-Nishizawa et al., 1996).

1.5 The ICE1 pathway to encounter cold stress

Chilling-tolerance divergence 1 (*COLD1*) in rice is essential for the regulation of the G-protein mediated influx of Ca^{2+} into the cytosol (Ma et al., 2015). It is located at the plasma membrane and in the endoplasmic reticulum (Ma et al., 2015). *COLD1* itself is hypothesised to be able to sense changes in the membrane fluidity and allow Ca^{2+}-influx. Whether or not Ca^{2+} regulates the *inducer of CBF expression 1* (*ICE1*) remains unclear. It is assumed that kinases bridge calcium signalling with ICE1 regulation (Chinnusamy et al., 2007). Calcium dependent kinases might play a role here, but so far no information are published. Ca^{2+} is known to accumulate in the cytosol of cold stressed plants (Knight et al., 1991). Cold acclimation alters the response of Ca^{2+} influx into the cytosol. Interestingly, the same response can be observed by exchanging the cold treatment after external application of hydrogen peroxide (Knight et al., 1996).

ICE1 itself is localized in the nucleus (Chinnusamy et al., 2003) and mostly regulated on protein level. The mRNA is present independently of cold or drought stress. ICE1 can be phosphorylated by open stomata 1 (OST1), which also supresses HOS1 (high expression of osmotically responsive

gene 1) (Ding et al., 2015). HOS1, an E3-ligase, ubiquitinates ICE1 and targets the transcription factor for proteosomal degradation (Dong et al., 2006; Kim et al., 2015). Additionally ICE1 can be sumoylated by the E3 SUMO-protein ligase SIZ1, which stabilises the protein under freezing conditions (Miura et al., 2007).

At the end of this long cascade, transduced via CBFs *cold regulated* (*COR*) genes are induced (fig. 1). They represent the actual functional proteins that are important to encounter chilling stress (Lissarre et al., 2010). *COR15A* is one of the *COR* genes. It is localized in chloroplasts and stabilizes membranes under freezing stress (Nakayama et al., 2007; Wang and Hua, 2009; Thalhammer et al., 2014; Thalhammer and Hincha, 2014; Wan et al., 2014).

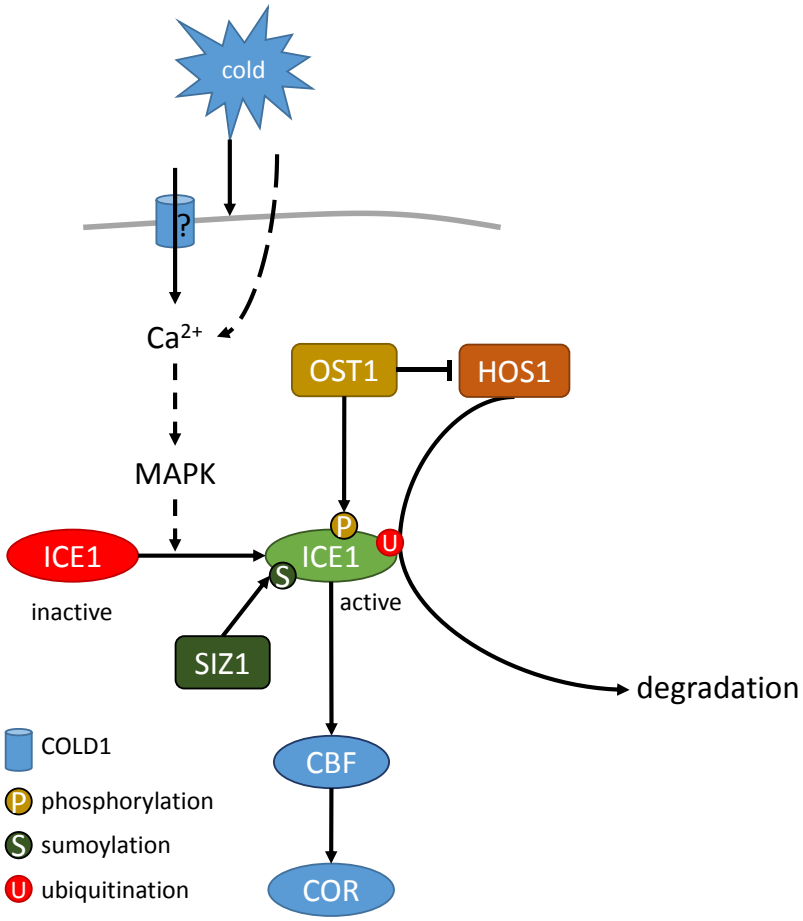

Figure 1: Model of the cold induction of *COR* genes. Cold decreases the membrane fluidity. Ca^{2+} influxes into the cytoplasm take place, possibly due to the function of a COLD1 homologue. Ca^{2+} activates the MAPK pathways. MAPKs activate already existing ICE1 protein. Activation is mediated by OST1 (phosphorylation) and SIZ1 (sumoylation). At the same time OST1 suppresses HOS1, which ubiquitinates ICE1 and targets it for proteosomal degradation. Active ICE1 induces the expression of *CBF* genes, which ultimately leads to an induction of *COR* genes. Modified after Lissarre et al. (2010).

Even though the logical order of ICE1-CBF-COR is discussed in most research article and reviews, the regulation of *COR* genes is not as simple. A recent publication showed that at least 25 % of *COR* genes are not only regulated by CBF transcription factors, but at least by one additional regulator such as heat shock transcription factor C1 (HSFC1) or salt tolerance zinc finger (ZAT10) (Park et al., 2015).

Cold stress does not only change kinetic properties of enzymes (Peterson et al., 2007), the membrane fluidity (Vigh et al., 1993; Vaultier et al., 2006) and hormonal signalling (Eremina et al., 2016), but also increases the production of ROS (see chapter 1.4 and O'Kane et al. (1996)). Because of the toxic nature of ROS, detoxification mechanisms need to be in place to reduce damage by ROS (Saed-Moucheshi et al., 2014).

1.6 Plastidic detoxification of ROS

Reactive oxygen species can be enzymatically detoxified by a variety of enzymes (fig. 2). Superoxide can be converted to hydrogen peroxide by superoxide dismutases (SOD) (Bowler et al., 1992). The existence of three superoxide dismutases is reported in chloroplasts: two iron dependent SODs (FSD2 and FSD3) and a copper dependent one (copper/zinc superoxide dismutase 2, CSD2) (Tsang et al., 1991). Under controlled growth conditions, the expression of FSD2 and FSD3 is negligible, only in case of copper depletion the expression of both isoform increases. If copper is available to the plant, CSD2 represents the major form of superoxide detoxification (Pilon et al., 2011). CSD2 is controlled on post-transcriptional level by the *miR398* (Sunkar et al., 2006).

In chloroplasts, hydrogen peroxide can be converted to water by ascorbate peroxidases, glutathione peroxidases, peroxiredoxins and thioredoxin peroxidases. Ascorbate peroxidases use ascorbate (asc) as an electron donor in the peroxide detoxification (Asada, 1999). In Arabidopsis, there are two genes coding for a stromal and a thylakoid-bound ascorbate peroxidase (Jespersen et al., 1997; Pitsch et al., 2010), while in tobacco both isoforms are generated from the same transcript by alternative splicing (Ishikawa et al., 1997). Both plastidic isoforms are highly sensitive to low ascorbate concentrations and are inactivated under such conditions (Miyake and Asada, 1996).

In chloroplasts, there are two glutathione peroxidases (GPX1 and GPX7) (Chang et al., 2009). For both plastidic GPXs, a role in fine tuning of the photooxidative stress response, signalling with respect to leaf morphology and immune defence has been suggested (Chang et al., 2009).

Peroxiredoxins (PRX) contain four subgroups of enzymes. The 1-Cys, 2-Cys, type II peroxiredoxins and PRXQ (Horling et al., 2002). 2-Cys peroxiredoxins (2CPs) are ubiquitously found throughout the tree of life (Baier and Dietz, 1996). They are highly expressed and contribute to protect the chloroplast from oxidative damage (Baier et al., 2000). As highly abundant H_2O_2 scavenging enzymes, the two 2CPs are thought to be involved in retrograde signalling (Baier and Dietz, 2005). PRXQ is found in the thylakoid lumen (Petersson et al., 2006). Overexpression of PRXQ supports plants to counter oxidative stress and fungal infections (Kiba et al., 2005). peroxiredoxin IIE (PRXIIE) is mostly expressed in reproductive tissue (Brehelin et al., 2003). Additionally, PRXIIE is transcriptionally down-regulated upon salt stress (Horling et al., 2002) and the protein accumulates during bacterial infection (Jones et al., 2004).

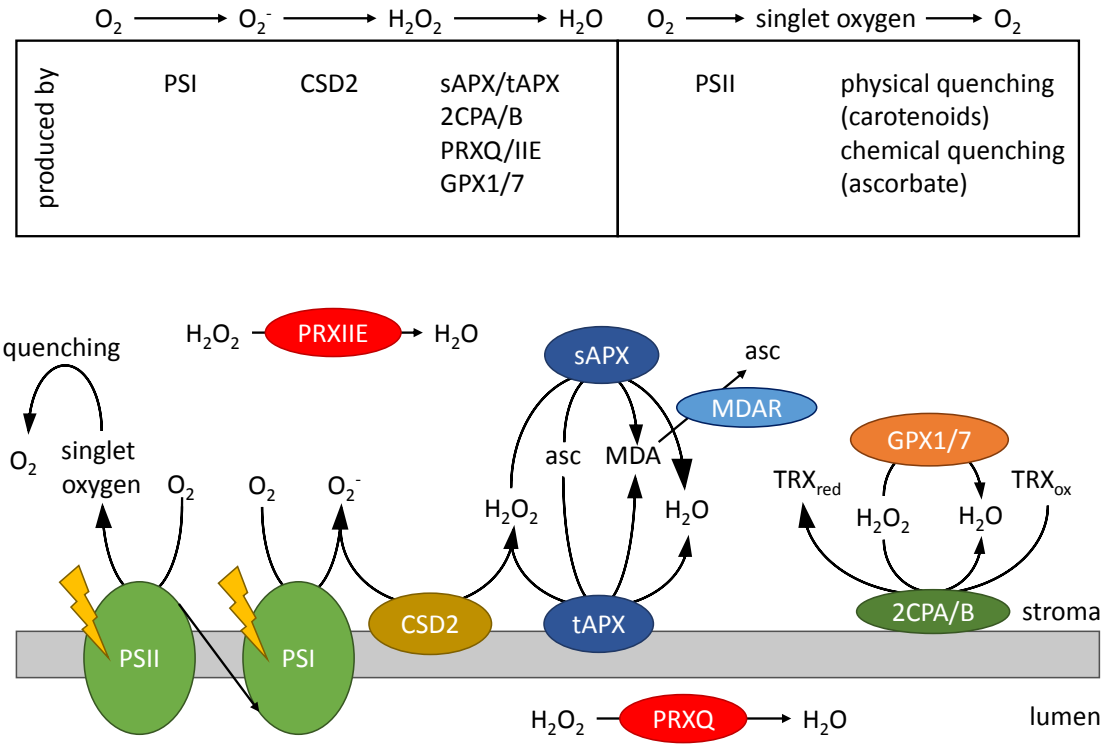

Figure 2: Simplified model of the plastidic antioxidant system. Photosystem (PS), cupper-zinc superoxide dismutase (CSD2), thylakoid bound/stromal ascorbate peroxidase (sAPX/tAPX), Peroxiredoxin Q (PRXQ), Peroxiredoxin IIE (PRXIIE), 2-Cys peroxiredoxin A/B (2CPA/B), glutathione peroxiredoxin 1/7 (GPX1/7), mondehydroascorbate (reductase) (MDAR), ascoarbate (asc), thioredoxin (TRX).

Two of the most important low molecular weight antioxidants in plants are ascorbate and glutathione (Noctor and Foyer, 1998). Ascorbate can accumulate to millimolar concentrations in plant tissue (Noctor and Foyer, 1998). 30 %–40 % of the total ascorbate is located in chloroplasts (Foyer et al., 1983). Despite its function in enzymatic reactions ascorbate can reduce free radicals spontaneously in aqueous solutions (Njus and Kelley, 1991). As ascorbate, glutathione can directly scavenge free radicals in aqueous solutions (Galano and Alvarez-Idaboy, 2011). Both, ascorbate and glutathione, are discussed to be involved in redox sensing and thus in transmitting information about the overall status of a plant to the nucleus to adjust nuclear gene expression (Foyer and Noctor, 2011).

1.7 Different ROS signalling pathways for chloroplast-nucleus signalling

Plants with a mutation in the *fluorescent in blue light* (*FLU*) gene are disturbed in a negative feedback loop in the tetrapyrrole biosynthesis. This results in the accumulation of protochlorophyllide in the dark (Meskauskiene et al., 2001). In the light, protochlorophyllide leads to an enhanced production of singlet oxygen. This leads to a rapid bleaching and ultimately to the death of the plants (op den Camp et al., 2003). If the *flu* mutant is grown in continuous light, it is viable (Meskauskiene et al., 2001). The protochlorophyllide is reduced to chlorophyllide, but the levels are constant and lethal level of protochlorophyllide cannot accumulate (Zhang et al., 2014). By modulation of the dark and light in rosette stage or older *flu* mutant plants, it was possible to specifically search for genes activated by singlet oxygen non-invasively. Via the *flu* mutant, 1206 genes were initially described to be responsive to singlet oxygen (op den Camp et al., 2003). In 2004, a screen to reverse the *flu* phenotype was successful. 15 mutations were found, which responded like wild-type to light-dark-light shifts. All mutations were found in one locus. This locus was named executer 1 (EX1) (Wagner et al., 2004). Wagner et al. (2004) could show, that the lethality in *flu* was not due to the toxic nature of singlet oxygen, but caused by activation of genetically controlled responses via singlet oxygen signalling. However, not all singlet oxygen induced responses were abolished in *executer1* mutants. A second gene, related to EX1 was found: executer 2 (EX2). As EX1, EX2 is localized in chloroplasts (Lee et al., 2007). A triple mutant *flu*/*ex1*/*ex2* nearly abolished the response of all genes normally induced by singlet oxygen in the *flu* mutant (Lee et al., 2007).

High concentrations of H_2O_2 protect plants during high light stress (Karpinska et al., 2000). During high light stress photoinhibition and photooxidative damage of PSII takes place. Thus, primarily singlet oxygen is produced (Laloi et al., 2007). Laloi et al. (2007) explained the protection of plants during high light stress by an antagonistic effect of O_2^-/H_2O_2 signalling on singlet oxygen signalling. This explanation was based on their and related findings by Karpinska et al. (2000). Karpinska et al. (2000) externally applied H_2O_2, while Laloi et al. (2007) used a tAPX overexpression line to reduce the H_2O_2 levels. The elevated H_2O_2 levels led to less singlet oxygen signalling (Karpinska et al., 2000), while the reduced level enhanced singlet oxygen levels (Laloi et al., 2007), measured by PCD and growth inhibition.

H_2O_2 is an important signalling molecule (Neill et al., 2002). In principle, H_2O_2 can pass membranes via aquaporins (Bienert et al., 2007) and reach the nucleus via the cytosol or by diffusion through stromules (Brunkard et al., 2015; Caplan et al., 2015). Once H_2O_2 reaches the nucleus it could modify redox sensitive transcription factors to impact nuclear gene expression, as it was shown for related to apetala 2A (RAP2.4A) (Shaikhali et al., 2008). Another discussed possibility is signalling by degradation products of oxidized proteins, however no such signals were described yet (Møller and Sweetlove, 2013). Additionally, the accumulation of H_2O_2 impacts on the redox potential of the chloroplasts. Changes in the redox potential can be used for retrograde signalling (Dietz and Hell, 2015; Noctor et al., 2016). However, no signalling components as EX1 and EX2 are known to determine H_2O_2 signalling in retrograde ROS signalling.

1.8 The plant memory

Memorizing requires the perception of a stimulus, the storage of the information and the ability to recall the stored information. The first incidents of perception, storage and recall in plants were found early in the twentieth century. The first review about the acquired physiological immunity in plants was published in 1933. Chester (1933) proposed an experimental schedule that consisted of the establishment of an infection of plant tissue (perception), a recovery from the primary infection, including a control that the plants react, in every aspect, as control plants before the second challenge (storage) and subsequently the determination of the degree of acquired immunity (recall). Thus, during the first infection something must change in

the plants recognition/signalling capabilities that induces the acquisition of immunity (Conrath et al., 2006). This process is called priming (review in Hilker et al. (2015)).

Whether the incident is called acquired physiological immunity (Chester, 1933) or priming (Conrath et al., 2006), it represents the question whether the plants experienced a related stress before and recall the information. If so, the response to the second stress stimulus (trigger) should be different to the one of naïve plants. No response would also be found if the priming stimulus is already 'forgotten'. Priming is generally defined to change the response to a second stimulus in dependence of the experience of an earlier stress and is beneficial if the two stress stimuli are related (Hilker et al., 2015).

Priming is consisting of two different aspects: a spatial and a temporal one. Even though a mixture of both aspects is common for most stresses.

I. *Spatial aspects of priming*

Not all stresses are acting on a global level, meaning not all stresses are perceived at the same time by the majority of or all plant cells. Cells that did not perceive the priming stimulus (warning queue) nevertheless show induced resistance after triggering. This phenomenon is mostly called systemic acquired resistance (SAR), as it is mainly examined in the field of phytopathology, taking advantage of the plant innate immune system (Conrath et al., 2015).

Ross (1961) reported that infection of half a leaf of tobacco with the tobacco mosaic virus for seven days leads to increased resistance in the other half of the leaf that was never in contact with the virus. Showing that a local infection can lead to immunity in distal plant tissue. The response of both local and systemic tissue can be described by an increased expression in *pathogen related* (*PR*) genes, that were first described by Van Loon and Van Kammen (1970). Furthermore SAR can be induced by treating plants with SA (White, 1979).

ROS signalling is discussed to be essential for the induction of SAR both on local and systemic level (Alvarez et al., 1998; Wang et al., 2014). One signalling component is well described in literature, the non-protein amino acid pipecolic acid (Pip) (Návarová et al., 2012). Pip accumulates in local

and distal tissue and more importantly in the petiole exudates. Mutants lacking Pip cannot induce SAR, a phenotype that can be rescued by externally applying the amino acid (Návarová et al., 2012). The response of Pip is independent of the SA response in SAR (Bernsdorff et al., 2015).

Systemic acquired resistance is not the only possible mechanism of spatial priming. A similar effect has been found for abiotic stresses and was named systemic acquired acclimation (SAA). Under controlled conditions the non-photochemical quenching (NPQ) of plants is highly variable. If single leaves are exposed to a high light treatment the NPQ of not high light treated plants synchronized the NPQ indicating a way of signalling and training non-stressed plant tissue (Karpinski and Szechynska-Hebda, 2010; Szechynska-Hebda et al., 2010). A most recent study shows that ROS are involved in the process of SAA to high light; mutations of apoplastic ROS producing enzymes (RBOH D/F) showed that apopastic ROS is required for the systemic signalling while tAPX function and executer 1/2 are important both for local and distal acclimation (Carmody et al., 2016). This kind of systemic signalling is not only taking place during high light stress, but is also existing for systemic acclimation to low temperature stress (Gorsuch et al., 2010).

One mechanism that could be the main force of this long distance signalling is the propagation of a H_2O_2 signal at the plasma membrane (Mittler and Blumwald, 2015). Already earlier, it was discussed that ROS-signals can move as a wave through the plant and by that spread locally acquired information systemically (Mittler et al., 2011). This kind of spatial distribution mechanism of information in plants enables them to integrate information coming from different parts of the plants, that might be contradicting and launch adequate responses to react optimally to the encountered needs. Even though the time scale is different to nervous stimulus conduction, the results indicate parallels.

II. *Temporal aspects of priming*

Most abiotic stresses are sensed by the plant with the majority of the tissue. They tends to be more uniform than biotic stress. Pathogen infections start at a single spot, if not by chance multiple independent infections occur at the same time. The situation is similar in herbivory. The herbivore starts consuming at one spot, maybe moving around while feeding on the plant tissue.

Thus, the signalling aspect of spatial priming is more important for biotic stresses to launch defences and maybe induce resistance.

Temperature stress reaches all the aerial tissues in similar intensities if the architectural design of the plants allows it. For example drought and salt stress in the soil are sensed first by the roots, after a time the signal reaches the leaves homogenously. High light falls on all of the tissues that are illuminated directly. Most research in the field of plant memory formation for abiotic stress was done elucidating the effect of time between two stimuli.

Temporal priming was already described for a range of abiotic stresses. For high temperature stress established experimental designs are available to test for thermomemory (Yeh et al., 2012). It could be shown that heat shock transcription factor A2 (HSFA2) is a heat inducible activator, which is essential to maintain heat tolerance over time (Charng et al., 2007). Later Stief et al. (2014) discussed the involvement of miRNAs as essential for thermomemory and discussed the role of the highly conserved *miR156*. The heat stress memory is also regulated on the level of chromatin structure. Maintaining the memory is associated with H3K4 methylation, which leads to a hyper-induction of heat stress related genes upon a reoccurring heat stress (Lamke et al., 2016).

According to heat stress memory, similar results for drought stress induced memory formation were found (Sani et al., 2013). A one day long stress treatment with NaCl allowed plants to survive two weeks of drought, even if both treatments were separated by as much as ten days. The tolerance went hand in hand with major changes in the chromatin structure of the plants. Regions of H3K27me3, associated with a lower transcriptional activity, were reduced in primed plants and these changes were kept over the ten days at control conditions, even though the produced gaps in the H3K27me3 landscape were subsequently ‘filled’ again. This might hint towards a mechanism of ‘forgetting’ that was demanded by Crisp et al. (2016).

1.9 Difference between priming and acclimation

Acclimation and priming are terms, used in the field of plant stress biology. Acclimation can be defined as a response induced by chronic changes of the environment (Woods and Harrison, 2002). Smith and Dukes (2013) used the term as well for biochemical and physiological responses of an individual to an environmental change. This is the same prerequisite as demanded by the definition of priming (Hilker et al., 2015).

If plants are tested for cold acclimation, standard procedures contain a step of acclimation at low, sub-lethal temperatures and an immediately following sub-zero temperature stress. This sub-zero stress is chosen in a way to be lethal for non-acclimated plants (Thomashow, 1999; Janska et al., 2010). During the acclimation process, free sugars and proline accumulate and protect plant cells against the freezing damage by preventing ice crystal formation in the cells (Steponkus, 1984; Uemura et al., 2003). In general, the beneficial effect of the non-lethal stress build up tolerance to a normally lethal stress. Recent findings show that the amount of free sugars is reduced to half within the first day after acclimation stopped, the same was observed for the accumulation of proline (Zuther et al., 2015). With the decrease in soluble sugars and proline, the lethal temperature 50 (temperatures, where 50 % of the ions leaked from the cells, LT50) decreased (Zuther et al., 2015). This shows that the effect that was build up by acclimation declines fast. The question arises, whether there is still a higher survival rate of acclimated plants, if the first stimulus (in this case acclimation) and the second stimulus (trigger by lethal temperatures) is separated by a longer period of time?

1.10 Cold priming

Cold, salinity and drought stress partly share common signals and signalling pathways (Xiong et al., 2002). For cold stress, it could be shown that a pre-treatment at 0 °C for one day lead to healthier plants, if the plants had to face freezing conditions three days later (Byun et al., 2014). Byun et al. (2014) proposed that the improved response to the freezing stress was related to strong induction in photosynthesis related genes during the phase in between the two stress stimuli. Additionally, it was found that the maximal photosynthetic quantum yield is higher in cold pre-treated plants compared to non-cold pre-treated plants after applying a cold trigger in winter

wheat (Li et al., 2014). Li et al. (2014) showed, that the total ascorbate peroxidase activity is higher if plants were pre-treated before the trigger was applied, although appropriate controls were absent in this publication. As already mentioned (chapter 1.4), ROS are produced during cold stress, mainly due to an imbalance of the excitation and consumption of exited electrons in the PET chain (Ensminger et al., 2006). The produced ROS can be partly detoxified by the plastidic antioxidant system (see chapter 1.6). ROS are important mediators of signals in plants (see chapter 1.7). Both, adjustments in the photosynthetic capacity (Byun et al., 2014; Li et al., 2014) and the increase in the total ascorbate activity (Li et al., 2014) link the formation of a cold memory to the ROS formation in chloroplasts. However, studies analysing the response of the plastidic antioxidant system in cold priming in-depth, and by this the limiting factor for ROS production, are missing.

1.11 Aim of the study

As already introduced, ROS are important signalling molecules (Chapter 1) and are produced in various organelles (chapter 1.2). The chloroplast is one of the most potent producers of ROS (Foyer and Noctor, 2003). Li et al. (2014) found that the ascorbate peroxidase activity in chloroplasts is increased in plants after two chilling stresses (priming and triggering), correlated with a lower accumulation of H_2O_2. What is missing in the study of Li et al. (2014) is the in-depth analyses of the response of the plastidic antioxidant system and the influence of the induced PAS capacity on ROS signalling.

The aim of this study is to characterize the response of the plastid antioxidant system during priming and to analyse to which extent repetitive cold stimuli impact on the retrograde ROS signalling. Analyses are made to quantify and categorize the response of the major enzymes of the plastidic antioxidant system (PAS) after the first cold stimulus (priming) and after the second one (triggering). To do so, a full factorial design was used to verify, that no changes were kept over the period of time between the two cold stimuli (lag-phase). The length of the used stresses was drastically decreased compared to Li et al. (2014) and Byun et al. (2014) to be able to focus on initial regulatory events of priming and not the overlap between priming and acclimation.

It will be evaluated whether the age of plants during the experimental course affects the ability to be primed (primability). Furthermore, the impact of the length of the time between the two stresses on priming will be elucidated. The specificity of priming will be addressed by using excess light stress as alternative trigger to chilling stress. Additionally, priming will be analysed in different natural accessions of *Arabidopsis thaliana* originating from different climates to evaluate the impact of adaption for priming.

All in all, this study will prove that cold priming is linked with changes in ROS signalling mediated by higher ROS levels in the chloroplast. It will analyse the role of the plastidic antioxidant system in the establishment of a priming effect in the retrograde ROS signalling pathway under chilling stress conditions.

2 Material and methods

2.1 Plant material, growth conditions and experimental design

The natural accessions (tab. 1), T-DNA lines and the GUS fusion line (tab. 2) were grown on Arabidopsis soil [70 volumes 'Topferde' (Einheitserde, Sinntal-Altengronau, Germany), 70 volumes 'Pikiererde' (Einheitserde, Sinntal-Altengronau, Germany), 25 volumes Perligran Classic (Knauf, Iphofen, Germany)] supplemented with 0.5 $g*l^{-1}$ dolomite lime (Deutsche Raiffeisen-Warenzentrale, Frankfurt am Main, Germany) in pots (6 cm diameter). The seeds were vernalized for three days in the dark at 4 °C on wet soil. Afterwards the pots were transferred to a growth chamber with a 10 h light/14 h dark growth regime (20 °C ± 2 °C/18 °C ± 2 °C, relative humidity of 60 % ± 5 %). Illumination was set to 100-110 µmol photons$*m^{-2}*s^{-1}$ supplied by L36W/840 Lumilux Cool White fluorescent stripes (Osram, Munic, Germany). Plants were separated to individual pots (6 cm diameter) at an age of 7-9 days. For pest control the plants were watered with 0.5 $g*l^{-1}$ Axoris Insekten-frei (COMPO, Münster, Germany) every three weeks.

Table 1: Name of used natural accessions, abbreviations and NASC IDs (http://arabidopsis.info/)

Name	**Abbreviation**	**NASC ID**
Columbia 0	Col-0	N1092
Moscow 0	Ms-0	N1376
Cape Verde Island 0	Cvi-0	N1096
Kashmir 1	Kas-1	N1264

Table 2: Name, stably inserted constructs and background of used mutant lines for this study

Name	**Construct**	**Background**	**Source**
$tAPX_{prom}$:GUS	pHGWFS7.0-$tAPX_{prom}$-GUS-GFP	Col-0	Cai (2014)
sapx (SALK_083737)	T-DNA insertion line (pROK2-T-DNA)	Col-0	Kangasjärvi et al. (2008)
tapx (SALK_027804)	T-DNA insertion line (pROK2-T-DNA)	Col-0	Kangasjärvi et al. (2008)

For the cold treatments, plants were transferred to a cold growth chamber set to constant 4 °C. The light intensity and light/dark cycle was similar to the standard growth conditions. For excess light treatments plants were illuminated with 250-300 µmol photons*m^{-2}*s^{-1}, representing 2.5 -3 fold higher light intensities, in a Percival CU 22L (CLF Plant Climatics, Wertingen, Germany). The illumination cycle and humidity were the same as those for standard growth conditions. All harvested plant material was immediately frozen in liquid nitrogen.

