The impact of cold priming on the transcriptomic response of *Arabidopsis thaliana* upon cold and high light

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Abbreviations

ABA	abscisic acid
AOC	allene oxide cyclase
AOS	allene oxide synthase
APX	ascorbate peroxidase
BAP1	bonsai associated protein 1
CAMTA	calmodulin-binding transcription activator
CBF	c-repeat binding factor
cDNA	reverse transcribed copy DNA
CDPK	calcium dependent protein kinases
CHS	chalcone synthase
Col-0	*Arabidopsis thaliana* var. Col-0
COR	cold regulated gene
ct	cycle threshold
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
dde-1	delayed dehiscence 1
DEG	differential expressed gene
DNA	deoxyribonucleic acid
ELIP	early light inducible protein
FDR	false-discovery-rate
flu	fluorescent in blue light
FPKM	fragments per kilobase million
FW	fresh weight
GABA	ɣ-aminobutyric acid
GO	gene ontology
H3K4me3	trimethylation of lysine 4 of histone H3
HD2C	histone deacetylase 2C
HL	high light
HOS	high expression of osmotically responsive gene
HPLC	high performance liquid chromatography
HSF	heat shock factor
HSP	heat shock protein
ICE1	inducer of CBF expression 1
Ile	isoleucine
iOE	inducible overexpression
IPT3	isopentenyl-transferase 3
iRNA	inducible RNA silencing
JA	jasmonic acid
JR	Jasmonic acid responsive gene
LOX	lipid oxygenase
MAPK	mitogen activated protein kinase
MDA	malondialdehyde

MEcPP	2-methyl-erythritol-2,4-cyclopyrophosphate
MS	mass spectrometry
MYC	myelocytomatose like helix-loop-helix binding protein
NPQ	non photochemical quench
NPR1	non-expresser of PR genes 1
NTC	no-template control
OD	optical density
OPDA	*cis*-(+)-12-oxo-phytodienoic acid
OPR	12-oxophytodienoate reductase
P	priming
PAL	phenylalanine ammonia-lyase
PAM	pulse amplitude modulation
PAP	3'-phosphoadenosine 5'-phosphate
PC	principal component
PCC	pathogen and circadian controlled gene
PCR	polymerase chain reaction
PQ	plastoquinone
PR	plant resistance gene
PSI / PSII	photosystem I / photosystem II
PT	primed and triggered
qP	photochemical quench
qPCR	quantitative PCR
RES	reactive electrophile species
RNA	ribonucleic acid
RNA-seq	RNA sequencing
ROS	reactive oxygen species
SA	salicylic acid
SAG13	senescence associated gene 13
sAPX	stromal located ascorbate peroxidase
SD	standard derivation
SOD	super oxide dismutase
T	triggering
TBA	thiobarbituric acid
TBRAS	TBA reactive substances
tDNA	transfer DNA
TGA	TGACG sequence specific binding protein
VSP2	vegetative storage protein 2
WRKY40	WRKY DNA binding protein 40
XVE	chimeric transcription factor
YLS8	yellow leaf specific gene 8
ZAT	zinc-finger transcription factor

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Summary

Several studies described in last decades, how plants can cope with different single stress events. But in nature, plants are rather exposed to different stress combinations and series of stress events. Under such fluctuating conditions, it is beneficial for the plant fitness to maintain information from a past transient stress, called priming stimulus, over a stress-free period to be prepared for a subsequent second stress event. One example is the process of cold priming where plants adjust their plastid antioxidant system after a first cold exposure and thereby adjust their response to a second cold stress, also both treatments are separated by stress free period.

In my thesis, I investigated the impact of cold priming on the cold and high light response of *Arabidopsis thaliana*. Comparative transcriptome analyses revealed that most of the cold priming imprints in the high light and cold response are stress type specific. Some genes showed even an inverse priming-regulation upon cold and high light, which indicates an active reprograming of the plant memory in response to both triggering stimuli.