All treatments started 2 ½ hours after the onset of light. To exclude circadian effects, all samples were harvested at the same time day on experimental days. Primed only (P) plants experienced chilling stress at the age of four weeks, triggered only (T) plants at the age of four weeks and six days, while primed and triggered (PT) plants perceived both stimuli (fig. 7). Additionally, control (C, unstressed) plants were used for the study. The time in between the two cold treatments was called lag-phase (L). Unless stated otherwise the lag-phase was five days long.

2.2 Phenotypical measurements

2.2.1 Measurement of plant diameter

For habitus, analysis digital images were taken seven days after triggering with a Nikon digital camera D3100 (Nikon, Tokyo, Japan). The plant diameter was measured using the software ImageJ (http://imagej.nih.gov/ij/). All images were taken from above the plant.

2.2.2 Plant fresh and dry weight determination

The fresh weights of plants were determined using a XA105 DualRange scale (Mettler Toledo, Columbus, USA) seven days after triggering. For dry weight analyses, plants were dried for three days at 60 °C prior to their weight determination.

2.3 Chlorophyll determination

For the determination of the chlorophyll levels, plant material (6-13 mg) was homogenized in 1 ml 80% acetone supplemented with 1-3 crystals $CaCO_3$. Extraction was executed at -20 °C overnight in darkness. To avoid distortion of the measurements by particles, the plant material was sedimented for 5 minutes at 14.000xg. The chlorophyll contents and chlorophyll a/b ratio were determined according to Porra et al. (1989)

$$Chlorophyll\ total\ (chl\ tot)\ [\mu g * mg\ FW^{-1}] = \frac{17.76 * A_{646.6\,nm} + 7.34 * A_{663.6\,nm}}{weight\ [mg]}$$

$$Chlorophyll\ a\ (chl\ a)\ [\mu g * mg\ FW^{-1}] = \frac{12.25 * A_{663.6\,nm} - 2.55 * A_{646.6\,nm}}{weight\ [mg]}$$

$$Chlorophyll\ b\ (chl\ b)\ [\mu g * mg\ FW^{-1}] = \frac{20.31 * A_{646.6\,nm} - 4.91 * A_{663.6\,nm}}{weight\ [mg]}$$

$$Chlorophyll\ \frac{a}{b}\ ratio\ (chl\ a/b) = \frac{Chl\ a}{Chl\ b}$$

2.4 Ascorbate measurements

Total and reduced ascorbate levels were measured as described in Foyer et al. (1983) with minor modifications according to Queval and Noctor (2007). Ground plant material (40 mg) was extracted with 400 µl 0.2 M HCl. After spinning down the sample for 5 minutes at 13.000xg, 250 µl of the supernatant was supplemented with 85 µl 0.2 M NaH_2PO_4. The pH was adjusted to 5-6 using 220 µl 0.2 M NaOH. Two equal fractions were produced from the mixture. One fraction (200 µl) was used for the measurements of total ascorbate. 200 µl 0.2 M NaH_2PO_4 (pH 5.6) and 0.12 M NaH_2PO_4 (pH 7.5) were added to the 200 µl of the first fraction. Additionally 40 µl of 25 mM DTT was supplied to reduce mono- and dehydroascorbate to ascorbate. After 30 minutes of incubation at room temperature the concentration of total ascorbate was measured, by adding 1 U of ascorbate oxidase (AppliChem, Darmstadt, Germany) and analysing the decrease in A_{265nm} spectrophotometrically, relative to the plants fresh weight. The quantity of ascorbate was determined by an ascorbate calibration series.

To determine the concentration of reduced ascorbate, the second fraction (100 µl) was mixed with 520 µl 0.2 M NaH_2PO_4 (pH 5.6), 375 µl distilled water and 1 U of ascorbate oxidase (AppliChem, Darmstadt, Germany) and the A_{265nm} was recorded. The reduction state was calculated by setting the amount of reduced ascorbate in relation to the total amount of ascorbate.

2.5 Determination of H_2O_2

The assay for the determination of H_2O_2 was done as described in Gay et al. (1999). For the quantification of H_2O_2 about 50 mg of plant material was harvested and ground in liquid nitrogen. Hydrogen peroxide was extracted by adding 200 µl 5 mM KCN and mixing thoroughly. Insoluble plant material was sedimented for 15 minutes at 13.000xg and 4 °C. The supernatant was used for the assay.

A standard curve was generated in the range of range 0 - 500 µM H_2O_2 in water. 10 Volumes of the working solution (100 volumes reagent B and 1 volume reagent A) were mixed with 1 volume of the sample (or standard dilution). After 15 minutes at room temperature, the absorbance at 560 nm was measured. H_2O_2 concentrations were calculated based on the standard curve and standardized on the plants fresh weight.

Reagent A: 25 mM Ammonium ferrous (II) sulphate
2.5 M H_2SO_4

Reagent B: 100 mM Sorbitol
125 µM Xylenol orange

2.6 GUS methods

2.6.1 Histochemical staining

For histochemical staining, according to Jefferson (1987), plants containing the $tAPX_{prom}$:GUS reporter gene construct (Cai, 2014) were vacuum infiltrated twice for 1 minute with ß-glucuronidase (GUS) staining buffer and incubated until the blue colour had developed to the desired intensity. The stained plants were repeatedly incubated in 70% ethanol until the plant tissue was dechlorophyllized. Digital images were taken.

GUS staining buffer: 1 mM X-GlcA cyclohexylammonium salt
0.2 % (v/v) Triton X-100
1 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$
50 mM Na_2HPO_4/NaH_2PO_4 (pH 7.2)

2.6.2 Activity assay

GUS-activity was determined by extracting GUS from 30-50 mg plant material. Material was ground and subsequently supplied with 300 µl GUS extraction buffer. Residual plant material was sedimented for 5 minutes at 14.000xg. 10 µl of the supernatant were mixed with 500 µl GUS reaction buffer. The mixture was incubated for 1 hour at 37 °C. The reaction was stopped by adding 500 µl 400 mM Na_2CO_3. Absorption at 405 nm (coefficient ε=18.300 $l*mol^{-}1*cm^{-1}$) was determined. Values were calculated in comparison to samples, where the reaction was stopped immediately. GUS activity was normalized to the total amount of protein in the sample (see 2.7.2).

GUS extraction buffer: 50 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0)
10 mM DTT
0.1 % (v/v) Triton X-100
0.1 % (v/v) SDS
10 mM EDTA

GUS reaction buffer: 20 mg p-nitrophenyl-ß-D-glucuronide in 20 ml GUS extraction buffer

2.7 Protein analysis

2.7.1 Protein extraction

Protein was extracted from ground material. Protein extraction buffer was added (approximately 300 µl extraction buffer to 100 mg plant material), samples were vortexed and sedimented for 10 minutes at 4°C at 14.000xg. The supernatant was used for further analyses. Samples were stored at -20°C.

Protein extraction buffer: 330 mM Sucrose
25 mM Hepes-KOH (pH 7.4)
10 mM $MgCl_2$
10 mM NaF
0.1 % (v/v) Triton X-100
0.1 % (v/v) SDS

2.7.2 Protein determination

The precise concentration of proteins in the samples was measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, USA). The Bio-Rad Protein Assay is using the Bradford method (Bradford, 1976) to quantify protein amounts. The Protein Assay Concentrate was diluted 1:5 with distilled water. A standard curve ranging from 0 – 1 mg/ml bovine serum albumin (BSA) was measured to calculate the precise protein concentration of the samples. 1 ml of diluted Protein Assay Reagent was mixed with 10 µl of protein sample or standard. After 15 min the absorption at 595 nm was quantified and the protein concentration determined.

2.7.3 SDS-Page

Proteins were analysed on 12 % (v/v) gels according to Laemmli (1970). The separating gel was polymerized between two glass plates and, to provide a straight edge, overlaid with isopropyl alcohol. After the polymerization the alcohol was removed and the stacking gel casted. A comb was inserted prior to the polymerization to produce pockets for the samples.

The protein samples were diluted to a concentration of 1 mg/ml with protein extraction buffer. 1.25 µl of the protein loading buffer was added to 5 µl diluted sample. The samples were heated to 95 °C for 5 minutes and subsequently cooled down for at least 2 minutes on ice. The whole volume of the samples were loaded into the gel pockets.

Gels were run at 40 V as long as samples were located in the stacking gel. The separation gel was run for approximately 120 minutes at 140 V until the desired separation was achieved.

Separating gel: 12 % (v/v) Rotiphorese Gel (37.5:1)

0.375 mM Tris-HCl (pH 8.8)

0.01 % (w/v) SDS

0.01 % (w/v) APS

0.1 % (v/v) TEMED

ad H_2O_2 to 8 ml

Stacking gel: 5 % (v/v) Rotiphorese Gel (37.5:1)

0.1 mM Tris-HCl (pH 6.8)

0.016 % (w/v) SDS

0.016 % (w/v) APS

0.0016 % (v/v) TEMED

ad H_2O_2 to 3 ml

Protein loading buffer (5x): 625 mM Tris-HCl (pH 6.8)

50 % (v/v) Glycerol

20 % (w/v) SDS

0.025 % Bromophenol blue

25 % (v/v) distilled water

Added freshly before usage: 1/3 volume ß-Mercaptoethanol

SDS running buffer: 200 mM Glycine
25 mM Tris
0.1 % (w/v) SDS

2.7.4 Western blots

Western blots were conducted according to Kyhse-Andersen (1984). Gel-Blotting-Paper (Schleicher&Schuell, Düren, Germany) and nitrocellulose membrane (Roti-NC, Roth, Karlsruhe, Germany) of the correct size were soaked in transfer buffer. Three layers of soaked Gel-Blotting-Paper were placed on the anode. To avoid air bubbles between the layers the papers were flattened with a roller after each layer. The nitrocellulose membrane was placed on top of the papers. Next the separating gel was carefully placed on the membrane followed by three layers of pre-soaked Gel-Blotting-Paper. A current of 2 mA/cm^2 of nitrocellulose membrane was applied for 35 minutes to transfer the proteins onto the nitrocellulose membrane.

For the protein transfer control, the membranes were incubated for 1 minute in Ponceau S staining solution (0.2 % (w/v) Ponceau S in 3 % (v/v) acetic acid). Subsequently, they were washed with water to remove unbound Ponceau S. The membranes were scanned on a desk scanner. The signal of the large subunit of RuBisCO (rbcL) was quantified using ImageJ (http://imagej.nih.gov/ij/) and used for normalization of western blot signals. The staining was washed away with water afterwards to prepare the membranes for the detection of specific proteins with antibodies (chapter 2.7.5).

Transfer buffer: 25 mM Tris-HCl (pH 8.3)
150 mM Glycine
10 % (v/v) Methanol

2.7.5 Detection of a specific proteins using antibodies

The blotted membranes were incubated overnight in 5 % (w/v) milk powder in TBS-T. The antibodies were diluted in TBS-T supplied with 5 % (w/v) milk powder (αtAPX 1:1.000, horseradish peroxide-conjugated anti-rabbit antibody 1:10.000). For detection of tAPX an antibody against the thylakoid-bound ascorbate peroxidase lacking the chloroplast import signal and the transmembrane helix was used. The first (αtAPX) and second (horseradish peroxide-conjugated anti-rabbit antibody, Sigma-Aldrich, St. Louis, USA) antibody was incubated for at least 1 hour. After the treatments with the antibody solution the membranes were washed at least 3 times for 5 minutes with TBS-T. As developing agent Western Blotting Substrate Pierce ECL (Thermo Scientific, Waltham, USA) was used, which emits light if it comes in contact with horseradish peroxidase. The light emitting substance in the ECL reagent is luminol. Luminol can be oxidized under alkaline conditions bringing it on an excited state. To reach the ground state, energy is transmitted as a photon. Pictures were taken with the ImageQuantTM LAS 4000 Mini Biomolecular Imager (GE Healthcare, Little Chalfont, Great Britain).

TBS-T: 10 mM Tris-HCl (pH 7.5)
150 mM NaCl
0.05 % (v/v) Tween-20

2.8 Transcript analysis

2.8.1 RNA isolation

RNA was isolated from plant material frozen in liquid nitrogen using the Universal RNA Purification Kit (Roboklon, Berlin, Germany) according to the manufacturer's instruction. The kit is based on the usage of silica columns and the high binding efficiency of nucleic acids to silica matrixes. To remove bound DNA, a digestion by DNaseI (Fermentas, St. Leon-Rot, Germany) was conducted.

RNA quantity was assessed with a NanoPhotometer P300 (Implen, München, Germany), by determining the absorption ratios of the RNA A_{260nm}/A_{230nm} and A_{260nm}/A_{280nm}, which indicate salt,

protein and phenol contaminations, respectively. Samples with ratios <2 were excluded from the analysis and re-extracted. For intactness analyses, the RNA was evaluated on a 1 % agarose gel (casted with 1x MOPS), supplied with 2.5 % (v/v) formaldehyde to verify the absence of ribosomal RNA (rRNA) degradation. For this, 1 µg of RNA in 3 µl distilled water was incubated for 10 minutes at 65 °C together with 3 µl 6x DNA loading dye and 4.8 µl 6x RNA loading buffer to linearize the RNA. To avoid reformation of the RNA secondary structure the samples were immediately transferred on ice and cooled until they were loaded onto a gel.

1x MOPS: 200 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS)
10 mM Na-acetate
10 mM EDTA
The pH was adjusted to 7.0

6x DNA loading dye: 10 mM Tris-HCl (pH 7.6)
60 % (v/v) Glycerol
60 mM EDTA
0.03 % (w/v) Bromophenol blue

6x RNA loading buffer: 60 % (v/v) Formamide
18 % (v/v) Formaldehyde
15 % (v/v) 10x MOPS
1.2 % (w/v) Ethidium bromide

2.8.2 Reverse transcription

Complementary DNA (cDNA) synthesis was done using the High-Capacity cDNA Reverse Transcription Kit from Life Technologies (Life Technologies, Ober-Olm, Germany) as described in the manual. The kit uses the MultiScribe™ MuLV reverse transcriptase to produce single stranded cDNA from RNA samples. As primer for the MultiScribe™ MuLV, oligodT_{16} primer were used (oligodT: $T_{16}V$) instead of the supplied random hexamer primers, to exclude cDNA synthesis from rRNA and transfer RNA (tRNA). The cDNA was tested for contaminations with genomic DNA (gDNA) by PCR using intron spanning primer for *BasOH* (*2CPA*, AtBas OH1 forward:

ATCACTCCTTCCTTGTCG, AtBas OH4 reverse: GACTTTACTTTCGTCTGC) and evaluating the size of the product. cDNA had a size of 285 bp while gDNA was 585 bp long due to two incorporated introns.

2.8.3 Quantitative reverse transcription real-time PCR

The transcript abundances of genes were determined using quantitative reverse transcription real-time PCR (qRT-PCR). Each reaction contained 16 mM ammonium sulphate, 100 mM Tris-HCl (pH 8.3), 0.01 % (v/v) Tween-20, 2 mM $MgCl_2$, 0.1 mM of each dNTP, 1x SYBR Green (Sigma-Aldrich, St. Louis, USA), 0.01 U/µl OptiTaq Polymerase (EURx, Gdansk, Poland), 30 µM of each primer (forward and reverse) and 50 ng cDNA in a total volume of 20 µl. Each biological replicate was measured in technical triplicate.

After a 3 minute long initiation period of the qRT-PCR at 95 °C 40 PCR cycles were executed consisting of: DNA denaturation at 95 °C for 15 seconds, primer annealing at 60 °C for 30 seconds and a DNA-elongation step at 72 °C for 30 seconds. A melting curve was recorded at the end of the qRT-PCR by increasing the temperature at a rate of 0.5 °C from 60 °C – 95 °C with a length of 5 seconds for each temperature change. The fluorescence was quantified after each step. A C1000 Thermal Cycler with a CFX96 Real-Time System (Bio-Rad, Hercules, USA) was used to execute the analysis. The cycle threshold value (Ct-value) was determined using the single threshold method of the Bio-Rad CFX Manager 3.1 (Bio-Rad, Hercules, USA).

Primers were designed using the QuantPrime software (Arvidsson et al., 2008). If applicable, primers were designed to be intron spanning (tab. 3).

Table 3: Primer sequences for the expression level determination used in this study. Grey highlighted genes were used as reference genes for transcript abundance normalization

Annotation	***AGI code***	**forward**	**reverse**
2CPA	At3g11630	CCCAACAGAGATTACTGCCT	ATAGTTCAGATCACCAAGCCC
2CPB	AT5G06290	TCATACCCTCTTCCTCGGCATC	ACCGACCAGTGGTAAATCATCAGC
ACT2	At3g18780	AATCACAGCACTTGCACCAAGC	CCTTGGAGATCCACATCTGCTG
APL3	AT1G27730	AAACCGAGAAGTGCCGGATTG	GTTGGATGCTGCATTCTCCCAAG
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
ORE1	AT5G39610	CTTACCATGGAAGGCTAAGATGGG	TTCCAATAACCGGCTTCTGTCG
PAL1	AT2G37040	GCAGTGCTACCGAAAGAAGTGG	TGTTCGGGATAGCCGATGTTCC
sAPX	At4g08390	AGAATGGGATTAGATGACAAGGAC	TCCTTCTTTCGTGTACTTCGT
tAPx	AT1G77490	GCTAGTGCCACAGCAATAGAGGAG	TGATCAGCTGGTGAAGGAGGTC
YLS8	AT5G08290	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT
ZAT10	AT1G27730	TCACAAGGCAAGCCACCGTAAG	TTGTCGCCGACGAGGTTGAATG

2.9 Statistical tests

For statistical analyses Microsoft Office Excel (Student's t-Test) and SPSS 22 (ANOVA (Tukey's HSD)) were used. For both tests a p-value <0.05 was set to determine significance.

3 Results

The plastidic antioxidant system is essential for the survival of plants. Its capacity to detoxify reactive oxygen species allows plants to counteract accumulation of ROS and thus reduce oxidative damage to DNA, proteins and lipids (Schieber and Chandel, 2014). The PAS consists of a variety of different antioxidant enzymes (see chapter 1.6). Besides the ROS being toxic, they also have beneficial aspects functioning as signaling molecules (Laloi et al., 2007; Choudhury et al., 2013). By modifying the composition of the various enzymes, the plants can control the plastidc ROS signature. This allows to transmit information about the status of the plant between a primary sensor of stress (photosynthesis) and the nucleus (Pfannschmidt, 2003; Foyer and Noctor, 2009). Here the role of the PAS in ROS signalling is investigated with special attention to memory formation in respect of chilling stress.

3.1 H_2O_2 accumulation under short term chilling stress

When plants are shifted from standard growth conditions to stress conditions and the delicate balance of plant metabolism is interfered with, higher amounts of reactive oxygen species are produced (Mittler, 2002). Chilling stress generates an imbalance between the excitation pressure generated by photosynthesis and the release of this pressure by correctly adjusted electron transport and metabolic usage of the excitation energy (Ensminger et al., 2006). In cold, the transfer and usage of excitation energy of the photosynthetic electron transport is lower, due to the deceleration of metabolic processes (ap Rees et al., 1988), especially the phosphate supply for photophosphorylation of the RuBisCO is limited (Sage, 2002). Upon strong excitation of the photosynthetic electron transport chain, excited electrons are transferred to molecular oxygen giving rise to ROS (Ensminger et al., 2006; Foyer and Noctor, 2009).

To estimate the net oxidative burden induced by a short (24 h) chilling stress and the production of ROS that is necessary for signalling, the accumulation of H_2O_2 was measured directly after priming and one day after (fig. 3).

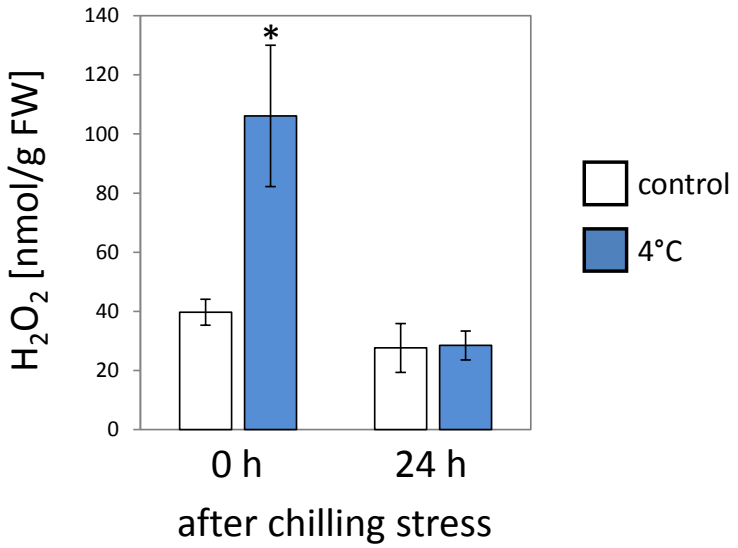

Figure 3: H_2O_2 levels directly and 24 h after a short (24 h) chilling stress at 4 °C in Col-0. n=10±SD, * Student's t-test p<0.05

The accumulation of H_2O_2 under chilling stress was 2.5 fold higher compared to control conditions (fig. 3). This showed that the disturbance triggered by the chilling stress was strong enough to lead to oxidative imbalances between production and consumption of H_2O_2. One day under control conditions was sufficient to reduce the amount of H_2O_2 back to control levels (fig. 3).

3.2 Ascorbate levels under short term chilling stress

Ascorbate is one of the main low molecular weight antioxidant molecules and the most abundant one (Mittler, 2002; Foyer and Noctor, 2009). It can spontaneously convert H_2O_2 to H_2O or be used by enzymatic reactions by ascorbate peroxidases for the H_2O_2 reduction (Asada, 1999, 2006). Ascorbate represents roughly 10 % of soluble carbohydrate under control conditions, this is in some cases more than chlorophyll (Noctor and Foyer, 1998). To exclude that the reduction in H_2O_2 after the short chilling stress (fig. 3) was due to an increase in ascorbate, the total amount and the percentage of reduced ascorbate were analysed (fig. 4).

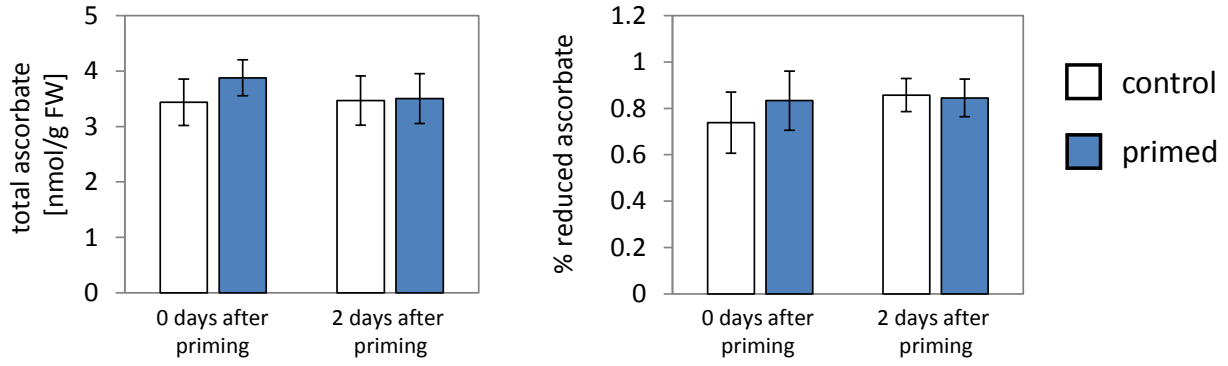

Figure 4: Total amount and the reduction state of ascorbate directly and two days after a short (24 h) chilling stress in Col-0. n=3±SD* Student's t-test $p<0.05$, no significant changes were found

Statistically significant changes were neither found for the amount of total nor for the percentage of reduced ascorbate. A slight trend for an increase directly after the chilling stress could be observed (fig. 4). A different response was described for priming with a longer chilling stress (14 days), which led to an induction of ascorbate (Cvetkovic, 2016; Juszczak et al., 2016). This suggests, that the reduction in H_2O_2 (fig. 3) was not caused by an induction of ascorbate to spontaneously convert hydrogen peroxide to water, but by an increase of enzymatic ROS detoxification.

3.3 The reaction of the plastidic antioxidant system under short term chilling stress

ROS accumulation can be antagonized by the biosynthesis of low molecular weight antioxidants and increased PAS activity (Asada, 1999; Mittler, 2002; Asada, 2006). One day at 4 °C was too short to induce substantial changes in the ascorbate content (fig. 4). Due to this, the reaction of the PAS to the chilling stress induced imbalance of ROS production and consumption was analysed (fig. 5).

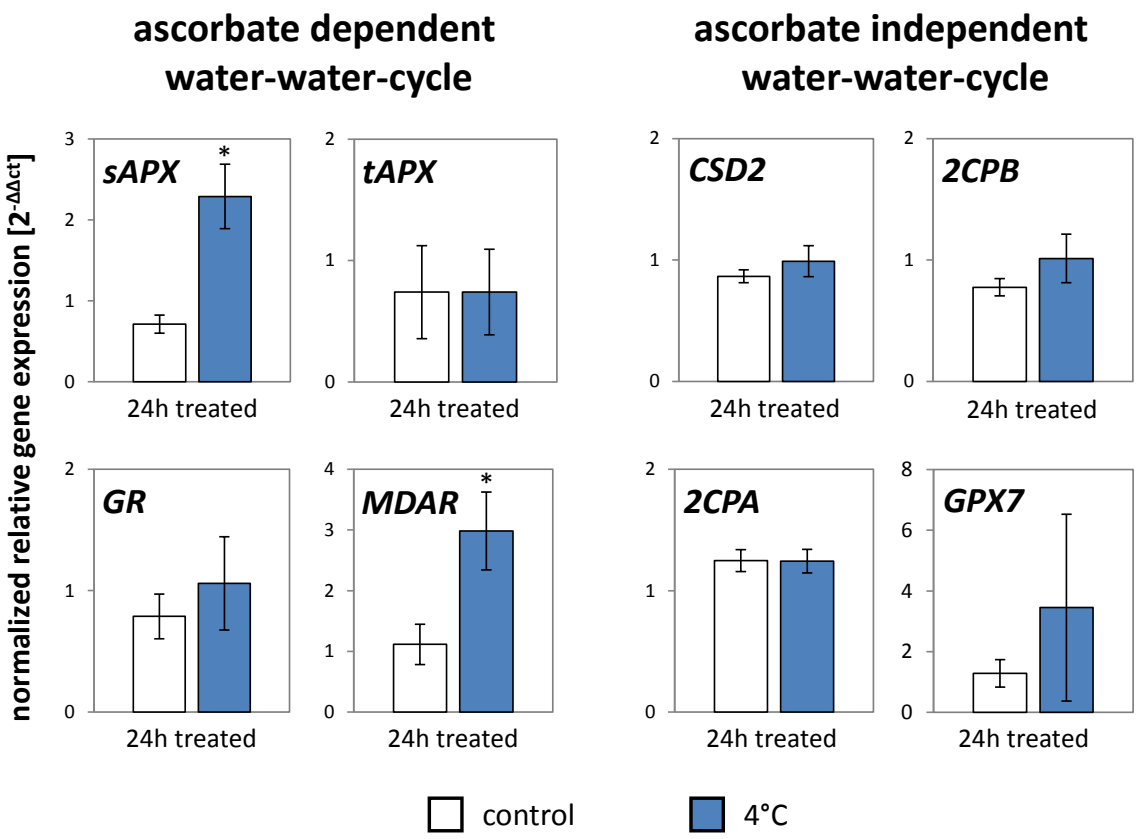

Figure 5: Transcript levels of the plastidic antioxidant system to a short (24 h) chilling stress (4 °C) in Col-0. * Student's t-test p<0.05

The plastidic isoforms of two enzymes of the ascorbate dependent water-water cycle were responsive to chilling stress. The transcript levels of the *stromal ascorbate peroxidase* (*sAPX*) and the *monodehydroascorbate reductase* (*MDAR*) were strongest responsive and more than two fold induced (fig. 5). Ascorbate peroxidases detoxify hydrogen peroxide to water at the expense of ascorbate, monodehydroascorbate (MDA) is formed. The other cold responsive enzyme, MDAR, recycles MDA to ascorbate (asc), making it again available for ascorbate peroxidases (APXs) and direct H_2O_2 detoxification. The transcript levels for the thylakoid bound ascorbate peroxidase (*tAPX*) did not show any cold induced increase in transcript abundance, demonstrating that the APX regulation was isoform-specific.

A trend for stronger induction was also observed for the *glutathione peroxide 7* (*GPX7*), *glutathione reductase* (*GR*) and *2-Cys peroxiredoxin B* (*2CPB*), indicating induction of various genes for PAS enzymes. The regulation, however, was not significant due to high variation in the

response levels. *Cu-Zn-superoxide dismutase 2* (*CSD2*) and *2-Cys peroxiredoxin A* (*2CPA*) were not affected after 24 h chilling stress at 4 °C, like *tAPX*, demonstrating that these genes were less sensitive to cold.

3.4 Expression levels of genes of the plastidic antioxidant system after the end of chilling stress

To analyse the persistence of cold inducible gene regulation, the transcript levels of the PAS genes were quantified one and five days after the stress, when plants were grown under standard conditions (fig. 6).

The post-cold response varied among the investigated genes. The induction of *sAPX* and *MDAR* subsided one day after the chilling stress, with a modest tendency towards upregulation in *MDAR*. The transcript abundance of *2CPA* was still unchanged. The *2CPB* and *GPX7* transcript levels were back to pre-cold conditions one day after the chilling, demonstrating attenuation. The transcript levels for *GR* were transiently downregulated. *CSD2* was slightly up-regulated 24 h after the chilling stress, demonstrating post-cold regulation. *tAPX*, which was also not regulated by cold, showed continuous transcript accumulation on the days after chilling stress, while transcript levels decreased slightly in control plants due to developmental regulation.

Notably, *tAPX* still had elevated mRNA levels, compared to untreated control plants five days after the initial chilling stress. This was contrary to M*DAR* expression, which was continuously decreased. This showed that *MDAR* and *sAPX* were highly correlated, while *tAPX* regulation was isoform-specific also after the chilling stress ended.

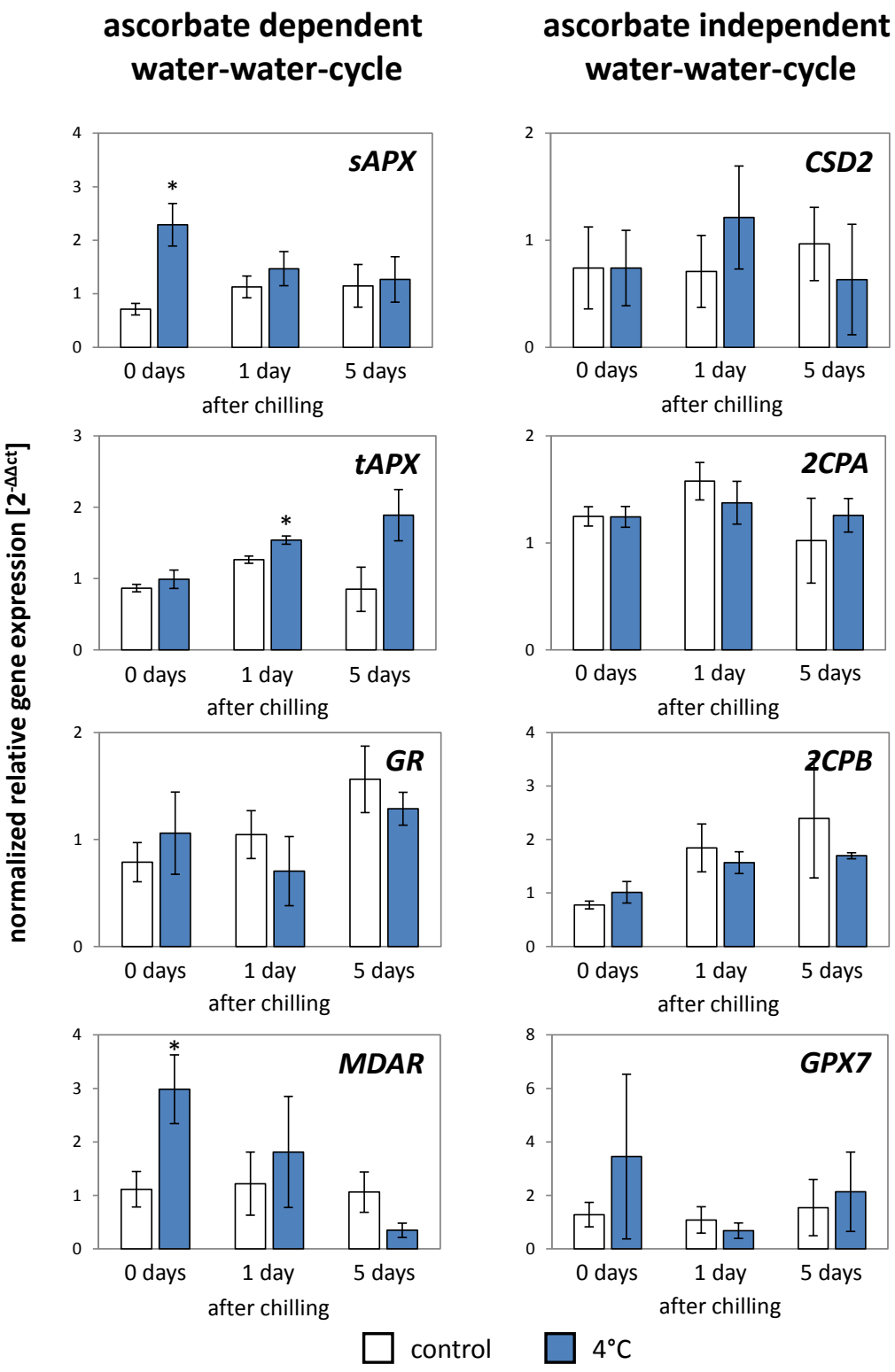

Figure 6: Transcript levels of genes of the plastidic antioxidant system to a short (24 h) chilling stress (4 °C), one and five days after in Col-0. n=3±SD, * Student's t-test p<0.05

3.5 The response of the plastidic antioxidant system to a second chilling stress

Priming is a phenomenon, where the prior experience of a stress induces changes in a plant's properties. These changes are beneficial when the plant is challenged by a similar stimulus after a period of time under controlled conditions (Bruce et al., 2007; Conrath et al., 2015).

To test whether the PAS is primable, stressed and non-stressed plants were exposed to a second identical chilling stress five days after the initial one. The two identical stresses led to four different combinations in a full factorial design. Plants either got stressed by the initial stress pulse (called priming) or not. Furthermore, plants could experience a second cold stress stimulus (triggering). At the time-point after triggering, plants could have never experienced stress (control, C), just the initial stress stimulus (primed only, P), only the second chilling stress (triggered only, T) or both cold pulses (primed and triggered, PT) (fig. 7).

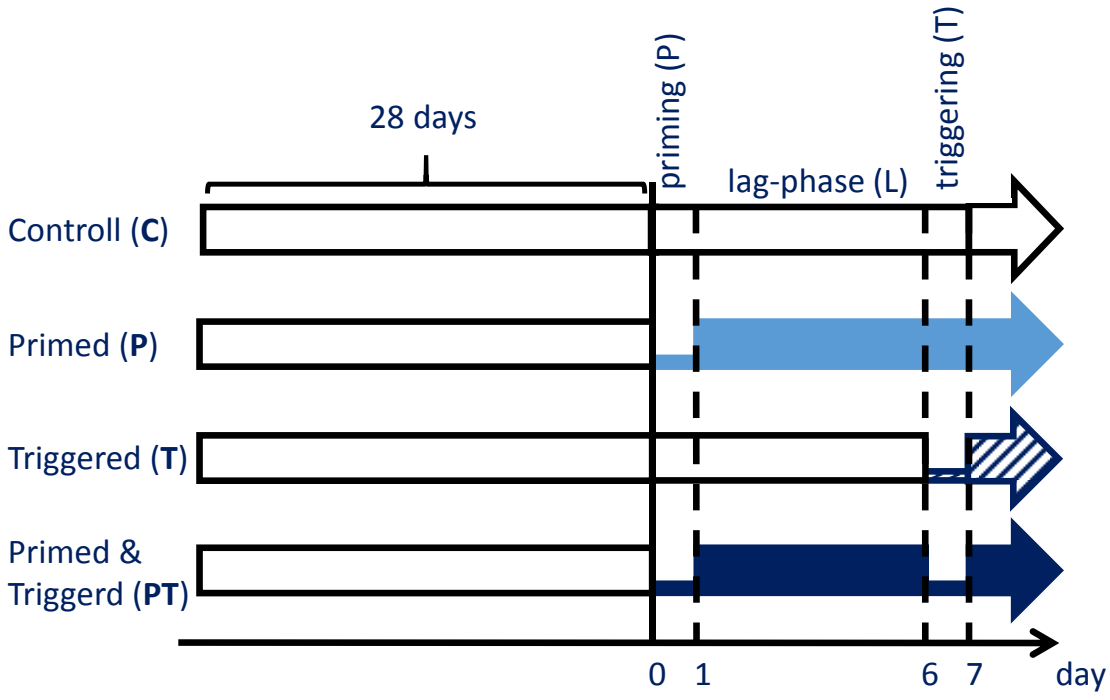

Figure 7: Experimental design used in this study with the four different treatments. Wide bars represent a temperature of 20 °C/18 °C (light/dark), small bars symbolize treatments at 4 °C/4 °C (light/dark)

The response of triggered plants after triggering resembled the one of primed plants after priming

The response of naïve plants after triggering were similar to the one directly after priming (fig. 5 and 8). *sAPX* and *MDAR* were up-regulated. The expression level of the thylakoid bound isoform of APX was again not affected by chilling stress. Also *CSD2* and *2CPA* did not respond after priming as well as after triggering in naïve plants. As before no significant changes were observed for *2CPB*, *GPX7* and *GR*. Of those three genes, only in *GR* was the trend for a slight up-regulation consistent after priming and after triggering. The minor changes in *2CPB* and *GPX7* could not be reproduced by the triggering stress. The similarity of the responses in T plants after triggering and P plants after priming of the PAS genes demonstrated that the majority of PAS gene regulation was not affected by developmental changes due to the shift of the chilling stress by five days.

The response of naïve and cold treated plants to the triggering stimulus

sAPX and *MDAR* were responsive to the triggering stimulus (compare C, T and PT plants, fig. 8). The transcript level was back to normal levels in case of *sAPX* and slightly induced levels in case of *MDAR* one day after the triggering. All the other tested genes were not responsive to the second chilling stress stimulus. *tAPX* transcript levels were induced two fold directly before the triggering stimulus was applied (fig. 6). Triggering reset the transcriptional response of *tAPX*, the transcript abundance directly after triggering was the same for all stress combinations. In primed only plants elevated, but not statistically significant, mRNA accumulation could be quantified in comparison to C plants. The thylakoid-bound ascorbate peroxidase could not be induced by chilling stress (fig. 5 and 8). But again, one day after the triggering stimulus was applied a slight induction of *tAPX* on transcriptional level was measurable, as it was the case after priming. This response was more pronounced if plants got primed before the triggering (fig. 8).

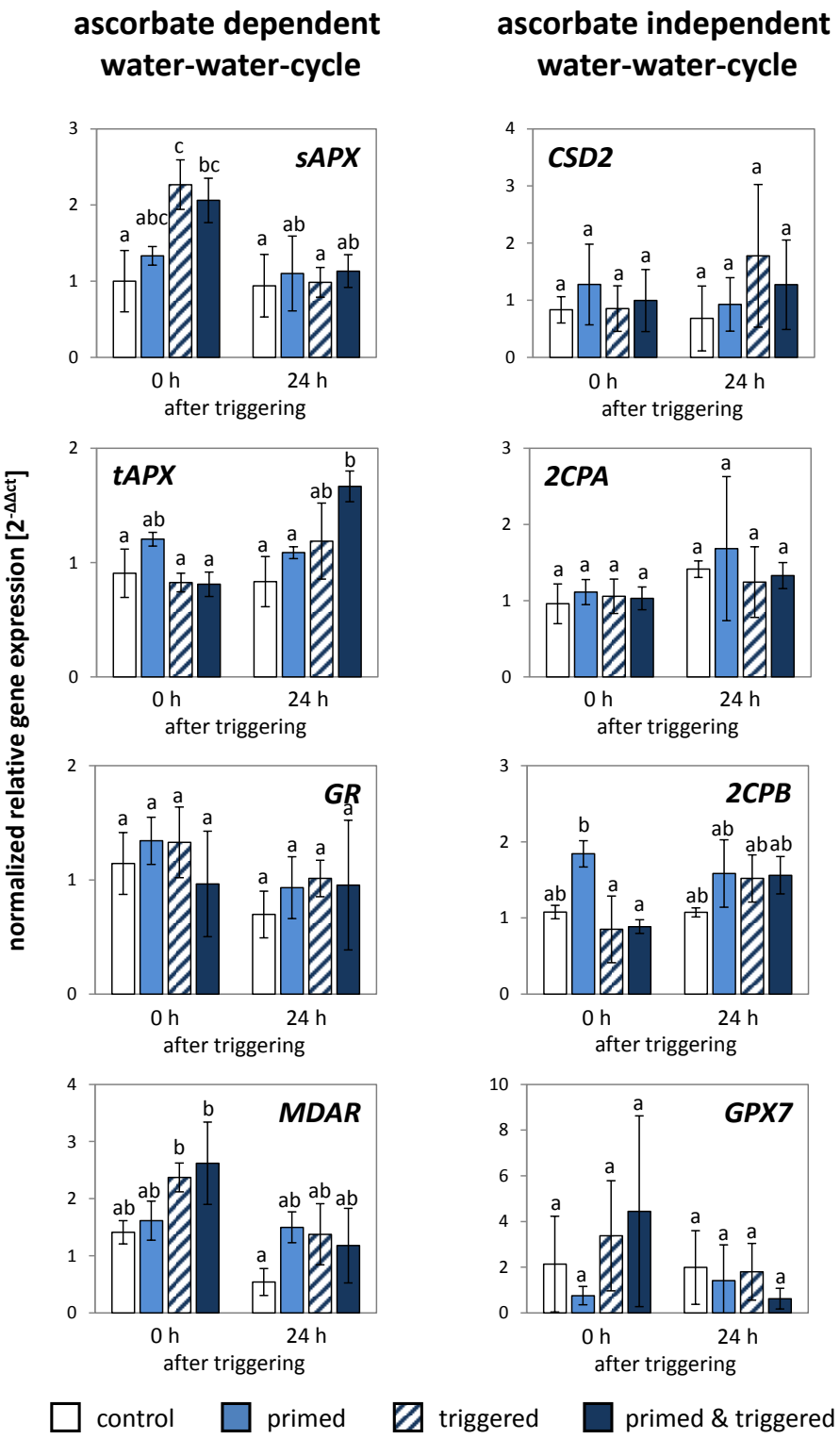

Figure 8: Response of the plastidic antioxidant system triggering (24h, 4°C) with all four possible combinations of stress/non-stress plants. n=3±SD, ANOVA (Tukey's HSD) p<0.05

The reaction of the PAS could be classified in three groups

Directly after triggering, the mRNA amounts of T and PT plants were similar for all testes genes, independent of the pre-treatment, demonstrating that none of the PAS genes was priming sensitive. Even the difference in *tAPX* transcript levels directly before triggering were reset by triggering rather than promoted. Either the genes did not respond to cold at all (e.g. *2CPA*) or they were responsive to cold, but did not show a difference in mRNA abundance between the T and PT treatment (e.g. *sAPX*).

In general, the genes involved in plastidic ROS detoxification could be grouped in three different classes:

- Non responsive genes

The majority of genes did not show changes after triggering for the different combination of stress treatments. *2CPA*, *CSD2*, *GR* and *2CPB* belonged to this group. The transcript abundances were kept almost unchanged during and after chilling stress. Even for *GR*, which showed a small tendency for an upregulation after priming, the changes were not significant. *2CPB* was slightly down-regulated directly after the triggering (T and PT) and slightly up-regulated after priming. One day after triggering expression levels normalized again. For *GPX7* an induction after priming was indicated, but internal variation was too high for the differences to be statistical significant.

- Cold responsive genes

sAPX and *MDAR* were both induced after chilling stress. The same trends were observed directly after priming and after triggering. Upon chilling stress transcript levels were increased. After the chilling stress stopped transcript abundance was nearly back to control levels within 24 h. This shows that both genes were responsive to chilling stress, but not primable.

- Delayed r*e*sponsive gene(s)

Directly under chilling stress conditions expression of *tAPX* was not altered. But after priming, transcript abundance increased one day after (fig. 6 and 8) and was kept elevated for five to six days (fig. 7 and 8). After triggering the delayed response was not as pronounced as after priming. This might reflect the developmental stage of plants and be a hint, that the reaction of the

ascorbate peroxidases are strongly affected by age and/or developmental status (Panchuk et al., 2005).

3.6 ROS marker genes showed different expression patterns upon triggering

Chilling induces ROS accumulation (Mittler, 2002; Juszczak et al., 2016). Here, 2.5 fold higher levels of H_2O_2 were detected after a 24 h long chilling stress (fig. 3). ROS are generated in all cellular compartments including the apoplast (Tripathy and Oelmüller, 2012). Measuring the precise levels organellar ROS is not possible (Noctor et al., 2016). To monitor the ROS signalling responses originating in the chloroplast, two marker genes were analysed, namely *salt tolerance zinc finger* (*ZAT10*) and *BON association protein 1* (*BAP1*) (Laloi et al., 2006). Both are connected to ROS signals coming from the chloroplast, but involved in different signalling pathways:

- ***ZAT10*** (AT1G27730) is a zinc finger transcription factor located in the nucleus and was shown to be responsive to salt and cold stress (Sakamoto et al., 2000; Sakamoto et al., 2004). ZAT10 activates expression of *APX1* and *APX2*. Induction of these extra-plastidic antioxidant enzymes helps to counteract oxidative stress upon a wide range of stresses (Mittler et al., 2006). *ZAT10* shows high correlation with *ZAT12* expression (Mittler et al., 2006), which plays a role in oxidative stress and is thought to decrease *CBF* induction (Davletova et al., 2005). ZAT12 also controls *APX1* transcription (Rizhsky et al., 2004). As with ZAT12, ZAT10 overexpression leads to higher transcription of oxidative and light stress inducible genes (Davletova et al., 2005; Mittler et al., 2006).
- ***BAP1*** (AT3G61190) encodes a small protein of 22 kDa with a calcium-dependent phospholipid-binding C2 domain that interacts with bonzai1 (BON1) (Hua et al., 2001; Yang et al., 2006). Together BAP1 and BON1 inhibit the hypersensitive response (HR) upon pathogen infection and programmed cell death (PCD) upon treatment with hydrogen peroxide (Yang et al., 2007). *BAP1* is under the control of the cold activated transcription factor ICE1 (Zhu et al., 2011). Even very small temperature changes lead to *BAP1* induction (Zhu et al., 2011). *BAP1* was found to be induced early in the *flu* mutant (Meskauskiene et al., 2001). *flu* lacks a feedback inhibition on the tetrapyrrolbiosynthesis and allows

protochlorphyllide accumulation after dark/light transition (op den Camp et al., 2003). As such, *BAP1* is responsive to increasing singlet oxygen levels.

By comparing *flu* mutants after a dark/light shift with *flu* mutants grown under continuous light that were treated with methyl viologen (MV), Laloi and co-workers (2006) showed that singlet oxygen induces a specific set of genes, which are different to the genes responsive to superoxide and hydrogen peroxide. *BAP1* was found to be upregulated by dark/light shifts, but not in the MV treatment, pointing out that it is predominantly responsive to singlet oxygen. In the same study, *ZAT10* was shown to respond mainly to superoxide/hydrogen peroxide and just to a minor extent to singlet oxygen. These two genes allow to deduce an induction of plastidic ROS upon stress treatments, furthermore they grant insights into the different response pathways of ROS signalling (Laloi et al., 2004).

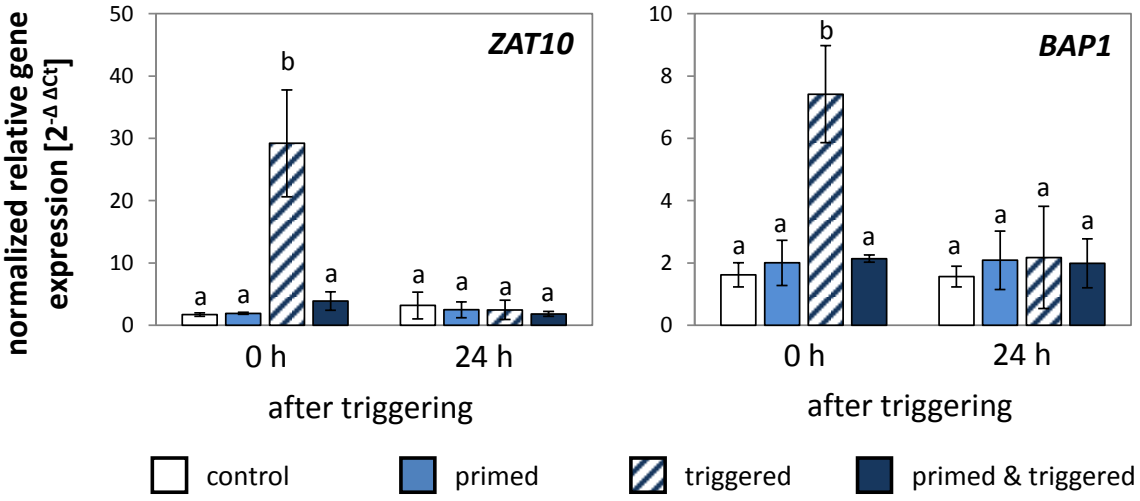

Figure 9: Relative normalized expression of *ZAT10* and *BAP1* in Col-0 directly and 1 day after triggering. n=3±SD, ANOVA (Tukey's HSD) p<0.05

In Col-0, both marker genes were induced strongly after triggering, if plants perceived chilling stress for the first time (T plants). On the other hand, primed and triggered plants displayed an expression as control plants did, indicating a different ROS accumulation/signalling pattern ultimately leading to different expression profiles of *BAP1* and *ZAT10* (fig. 9).

3.7 COR15A was non priming responsive

COR15A inhibits protein aggregation under freezing stress (Nakayama et al., 2007) and stabilizes membranes (Thalhammer et al., 2014; Thalhammer and Hincha, 2014). Over-expression of *COR15A* and *CBF3* in eggplant led to enhanced chilling stress tolerance (Wan et al., 2014).

In this study, *COR15A* was used as a cold marker gene to prove that cold was perceived by plants after chilling stress treatments. After triggering, *COR15A* transcript abundance differed between cold and non-cold treated plants (fig. 10, C and P vs. T and PT). As such *COR15A* represents the response of a cold inducible, but non-primable gene. All responses found were very transient; one day after the transfer from chilling back to standard growth conditions inductions were abolished.

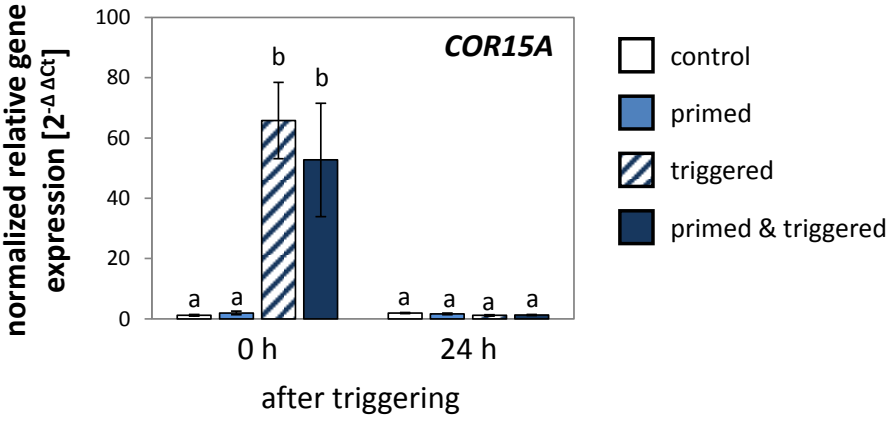

Figure 10: Relative normalized expression of *COR15A* in Col-0 directly and 1 day after triggering. n=3±SD, ANOVA (Tukey's HSD) $p<0.05$

The contrasting response of *BAP1* and *ZAT10* on the one and *COR15A* on the other hand depict specific impacts of priming on signalling after chilling stress. While the ROS-responsive genes were primable, the cold-inducible gene *COR15A* was not.

3.8 Chilling stress did not affect the phenotype of treated plants

In this study, investigation of the regulatory effects underlying the formation of memory and the consequences of priming have been analysed. Short (24 h) 4 °C cold treatments were chosen for their mildness, to establish an experimental setting, where the focus was on regulatory

mechanisms and not acclamatory events or harsh stress side effects appearing due to the stress. To analyse plants for an impact of growth conditions on the whole plant, morphologically related parameters were determined seven days after triggering in C, P, T and PT plants:

3.8.1 Growth

The short stress pulses caused no significant changes on fresh and dry weight of the plants (fig. 11). Variations between individual plants were larger than the ones between the treatments, demonstrating, that the treatments did not cause severe alteration in growth. Groups of plants that were cold triggered (chilling stress seven days before the measures were taken) had lower standard deviations for fresh and dry weight, compared to C and P plants. This implies that the chilling stress synchronized growth of individual plants and superimposed weak environmental cues that lead to high variation between individuals. A small growth defect could be seen seven days after triggering if plants were treated with two chilling stimuli (fig. 11).

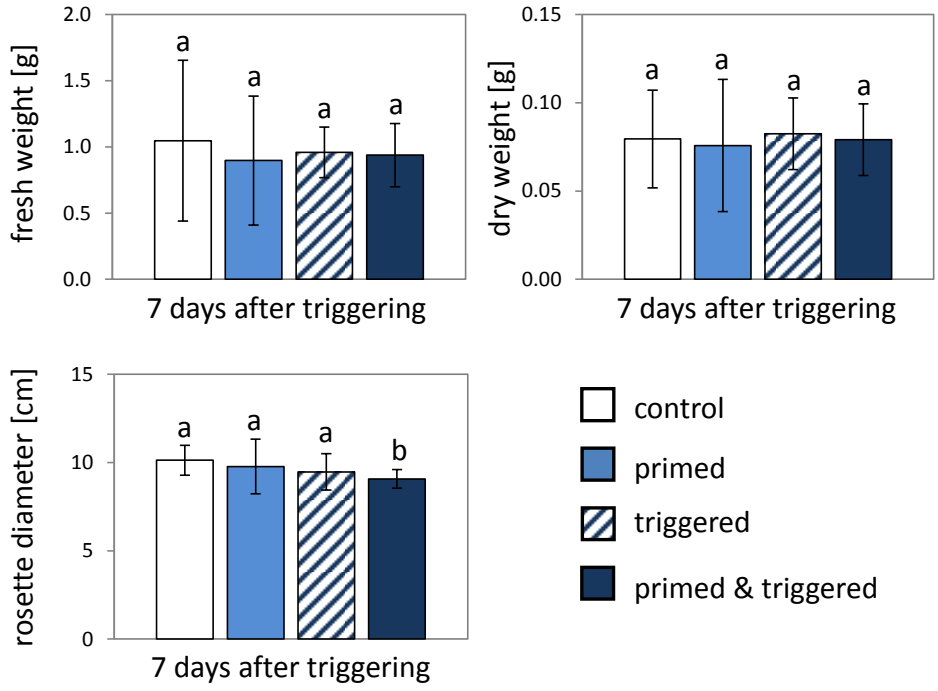

Figure 11: Fresh weight and dry weight and rosette diameter of Col-0 plants measured seven days after triggering. n=20±SD (2 independent propagations), ANOVA (Tukey's HSD), $p<0.05$.

When plants are transferred for longer time periods to non-optimal chilling conditions, growth is widely arrested. At the beginning, the arrest is mainly due to a large decrease in net photosynthesis per unit area (Atkin et al., 2006). During prolonged cold, the plants increase the growth rate again due to acclimation, which leads to changes in the plant metabolome and to changes in already existing and newly emerging leaves (Atkin et al., 2006). Additionally salicylic acid (SA) accumulates when plants perceive cold (Scott et al., 2004). In plants with lower levels of a SA hydroxylase growth at 5 °C is partially restored, demonstrating the impact of SA on growth. Application of external SA leads to retarded shoot growth and decreased leaf epidermal cell size (Dat et al., 2000; Scott et al., 2004). After 10 days under chilling conditions photosynthesis is re-established and the enzymatic activity of e.g. RuBisCO is even higher than under control conditions (Strand et al., 1999). Savitch et al. (2001) found that three days were not sufficient to acclimate plants to chilling conditions to support photosynthesis to the level of cold acclimated plants. Strengthening again the fact that the observed changes in the PAS regulation (chapter 3.3-3.5) were not influenced by the induction of acclimation.

3.8.2 Chlorophyll

To analyse the plants for an effect on photosynthesis, chlorophyll levels and their ratio were recorded as an indicator for changes in photosystems and light harvesting complexes (fig. 12 and 13).