One of the few triggering type independent effects of cold priming was the downregulation of genes of the jasmonic acid (JA) metabolism upon the first hour of high light and cold application. The impact of cold priming on stress responsive JA metabolism was further investigated upon cold triggering and showed that priming impacts on the cold inducible lipid oxidation. Genetic analysis dissected the role of JA metabolism and indicates that cold priming responsive genes are regulated by the JA precursor *cis*-(+)-12-oxo-phytodienoic acid (OPDA). A casual connection between the known cold priming effects and the cold response of OPDA metabolic genes could be shown by transient expression of the thylakoid located ascorbate peroxidase (*tAPX*).

In summary, my thesis showed a strong impact of OPDA in the cold priming regulated cold response. The comparative transcriptome analyses indicate that cold priming effects also the high light response of OPDA metabolic genes. However, OPDA responsive genes showed triggering type specific priming regulation, which points to a cold and a high light specific impact of the cold priming reduced OPDA metabolism.

Zusammenfassung

Zahlreiche Studien untersuchten, wie Pflanzen auf einzelne Stressfaktoren reagieren. In der Natur treten allerdings eher Stresskombinationen und sich wiederholende Stressbedingungen auf. Unter solchen Konditionen ist es für die Pflanze vorteilhaft, Information eines ersten vergangenen Stresses, dem *Priming*-Stimulus, zu nutzen, um ihre Antwort auf zukünftige Stressfaktoren anzupassen. Ein Beispiel ist Kälte-*Priming*, wo eine erste Kältebehandlung zu einer Anpassung des antioxidativen Schutzsystems in Chloroplasten führt und dadurch die Antwort auf einen zweiten Kältestress beeinflusst, auch wenn bei Behandlungen durch eine stressfreie Phase getrennt sind.

In der hier vorgelegten Studie wurde der Einfluss von Kälte-*Priming* auf die Kälte- und Lichtantwort von *Arabidopsis thaliana* untersucht. Vergleichende Transkriptom-Analysen zeigten, dass Kälte-*Priming* unterschiedlich auf die Regulation der Kälte- und Lichtantwort einwirkt. Manche Gene zeigten sogar eine inverse Regulation durch *Priming* nach beide Behandlungen, was darauf hinweist, dass das Gedächtnis der Pflanze aktiv an verschiedene Stressarten angepasst wird.

Einer der wenigen stresstyp-unabhängigen Effekte von Kälte-*Priming* war die reduzierte Licht- und Kälteregulation von Genen des JA-Metabolismus. Analysen des kälteinduzierten JA-Metabolismus zeigten, dass Kälte-*Priming* die kälteinduzierte Oxidation von Lipiden beeinfluss. Genetische Analysen weisen darauf hin, dass die Kälteantwort von *priming*-regulierten Genen von der JA Vorstufe OPDA abhängt. Mittels transienter Expression der tAPX konnte ein Zusammenhang zwischen bekannten Effekten von Kälte-*Priming* und der Kälteinduzierten OPDA Biosynthese hergestellt werden.

Zusammenfassend, zeigt die hier vorgelegt Studie einen starken Einfluss von Kälte-*Priming* auf die Regulation von OPDA während eines zweiten Kältestresses. Transkriptom-Analysen legen nahe, dass auch die Lichtantwort von OPDA durch Kälte-*Priming* beeinflusst wird. Allerdings zeigen OPDA-regulierte Gene eine stress-spezifische Regulation durch Priming, was auf licht- und kälte-spezifische Effekte der *priming*-regulierten OPDA Biosynthese hinweist.

Introduction

1.1 Adaptation of phototrophic organisms

Light is the prerequisite for autotrophic growth of photosynthetic organisms. Photosynthesis is a highly sensitive process and unfavourable changes in conditions can lead to an imbalance between the amount of absorbed light energy and energy consumption (Ensminger et al. 2006). This imbalance can cause overexcitation of the photosystems and in extreme cases the inhibition of photosynthesis or even death of the whole plant (Aro et al. 1993; Ort 2001; Mittler 2002). Therefore, evolutionary pressure led to mechanisms to avoid overexcitation during perturbed energy consumption (Bailey et al. 2001). The result is a selection of genotypes that provide phenotypic plasticity, which fit to the everchanging conditions in their habitat, a process that is called adaptation (Barrett and Schluter 2008). The genetic manifestation by adaptation enables acclimatization, a process of adjustment to transient environmental changes within an organism´s life, like the active rearrangement of the photosystems upon light intensity changes (Berry and Bjorkman 1980).