Within minutes after a transfer to chilling conditions, a re-localization of light harvesting complexes (LHC) takes place, a process called state transition (Minagawa, 2011). This is required to adjust photosynthetic electron transport and to balance the excitation pressure of photosystem II and photosystem I (Ensminger et al., 2006). In cold treated pine the chlorophyll levels decline within 24 h, while in wheat no changes are observed, demonstrating species-specific regulation. The chlorophyll (chl) a/b ratio is reported to not change in various species (Savitch et al., 2002). Consistent with Savitch et al. (2002) only minor changes concerning the chlorophyll a/b ratio could be monitored through-out the experiment (fig. 12).

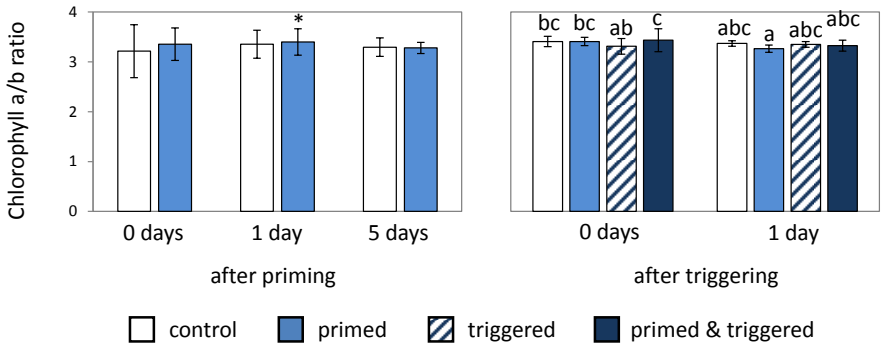

Figure 12: Ratio of chlorophyll a to b after priming (left) and triggering (right) in Col-0. n=30±SD (3 independent propagations) after priming Student's t-test * p<0.05 between P and C, after triggering ANOVA (Tukey's HSD), p<0.05

A slight decline in chlorophyll a and b was determined after priming and due to this in total chlorophyll. After triggering no general trend could be observed (T slightly up-regulated and in PT slightly down compared to C, fig. 13).

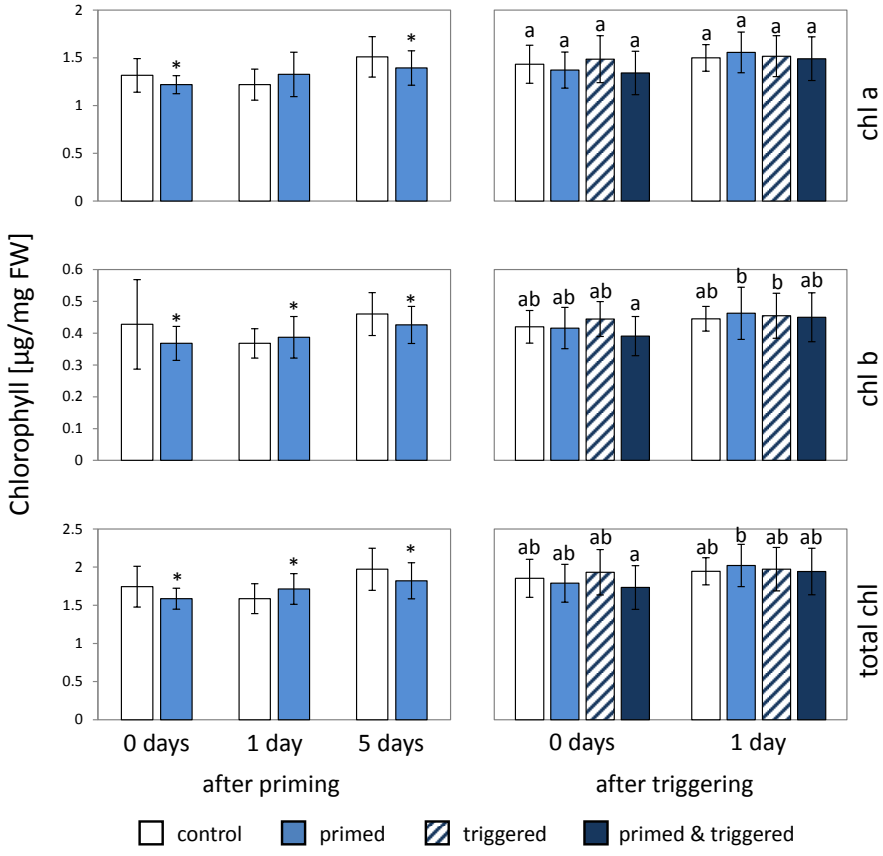

Figure 13: Chlorophyll levels (chl a, chl b, total chlorophyll) as time course over the experiment, left side after priming, right side after triggering in Col-0. n=30±SD (3 independent propagations) after priming Student's t-test * p<0.05 between P and C, after triggering ANOVA (Tukey's HSD), p<0.05

3.8.3 Ascorbate

After priming, no significant changes in the total amount and percentage of reduced ascorbate could be quantified (fig. 4). To analyse the effect of triggering on the ascorbate status of the plants, the total amount and percentage of reduced ascorbate were measured after triggering (fig. 14).

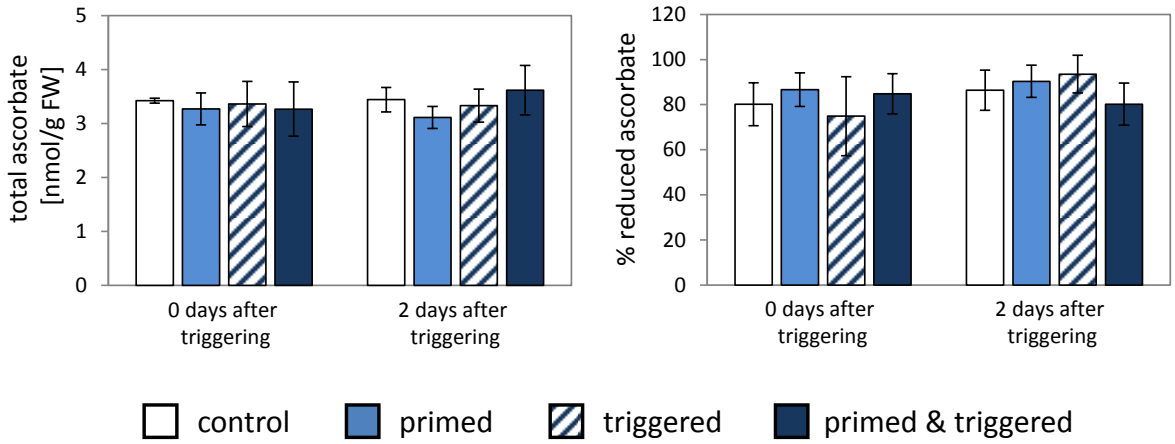

Figure 14: Total ascorbate amount for plants throughout the experiment (top) and percentage of reduced ascorbate (bottom) in Col-0. n=3±SD, ANOVA (Tukeys' HSD), $p<0.05$, no significant changes were found

No statistically significant changes were found for ascorbate levels, because of the mild treatments used in this study (fig. 4 and 14). This further confirms, that no strong changes were induced, such as shifting plant metabolism in the direction of stress acclimation (Zuther et al., 2015). Even though ascorbate is dependent on the supply of carbon skeletons, this is unlikely to be playing a role in the lack of induction of ascorbate as observed here (fig. 4 and 14). Under control conditions the production of glucose by photosynthesis is calculated to be three magnitudes higher than the need to replace ascorbate (Bulley and Laing, 2016). This would speak for a decrease of ascorbate under chilling conditions, before the activity of RuBisCO is adjusted to the changed conditions (Strand et al., 1999). But after one day at chilling conditions (priming) a slight trend was found for an increased total amount and percentage of reduced ascorbate, respectively (fig. 4), demonstrating, that a sufficient supply of carbon skeletons was guaranteed. The slight up-regulation of ascorbate levels could not be made after triggering (fig. 14).

In summary the data showed, that there was:

- no effect on the growth parameters fresh and dry weight and rosette diameter,
- no strong effects on chlorophyll levels or the chl a/b ratio and
- no changes in ascorbate levels neither in the amount nor the percentage of reduced ascorbate.

This suggests that the limitation of *ZAT10* and *BAP1* induction by triggering (fig. 9) was changed due to a different response in primed and triggered plants and not caused by changes in the general status of the chloroplasts, but a result from regulation caused by priming.

3.9 *tAPX* was induced during the lag-phase

On transcript level the strongest change in PAS regulation was the long lasting induction of *tAPX* after priming (fig. 6). Transcript accumulation can be caused by higher promotor activity or higher mRNA stability such as by decreased degradation. A *tAPX* promotor GUS-GFP fusion line was used to assess promotor activity during and after the first chilling stress (fig. 15).

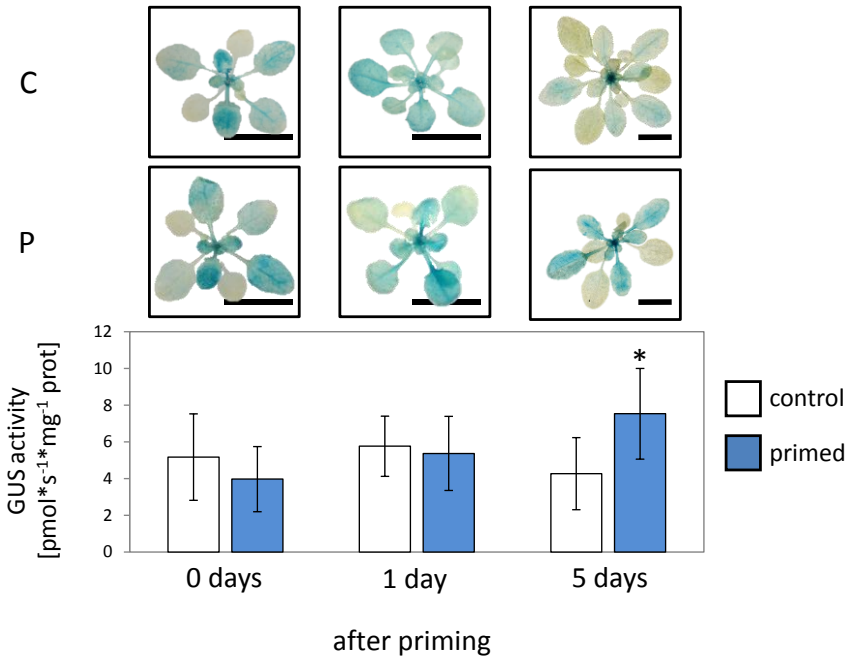

Figure 15: Promotor activity measurement in a stable *tAPX*$_{prom}$:GUS-GFP fusion line (Col-0 background). GUS staining (top) and GUS activity assay (bottom) were conducted. Black bars represent 1 cm. Statistics for the GUS activity assay n=20±SD (2 independent propagations) was analysed by Student's t-test * p<0.05

Priming decreased the promotor activity slightly upon chilling stress. The promotor activity increased again to control level within the next 24 hours. Five days after the initial chilling stress treatment, the promotor activity of *tAPX* was significantly higher as compared to non-treated plants. This showed, that the *tAPX* mRNA accumulation five days after priming (fig. 6) can be explained by stronger promotor activity. The distribution over the rosette was the same in C and P plants (fig. 15 top). Consequently, differences in GUS activity were caused by higher activity and not by a larger area with GUS activity in primed plants.

To analyse whether the *tAPX* accumulation on mRNA level had an actual impact on the functional level, the protein abundance was quantified. The protein is ultimately responsible for the detoxification of H_2O_2. A polyclonal antibody against the mature protein of tAPX detects tAPX and its close homologue sAPX (<88% homology) and to a minor extent the cytosolic isoforms of the ascorbate peroxidases. To quantify tAPX over the experimental course, equal amounts of total protein were analysed by Western blots (fig. 16). For quantification, the protein level was standardized on that of the large subunit of the RuBisCO (rbcL), which is the most prominent plastidic protein.

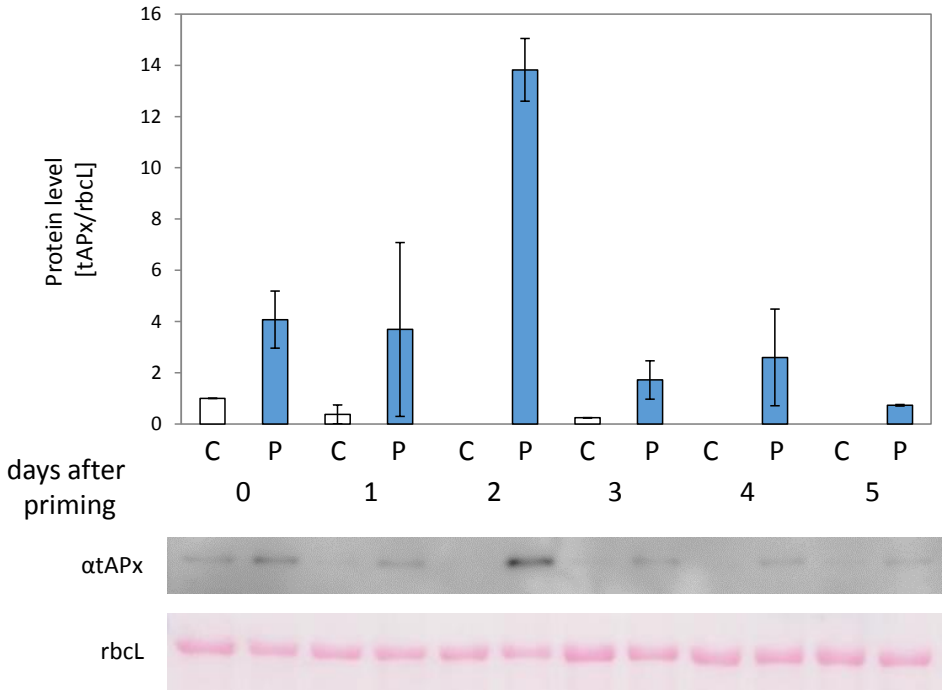

Figure 16: Mean of Western blots of tAPX, in relation of the specific αtAPX antibody to the amount of rbcL (top). Exemplary western blot and Ponceau S staining for detection of rbcL (bottom). 5 µg total protein were loaded, n=4±SD

Primed plants had higher protein levels of *tAPX* during the entire lag-phase. The difference was strongest two days after priming. Accumulation of tAPX altered the composition of the chloroplast antioxidant system. This resulted in different levels of tAPX at the time when the plants were triggered. This demonstrates that the capacity of ROS detoxification was increased at the thylakoid membrane. Higher tAPX-mediated ROS-detoxification could have antagonized the induction of *ZAT10* and *BAP1* after triggering (fig. 9). This hypothesis was subsequently tested in knock-out lines of the plastidic ascorbate peroxidases.

3.10 The priming response of *ZAT10* was altered in plastidic ascorbate peroxidase knock-out lines

If *tAPX* was responsible for changes in *ZAT10* transcript abundance after triggering, the response of the plastidic ROS marker gene should be different in tAPX knock-out lines (*tapx*). The expressional response in *tapx* lines was analysed. As a control, a line with a dysfunction in the expression of the close homologue of *tAPX*, namely *sAPX* (*sapx*), was investigated (fig. 17).

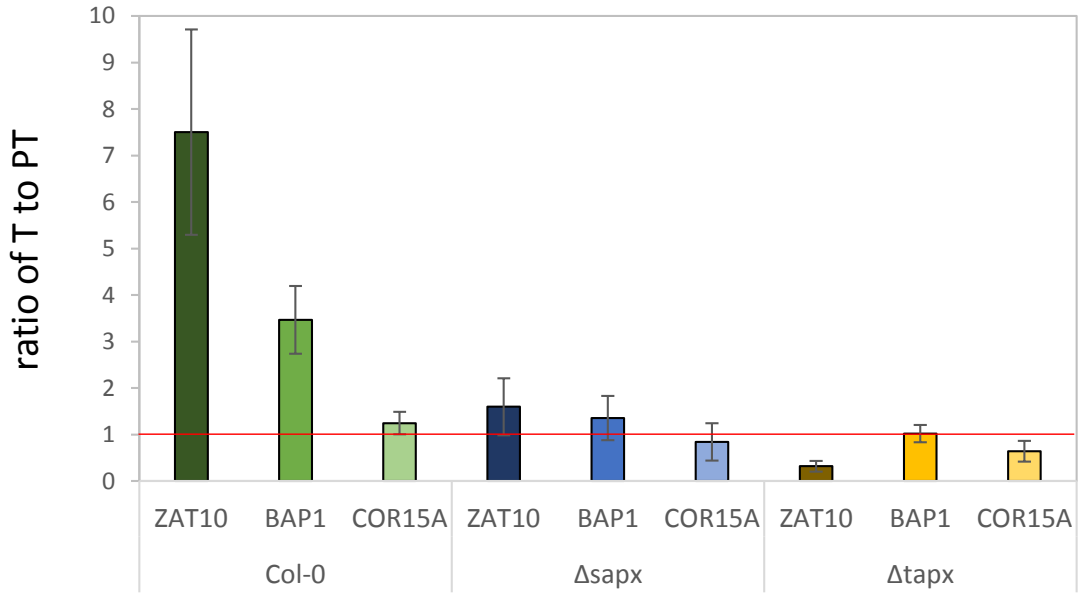

Figure 17: Transcript abundance ratio of T to PT plants at the end of triggering for *ZAT10*, *BAP1* and *COR15A* in Col-0, *sapx* and *tapx*. The red line indicates T=PT=1. n=3±SD

The induction intensities for *ZAT10* and *BAP1* and (to a lesser extent) *COR15A* were lower in the knock-out lines compared to Col-0 (data not shown). Larger differences were observed for the

mutant line missing the thylakoid bound isoform of the plastidic ascorbate peroxidase. *BAP1* and *COR15A* in *tapx* plants had a ratio of 1 or close to 1, respectively. A strong difference was found for the transcript level of *ZAT10*. Expression levels of PT plants where three times higher than those of T plants (fig. 17). This was invers to the response in wildtype Col-0 plants (fig. 9 and 17) and demonstrates the importance of tAPX for the primability of *ZAT10* in Col-0. The singlet oxygen marker gene *BAP1* responded in *tapx* plants as in *sapx* plants. The data suggest that tAPX is a main reason for the non-induction of *ZAT10* in Col-0, while *BAP1* regulation is sAPX and tAPX controlled. For *ZAT10* and *BAP1* in *sapx* plants, a ratio slightly above 1 was observed. They have had a stronger induction in T compared to PT plants, but compared to Col-0 the effect strength diminished and was not statistically significant anymore.

Expression pattern of COR15A was not affected by the knock-out of plastidic ascorbate

In general a weaker expression of *COR* genes was reported, by induction of plastidic H_2O_2 by an inducible knock-down line of *tAPX* (Maruta et al., 2012). The same effect was seen by increased levels of extraplastidic H_2O_2 in a catalase deficient mutant (Vanderauwera et al., 2005). This phenomenon could also be observed here (data not shown). So the induction intensity of *COR15A* was decreased by changing the plastiditic capacity for ROS detoxification, but the ratio of expression of T to PT for *COR15A*, and with that the general regulation pattern, did not differ dramatically (fig. 17). *COR15A* still resembled the expression pattern of a chilling stress inducible, but not primable gene.

3.11 The genes for the regulation of the phenylpropanoid pathway were ‘positively primable’

Phenylalanine ammonia-lyase 1 (*PAL1*) plays a role in a variety of stress situations, both biotic and abiotic ones; it can be induced by fungal elicitors (Lawton et al., 1983; Habereder et al., 1989) and bacterial pathogens (Mishina and Zeier, 2007). Additionally mutants lacking various *PAL* isoforms are highly sensitive to UV-B radiation (Huang et al., 2010). *PAL1* is also upregulated under

cold stress (Olsen et al., 2008) and PAL activity regulation under stress conditions is plant species specific (Rivero et al., 2001).

Like *PAL1*, the *chalcone synthase* (*CHS*) can be induced by fungal pathogens (Nagy et al., 2004), UV-B radiation (Bieza and Lois, 2001; Wolf et al., 2010) and under chilling stress (Catala et al., 2011). As such *PAL1* and *CHS* are responsive to the same range of stimuli. The most expressed isoform of *PAL* (*PAL1*) and *CHS* were tested for priming and triggering responses to analyse the regulation of additional strongly stress and ROS responsive genes (fig. 18). The *PAL1* and *CHS* promotors both contain AC-rich motifs, which are bindings sides for MYB-transcription factors (reviewed in Zhang and Liu (2015)). Additionally *CHS* is under control of Hy5, a basic leucine zipper transcription factor (Catala et al., 2011). *PAL1* is phosphorylated by CPK1, linking Ca^{2+} to the regulation against pathogenic attacks (Cheng et al., 2001), and feedback inhibited by flavonols (Yin et al., 2012). By expressing the H_2O_2 producing glycolate oxidase in chloroplasts of Arabidopsis, Fahnenstich et al. (2008) could show a negative relationship between H_2O_2 and the expression of *PAL1* and *CHS*. The more H_2O_2 was induced by the glycolate oxidase in combination with a high light treatment the less *PAL1* and *CHS* were expressed.

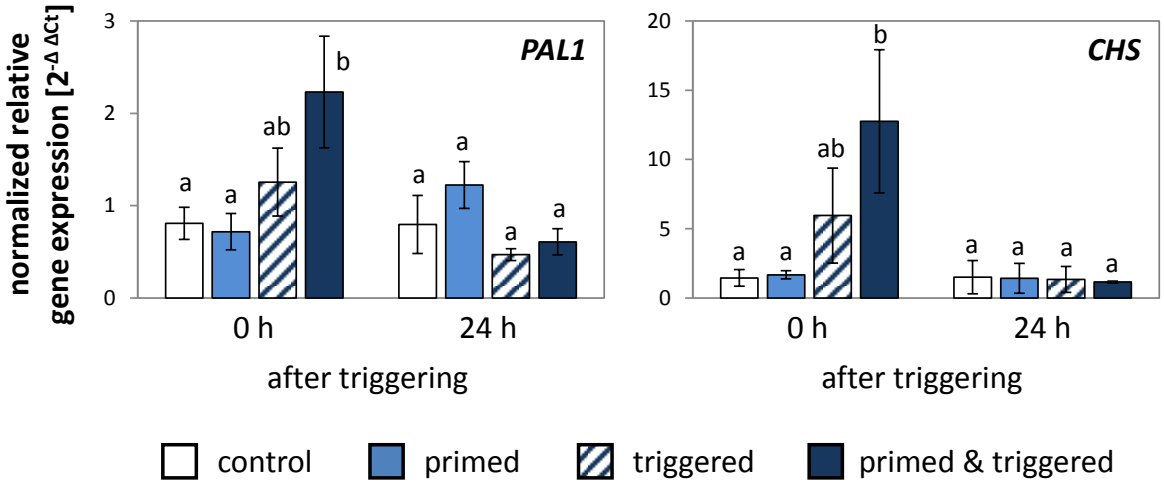

Figure 18: Transcript abundance relative to control levels before start of experiment for *PAL1* and *CHS* in Col-0. n=3±SD ANVOA (Tukey's HSD) $p<0.05$

PAL1 and *CHS* do not only respond to the same stresses, but also their response towards two consecutive chilling stresses were alike (fig. 18). Both are reactive to the short chilling stress pulses. Induction levels of primed and triggered plants were elevated significantly compared with

primed only and control plants. Triggered only plants displayed an intermediate response. Priming enables plants to have higher induction of *PAL1* and *CHS* after triggering. If there is a negative correlation of plastidic ROS and *PAL1* and *CHS* expression, as suggested by Fahnenstich et al. (2008), this would explain the response of *PAL1* and *CHS* in PT plants. After priming the levels of tAPX increased (chapter 3.9). This leads to a reduction of the hydrogen peroxide burden in chloroplast (see *ZAT10* expression in PT plants, fig. 9), the H_2O_2 accumulation in T plants is higher than in PT plants (fig. 9). This would lead to a stronger induction of *PAL1* and *CHS* in PT plants, due to lower inhibition of expression by plastidic H_2O_2 compared to T plants.

3.12 Accessions from warmer and colder environments differ in their primability

3.12.1 Origin of the accessions

The predictive value of a stress stimulus for a following stress event must be sufficiently high to evolve priming mechanisms (Hilker et al., 2015). Even though costs of priming are expected to be lower compared to acclimation, they are still existing. If plants are stressed and memorize the event to prepare themselves for future challenges and this predicted future stress does not occur on a frequent base, the priming responses might 'waste' energy. Ultimately the plant will have a disadvantage competing with neighbouring plants, which do not share this trait. However, for one species or naturally occurring accession primability in context of cold could be advantageous, for other stresses it would lead to a lower overall performance. As such, the trait must be considered in context of a specific stress or group of stresses which share certain features and the natural habitat of plants.

For Arabidopsis, huge collections of accessions are available from a wide range of different habitats (Scholl et al., 2000; Joshi et al., 2012). Some accessions come from similar climate zones and environments, even from locations close to each other. Others come from habitats, which differ tremendously. It was shown, that a variety of accessions differ in their capacity to develop freezing tolerance (Zuther et al., 2012; Zuther et al., 2015). The ability to accumulate sugars, proline and flavonoids as protectants against freezing stress during a long period with chilling stress differs between accessions from northern and southern habitats (Hannah et al., 2006;

Zuther et al., 2012; Zuther et al., 2015). Additionally, differences between accessions in the composition and regulation of the plastidic antioxidant system were recorded (Juszczak et al., 2012; Juszczak et al., 2016).

To test if chilling stress primability is a trait unique to Col-0, other natural occurring accessions were analysed. The decision was made based on different environmental conditions in their natural habitat and previously observed regulatory patterns of PAS genes (Juszczak et al., 2012; Juszczak et al., 2016). Kas-1 for example has the highest protein amount of CSD2 and levels in Ms-0 and Cvi-0 are higher compared to Col-0 (Juszczak et al., 2012). Ms-0 and Kas-1 seem to invest more resources in the recycling of MDA indicated by higher basal mRNA levels of *MDAR* (Juszczak et al., 2016). Additionally both accessions from colder climate invest less in chloroplast protection by *tAPX*, as indicated by lower mRNA abundance, compared to Col-0 and Cvi-0 (Juszczak et al., 2012). In contrast Cvi-0 puts a lot of resources into the plastidic ascorbate peroxide system.

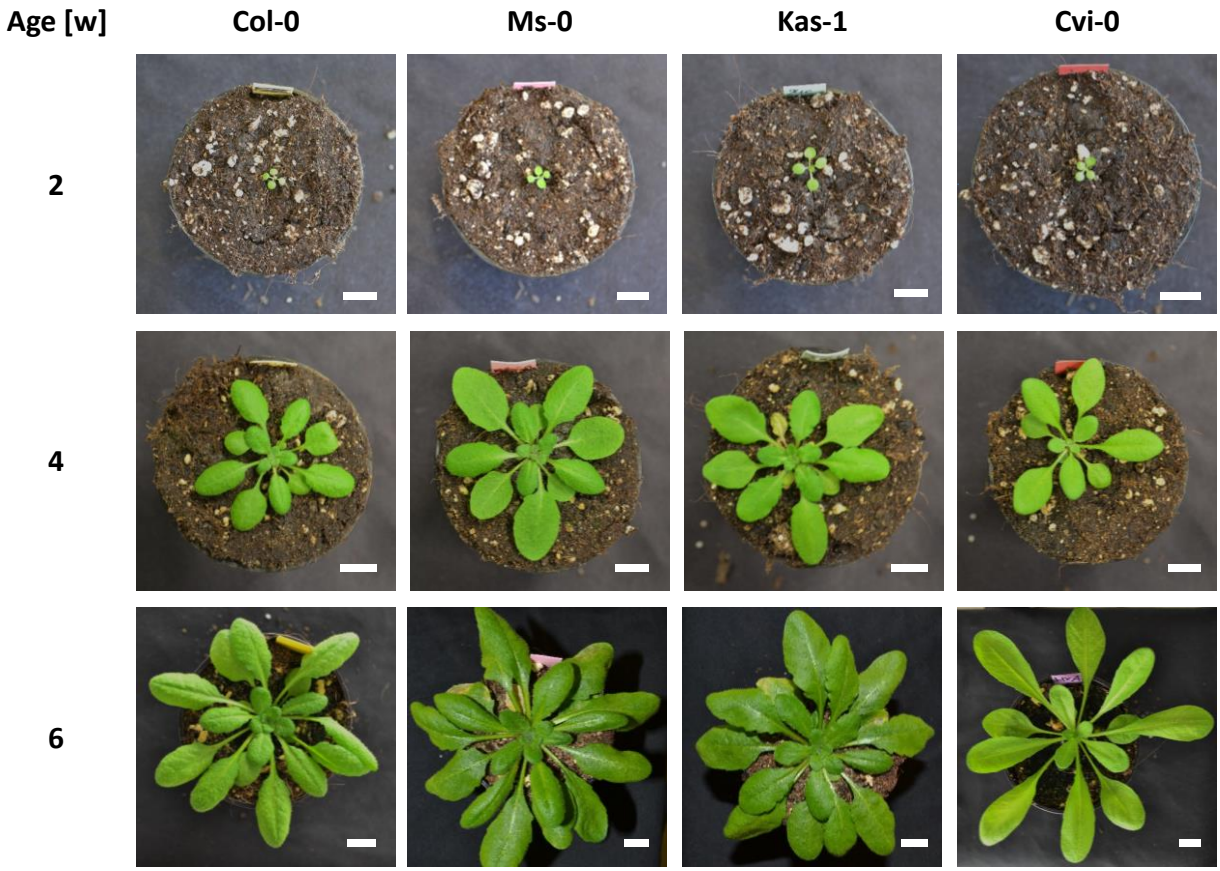

Figure 19: The four accessions of *Arabidopsis thaliana* used in this study. Photos were taken every two weeks, during a six weeks period. White bars represent 1 cm.

I. *Ms-0*

Moscow 0 (Ms-0, NASC N1377, Latitude: 55.7522, Longitude: 37.6322) was collected in Moscow, Russia. As such its natural habitat is in general stricter than that of Col-0 growing in a hilly region in southwest Poland. Ms-0 grows on slightly elevated altitude (167 m). More important are the climatic conditions. Moscow has a strict continental climate. As such, in summer, temperatures reach a maximum comparable to central Europe and at times even higher, but in winter temperatures are harsher given the continental climate of Russia. Generally, in spring temperatures are lower compared to those Col-0 experiences. For example, the mean March temperatures are lower (fig. 20). The date after which night temperatures are > 0 °C is later and the cold period in spring is longer compared to central Europe. This increases the chance that plants experience temporarily separate chilling stress stimuli frequently; the prerequisite described before to develop primability.

Ms-0 plants grew faster than Col-0 and had a larger leaf area and higher rosette diameter (fig. 19). The leaves were more planar than those of Col-0. After six weeks first signs of senescence occurred in older leaves, as chlorophyll degradation started and leaf tips turned yellowish brown.

II. *Kas-1*

Kashmir 1 (Kas-1, NASC N1264, Latitude: 35, Longitude: 77) was collected in India in the Kashmir region, which is a transition zone between arid central Asia and the sub-tropic Indian region. Because of the high altitude (1580 m) in winter months temperatures drop below -10 °C (fig. 20). In this region precipitation is very variable, so environmental conditions for Kas-1 are even grimmer compared to Ms-0.

Like Ms-0, Kas-1 plants grew faster than Col-0, which might be an adaption to the shorter vegetation period (fig. 19). The average leaf area was bigger, as well as the rosette diameter at any given time. Already after six weeks signs of senescence were observable (fig. 19). The shoot was significantly shorter compared to the three other accessions and the seed amount was dramatically decreased under greenhouse conditions (Cvetkovic, 2016).

III. *Cvi-0*

The accession Cape Verde Island 0 (Cvi-0, NASC N1096, Latitude: 15, Longitude: -23) comes from the Cape Verde Islands in the central Atlantic. The accession was collected at a high altitude (1200 m). The temperature on the islands vary less than in continental habitats. By average it is around 22 °C (fig. 20). On the Cape Verde Islands, Cvi-0 hardly ever experiences chilling stress periods. Even during nights, the temperatures barely decreases below 10 °C.

Leaves of Cvi-0 were lancet-shaped. The widest part of the leave was close to the tip, not in the middle, as in the other accessions. The leaves were orientated upwards. Leaves were normally not in contact with the soil. As in Col-0, chlorophyll degradation had not started after six weeks (fig. 19). Cvi-0 needed six to seven weeks to develop a shot, even under the 10 h light 14h dark cycle as used as standard growth conditions.

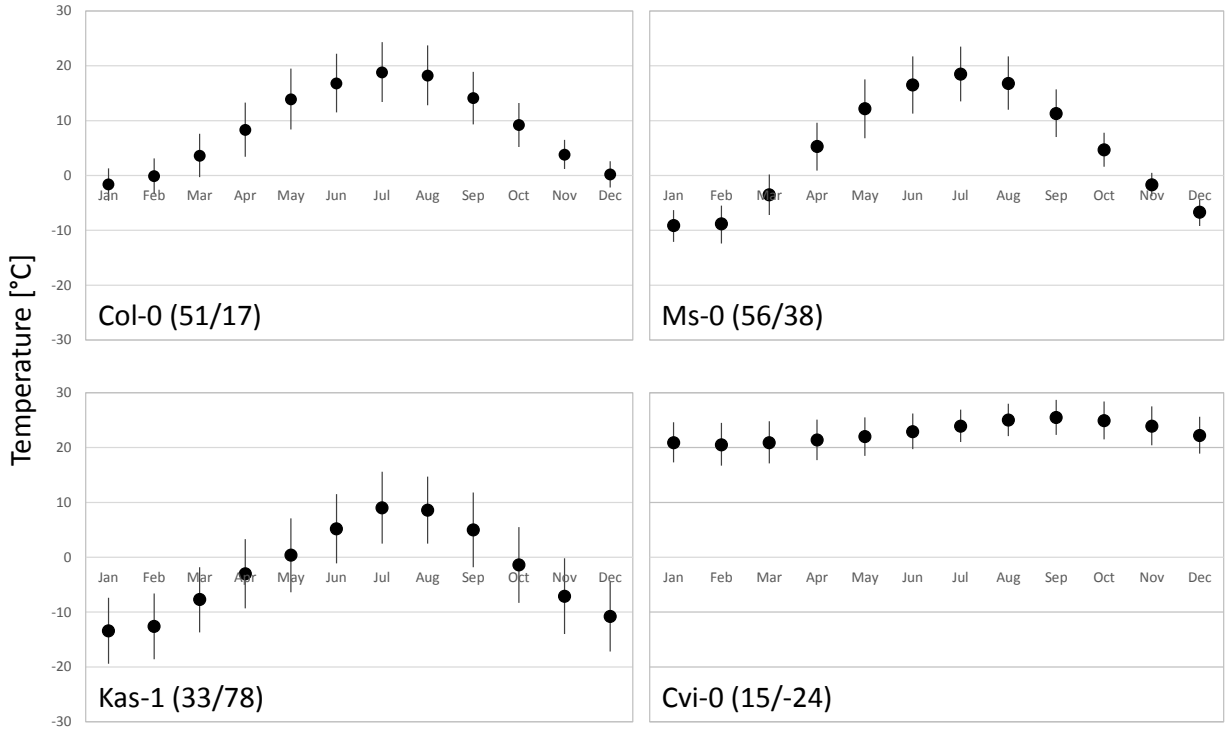

Figure 20: Temperature data for the four used accessions in this study. Shown are the average mean temperature ± the average maximal deviation. The numbers after the accession name correspond to the latitude and longitude of the closet meteorological station to the place of origin of the accession. The data are taken from http://www.globalspecies.org/

3.13 The trait primability was not shared by all tested accessions

Col-0 showed negative primability of *ZAT10* and *BAP1* (fig.9). In contrast to that, *COR15A* was not primable with a short chilling stress stimulus (fig. 10), but could be positively primed with a 14 day long priming period (Cvetkovic, 2016). Additionally, the response of *PAL1* and *CHS* were positively primable by a short priming stimulus (fig. 18). Primability was assessed directly after triggering using all different combination of priming and triggering. In all tested accessions the response of *COR15A* was not showing a pre-treatment dependent effect, but the induction of *COR15A* was more pronounced in accessions coming from colder areas (Ms-0 and Kas-1) compared to Col-0 and Cvi-0. Transcript levels were induced upon chilling treatment (T- and PT-plants, fig. 21). Demonstrating that *COR15A* was not primable under short term priming conditions in any of the accessions.

In Col-0, as response to the triggering the mRNA levels of *ZAT10* were increased (fig. 9). This response could be verified in all accessions, except Cvi-0 (fig. 21). 24 h of cold priming fully blocked *ZAT10* induction in Col-0 (fig. 9). The response intensities in PT plants between the accessions varied. Cvi-0, the accession from a warm habitat, responded to the chilling stress; the pre-treatment did not influence the *ZAT10* induction after triggering (PT and T were equally induced, fig. 21). As already mentioned the minimal daily average temperature per month on the Cape Verde Islands is +20°C or higher. These conditions do not provide selective pressure to memorize chilling stress.

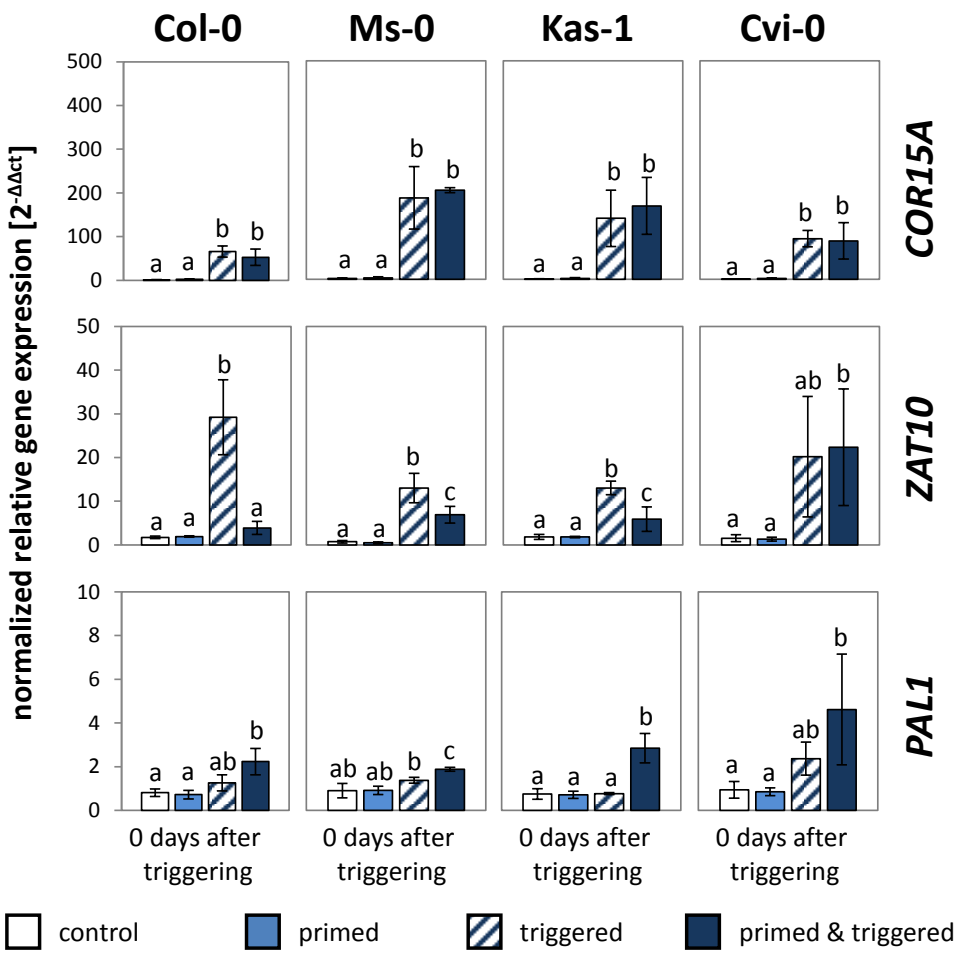

Figure 21: Transcript abundance analysis normalized to expression levels before the experiment started of the four accessions in comparison for *COR15A*, *ZAT10* and *PAL1* in Col-0, Ms-0, Kas-1 and Cvi-0 directly after triggering. n=3±SD, ANOVA (Tukey's HSD) $p<0.05$

Both accessions from habitats with lower spring temperatures than Col-0, namely Ms-0 and Kas-1, were sensitive for the pre-treatment and responded differently to the stimulus dependent on whether they were primed before or not. The expression levels of PT plants reached half maximal levels of T plants (fig. 21) compared to Col-0, where *ZAT10* induction was fully blocked in PT plants. The incomplete, but significant, induction of Zat10 after priming and triggering could have two different reasons: (1) In Ms-0 and Kas-1, *ZAT10* is in principle primable, but the signalling was not as severely impaired as in Col-0 by the temperature stress leading to a weaker priming. Or (2) as both accessions originally grow in environments with low spring temperatures a stronger response of pathways inducing freezing tolerance is induced, reducing the ability for priming (fig. 21).

In Col-0 (chapter 3.11), *phenylalanine ammonia-lyase 1* expression was positively primable (fig. 18 and 21). Cold itself induced expression slightly (T), but in combination with the pre-treatment (PT) mRNA levels were significantly induced compared to C plants. In Cvi-0 the same regulation was observed (fig. 21). As Cvi-0, the profile of Ms-0 resembled the one of Col-0.

Even though the response after triggering was similar for Ms-0 and Kas-1 concerning *ZAT10*, the accessions differed in their response to chilling stress in terms of *PAL1* regulation. In Kas-1, triggering alone did not lead to an induction of *PAL1*. A response to the triggering stimulus was shown only in combination with a pre-treatment five days before. This suggests that Kas-1 had been prepared by the priming stimulus before a response was initiated. Alternatively in Kas-1, a higher number of stress repetitions is needed to initiate *PAL1* induction. Something similar was found for Arabidopsis regarding abscisic acid and stomata opening, were multiple abscisic acid stimuli induced stomata opening (Goh et al., 2003). In response to mechanical stress the *touch* genes are strongly induced. 30 minutes after a mechanical stress, if the plants were challenge before by a repetition of bending events compared to a single mechanical damage the induction of *touch* genes was more pronounced (Braam, 1992). Epigenetic changes are needed to maintain the stronger response of *touch* genes, as mutants in *set domain group 8* showed altered responses to mechanical damage (Cazzonelli et al., 2014).

In Col-0, *tAPX* levels were increased on promotor activity, expression and protein level (promotor fig. 15, mRNA fig. 6, protein fig. 16). In *tapx* the regulation of *ZAT10* after priming and triggering was altered and led to stronger induction in PT relative to triggering only plants (fig. 17). In none of the three accessions, *ZAT10* expression was primable to the same extent as in Col-0 (fig. 21). Ms-0 and Kas-1 were compromised in their primability, while in Cvi-0 no hints for chilling stress priming could be found. This raises the question, whether changes in *ZAT10* expression profiles in Ms-0 and Kas-1 were caused by alterations in the plastid antioxidant system, which would explain a decrease of the ROS-inducible marker gene by better protection against oxidative stress, in a similar fashion to Col-0.

3.13.1 The response of the ascorbate peroxidases in the different accessions

The ascorbate peroxide system of the three accessions was tested. The stromal ascorbate peroxidase responded to the chilling stress. In all treatments connected to chilling (directly after priming and after triggering T and PT) expression was induced to a constant level of about 3 fold more transcript compared to the time point before the experiments were started for all accessions tested (fig. 8, 22, 23 and 24). Additionally, changes could be observed in the expression pattern of *tAPX*.

I. *Ms-0*

In Ms-0, directly after priming *tAPX* expression was down to 65 % of the expression level in control plants, demonstrating a stronger cold suppression than in Col-0. 24 hours after the priming, the mRNA level was significantly higher compared to control plants. This may lead to an increased protection of the thylakoid membranes during the early phases of readjustment to control conditions. Five days after priming, expression levels normalized, possibly aligning tAPX protein levels between control and primed plants (fig. 22).

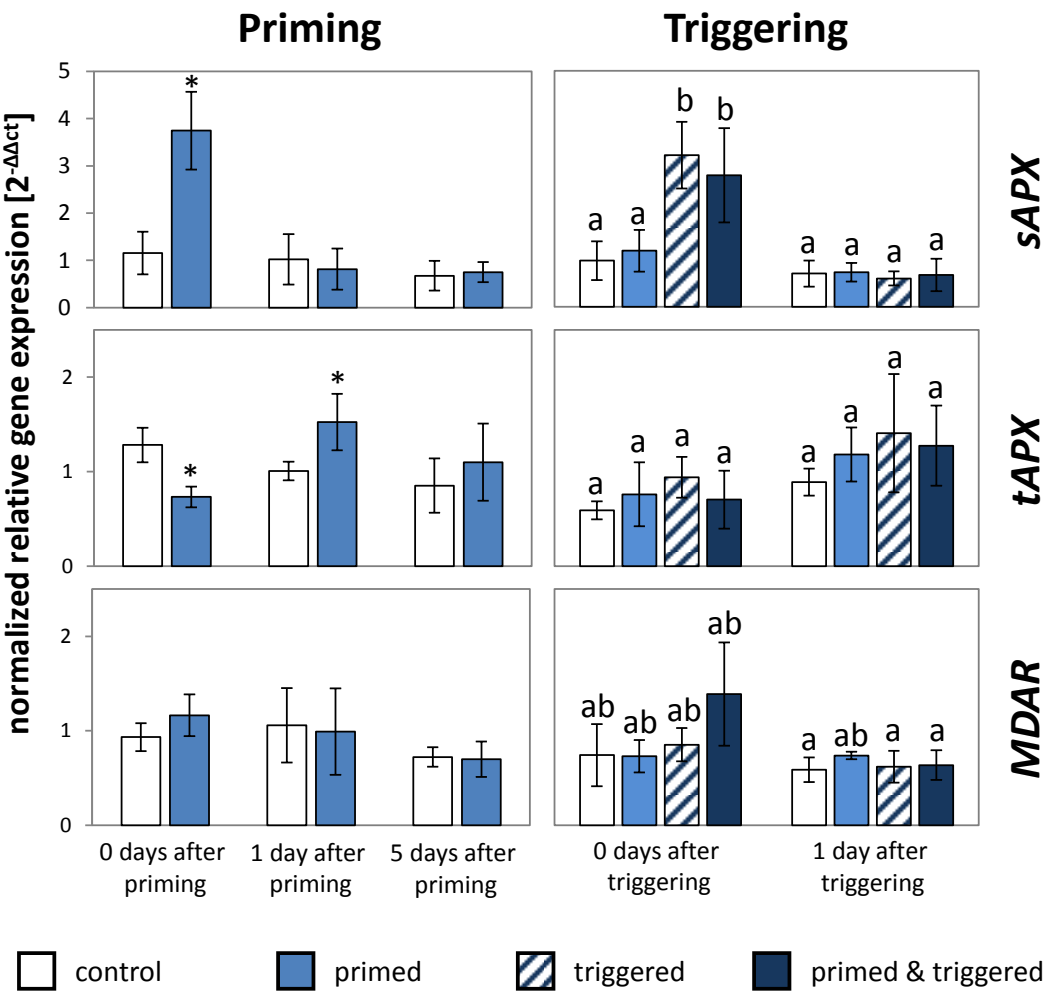

Figure 22: The response of enzymes of the ascorbate dependent water-water cycle in Ms-0. Transcript data were measured after priming (left) and triggering (right) and normalized to values before experiments were started. n=3±SD, * Student's t-test p<0.05, ANOVA (Tukey's HSD) p<0.05

Plastid ascorbate peroxidases are sensitive to inactivation by H_2O_2, if the ascorbate concentration is low (Miyake and Asada, 1996). The major enzyme converting monodehydroascorbate back to ascorbate and stabilizing the ascorbate availability upon stress is the *MDAR* (Asada, 1999). After priming, no change of *MDAR* was monitored in Ms-0 (fig. 22). After triggering however, PT plants showed a trend for induction of transcription. In contrast to Col-0 no induction in T plants occurred (fig. 8).

II. *Kas-1*

Kas-1 responded similarly to Ms-0. Immediately after priming, *sAPX* was upregulated, but due to strong quantitative differences the effect was not significant. *MDAR* showed a trend for higher expression levels (fig. 22 and 23). The responses were similar after triggering. However, in Kas-1 no changes in *tAPX* mRNA abundance were determined between the four different treatments.

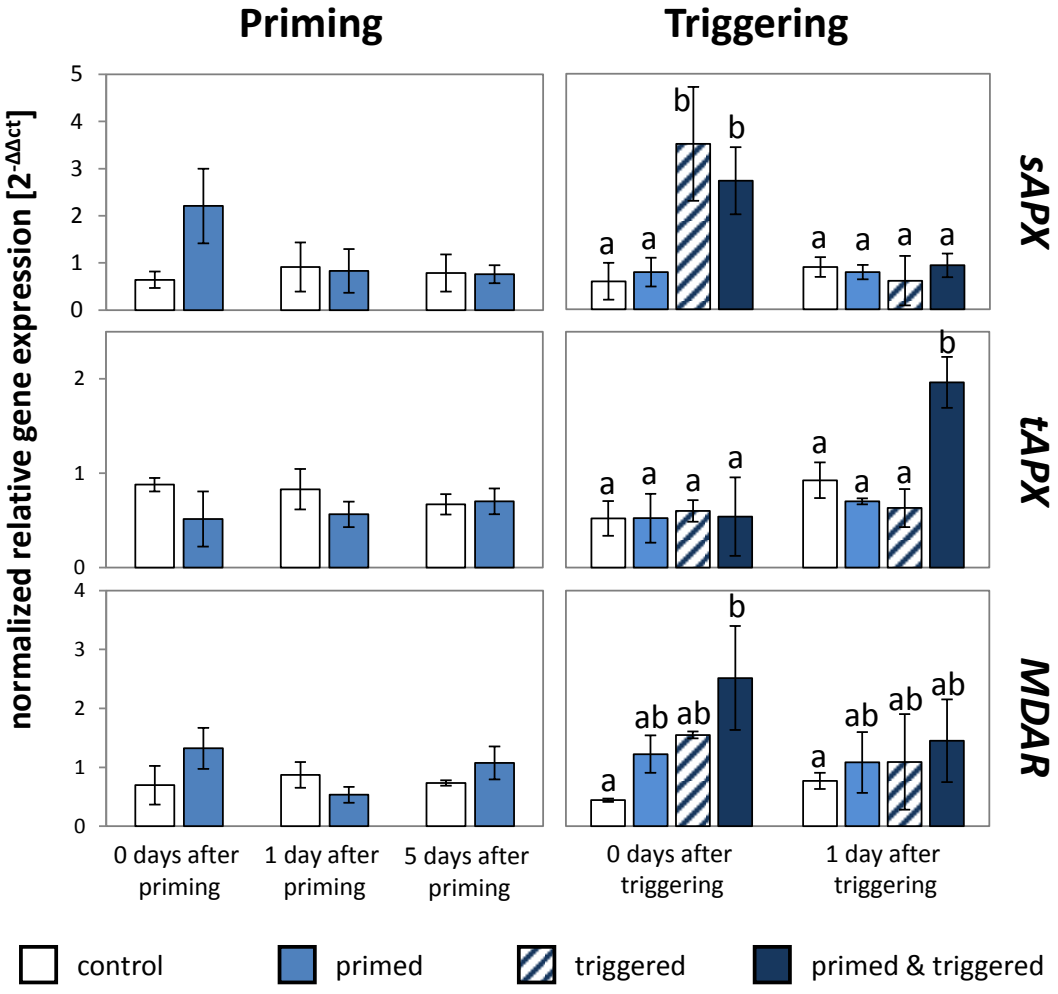

Figure 23: The response of enzymes of the ascorbate dependent water-water cycle in Kas-1. Transcript data were measured after priming (left) and triggering (right) and normalized to values before experiments were started. n=3±SD, * Student's t-test p<0.05, ANOVA (Tukey's HSD) p<0.05

One day later the level of *tAPX* was increased, but only in primed and triggered plants (fig. 23). This response was comparable to Ms-0 one day after priming, as well as Col-0 after priming. This data indicates that Kas-1 needs at least a second or a longer lasting stress event to induce *tAPX* transcription.

III. *Cvi-0*

In Cvi-0, cold also induced *sAPX* expression directly after priming in P and directly after triggering in T and PT plants (fig. 24). *sAPX* responded with a transient induction in response to chilling stress as described for the other accessions (fig. 24).

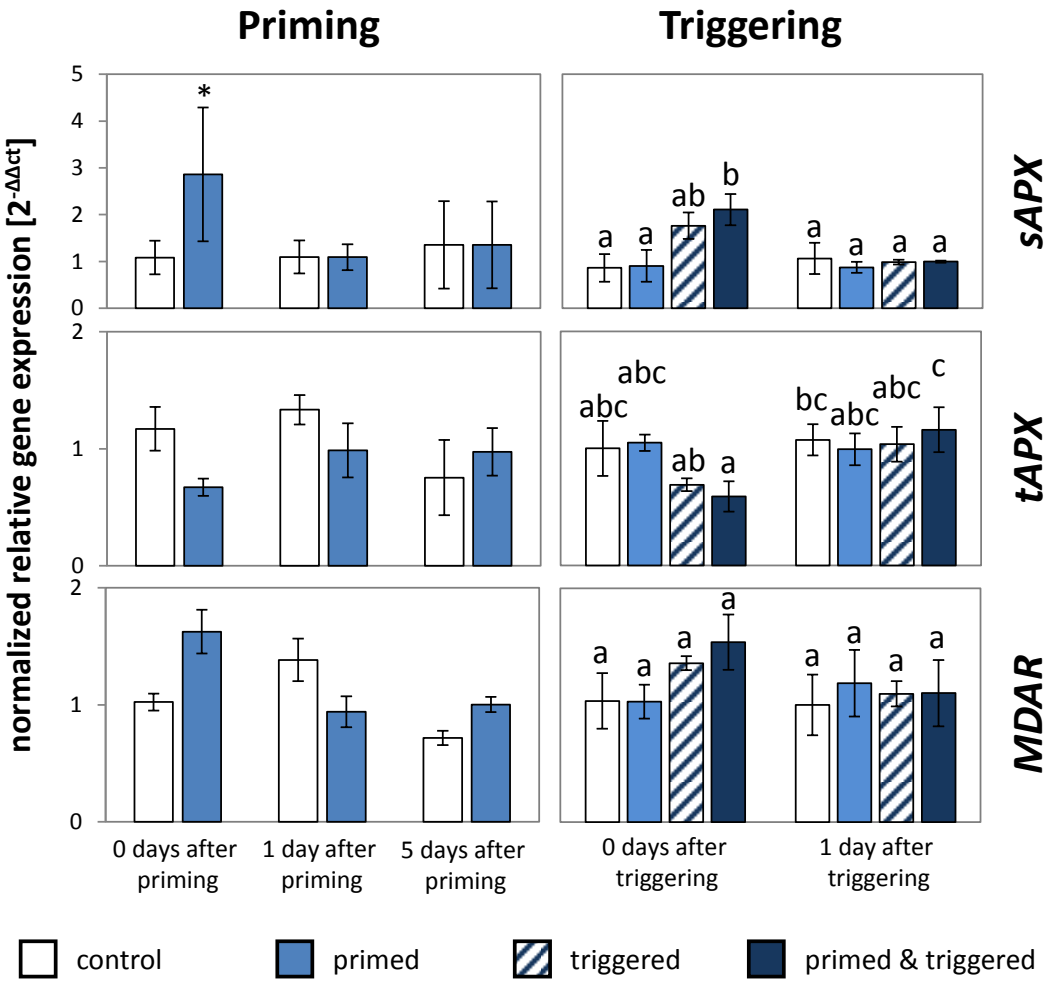

Figure 24: The response of enzymes of the ascorbate dependent water-water cycle in Cvi-0. Transcript data were measured after priming (left) and triggering (right) and normalized to values before the experiments were started. n=3±SD, * Student's t-test p<0.05, ANOVA (Tukey's HSD) p<0.05

tAPX levels were not changed significantly during the course of the experiment. A slight down regulation was apparent after every chilling stress, but transcript levels normalized already one day after in all cases (fig. 24). Consistent with *sAPX* profiles, *MDAR* had higher expressions levels after priming and triggering, compared to triggered only plants.

Comparison of accessions

Over all, no profile comparable to the one in Col-0 was observed. *tAPX* was deregulated directly after priming, readjustments to control levels during the lag-phase occurred. In all accessions *sAPX* was cold responsive. But changes were transient and did not outlast the actual cold treatment. The response of *tAPX* was versatile. In Cvi-0 a suppression could be monitored upon chilling stress, while for Ms-0 and Kas-1 this was only the case after priming. In Ms-0 the expression one day after priming was elevated in P plants, as it was observed in Col-0. But only in Col-0 the five days long lasting induction could be seen.