Due to anthropogenic climate change, it is expected that the environmental fluctuations will be less predictable (IPCC 2007). Several examples documented, that the climate change already impacts agriculture, for instance shown by a shifted grape harvest time in Australian vine yards (Jarvis et al. 2019) or by 30 % less corn yield in the area of Brandenburg due to summer heat waves (Harvesting report 2018, German Federal Ministry of Food and Agriculture). Climate models predict that average risk of yield losses will double until the year 2050 (Li et al. 2009a). At the same time agriculture productivity must be increased by 70 %, to feed additional 2.3 billion people in the year 2050 when taking in account current food consumption trends (Tilman et al. 2011). To optimize the crop yield under such unfavourable conditions, it is important to understand how photosynthetic organism sense environmental cues and how plants integrate different environmental information into a precise response network. Higher understanding of common environmental response hubs, but also of the mechanisms that underpin stress specificity will improve the agriculture processing in the face of frequent climate extremes (Murchie et al. 2009).

1.2 Acclimation and common stress response hubs

The immediate cellular perturbations caused by unfavourable conditions are often similar for several types of stresses, like membrane disruption during freezing stress, drought and herbivory (Steponkus 1984; Zebelo and Maffei 2014) or the disturbance of the photosynthetic capacity by toxic ozone, temperature changes and drought (Huner et al. 1998; Ensminger et al. 2006). The logical outcomes are shared protective traits in response to different stress types. Comparison of transcriptional changes in response to different single stress events revealed general plant stress elements, so called common stress hubs (Fig. 1).

The activation of the general stress response relies on three main-drivers: the redox state in the cell compartments, especially of the photosystems (Huner et al. 1998), the calcium signature, especially in the cytosol (Steinhorst and Kudla 2013) and the electrical membrane potential (Gilroy et al. 2016). Pathways, like the mitogen activated protein kinase (MAPK) cascade (Jonak et al. 2002), the redox dependent accumulation of phosphoadenosine-5-phosphate (Estavillo et al. 2011), phytohormone signalling (Shinozaki and Yamaguchi-Shinozaki 2007) and Ca^{2+} dependent phosphor kinases activity (Schulz et al. 2013) are known to translate the respective stimuli into a cellular response (Bjornson et al. 2017). Thereby, several of these transmitting pathways are shared between different types of stresses, like activation of the mitogen activated phosphor kinase *MPK6* (AT2G43790) in response to chilling stress, osmotic stress and pathogen infection (Teige et al. 2004; Jonak et al. 2002). The transmitted signal activates the transcriptional reprogramming, which enables acclimation (Vogel et al. 2014). In the model plant organism *Arabidopsis thaliana*, Ma and Bohnert (2007) defined 197 genes, which are transcriptional activated in response to several biotic and abiotic stress events. Further meta-analysis of genome wide imprints in abiotic and biotic stress responsive transcription revealed a core set 56 transcription factors, which are commonly activated in transcription upon the first hour of stress application (Hahn et al. 2013; Bjornson et al. 2017). The activated core stress response led to a readjustment of the metabolism, DNA repair mechanisms, chromatin structure and post-transcriptional modifications to resist the environmental perturbations.