In this study, *ZAT10* and *BAP1* were used as readout parameters for chloroplast ROS signalling. Three different responses of *ZAT10* were monitored using the four accessions from different habitats (fig. 21):

In Col-0 the induction of *ZAT10* was fully blocked in primed plants upon triggering (fig. 9) correlated with an induction of *tAPX* in the lag-phase (fig. 6, 15 and 16). The knockout of tAPX lead to a reversal of induction patterns, showing that *tAPX* is causal for the priming effect (fig. 17). Ms-0 and Kas-1 induced *ZAT10* in primed and triggered plants (in contrast to Col-0). Even though induction levels of PT and T plants differed significantly. Both accessions induced *ZAT10* to half maximal levels in T compared to PT plants. Both accessions showed a tendency for down-regulating *tAPX* during priming, but not triggering (fig. 22 and 23). Neither Ms-0 nor Kas-1 developed the same long lasting induction as seen in Col-0 (fig. 6). The third mode of action was seen in Cvi-0. No significant differences between T and PT plants concerning *ZAT10* were measured (fig. 24). Again no long lasting induction of *tAPX* was found. The regulation of the stromal isoform however, was alike in all four tested accession (fig. 6, 8, 22, 23 and 24).

This indicates, that the stromal ascorbate peroxidase does not play an important role in short term cold priming, as *sAPX* response in T and PT plants is similar. However, if the priming stimulus is prolonged to 14 days, the stromal APX is still induced in the lag-phase and takes over the reduction of the plastidic ROS burden to ensure primability (Cvetkovic, 2016). The thylakoid-bound isoform was strongly induced in the post-stress phase in Col-0 (fig. 6). In the other three accession no such response could be monitored, implying a regulatory connection between the

regulation of *tAPX* and the degree of primability. It cannot be excluded, that the *sAPX* is stabilized post-translationally in Ms-0 and Kas-1 and thus takes over part of the H_2O_2 detoxification, which is provided by *tAPX* in Col-0. All in all, the findings consolidates the important role for *tAPX* in priming to facilitate the maximal priming intensity.

3.14 Plant age and length of the lag-phase altered ROS marker gene expression after triggering

The transcriptome, proteome and metabolome change during leaf development. During early development or the formation of new leaves, the leaves are sink organs (Turgeon, 1989). Later photosynthesis will produce more resources than the leaf requires and the leaves become source and storage organs (Lemoine et al., 2013). All developmental stages of leaves require different supply with nutrients. Already after 28 days first age-associated cell death takes place in leaves, controlled by *oresara 1* (*ORE1*) (Vogelmann et al., 2012) and plants switch from the vegetative to the reproductive state. It is tempting to assume that differences in sink/source distribution impact on priming.

To analyse the impact of age, the previously applied experimental set-up was modified: two and six weeks old plants were included to study developmental regulation of priming. In a second set-up the length of the lag-phase between the priming and the triggering cold stimulus was modified to analyse, the impact of memorizing and ‘forgetting’.

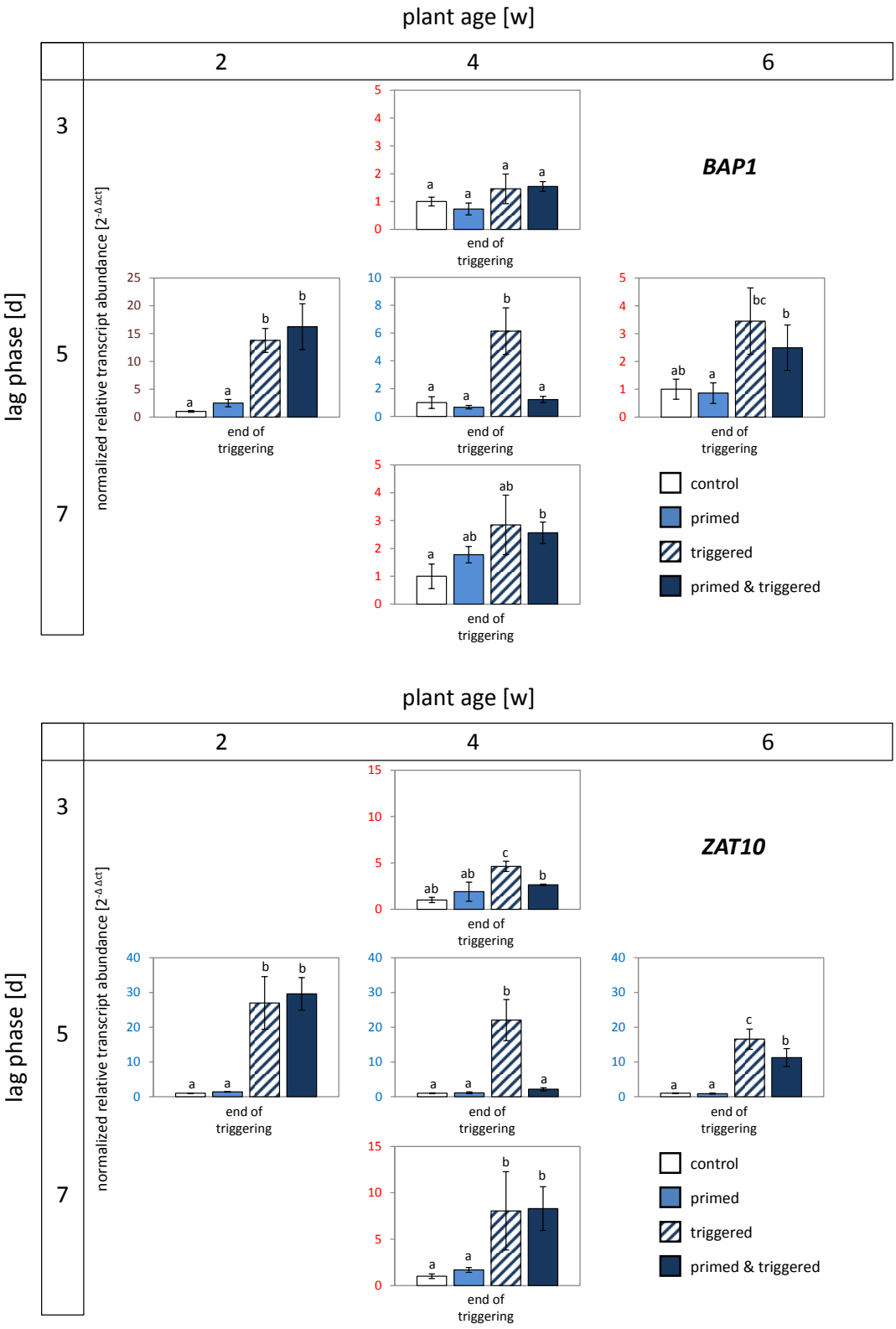

Figure 25: *BAP1* (top) and *ZAT10* (bottom) transcript abundance directly after triggering using various plant ages (2 w, 4 w, 6 w) and lag-phases (3 d, 5 d, 7 d). Different colours on the y-axis indicate different scales. n=3±SD, ANOVA (Tukey's HSD) $p<0.05$, values are normalized to control levels after triggering

I. *BAP1*

In the first part of the study (chapter 3.6) it was shown that priming blocks cold induction of *BAP1* and *ZAT10* in Col-0 (fig. 9). Comparison of *BAP1* regulation in two, four and six week old *Arabidopsis thaliana* var. Col-0 showed that the priming effect was age dependent (fig 25 top). Regulation of *tAPX* during the five day long lag-phase suggested that manifestation of priming was dependent on activation of chloroplast ROS detoxification (fig. 6) at the thylakoids. Western blots suggested that the priming memory is lost after some time (fig. 16). To test this hypothesis, the five day long lag-phase was compared with a three and seven day long lag-phase in four week old Arabidopsis (fig. 25 top). When the lag-phase was shortened to three days or prolonged to seven days, no difference between T and PT plants was observed (fig. 25 top).

Younger plants showed no differences between T and PT plants, the priming effect was not established. In six week old plants the chilling treatment (T plants) resulted in only 50 % induction of *BAP1* compared to four week old plants. Triggering led to no significant priming effect dependent of the pre-treatment, but a slight trend for a weak priming effect was observed (fig. 25 top). The results demonstrated that the intensity of the priming effect was developmentally regulated.

II. *ZAT10*

In general, *ZAT10* (fig. 25 bottom) responded similar to *BAP1* (fig. 25 top) concerning the memory strength using different lag-phases. If the length of the lag-phase was altered the memory strength was reduced (three days) or vanished (seven days) (fig. 25 bottom). By shortening the lag-phase the induction level of *ZAT10* in PT plants reached 60 % of the expression in T plants. With a lag-phase of five days the PT induction levels were only 10 % of the maximal induction of triggered only plants.

As for *BAP1*, two week old plants were not primable. The *ZAT10* induction in T plants was similar to the one in PT plants. In six week old plants a memory effect was measurable. But the effect strength was decreased in comparison to the effect in four week old Arabidopsis. This shows that the primability of *ZAT10* was influenced by the developmental stage of the plant.

III. *Cor15A*

As controls for this experiment the transcript abundance of *COR15A* was measured for all combinations of analysed lag-phases and plant ages (fig. 26). The response of the cold marker gene did not show any changes between primed and triggered and triggered only plants, just the induction caused by chilling stress. Induction intensity was lower in six week old plants.

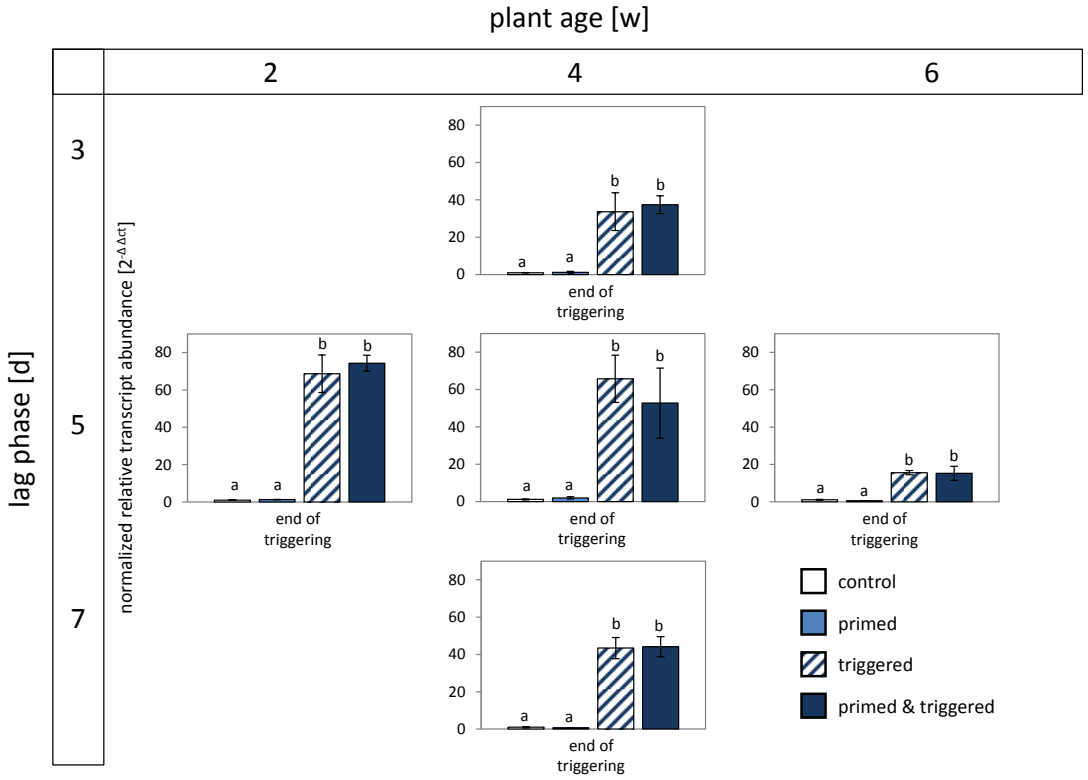

Figure 26: *COR15A* transcript abundance directly after triggering using various plant ages (2 w, 4 w, 6 w) and lag-phases (3 d, 5 d, 7 d). n=3±SD, ANOVA (Tukey's HSD) $p<0.05$, values are normalized to control levels after triggering

3.14.1 Timing of priming

The data (chapter 3.14) show that plants require some time to develop the priming competence (three days lag-phase compared to five days lag-phase) and that the memory of the previous chilling stress stimulus is weakened by increasing the length of the lag-phase (five days lag-phase compared to seven days lag-phase). Plants were not able to establish a memory at the

age of two weeks, where strong efforts are made to increase biomass and induce enhanced leaf formation. To the same extent primability decreased with plant age (six weeks).

3.15 The impact of leaf development and sugar relocation on priming

All leaves in Arabidopsis originate from the shoot apical meristem (Braybrook and Kuhlemeier, 2010). Leaves are produced in a spiral with an angle between leaves of around 120°. This puts as much distance between the newly developing leaves as possible. This is also known as the Hofmeister rule (Hofmeister, 1866). Leaf formation is supported by polar auxin transport (Reinhardt et al., 2000). This polar transport of auxin is realized by the pinformed proteins (PINs) (Jönsson et al., 2006). PINs are constitutively cycling between vesicles and the plasma membrane. The endocytosis of PINs is inhibited by high auxin concentrations. By this mechanism a directed concentration gradient can be established (Paciorek et al., 2005). Growth of leaves is promoted in the first stages of leaf development by cell proliferation, after some time cell proliferation slows down, meristemoid division is taking place and cells differentiates into e.g. stomata guard cells or vascular cells. At later stages, growth is realized by cell expansion (Gonzalez et al., 2012). All chloroplasts derive from proplastids from the meristematic tissue (Lopez-Juez and Pyke, 2005). New plastids are generated by a division as it is known for bacteria (Yang et al., 2008). In cotyledons of seedlings, it takes two to three days to accumulate starch, which can be partially used for the synthesis of ascorbate (Pena-Ahumada et al., 2006). During the early stage of seedling, up to three days after radicle emergence, the plastidic peroxiredoxins are the main contributors to plastidic ROS detoxification, only after some time, when the fat metabolism decreases and starch starts to accumulate, plastidic ascorbate peroxidases can properly function and are expressed (Pena-Ahumada et al., 2006). These findings hint towards the fact, that the ascorbate dependent water-water cycle is only stronger induced if sugars are produced in excess. Especially in younger plants most energy is invested into growth, suggesting a higher detoxification capacity of peroxiredoxins in seedlings compared to rosette stage plants (Baier et al., 2004).

tAPX levels tended to be lower in younger plants (fig. 27). As observed in *tapx* plants *tAPX* is crucial for the full induction of the priming effect (chapter 3.10). The low expression levels may support

the non-primable expression profile in young Col-0 plants (fig. 25). The steady state levels of *tAPX* vary strongly in older plants. In younger tissues of old plants expression is still high, while in older tissue of old plants the transcript abundance is nearly zero (Panchuk et al., 2005). The findings of Panchuk et al. (2005) could be verified in four week old $tAPX_{prom}$:GUS fusion lines (fig. 15 top). The promotor activity was highest in younger leaves, the older the leaves were the lower was the promotor activity of *tAPX*. The loss of primability in older plants could be seen in the context of the onset of senescence and the re-localization of nutrients connected to the transition from vegetative to the reproductive stage.

To test this the expression of *ORE1*, a senescence related gene, was measured in plants at different ages. Plants with a higher developmental age (six weeks) had significantly higher levels of *ORE1*. This indicated the onset of senescence, even though chlorophyll degradation was not yet visible.

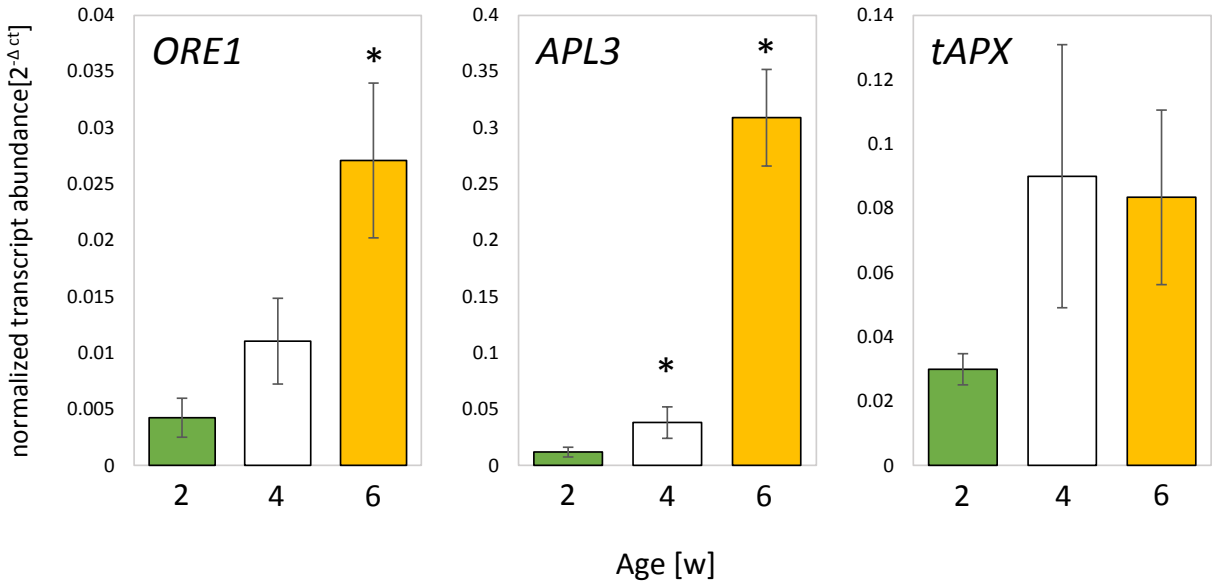

Figure 27: Expression levels of *ORE1*, *APL3* and *tAPX* in Col-0 at different ages: two, four and six week old plants. n=3±SD, * Student's t-test $p<0.05$ compared to two week old plants

In addition to *ORE1*, *ADP-glucose pyrophosphorylase 3* (*APL3*) transcript abundance increased with age (fig. 27). *APL3* is constitutively expressed in every plant tissue at a low steady state levels, with a strong induction of *APL3* by high levels of excess sugars. APL3 is the predominant regulatory APL subunit of APL in sink organs (inflorescence, fruit and root) (Sokolov et al., 1998) and can be

induced by feeding the plant with sugar mimicking excess sugar conditions (Rook et al., 2001; Crevillen et al., 2003; Crevillen et al., 2005). This strengthened the hypothesis that plants were already switching from rosette growth to the mobilization of nutrients for shot and inflorescence development.

3.15.1 The effect of leaf age in old rosettes on the priming intensity

Senescence starts in old leaves (Zentgraf et al., 2004). However, the first molecular signs of senescence, i.e. the increase in *ORE1* expression (fig. 27), could be observed already after four weeks under short day conditions. To further investigate the relationship between the onset of senescence and the reduction in priming efficient, different developmental stages of leaves of six week old plants were analysed for their primability (fig. 28).

The expression of *ORE1* was not only increasing with plant age (fig. 27), but also with leaf age (fig. 28). This increase in basal expression could not be observed for *ZAT10* or *COR15A*. This clearly shows that, even though chlorophyll degradation had not started, the first steps in senescence were already launched in older leaves. Additionally, it was apparent that *ORE1* expression was independent of chilling stress and priming.

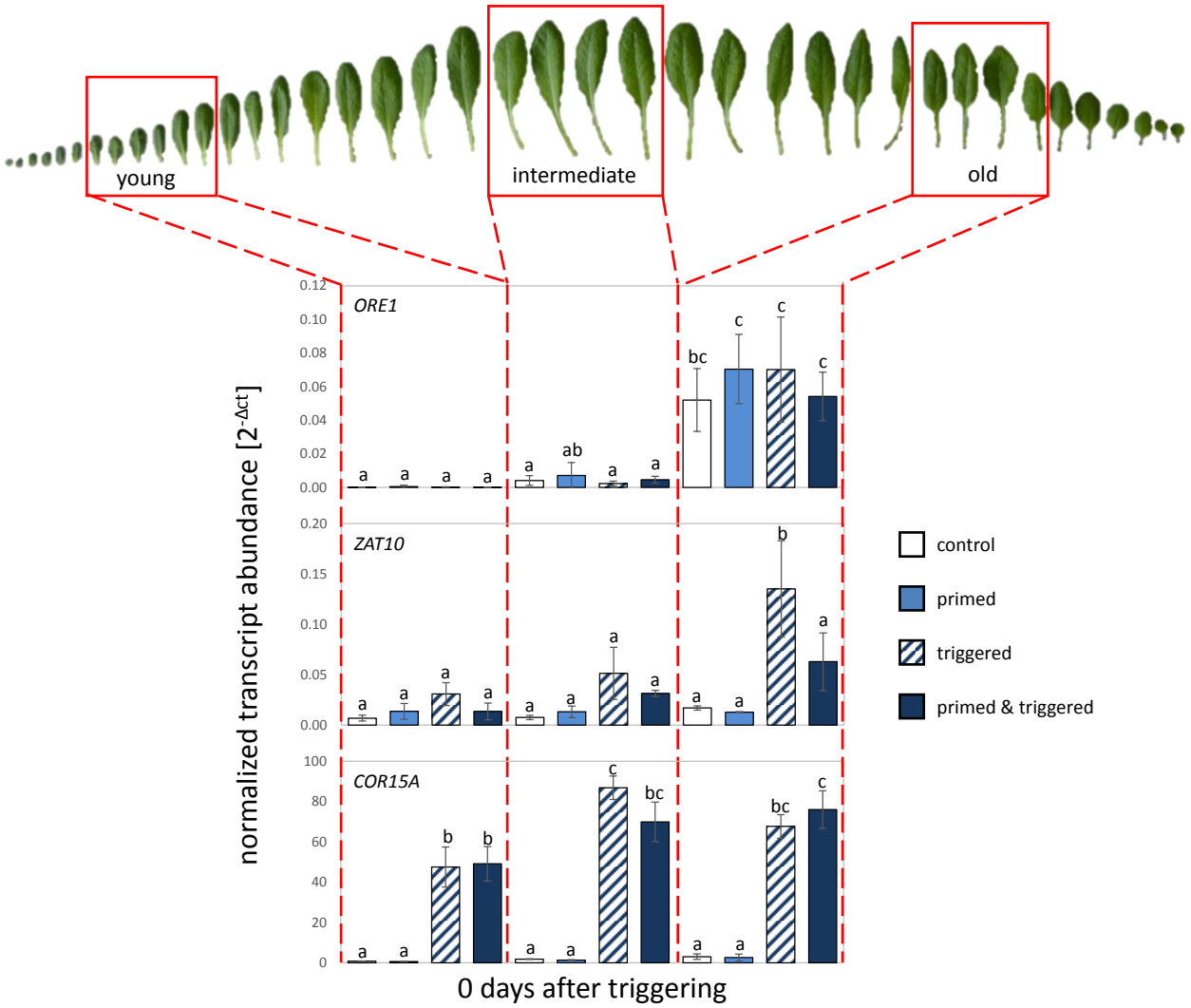

Figure 28: Transcript abundance of *ORE1*, *ZAT10* and *COR15A* in different developmental leaf stages of six week old plants directly after triggering. n=3±SD, ANOVA (Tukey's HSD) p<0.05

Even though the basal expression of *ZAT10* in different developmental stages of leaves did not change, the inducibility did (fig. 28). Only in the older leaves a significant increase in triggered plants was manifested. In young and intermediate leaves an induction was observable, but the changes were not statistically significant. Still, it was evident that there was a pre-treatment dependent regulation of *ZAT10* in intermediate and old leaves, that was comparable to the one observed in whole rosette of older plants (fig. 25 bottom). The transcript abundance for *ZAT10* in PT plants was higher compared to C plants, but lower in comparison to that of T plants. In younger leaves a trend for induction in T plants, without an induction in PT plants could be seen. This indicates that the response of the whole plant was determined mainly by intermediate and old

leaves, due to their large biomass in comparison to young leaves and that the reduction in primability was not correlated with an accumulation of *ORE1*.

COR15A in all tested developmental stages of the leaves was responsive to chilling stress. And was, as seen before (fig. 10 and 28), not sensitive to a chilling stress pre-treatment.

3.16 The priming effect on *ZAT10* is trigger-specific

To address the question whether priming is trigger-specific, in addition to chilling stress triggering, excess light was used as a triggering stimulus. Excess light was applied by shifting plants to 2.5-3 fold higher light intensities (250-300 µmol quanta*s^{-1}*m^{-2}) compared to control growth conditions. Both chilling stress and excess light differentially impact on the photosynthetic electron transport chain. Excess light stimulates the transport of excited electrons through the PET chain. This can be countered by non-photochemical quenching (Müller et al., 2001). There are three different mechanisms of NPQ:

First, if plants are challenged by light intensities that are higher than usual the pH gradient over the thylakoid membrane increases. This activates the violaxanthin de-epoxidase. This enzyme is responsible for the production of zeaxanthin from violaxanthin. The amount of zeaxanthin is correlated with photoprotection, based on fluorescence measurements (Eskling et al., 1997). This is mediated by the enhanced quenching of the chlorophyll triplet stage (Dall'Osto et al., 2012). Thus excitation pressure can be released by transferring energy to zeaxanthin. Subsequently, zeaxanthin can dissipate excess energy as heat (Horton et al., 1996). This reduces the transfer of energy to molecular oxygen and by that reduces the production of singlet oxygen.

The second mechanism is called state transition. In state transition the light harvesting complexes are relocated between PSII and PSI to balance the excitation pressure on both PS (Minagawa, 2011). This is realized by phosphorylation of light harvesting complexes and photosystems by the redox regulated state transition 7 and 8 (STN7 and 8) (Depège et al., 2003; Bellafiore et al., 2005; Bonardi et al., 2005).This allows an equal distribution of electrons over the PET chain leading to a higher photosynthetic yield and due to this, less formation of ROS.

The third process occurs only after prolonged severe light stress. This process is characterized by components of the two other mechanisms, but the reversion when plants experience non stressed conditions is much slower (Müller et al., 2001).

Chilling stress slows down enzymatic reactions, while the uptake of light and thus the excitation of pigments is hardly effected (Ensminger et al., 2006). In light, the excitation of electrons is enhanced, but the speed of enzymatic reactions is not altered. In response to both treatments the excitation pressure on the photosynthetic electron transport chain is increased as the consumption of excited electron is lower than their production. A condition that raises the levels of reactive oxygen species (McDonald et al., 2011). If cold priming is caused by a general protection against ROS, it should cause priming effects on excess light triggering.

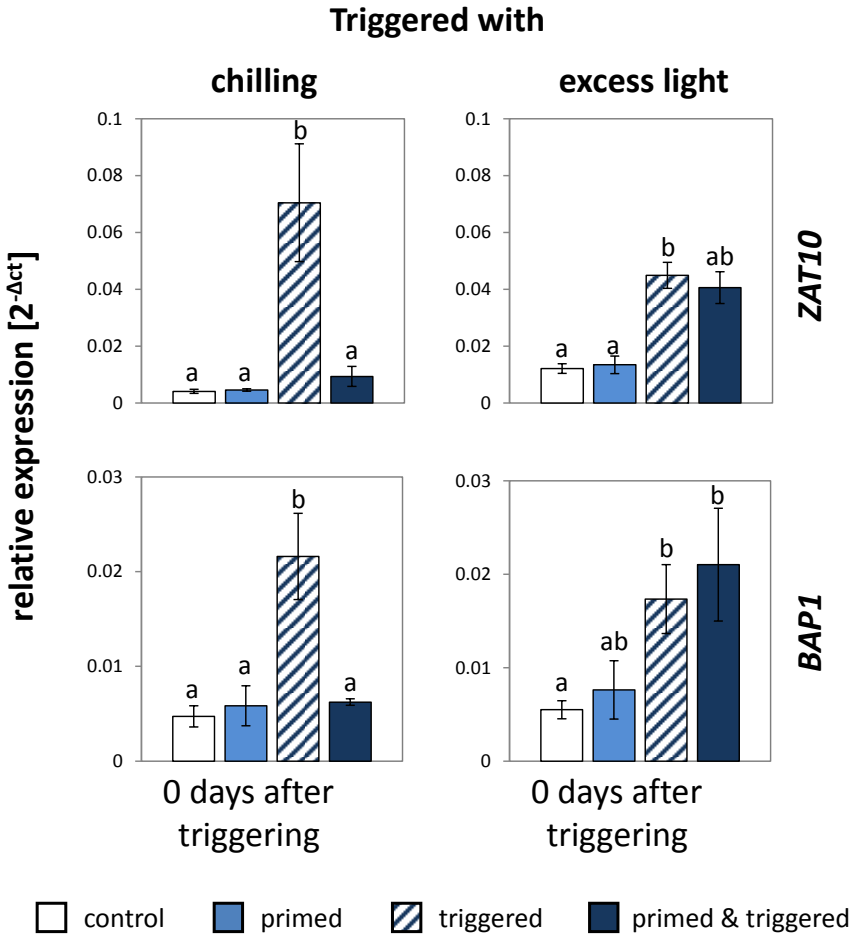

Figure 29: Test for the specificity of triggering in Col-0 triggered with chilling stress (left) and excess light (right). ROS marker genes *ZAT10* (top) and *BAP1* (right) were analysed n=3±SD, ANOVA (Tukey's HSD) p<0.05

Figure 29 displays that in contrast to triggering with chilling stress, excess light triggering indicated no differences in the expression levels of the ROS marker genes *ZAT10* and *BAP1* between T and PT plants. This finding was contrary to the response of the cold/cold combination as priming/triggering, where the induction of *ZAT10* and *BAP1* was supressed in PT plants. Excess light triggering led to a similar expression intensity as triggering with chilling stress did, showing that the ROS stress of chloroplast was essentially the same between the two different stresses (fig. 29).