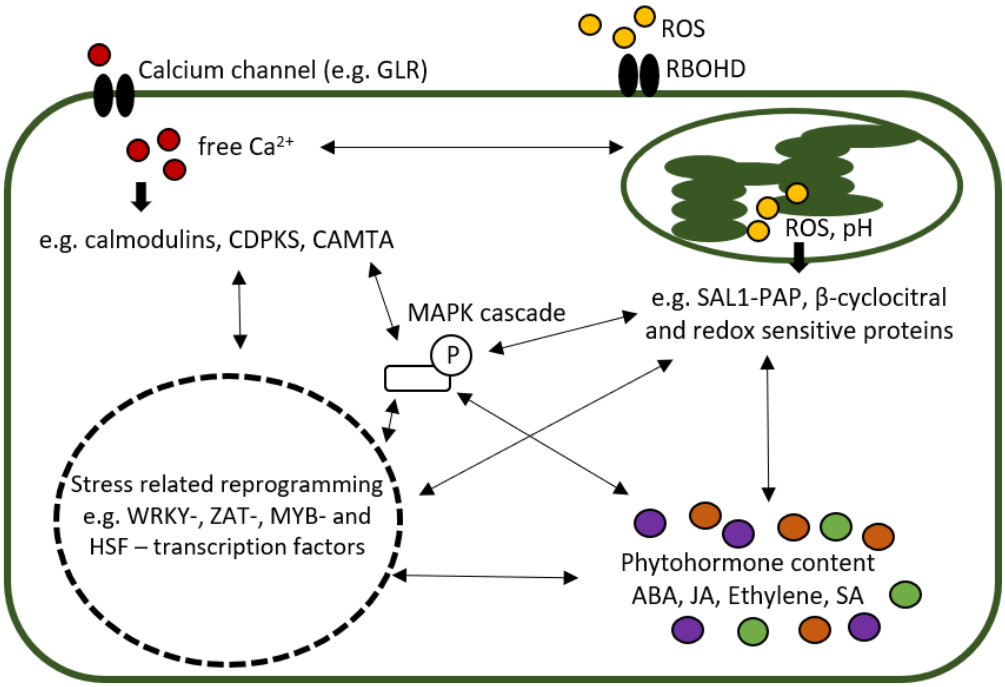

Figure 1: Model of the central stress response hubs and their interactions: *Environmental perturbations result in free Ca^{2+} (red) and redox changes (yellow). Both messengers are sensed and transmitted by central stress hubs, e.g. by the activation of the mitogen activated protein kinases (MAPK), conformational changes of calcium-dependent protein kinases (CDPK) or by retrograde pathways. This results into the transcriptional reprogramming and the activation of the core stress response, which includes induced expression of ZAT-, WRKY-, MYB- and heat shock transcription factors. The transcriptional reprogramming is often managed in crosstalk with different phytohormone pools, e.g. with absicic acid (ABA), jasmonic acid (JA) and salicylic acid (SA). The model is based on meta-analyses of single stress events in Arabidopsis thaliana (Ma and Bohnert 2007; Bjornson et al. 2017).*

1.2.1 Specificity of the stress response

As various stress hubs are common to different types of stresses, it is important to understand how plants distinguish between different stressors for an appropriate response. One hypothesis, is based on different amplitudes and kinetics of second messengers specific to each stress type (Dodd et al. 2010). For example, free cytosolic Ca^{2+} influx depends on the type of perturbation (Kiegle et al. 2000), the fortitude of the stress (Plieth et al. 1999) and how often the plant faced such stress conditions in the past (Knight et al. 1996). A second hypothesis, how plants provide stress specificity, involves the diversity within second messenger decoding pathways. In mammalians for example, micro-domain composition and localisation of calcium responsive protein clusters play an important role in maintaining stress specific adjustments

(Good et al. 2011). *A. thaliana* encodes approximately 250 proteins with Ca^{2+} responsive EF-hands motifs (Day et al. 2002). This complexity of Ca^{2+} modulated proteins can provide compartment-, tissue- and stress type dependent responses. For instance, Benn et al. (2016) reported a plastid specific regulation of the calcium response: Oxidative stress treatments triggers the accumulation of the retrograde signalling molecule 2-C-Methyl-d-erythritol-2,4-cyclopyrophosphate (MEcPP, Xiao et al. (2012)). The MEcPP disperses into the cytosol, where it acts as a rheostat for calcium dependent activation of the calmodulin-binding transcription activator *CAMTA3* (Benn et al. 2016).