ROS signals are in general not specific to a stress, but are induced by a huge variety of environmental conditions. (Møller and Sweetlove, 2013). Cold and elevated light share a high excitation pressure on the PET chain (Huner et al., 2012). A large proportion of genes is inducible by cold (Lissarre et al., 2010). 50 % of the genes are only induced in the light under cold conditions, 25 % percent only when plants are cold treated in darkness and the residual 25 % are induced under both conditions (Soitamo et al., 2008). This shows that beside the high excitation pressure others signals must specify which kind of stress is affecting the PET chain. *ZAT10* was found to be regulated independently of light or darkness, showing that the cold stress itself is the main regulatory force (Soitamo et al., 2008). This however, does not mean that *ZAT10* is not responsive to high light stress, as could be shown e.g. by Rossel et al. (2007). Thus it might be that *ZAT10* was predominantly responsive to the cold stimulus and affected differently by the excess light triggering, preventing the priming effect, because of the activation by different signalling cascades.

Light perception falls into two categories, first the light quality perception by photoreceptors (Galvão and Fankhauser, 2015) and second the light quantity perception by e.g. changes in the redox state of the plastoquinone pool (Li et al., 2009), but light quantity changes can also be detected by phototropin via excess of blue light (Jarillo et al., 2001; Li et al., 2009). Thus the specificity for priming can be achieved by a combination of different signalling cascades. While in cold treatments signalling via the PET chain (e.g. ROS) signalling occurs, at the same time IEC1-CBF-COR reactions are induced. Under excess light, as in cold, signalling via the PET chain is present, but additionally light intensity reaction could be launched e.g. via phototropins. In these cases ROS production is the necessary condition for priming, as ROS signalling is shared by all

stresses. The sufficient condition, which provide specificity, are ROS independent signals, e.g. via CBFs or phototropins. This two component system would allow to identify stress first via an induction of plastidic ROS, which is shared by a variety of stresses. The second signal would be integrated via stress specific responses, e.g. Ca^{2+} with stress specific amplitudes (Kiegle et al., 2000) or the induction of the ICE-CBF-COR regulon.

4 Discussion

In this study, the term priming is referred to as the perception, maintenance and access of information. Storage and access of information is also realized in cold stress acclimation (Xin and Browse, 2000), but still priming is not to be mistaken with acclimation. For acclimation a lot of focus was given on gene regulation of the ICE-CBF-COR regulon (Thomashow, 1999; Zhu et al., 2007; Lissarre et al., 2010). In typical acclimation experiments, the initial mild stress condition is immediately followed by a lethal or harsh sub-lethal stress. As a result acclimated plants have higher survival rates or are able to endure higher stress levels and such as a reduced leakage of electrolytes, indicating less membrane damage compared to non-acclimated individuals can be observed (Hannah et al., 2006; Zuther et al., 2015).

Recently it was discussed that *CBF1* and *CBF2* can be induced systemically by cold treatment of only a subpopulation of leaves (Gorsuch et al., 2010), a process called systemic acquired acclimation (SAA). Furthermore SAA was observed after a high light treatment (Karpinski et al., 1999; Rossel et al., 2007; Karpinski and Szechynska-Hebda, 2010; Szechynska-Hebda et al., 2010), elevated CO_2 (Lake et al., 2001) and in combination of shading and high CO_2 concentrations (Coupe et al., 2006). In systemic acquired acclimation an induction of *ZAT10* in non-stressed systemic tissue was detected after exposure to high light (Rossel et al., 2007) and cold temperatures (Gorsuch et al., 2010). Mittler et al. (2011) proposed that 'ROS waves' can distribute the information of a local stress to systemic tissue.

Both acclimation and systemic acquired acclimation require the storage and access of information, within the exposed or in distant tissue. In priming, the two different stimuli are separated temporally which makes the maintenance of information an essential requirement. Cold acclimation is achieved after a couple of days to weeks dependent on the species used (Guy et al., 1985; Livingston III et al., 2007). Short temperature drops (2h) do not lead to acclimation to freezing stress, even if repeated for a period of six days (Stavang et al., 2008).

In order to test *Arabidopsis thaliana* for the capability to establish a 'real memory', and as opposed to merely a preparation to an immediately subsequent stress, in this study a five days lag-phase was introduced in between the two cold stress treatments. Livingston III et al. (2007)

could show that one day at low temperature is not enough to induce freezing tolerance. Because of this the length of the priming stimulus was reduced to one day to reduce metabolic reprogramming to a minimum and to avoid the resulting acquisition of freezing tolerance. To take into account, that no freezing tolerance could be induced, the second cold stimulus was not lethal, but had the same intensity as the first one (fig. 7).

Ultimately, priming without triggering is suggested to lead to a decrease in fitness (Hilker et al., 2015). Consequently the ability to forget is as crucial as the initial step of memorizing. An 'endless memory' would carry the risk that the predictive value of the stress cue itself might be decreasing over time, so a disadvantageous stage would be maintained without a later benefit (Crisp et al., 2016). So energy is invested without a return of the earlier investment. It is claimed, that the forgetting is more often used than the maintenance of priming (Crisp et al., 2016). Contradicting to this are the findings mainly in *Arabidopsis thaliana* for priming effects for a broad range of stresses (see chapter 1.8).

The stress treatments used in the present experimental setup resemble comparatively mild stress conditions for *Arabidopsis thaliana*. They are encountered by most accessions regularly in spring without strong phenotypical interference, provided they persist for only 24 h. These permissive conditions were used to distinguish regulatory effects and reduce pleiotropic effect to a minimum.

4.1 Priming does not cause extensive cellular reprogramming

A 24 h long stress treatment at 4 °C was sufficient to increase the overall H_2O_2 level nearly threefold (fig. 3) and to induce a strong transcriptional response of cold regulated *COR15A*, *ZAT10* and *BAP1* (data not shown), as well as various members of the plastidic ROS detoxifying enzymes (fig. 5). Under chilling stress conditions, plants arrest growth and reprogram their metabolism (Atkin et al., 2006). Seven days after triggering, primed and triggered plants were significantly smaller than that of other treatments (fig. 11). The growth difference between control and primed and triggered plants was 10 %. This effect was pronounced, considering the fact that the PT plants only experienced 48 h at 4 °C in total. In general, cold treated plants were slightly smaller than control plants. The longer the cold stimulus was past, the smaller was the growth defect after

triggering (fig. 11, compare P to T or PT plants). The small growth defect was additive, the growth difference between C and P plus C and T plants resembled the difference in diameter between C and PT plants. However the smaller rosette diameter could not be correlated to changes in fresh or dry weight (fig. 11). This might be explained through an increased leaf thickness. In this case there would no growth delay, but a shift in growth parameters from leaf enlargement to increased leaf thickness (Ensminger et al., 2006).

Very subtle adjustments of the chlorophyll levels could be observed in Col-0 during the experimental course (fig. 12 and 13). The ratio of chlorophyll a to chlorophyll b was stable throughout the experiments, which is in congruence with the results of Savitch et al. (2002) during cold stress. This could be taken as a hint, that balance of the photosynthetic electron transport chain was not altered heavily. The total chlorophyll amount (and the amount of chl a and chl b) was only slightly altered during the course of the experiment.

Ascorbate accumulates upon long chilling periods (Juszczak et al., 2016) utilizing the increase in carbohydrate levels by increased activity of enzymes of the Calvin-Benson cycle (Strand et al., 1999). Ascorbate is used by ascorbate peroxidases to detoxify H_2O_2 to water (Asada, 1999; Mittler, 2002; Asada, 2006). Plastidic ascorbate peroxidases need high levels of ascorbate to avoid inactivation (Miyake and Asada, 1996; Shigeoka et al., 2002; Liu et al., 2008). So if oxidized ascorbate becomes depleted under stress conditions, the half-life of functional tAPX does not exceed 15 seconds (Miyake and Asada, 1996). After the application of a priming stimulus a slight trend for an up-regulation of the total amount and the percentage of reduced ascorbate was monitored (fig. 4), after triggering no such effect was observed (fig. 14), none of the changes were significant. This underlines the assumption that the short chilling stress pulses were not sufficient to cause a major reprogramming of the cellular metabolism to reach an alerted state, which would be reflected in the level of ascorbate.

Yet, all these data point out that the chilling stress is perceived by the plants and first defence responses were initiated (e.g. the up-regulation of *sAPX* and *MDAR*, fig 5). But the stress did not affect the phenotype severely. Neither dramatic changes in chlorophyll nor in ascorbate levels were quantified. This makes the system suitable to investigate changes in regulation without measuring side effects by inducing acclimation.

4.2 Most genes of the water-water cycle are insensitive to either chilling stress or priming

To counteract ROS accumulation under stress conditions antioxidant enzymes are frequently induced (Asada, 1999, 2006). One of the main production sites of ROS in plants are chloroplasts (Foyer and Noctor, 2003). Immediately after the cold treatments no significant changes for enzymes of the ascorbate independent water-water cycle could be detected (fig. 5, 6 and 8). *GPX7* and *2CPB* were slightly, but not significantly, induced upon priming (fig. 5) providing some protection to the chloroplast, but not after triggering (fig. 8). Six week old plants, exposed to a fourteen day chilling stress treatment showed a downregulation of *2CPA* and *2CPB*. Expression of *GPX7* and *CSD2* remained unchanged, while *GR* is clearly up-regulated (Juszczak et al., 2016). This shows that the regulation of the ascorbate independent water-water cycle is dependent on the length of the cold stimulus and the age at which plants are treated with cold. It appears that longer periods of chilling stress inhibits parts of the plastidic antioxidant system, while short stresses left the expression profiles mainly unchanged (fig. 5, 6 and 8).

In eight day old plants, promotor activity of *2CPA* is high (Baier et al., 2004). Cold treatment in the light, but not in the dark leads to a higher promotor activity of *2CPA* in younger leaves, yet this cannot be seen in older ones (Baier et al., 2004). Approximately 70 % of 2CPA attaches to the thylakoids under control conditions. Under cold stress of a few hours, a larger proportion of 2CPA accumulates in the inactive monomeric form (König et al., 2003). In this study, no induction could be observed at the transcript level, showing that *2CPA* regulation at this developmental stage was insensitive to cold stress (fig. 5 and 8). The non-accumulation of 2CPA does not exclude 2CPA to act as a redox sensor. Inactive, fully oxidised 2CPA oligomers accumulate at the thylakoid membranes. These oligomers are enzymatically inactive and could be stored as a signal for oxidative damage (König et al., 2003; Dietz et al., 2008). Large accumulation of 2CPA at the thylakoid membranes can only be found by application of as much as 5 mM H_2O_2 (König et al., 2003). This level of hydrogen peroxide was likely not achieved after a 24 h long chilling stress. No increase of *2CPA* promotor activity could be observed during or after cold stress (data not shown) and on transcript level (fig. 5 and 8). This makes it unlikely for 2CPA to be involved in shaping the

plastidic ROS response upon triggering in this study and thereby reducing the activation of *ZAT10* and *BAP1* in primed and triggered plants by mitigating plastidic ROS (fig. 9).

Not much is known about the regulation of *2CPB*. But its down-regulation upon cold stress is reported after a treatment as short as two days (Bondino and Valle, 2009) and after 14 days (Juszczak et al., 2016). After one day of chilling stress only minor differences in regulation were quantified (fig. 5, 6 and 8). This makes *2CPB* unlikely to be a main force in ROS regulation in priming.

GPX7 transcript accumulated only to a smaller amount compared to *2CPA*. Their Ct-values differ by more than six cycles. This translates into more than 60 times less mRNA, which is likely to affect the protein abundance. Depletion of *GPX7* was correlated with higher levels of salicylic acid and improved resistance against Pseudomonas (Chang et al., 2009). Additionally, the lack of *GPX7* alters the root length, formation of lateral roots and affects rosette growth (Passaia et al., 2014). The high internal variation of the samples did not allow significant statements about the regulation of *GPX7* (fig. 5, 6 and 8). These highly variable results were unlikely to be the reason of the consistent priming effect on two ROS marker genes for the H_2O_2/O_2^- and singlet oxygen pathway (*ZAT10* and *BAP1*, fig. 9).

CSD2 was reacting neither to priming nor to triggering (fig. 5, 6 and 8). Only a very subtle, not significant, increase could be seen one day after priming (fig. 6). Under heat stress *CSD2* is down regulated. For heat acclimation, control by the *miR398* is essential (Guan et al., 2013; Lu et al., 2013). Also upon oxidative stress, *CSD2* transcript abundance is affected indirectly by down-regulation of its miRNA (Sunkar et al., 2006). However, the correlation between *miR398* and *CSD2* is weak in Col-0 and *CSD2* is transcriptionally rather down-regulated upon chilling stress independent of *miR398* regulation (Juszczak et al., 2012; Juszczak, 2013).

The glutathione reductase plays a crucial role in regeneration of glutathione. Glutathione is, besides ascorbate, the second major low molecular weight antioxidant in plants (Asada, 1999). *GR* is important for maintenance and repair of PSII under excess light (Ding et al., 2016a). The artificial knock-down of *GR* leads to early onset of senescence (Ding et al., 2016b). In various different Arabidopsis accessions glutathione reductase activity was found to be increased by

chilling stress, but not after as little as one day (Distelbarth et al., 2013). After 14 days *GR* activity was increased (Distelbarth et al., 2013) as was the transcript abundance (Juszczak et al., 2016). Here, after a 24 h long cold stimulus no significant up regulation of *GR* could be found after either priming or triggering. Taken together with the results from Distelbarth et al. (2013), this suggests that it is unlikely for *GR* to be involved in the priming effect of *ZAT10* and *BAP1* reported in figure 7.

4.3 Only two genes of the PAS are clearly inducible by 24 h chilling stress

The analyses revealed, that chilling stress only induced two of the eight enzymes of the plastidic antioxidant system (fig. 5, 6 and 8): the stromal ascorbate peroxidase and the monodehydroascorbate reductase after both the priming and the triggering cold stimulus.

I. *sAPX*

The stromal ascorbate peroxidase detoxifies H_2O_2 to water in an ascorbate-dependent manner (Asada, 1999, 2006). Like tAPX, sAPX is inactivated by a low supply with ascorbate and high concentrations of H_2O_2 (Miyake et al., 1993; Miyake and Asada, 1996). A low supply of ascorbate leads to the oxidation of a tryptophan of the apoprotein via the haem group at the catalytic site. This ultimately inactivates plastidic APXs (Kitajima et al., 2008). Responsible for the inactivation are two cysteine residues and one tryptophan residue. Mutation of this residue can increase APX tolerance to H_2O_2 (Kitajima et al., 2008). Another reason for the easy inactivation is provided by a small 16 amino acid residue loop that could not be found in hydrogen peroxide tolerant forms. Additionally its deletion lead to higher tolerance against H_2O_2 (Kitajima et al., 2010).

Expression of *sAPX* was always induced while experiencing chilling conditions (fig. 5, 6 and 8). The transcript abundance was induced, by the triggering cold stimulus, independent of the pre-treatment (fig. 8). A number of different ROS scavenging enzymes are located directly at the site of ROS production, the thylakoid membrane, namely the CuZn superoxide dismutase (Asada, 2006), thylakoid bound ascorbate peroxidase (Asada, 2006) and a large proportion of 2-Cys peroxiredoxin A (König et al., 2003). *sAPX* belongs to the ‘second line of defence’, which protects

if hydrogen peroxide slipped through the initial defence mechanisms at the thylakoids (Asada, 2000). Induction of sAPX is part of the response to cold stress, but its regulation showed no indications for a priming-related response. The knock-out of sAPX was sufficient to abolish the primable response of two plastidic ROS marker genes (fig. 15). However, the effect was less severe than the effective inversion of *ZAT10* in *tapx* plants. In long term priming sAPX is the driving force of the plastidic ROS signalling (Cvetkovic, 2016). In short term priming it seems to support the signalling, but it is not the main contributor (see chapter 4.5).

II. *MDAR*

The monodehydroascobrate reductase reduces MDA to ascorbate and regenerates asc for plastidic ascorbate peroxidases (Asada, 1999, 2006). *MDAR* is important for the function of plastidic ascorbate peroxidases, which are easily inactivated by low supply with ascorbate (see 4.3 I.). Overexpression of *MDAR* in *Lycopersicon esculentum* leads to lower H_2O_2 accumulation under temperature stress (4°C and 40°C) as well as higher tolerance to methyl viologen (MV) (Li et al., 2010). Like *sAPX*, *MDAR* was induced directly upon chilling stress, but no differences could be monitored after 24 h (fig. 6).

MDAR and *sAPX* mRNA levels, but not *tAPX* expression, were highly correlated under long lasting chilling stress (Juszczak et al., 2016). The same observation was made in this study (fig. 5, 6 and 8). Even though the induction amplitude were lower compared to *COR15A*, the expression profiles upon triggering were alike (fig. 8 and 10). This shows that *MDAR* and *sAPX* were directly responsive to chilling stress. Neither the length of the chilling stress, nor the age of the plants seem to play an important role for induction (fig. 8, Cvektovic, 2016, Juszczak et al., 2016). Both enzymes are important parts of the plastidic antioxidant system. The lack of studies investigating the response of the ascorbate dependent water-water cycle on different kinds of stressors does not allow to deduce whether the up-regulation of these two enzymes is due to changes in the redox environment within the chloroplasts or whether it is specific to chilling stress. But both gene show no priming effect, thus they are unlikely to modulate the gene expression of *ZAT10* and *BAP1* in a priming-dependent manner.

Detoxification of ROS in chloroplasts is realized by different layers of defence, directly at the thylakoids and in the stroma. The results obtained show an induction of the 'second line of defence', the stroma, as it was described in Juszczak et al. (2016). Not a single one of the thylakoid bound or associated genes reacted directly to chilling stress (fig. 5 and 8).

4.4 The ROS detoxification capacity at the thylakoids is increased after chilling stress

There are a number of reports that altering expression of *tAPX* leads to altered stress resistance, such as elevated tolerance against high light in tobacco (Yabuta et al., 2002). *tAPX* overexpression in Arabidopsis leads to higher tolerance against MV, but not against temperature stress (Murgia et al., 2004). Furthermore, *tAPX* overexpression also delays the symptoms of the hypersensitive response in Arabidopsis (Yao and Greenberg, 2006). In tobacco, *tAPX* leads to better performance under temperature stress (Sun et al., 2010) and overexpression of *Suaeda salsa (seepweed) tAPX* was accompanied by higher tolerance against high light (Pang et al., 2011). A knockdown of *tAPX* in Arabidopsis correlated with higher sensitivity to MV (Tarantino et al., 2005) and in tomato *tAPX* a knockdown by RNAi decreased the tolerance against chilling stress (Duan et al., 2012).

On the one hand *tAPX* overexpression enables plants to survive a variety of different stress situations, but on the other hand transcription of *tAPX* was not affected during chilling stress (fig. 5 and 8). Similar results were obtained by studies applying longer chilling stress treatments, after which *tAPX* was found to be down-regulated (Juszczak et al., 2012; Juszczak et al., 2016). This findings suggests, that the protective function is not available when plants are cold treated. This indicates that the circumstances, where the protection is needed and were it occurs are not identical.

Instead of being induced upon chilling stress, *tAPX* levels increased in the post-stress phase. Promotor activity was still significantly induced five days after priming (fig. 15), mRNA levels were elevated (fig. 6). On the protein level an induction compared to C plants was observed (fig. 16).

Without any externally supplied stimuli the alteration of *tAPX* was kept for at least five days. This allows for two different interpretations:

- the shift from chilling to control conditions stressed the plants and *tAPX* was induced to cope with this situation or
- concomitantly with the hypothesis of priming, energy is invested into priming, so plants prepared themselves for a future stress.

In part the first assumption conflicts with the fact that plants were grown for four weeks prior to priming at standard growth conditions. Chilling conditions were applied only for 24 h before the shift back to the standard growth environment. The stress length and intensity were chosen because they did not induce defects in growth, chlorophyll accumulation or ascorbate levels (4, 11, 12, 13 and 14). Additionally levels of *sAPX* and *MDAR* were only up-regulated upon stress and not one day later (fig. 6 and 8). Even if the transfer from chilling stress to standard growth conditions is connected to cellular rearrangements, they should not last as long as five days. This factors seem to make the second interpretation more likely.

As already discussed above, high levels of *tAPX* are beneficial under a variety of stress conditions. The long lasting induction could fulfil two purposes: it could be a preparation induction for a future stress or it could give a measure for the time passed since the stimulus was perceived, respectively.

The promotor activity and the protein levels of *tAPX* were induced for at least five days (fig. 15 and 16). On transcript abundance level, *tAPX* expression was normalized after seven days (fig. 8). So directly before triggering plastidic ROS protective capabilities were elevated in primed plants compared to their control.

Two different studies have assessed the signalling function of plastidic APXs. In rice both forms of plastidic *APX* (*sAPX* and *tAPX*, *OsAPX7* and *OsAPX8*) were knocked down to roughly 50 % in transcript and overall plastidic APX activity. A proteomic approach showed, that up-regulated proteins were mainly associated with photosynthesis (~50 %) and response to oxidative stress (~25 %) (Caverzan et al., 2012). In Arabidopsis *tAPX* was repressed by RNAi using an estradiol inducible system (Maruta et al., 2012). When *tAPX* was repressed, lower transcript levels of *CBF1*,

an important regulator of the cold response, was found. Additionally more than 700 genes were deregulated after the estradiol mediated RNAi induction, 25 % of them are involved in signalling (transcription factors and protein kinases). Both papers show that tAPX is crucial for the proper functioning of chloroplasts. And if the expression is disturbed major re-adaptions of the cells are conducted.

High ROS levels interfere with the PET chain, reducing the level of D1 protein under oxidative stress (Nishiyama et al., 2001). The D1 protein is located at the center of PSII, as its close homologue D2 (Edelman and Mattoo, 2008). Elevated ROS levels, especially singlet oxygen, inactivate the *de novo* synthesis of D1 (reviewed in (Nishiyama et al., 2011)). This shows that ROS have an impact on the PET chain. Increased level of tAPX can reduce the ROS burden, especially at the thylakoids. Hence by increasing tAPX levels the plant might be able to reduce the inhibitory effect of ROS on PSII repair.

The redox state of the plastoquinone pool is an important factor for retrograde signalling (Pfannschmidt et al., 2001; Bräutigam et al., 2009; Pesaresi et al., 2010) and since the rate at which PSII reduces PQ strongly exceeds the rate of intersystem electron transport to PSI, its relative reduction level is mostly determined by PSII excitation pressure, i.e. the proportion of closed PSII reaction centres. Thus the protection of the D1 repair cycle from oxidative damage might be one mode of action that is impacted on by the increase in tAPX and can affect retrograde signalling by keeping the PQ pool in a more oxidized state.

4.5 *tAPx* induction in Col-0 is associated with a decrease of plastidic ROS marker genes

A priming-dependent change in two different marker genes for two different ROS signalling pathways was found (fig. 9), the singlet oxygen and the H_2O_2/O_2^- pathway. *BAP1* is mainly responsive to singlet oxygen, while *ZAT10* reacts predominantly to the production of H_2O_2 and O_2^- (Laloi et al., 2004). The reaction of both genes in triggered only plants proceeded as expected. A short period of chilling stress lead to the formation of ROS (fig. 1 for H_2O_2), which was in part produced in the chloroplasts and both ROS marker genes were responsive (fig. 9). If plants

perceived a warning cue (priming), five days before, no induction compared to control plants could be observed (fig. 9 compare T and PT plants). The lack of induction of *ZAT10* matches with the fact that the level of *tAPX* was increased (fig. 6, 15 and 16).

A decrease in *ZAT10* stands exemplary for a reduction of hydrogen peroxide and/or superoxide in chloroplasts (Laloi et al., 2006). In general, there are a lot of problems to define the required experimental proof to define a retrograde signal (Pfannschmidt, 2010). Especially as there are many different modes of actions conceivable (reviewed in Leister (2012) and Pfannschmidt (2010)). . Also, Ca^{2+} signals are known to play a role here, as well as tetrapyrols the strongest focus of discussion constitute metabolites having a function as retrograde signal, e.g. the signalling function of 3'-phosphoadenosine 5'-phosphate (PAP) found by Estavillo et al. (2011), ROS (mostly H_2O_2) (Apel and Hirt, 2004) or the redox state of the plastids (Baier and Dietz, 2005).

4.5.1 Signal transduction from the chloroplast to the nucleus

How the priming signal, which causes elevated levels of *tAPX*, is transduced from chloroplasts to nucleus, to repress *ZAT10* expression, remains unclear. As already discussed, it could be transmitted by changes of the D1 protein homeostasis. Alternatively ROS (mainly H_2O_2) signalling might cause the repression of the *ZAT10* expression in PT plants by the dislocation of plastidic H_2O_2 to the nucleus via the cytosol. Hydrogen peroxide is able to pass membranes e.g. via the tonoplast intrinsic protein 1 and 2 (TIP1 and 2) in Arabidopsis (Bienert et al., 2007). Under high light conditions, up to 5 % of the plastidic H_2O_2 is able to escape the PAS and be translocated outside of the chloroplast (Mubarakshina et al., 2010). Even the more reactive singlet oxygen is discussed to reach the nucleus by crossing the cytosol (Fischer et al., 2007). ROS may also reach the nucleus via chloroplast stromules. Stromules are stroma filled, tubular membrane extension of the chloroplast. They are produced during the innate immune response (Caplan et al., 2015), by exogenous application of H_2O_2 (Caplan et al., 2015), by stress pathways acting through ABA (Gray et al., 2012) and plastidic redox signals (Brunkard et al., 2015). Stromules may represent a way to transport ROS directly from chloroplasts to the nucleus without shuttling them through the cytosol. A third possible way would be signals transduced by 3'-phosphoadenosine 5'-

phosphate (Estavillo et al., 2011). Under high light and drought stress the enzyme dephosphorylating PAP (SAL1) is suppressed allowing PAP to travel to the nucleus and induce *ZAT10* expression (Estavillo et al., 2011). If the signal suppressing SAL1 is related to enhanced excitation pressure this pathway may also work under chilling stress conditions.

4.5.2 Knock-out lines prove a causal relationship between tAPX expression and the priming response

Examination of a knockout line of *tAPX* showed a link between the induction of tAPX and the reduced induction of *ZAT10* (fig. 17). Already characterized *sapx* and *tapx* lines (Kangasjärvi et al., 2008) underwent 24 h 4 °C priming and triggering (chapter 3.10). The *sapx* line was used as a control for this experiment. Like *tAPX*, *sAPX* is important under redox stress, but was not primable under repeated chilling stress conditions (fig. 8). The usage of this line shows the basal disturbances of the system, when a ROS scavenging enzyme is missing under oxidative stress conditions, even though other peroxidases are able to compensate its absence (Kangasjärvi et al., 2008). The knockdown of *tAPX* was found to reduce levels of *CBF1* leading to a decrease in transcript abundance of *COR* genes (Maruta et al., 2012). Expression of *COR15A* is controlled by CBF1 via C repeat/dehydration-responsive (CRT/DRE) elements (Wang and Hua, 2009). In both genotypes expression levels of *COR15A* were diminished compared to Col-0 (data not shown). Independent of the induction strength, the ratio of induction from T to PT plants were around 1 in Col-0 and both mutant lines (fig. 17). This suggests, that the induction intensity of *COR15A* is only affected by cold stress and not by priming or the knockout of either of the two plastidic ascorbate peroxidases.

In contrast to *COR15A*, the regulation of *ZAT10* and *BAP1* was altered in *sapx* and *tapx* lines (fig. 17). In both ascorbate peroxidase knockout lines the difference of induction between T and PT of *BAP1* vanished. As for *COR15A*, the T to PT ratio was 1. Changes of the singlet oxygen responsive gene *BAP1* were probably due to pleitropic effects, because of the reduction of ascorbate peroxidases and the induction of different plastidic ROS scavengers, modulating the ROS signature (Kangasjärvi et al., 2008).

The priming effect on *ZAT10* was also lost in *sapx* plants. In *tapx* plants the regulation of *ZAT10* was inverted, the induction in PT plants was more pronounced compared to T plants (fig. 17). In primed and triggered plants expression of *ZAT10* was 3 times higher compared to triggered only plants. This strengthens the hypothesis that the induction of *tAPX* is essential for the priming effect of *ZAT10* in Col-0.