Another stress hub, that could provide stress specific adjustments, is the group of mitogen activated protein kinases (MAPK). MAPK cascades are involved in developmental-, hormonal- and stress homeostasis (Cardinale et al. 2002; Bigeard and Hirt 2018). Like the stress-specific second messenger signature of Ca^{2+}, the duration of the MAPK cascade activation plays a role in stress specific adjustments. Wounding of Arabidopsis leaves leads to a transient activation of MPK6 and thereby to a transient production of ethylene (Zhang and Klessig 1998), whereas hypersensitive cell death leads to a long lasting *MPK6* activation (Zhang et al. 1998). Further stress specificity can be obtained by the diversity within the MAP-kinase cascade. The sum of MAPK encoding genes in Arabidopsis could lead to thousands of possible MAPK combinations and thereby could confer specificity (Cvetkovska et al. 2005). Alternative splicing of MAPKs can provide further complexity: For example, maize (*Zea mays*) plants can express four different splice variants of the kinase *MPK4* depending on the plants age, resulting in developmental stage specific stress regulation (Castells et al. 2006).

Besides the complexity within one stress hub, the interaction between several stress hubs is considered to promote specificity of the plants response (Teige et al. 2004). Therefore, signalling networks are rather complex systems with overlaps, but also separated branches (Knight and Knight 2001). The interaction of a small number of signal-transmitting steps can already provide complex adaptive traits. One stunning example is the well described process of chemotaxis in bacteria (Rao et al. 2008), which is a network consisting of six signal transduction steps. The interaction between the six processes results in complex traits like stress sensing, acclimation and even stress memory (Vladimirov and Sourjik 2009).

1.2.2 The redox state of the photosystem as a common stress hub

One important factor in several biological processes, like in development, differentiation and acclimation are reactive oxygen species (ROS, Foyer and Shigeoka (2011), Mignolet-Spruyt et al. (2016), Mittler (2017). ROS are reduced or excited oxygen derivates with a higher reactivity compared with oxygen molecules (O_2) including the superoxide anion ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\bullet}$) as well as singlet oxygen ($^1O_2$). Each kind of ROS has its own unique chemical properties, that trigger specific targets and signalling pathways (op den Camp et al. 2003; Vaahtera et al. 2013; Laloi et al. 2007; Gadjev et al. 2006). ROS arise passively due to electron transport reactions, like photosynthesis in chloroplasts and photorespiration in peroxisomes (Mittler 2017), or actively by enzymatic reactions in the apoplast (Keller et al. 1998).

Photosynthesis is a strong source of reducing power and thereby one of the major production sites of ROS in photosynthetic organisms (Ort 2001; Pospíšil 2016). A range of stresses such as low temperatures and drought support the production of ROS by limiting utilization of the light energy, for example by slowing down the Calvin-Benson cycle (Prasad et al. 1994; Apel and Hirt 2004; Ensminger et al. 2006). If the amount of absorbed light energy exceeds the capacity for photosynthetic energy consumption, overexcitation led to the increased formation of ROS (Li et al. 2009b; Demmig-Adams and Adams 1992). In detail, photosensitization of photosystem II results in energy transfer to O_2. The energy allows electrons to overcome spin restriction, which results into the formation of singlet oxygen (Krieger-Liszkay 2004). The high electron pressure due to photosystem overexcitation can further lead to the reduction of O_2 through the Mehler-reaction (Mehler 1951) and, thereby, to the formation of superoxide anions. The redox shift by the ROS formation can oxidize and damage lipids, proteins, and other components. In the worst case lead the ROS formation to photoinhibition and cell death if the rate of oxidation damage exceed the rate of photosystem repair (Aro et al. 1993).