The de-regulation of *ZAT10* can be expected to influence stress protection in the entire cell, as *ZAT10* controls *APX2* expression (Mittler et al., 2006). Together with *APX1*, *APX2* function protects the cytosol against oxidative damage (Suzuki et al., 2013). *APX2* expression is induced by both H_2O_2 and redox changes in the plastidic electron transport chain (Yabuta et al., 2004). But *APX2*, is mostly discussed in context of high light stress (Karpinski et al., 1999; Mullineaux and Karpinski, 2002; Fryer et al., 2003; Rossel et al., 2006), even in response to wounding stress *APX2* upregulation is discussed to be PET chain controlled (Chang et al., 2004). The priming effect on *ZAT10* was strong, while in contrast *APX2* transcript levels were non-detectable (Ct-values were within the range of the negative control, data not shown). Under non-stressed conditions, *APX2* is expressed at a very low level, which was demonstrated when the APX2 promotor fused to luciferin failed to give a signal under control conditions (Fryer et al., 2002). This supports the notions that (a) the used stress conditions were possibly to mild to lead to an induction of *APX2* and (b) while an induction of a plastidic ascorbate peroxidase (*sAPX*, fig 5 and 8) took place, the cytosolic ascorbate peroxidase system was not affected. This supports the fact that the ROS burden is compensated by the chloroplasts and not in the cytosol and as such signals controlling the primability of the *ZAT10* and *BAP1* response originates in the chloroplast.

4.6 Differences in the effect intensity of priming and the primability in natural *A.thalina* accessions

A comparison of the four Arabidopsis accessions Col-0, Ms-0, Kas-1 and Cvi-0 showed that primability is a trait adopted in different climate zones (see fig. 21).

I. *Warm adapted accession*

In Cvi-0, *ZAT10* and *BAP1* were not cold primable (fig. 21). The response of *ZAT10* was the same as in PT and T plants, comparable to the response of *COR15A*. This indicates, that a mechanism enabling primability in response to chilling stress is absent in this accession. Cvi-0 originates from the Cape Verde Islands, where chilling period are very rare, even during the nights (fig. 20). Thus, some of the essential prerequisites for being primable could be lost during adaptation when Arabidopsis colonized the Cape Verde Islands.

Southern Arabidopsis accession where found to have lost the ability for cold acclimation if plants got challenged by two nights in a row with temperature below -10°C and a day temperature of 4°C (Zhen et al., 2011). For Cvi-0, it has been described that *CBF1* is only very weakly expressed (Barah et al., 2013). Additionally, it was found by several groups, that the latitude of origin (Zhen and Ungerer, 2008a; Zuther et al., 2012) and the average winter temperature (Hannah et al., 2006) correlates with the ability to acquire freezing tolerance, a phenomenon potentially caused by a relaxed selection on *CBF/DREB1* in more southern accessions (Zhen and Ungerer, 2008b). Nothing is known about how ROS signalling differs in Arabidopsis accessions. *BAP1* however, is not only induced by singlet oxygen, but the response of *BAP1* was found to be mediated by ICE1 (Zhu et al., 2011). This could mean, that the singlet oxygen signalling derived from the chloroplasts is still intact, but the response of ICE1 is changed in Cvi-0, as is the CBF response (Barah et al., 2013). *ZAT10* expression is also connected to cold signalling. Enolase 2 (LOS2) can bind to the promotor region of *ZAT10* and represses the induction of *ZAT10* and its activating the response essential for freezing tolerance (Lee et al., 2002). The overexpression of *ZAT10* had little effect on the ability to acquire freezing tolerance (Park et al., 2015), so the reduction of transcript levels of *ZAT10* may be to fine-tune the response to chilling stress to gradual changes in temperature, rather than playing a role in the massive impact of a lethal freezing stress.

This indicates that the necessary condition for priming may still be fulfilled (plastidic ROS signalling), but the sufficient condition (correct processing of the cold stimulus) could be altered in Cvi-0 due to a relaxation of the selective pressure on cold signalling.

II. *Cold adapted accessions*

In the accession, Ms-0 and Kas-1, *ZAT10* expression was primable (fig. 21). The induction of *ZAT10* in primed and triggered plants was lower compared to the one in triggered only plants. Compared to Col-0 the block of *ZAT10* induction after triggering (PT plants) was weaker. In Col-0 hardly any induction of *ZAT10* in PT plants was monitored, while in Ms-0 and Kas-1 it reached roughly half maximal level. This raise the question why accession from climates with a longer winter and colder spring (fig. 20) showed higher accumulation of ROS marker gene *ZAT10* in PT plants compared to Col-0.

The regulatory circuits in both accessions from colder, harsher climate are different from the one in Col-0. Gabruk et al. (2016) discussed that accessions from northern habitats have a higher ratio of hydroxy-plastochromanol to plastochromanol, which is correlated to higher levels of singlet oxygen. This might induce stronger signalling in comparison to the more southern accessions. Overall this finding (Gabruk et al., 2016) might not only expand to northern and southern accession, but also cause effects when comparing northern with intermediate accessions. After 14 day long acclimation period to cold stress northern accession accumulate more soluble sugars and proline as osmoprotectants compared to Col-0 (Zuther et al., 2015). Additionally the expression of *COR* genes is stronger, caused by stronger activation of *CBF1* (Zhen and Ungerer, 2008b).

Taken together Ms-0 and Kas-1 probably invest more energy in stronger and faster accumulation of the ICE-CBF-COR-regulon and osmoprotective metabolites than Col-0 does. The comparison of the 24 h cold priming presented here, with data from 14 days long chilling stress as the priming stimulus (Cvetkovic, 2016) lead to the hypothesis that primability is negatively affected by acclimation. Under long term priming (14 d) the response of *COR15A* and the two plastidic ROS marker genes was primable. However *ZAT10* and *BAP1*, were not primable to the same extent as after short term priming. The finding that the induction of *COR15A* under chilling stress conditions was nearly twice as pronounced as in Col-0 supports the idea of a invers relation between acclimatisation and priming (fig. 21).

In summary the presented data point out that accession with an intermediate ability to induce freezing tolerance by acclimation have a higher capability for priming compared to cold adapted accessions.

4.7 Primability is age dependent in Col-0

All Accessions used in this study are facultative winter annuals (Michaels et al., 2005; Shindo et al., 2007; Penfield and Springthorpe, 2012; Footitt et al., 2014; Huang et al., 2014). The vegetation period of Ms-0 and Kas-1 are too short for growth and seed production in summer. The plants over-winter in the rosette stage. Seeds are produced in late spring and germinate in beginning of autumn (Shindo et al., 2007). Due to this peculiar life-cycle not only different accessions perceive different climatic conditions, but also different developmental stage throughout the life cycle are challenged with diverse environmental circumstances.

Here, the priming effect was analysed in two, four and six weeks old plants (chapter 3.14), Col-0 at the age of two weeks responded exactly like Cvi-0 (fig. 21) concerning the priming response of *ZAT10* (fig. 25 bottom). The levels of *ZAT10* were induced in T and PT plants but no difference could be observed between the two treatments. The same expression profile was recorded for *BAP1* (fig. 25 top) and *COR15A* (fig. 26). Plants at the age of 14 days were in the 4 leaf stage (stage 1.02 according to Boyes et al. (2001), 2 rosette leaves larger than 1 mm, fig. 19). The main portion of the seedlings nutritional and energy resources are invested into growth and the signalling mechanism needed for priming might not be in place yet, to avoid interference with the increase of biomass. Additionally, as winter annuals, the predictive value of the warning cue (priming) may not be high enough for plants at the young stage 14 days after germination to gain a fitness benefit, establishing primability (fig. 30). In literature no studies are to be found, whether freezing tolerance can be achieved at that age. Even the low costs of priming could have been uneconomic and could in the long run decrease fitness more than the lack of primability.

When climate conditions shifts towards late autumn and early winter, periods chilling stress become more and more frequent, especially during the nights. Under these conditions the predictive value of chilling stress increases (fig. 30). The chance that the priming stimulus is actually followed by a trigger; the chance of the invested resources being beneficial in the long

run, is higher. When the plants were four weeks old, they reached the 1.14 stage (14 rosette leaves larger than 1mm according to Boyes et al. (2001), fig. 19). That age enabled plants to be primed by chilling stress (fig. 9 and 25).

Six week old plants were in between stage 3.50 and 3.70 in terms of rosette growth (according to Boyes et al. (2001), rosette reached 50 % -70 % of its final size, fig. 19). Even under the modified short day conditions in this study (10 h light, 14 h dark) six week old plants are shortly before developing a stem, which marks the transition from vegetative to reproductive growth. This stage is associated with spring, when plants start to bolt. At that stage chilling temperatures are still frequent, even though the frequency is declining. Thus it is likely that plants are challenged by a series of weather conditions mimicked by the priming setup used in this study. Results for *BAP1* (fig. 25 top) and *ZAT10* (fig. 25 bottom) suggest primability for 6 week old plants.

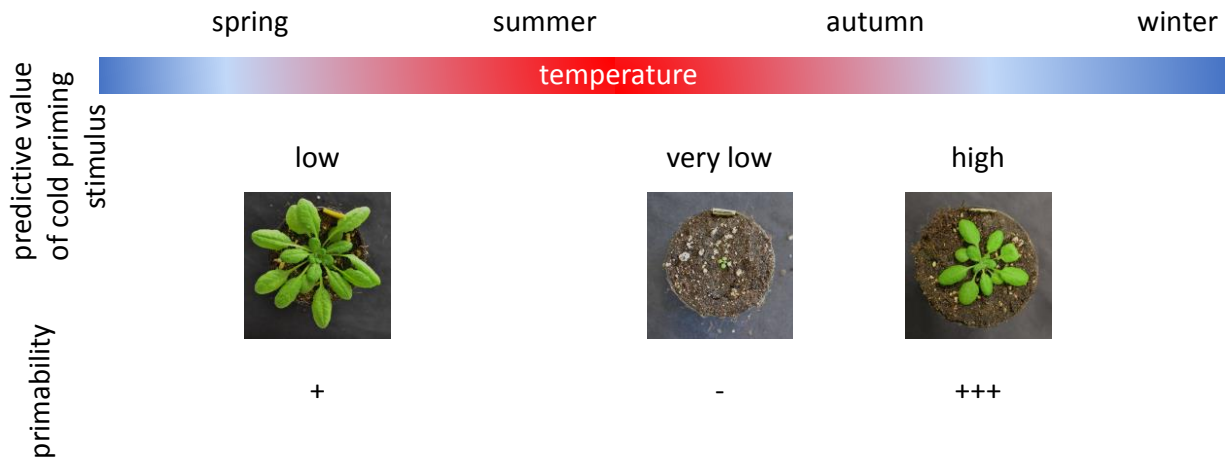

Figure 30: A model for timing of priming and the connection between the average temperature (blue low temperature, red high temperature), the predictive value of the cold priming stimulus and the primability.

The phenomenon of reduced primability correlates with the onset of induction of the senescence marker gene *ORE1* (fig. 27). This is a hint that the onset of senescence could interferes with signalling pathways or underlying mechanism that are needed for priming and that the general scope is more focussed at this developmental stage on reproduction, rather than better survival. However the induction of senescence is not important for the decrease in primability (fig. 28). The higher induction in six week old PT plants compared to four week PT plants is not correlated with the induction of *ORE1* (fig. 28). The reduction of the priming effect can also be seen in

intermediate leaves of older rosette, in which the induction of *ORE1* is still low (fig. 28). Thus the increase of *ZAT10* in six weeks old PT plants was not related to the onset of senescence, but must be connected to a switch that is active in intermediate and old leaves.

4.8 The length of the lag-phase modulates the priming effiency

An important factor for transducing the signal from chloroplasts to the nucleus is *tAPX* availability (see chapter 3.9 and 3.10). After seven days its expression levels in primed plants were statistically indistinguishable from control plants (fig. 8). The primability of *ZAT10* was disabled if plants were triggered after a seven day long lag-phase (fig. 25 bottom). This is again connecting the regulation of *tAPX* to the overall primability concerning chilling stress in *Arabidopsis thaliana*.

After the initial chilling stress, *tAPX* levels were elevated (promotor fig. 15, mRNA, fig. 6, protein fig. 16) and remained elevated over a long period of time. The reduction of *ZAT10* and *BAP1* was recorded due to a better protection at the thylakoids against ROS (fig. 9). When the levels of tAPX fell below a certain threshold the effect of better protection diminished. This indicates a role of tAPX as a timekeeper. After chilling stress the level of *tAPX* increased in the lag-phase, but only if no acclimatory response, by a 14 days long priming stimulus, was induced (compare fig. 6 and Cvektovic, 2016). While the levels of *tAPX* were elevated, primability was observed (fig. 25). Plants did not invest continuously in the better protection of the thylakoids (fig. 8 P plants). The *tAPX* transcript abundance could define a window, in which priming can occur.

Analysis of Arabidopsis after one week of cold stress showed no deregulation of *tAPX* (Juszczak et al., 2012). In six weeks old plants, after two weeks of cold stress the expression level of *tAPX* was decreased under the chilling stress itself and in the day after the stress treatment (Juszczak et al., 2016). The same response was observed in four weeks old plants after two weeks of cold stress (Cvetkovic, 2016). This raises the question how long plants must be treated with chilling stress to induce the tAPX expression and how long the treatment can last before the inducible response is not executed anymore.

By reducing the lag-phase to three days, the efficiency of the decreased induction of *ZAT10* was reduced (fig. 25 bottom). This could indicate that *tAPX* might work as a timekeeper, but besides

tAPX there is another factor establishing the priming effect. This factor could need more than three days to fully develop his effect.

All in all, the priming effect was diminished when the lag-phase deviated from the five day standard pattern. This allows the hypothesis that (I) tAPX levels are crucial for maintaining the maximal possible priming response and (II) there is a second factor influencing the primability that needs longer to ramp up.

4.9 Priming is trigger specific

Priming by short term chilling stress is stressor specific (fig. 29). By substitution of the cold triggering stimulus by an elevated light pulse no priming response of *BAP1* and *ZAT10* could be observed: This suggests that at least one other than the ROS signalling pathway must be involved in the signalling process providing specificity for the stressor.

PSII is discussed to be a 'photon counter' (Park et al., 1996; Melis, 1999). A linear relationship between absorbed photons and photodamage of PSII is described (Kornyeyev et al., 2010). Cold stress induces the ROS production by a strong excitement of the pigments in PSI and PSII. High light conditions result in excess excitation energy for the PET chain, which then increases the number of damaged PSII reaction centers, due to the higher number of arriving photons. The lack of a protease degrading damaged PSII leads to high level of plastidic ROS in variegated mutants (Miura et al., 2010). The increased amounts of damaged PSII in excess light triggered plants could lead to a temporary disproportion of the PSII degrading enzyme and the amount of damaged PSII and as such increase the amounts of produced ROS above a threshold, which is sufficient to restore the abundance of H_2O_2/O_2^- to a level necessary to equalize *ZAT10* expression between cold triggered, excess light triggered and cold primed and excess light triggered plants.

Under excessive light stress changes are induced in the thylakoid membrane dynamic: stacked granas turn into unstacked thylakoids (Yoshioka-Nishimura, 2016). This could be another explanation for the lack of the priming effect in excess light triggered plants. Changes in the spatial arrangement of the thylakoids could disturb the package of tAPX in respect to the photosystems

and might thereby influence the rate of escaped ROS in cold primed and excess light triggered plants.

Additionally both plastidic ROS marker genes are connected to cold stress (chapter 4.6 I.) (Lee et al., 2002; Zhu et al., 2011). This could provide the observed specificity as a signalling pathway of both plastidic ROS and the cold reaction have influence on the expression of *ZAT10* and *BAP1* (fig. 31).

5 Conclusion

Chloroplasts are affected by cold in multiple ways. By perturbing photostasis, an imbalance between the emergence and reduction of excitation energy is introduced (Ensminger et al., 2006). This imbalances can cause an strong reduction of the PQ pool and can change gene expression (Escoubas et al., 1995). On possible way to resolve the strong reduction of the PQ pool is by state transitions, the transfer of light harvesting complexes from PSII onto PSI by the action of a protein kinase (Lunde et al., 2000). Additionally more excited electrons can be transferred to molecular oxygen and give raise to ROS. H_2O_2 is discussed to have two activities in cells: first it is a cytotoxin and second as messenger in cellular signalling (Vanderauwera et al., 2005; Barajas-López et al., 2013). The bivalent function was also shown for other kind of ROS, namely singlet oxygen and superoxide (Laloi et al., 2004; Baxter et al., 2014). In the chloroplast ROS are counteracted by a complex network of antioxidant enzymes and low molecular weight antioxidants (Mittler, 2002).

This study verifies the important role of the plastidic antioxidant system in the regulation of the response to abiotic stress and it highlights the capacity to act as a sensor and simultaneously signal transducer with the ability to induce an extensive reprogramming of nuclear gene expression. Two cold stimuli separated by a short period at standard growth conditions (fig. 7) led to the modulation of the ROS signalling deriving from chloroplasts (fig. 9). It was found, that during and after a short chilling pulse most of the PAS was not altered. Only three genes, namely *sAPX*, *tAPX* and *MDAR*, showed a clear response to the cold stimuli (fig. 5, 6 and 8), yet none of them were primable. The thylakoid bound ascorbate peroxidase accumulated in the lag-phase for up to five days (fig. 6, 15 and 16). This indicates that primed plants memorize the previous stress by building up a higher capacity for protection of the thylakoids against ROS. If primed plants were challenged for the second time, the higher thylakoid protection presumable led to reduced retrograde ROS signalling, measured by a lower induction of plastidic ROS marker genes *ZAT10* and *BAP1* (fig.9). In APX knockout lines, the lack of tAPX inverted the response, *ZAT10* was stronger expressed in PT compared to T plants (fig 17). *BAP1* was not regulated in the same manner, it did only show the same variation of regulation which was observed in *sapx*, showing the strong impact of tAPX on H_2O_2/O_2^- regulated gene *ZAT10*, and lesser influence on the singlet oxygen regulated gene *BAP1* (Laloi et al., 2004; Laloi et al., 2006). Under conditions, where the

tAPX levels were not induced in the lag-phase directly before triggering (in Ms-0 and Ks-0, fig. 22 and 23 and after prolonging the lag-phase to seven days, fig. 8) the priming effect could not be developed to the maximal level for the two plastidic ROS marker genes as in Col-0 (fig. 21 and 25). *PAL1* and *CHS* were 'positively' primable. This is related to a negative correlation of *PAL1* and *CHS* mRNA abundance and the level of plastidic ROS (Fahnenstich et al., 2008).

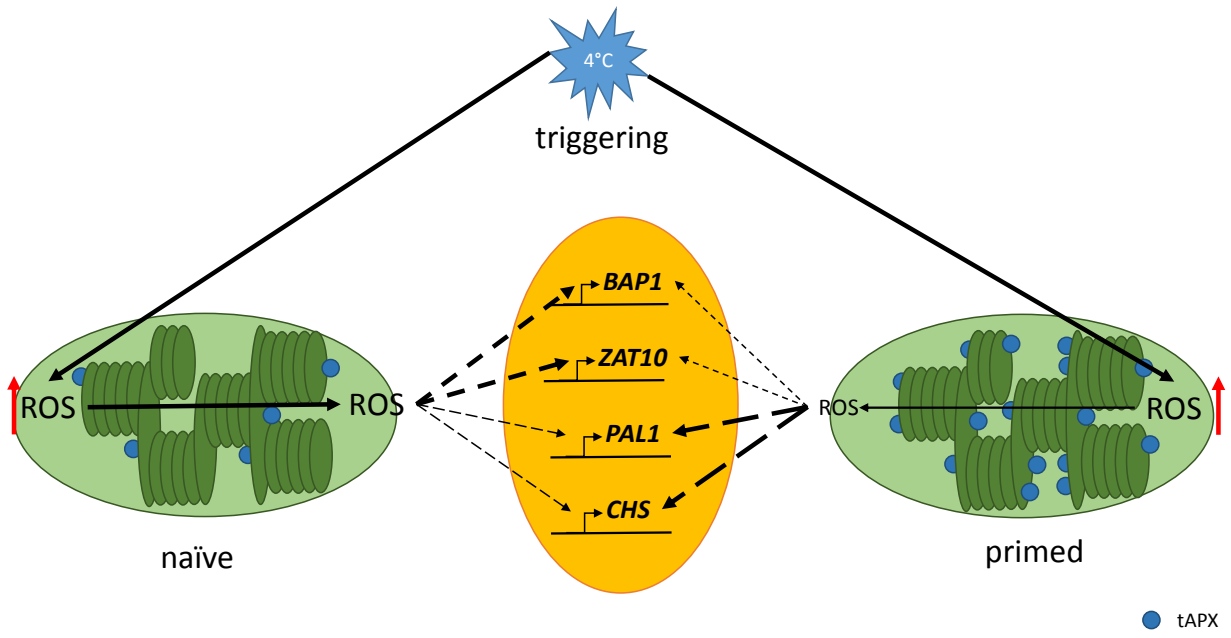

Figure 31: Model of the impact of tAPX on the expression of the nuclear ROS marker gene *ZAT10* and *BAP1* and of *PAL1* and *CHS*. In naïve plants, which are triggered, no preparation were induced to counteract the cold induced ROS production. By chloroplast-to-nucleus signalling *ZAT10* and *BAP1* expression is initiated. By priming an accumulation of tAPX during the lag-phase in chloroplasts takes place. If primed plants are triggered the increased tAPX abundance reduce the amounts of ROS accumulating and as such reducing the ROS signalling to the nucleus, decreasing the induction of *ZAT10* and *BAP1*. For *PAL1* and *CHS* a negative correlation between plastidic ROS and transcript abundance was shown by Fahnenstich et al. (2008) explaining the 'positive' priming response.

This shows, that the thylakoid-bound ascorbate peroxidase mediates the priming effect of *ZAT10*, *BAP1*, *PAL1* and *CHS*. The priming effect is not dependent on the differential expression of *sAPX*, linking the priming effect directly to a better protection against oxidative stress of thylakoids during triggering. This highlights the extensive effect of the plastidic antioxidant system, especially the thylakoid bound ascorbate peroxidase, for ROS based chloroplast-to-nucleus signalling (fig. 31).

6 Summary

Reactive oxygen species (ROS) are produced as natural by-products of aerobic metabolism. The production is strongly increased by stress. They can cause damage to DNA, protein and lipids and are important signalling molecules in stress regulation and plant development. The toxic nature of ROS demands for mechanisms for detoxification. All cellular compartments are protected by a variety of antioxidant enzymes. In plants, a high proportion of ROS is produced in chloroplasts. Superoxide dismutases, ascorbate peroxidases, glutathione peroxidases and peroxiredoxins antagonize ROS levels in chloroplasts, prevent oxidative damage and restrict ROS based chloroplast-to-nucleus signalling.

Experimental evidence for priming of chloroplast-to-nucleus ROS signalling by a short (24 h) 4 °C stimulus in *Arabidopsis thaliana* was found for the first time. In naïve plants, plastidic ROS marker (*ZAT10* and *BAP1*) were strongly induced. A short cold pre-treatment (priming), five days prior to the chilling trigger, abolished accumulation of *ZAT10* and *BAP1*. The influence of the plastidic antioxidant system on ROS signalling was analysed in response to short term cold priming. The priming effect on *ZAT10* and *BAP1* depends on a slow, but long lasting induction of the thylakoid bound ascorbate peroxidase (tAPX) initiated by the priming stimulus. Regulation of *tAPX* took place on the level of promotor activity and was maintained on transcript abundance and protein level. A causal relationship between the presence of tAPX and the priming effect on the plastidic ROS marker was confirmed in a tAPX knockout line.

Furthermore, it was shown that the priming effect of *ZAT10* and *BAP1* is trigger specific. Experiments with excess light as trigger instead of cold, showed no priming specific response of the two plastidic ROS marker genes for a cold/excess light combination, as was shown for cold/cold treatments. Primability of *ZAT10* was not only observed in Col-0, but additionally in two accessions from colder climate, namely Ms-0 and Kas-1. A representative of a warm habitat (Cvi-0) was not cold primable, showing that primability was lost during adaptation to this habitats. Further analysis demonstrated that priming is developmentally regulated. Young plants are not able to establish priming and the capacity for priming decreases in old plants.

6.1 Zusammenfassung

Reaktive Sauerstoffspezies (ROS) werden als natürliches Nebenprodukt des aeroben Stoffwechsels gebildet. Ihre Produktion wird unter Stress stark erhöht. Sie können DNA, Proteine und Lipide schädigen und sind wichtige Signalmoleküle in der Stressregulation und pflanzlichen Entwicklung. Aufgrund der toxischen Wirkung von ROS sind Mechanismen für ihre Entgiftung unerlässlich. Alle zellulären Kompartimente sind durch eine Vielzahl an antioxidativen Enzymen geschützt. In Pflanzen wird ein großer Teil an ROS in Chloroplasten gebildet. Superoxiddismutasen, Ascorbateperoxidasen, Glutathionperoxidasen und Peroxiredoxine wirken ROS entgegen, verhindern oxidativen Schaden und schränken die ROS-basierte Weiterleitung von Signalen zwischen dem Chloroplasten und dem Zellkern ein.

Es wurden erstmals experimentelle Belege für Priming der ROS-basierten Weiterleitung von Signalen aus dem Chloroplasten zum Zellkern nach kurzen (24 Std) 4 °C-Stimuli in *Arabidopsis thaliana* gefunden. In unbehandelten Pflanzen wurden die plastidär gesteuerten ROS-Markergene *ZAT10* und *BAP1* stark induziert. Eine kurze Kältebehandlung (Priming) fünf Tage vor dem Kältetrigger verhindert die Induktion von *ZAT10* und *BAP1*. Der Einfluss des plastidären antioxidativen Schutzsystems auf die ROS-Signalweiterleitung wurde untersucht. Es konnte gezeigt werden, dass der Primingeffekt auf *ZAT10* und *BAP1* von einer langsamen, langanhaltenden Induktion der thylakoidmembran gebundenen Ascorbateperoxidase (tAPX) abhängt, welcher vom Primingstimulus ausgelöst wurde. Die Induktion der tAPX wurde durch eine erhöhte Promotoraktivität ausgelöst. Diese wirkte sich über die Transkript- auf die Proteinebene aus. Ein kausaler Zusammenhang zwischen dem Primingeffekt auf *ZAT10* und *BAP1* konnte durch den Einsatz von tAPX Knockoutpflanzen bestätigt werden.

Zusätzlich konnte gezeigt werden, dass der Primingeffekt auf die beiden ROS-Markergene triggerspezifisch ist. Experimente mit erhöhten Lichtbedingungen als Trigger zeigten keinen Primingeffekt auf die ROS-abhängige Signalweiterleitung, der für Kälte/Kälte-Behandlungen gezeigt werden konnte. Die Primbarkeit von *ZAT10* konnte nicht nur in Col-0 festgestellt, sondern auch in zwei Akzessionen aus Regionen mit kälterem Klima nachgewiesen werden (Ms-0 und Kas-1). Eine Akzession aus einem warmen Lebensraum (Cvi-0) war im Vergleich nicht primbar. Dies zeigt, dass die Primbarkeit während der Adaptation an dieses Habitat verloren ging. Zudem

zeigten weitere Analysen, dass Priming entwicklungsabhängig reguliert wird. Junge Pflanzen konnten nicht geprimt werden, in alten Pflanzen war die Primbarkeit stark reduziert.

7 Literature

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8 List of publications

Paper

van Buer J.[§], Cvektovic J.[§], Baier M. (2016). Cold regulation of plastid ascorbate peroxidases serves as a priming hub controlling ROS signaling in Arabidopsis thaliana, *BMC Plant Biology 16, 1-20*

§ equally contributed

Conference contributions

talk

2016 Tagung Molekularbiologie der Pflanzen (Dabringhausen)
The chloroplast antioxidant system as a priming hub under cold stress

2015 Botanikertagung 2015 'From Molecules to the Field' (Munic)
The chloroplasts antioxidant system and its role in plant stress memory

poster

2015 26. Scandinavian Plant Physiology Society Congress (Stockholm)
van Buer J.[§], Cvektovic J.[§], Baier M., *Chloroplast antioxidant system under temperature stress and medium term memory development in Arabidopsis accessions*

2014 Plant Biology Europe FESPB/EPSO 2014 Congress (Dublin)
van Buer J.[§], Cvektovic J.[§], Baier M., *The chloroplast antioxidant system-Priming the system for cold?*

2013 Botanikertagung 2013 (Tübingen)
van Buer J., Baier M.*, The chloroplast antioxidant system in priming stress* responses – *short cold challenges for inducing cold memory*

§ equally contributed

9 Curriculum vitae

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