1.2.3 Signalling function of reactive oxygen species

Besides the threat of oxidation, increased ROS production and changes of the oxidative milieu can be sensed as initiation signal for multiple stress response pathways (Gadjev et al. 2006; Laloi et al. 2004; Mittler 2017). Studies of knockout and antisense lines of several ROS scavengers revealed a complex ROS response network, that comprises at least 152 genes (Mittler et al. 2004). Thereby, type and source specific ROS signalling pathways were proposed (Gadjev et al. 2006). Hydrogen peroxide (H_2O_2) is considered as the predominant ROS signal, due to its stable nature (half-life > 1 ms) compared to other ROS (Mattila et al. 2015). Recent reports presented evidence that H_2O_2 can be directly delivered from the plastid to the nucleus mediated most likely by the formation of stromules (Exposito-Rodriguez et al. 2017). Tian et al. (2016) demonstrated an active H_2O_2 translocation via the transmembrane protein PIP1:4 (AT4G00430), which gives further evidence that H_2O_2 directly impacts several cell compartments. Redox sensitive proteins, like the transcription factor RAP2.4a (Shaikhali et al. 2008) and oxidation-by-products, like the oxidation of β-carotene to the volatile β-cyclocitral (Ramel et al. 2012) translate the ROS signature into a cellular response. Estavillo et al. (2011) reported for instance, that the redox dependent inactivation of the chloroplast located 3'-phosphoadenosine 5'-phosphate (PAP) phosphatase (AT5G63980) leads to the accumulation of PAP. The elevated PAP content activates the nuclear 5'-to-3'exoribonucleases (*XRN2-4*) which promotes the acclimation to excess light intensities (Estavillo et al. 2011).

ROS signalling is strongly interconnected with other signalling pathway including the response to SA and JA (Mhamdi et al. 2010) as well as to ABA and Ca^{2+} (Pei et al. 2000). One example is the $^1O_2$ activated production of oxylipids: The conditional fluorescent (*flu*) mutant accumulates proto-chlorophyllide when moving the plants into the darkness. Chlorophyllide, which is produced from proto-chlorophyllide, leads to excessive $^1O_2$ production after shifting the plants back to light conditions (op den Camp et al. 2003). The production of $^1O_2$ promote the accumulation of the phytohormone JA and its precursor OPDA (Ochsenbein et al. 2006)). Genetic analyses in the *flu* background revealed, that the JA accumulation depends not directly on $^1O_2$, but on Executer 1 (AT4G33630) mediated retrograde signalling activated by excess $^1O_2$ production in the chloroplast (Przybyla et al. 2008).

1.2.4 The antioxidant system governs reactive oxygen signalling

ROS production and thereby ROS signalling is under the control of several enzymatic and non-enzymatically antioxidant mechanisms (Noctor and Foyer 1998; Asada 1999; Sewelam et al. 2016). Ascorbate is the most abundant non-enzymatic antioxidant in plant cells and detoxify superoxide, hydroxyl radicals and singlet oxygen (Noctor and Foyer 1998). Besides ascorbate, also glutathione plays a pivotal role in ROS scavenging. Further, glutathione reduces oxidised ascorbate and thus, regenerates the ascorbate pool via the Asada-Foyer-Halliwell pathway (Asada 1999; Noctor et al. 2012). Several other metabolites with antioxidant properties are described, e.g. tocopherols, carotenoids and flavonoids (Ksas et al. 2015).

Besides antioxidant metabolites, antioxidant enzymes are present in different cellular compartments including ascorbate and glutathione peroxidases (APX and GPX), catalases (CAT), superoxide dismutase (SOD), thioredoxins and peroxiredoxins as well as dehydroascorbate-, monodehydroascorbate- and glutathione reductases (Mittler 2002). The main ROS-scavenging mechanism is driven by the superoxide dismutase (SOD), which is present in nearly all aerobic organism and converts $O_2^{\bullet -}$ to H_2O_2 (Bowler et al. 1992). The number of SOD isoenzymes differs between plants species, but in general three different SOD types have been described: copper/zinc-, manganese-, and iron-containing SODs (Bowler et al. 1992). The ascorbate peroxidases (APX) as well as the catalases (CAT) scavenge H_2O_2 and thus control the H_2O_2 concentration (Asada 2006). Due to different affinities for H_2O_2 and different subcellular localisations, distinct functions for the APX and catalase enzymes are proposed (Mhamdi et al. 2010).

In Arabidopsis, three different APX isoenzymes are located in plastids: the stromal (*sAPX,* AT4G08390), thylakoid membrane (*tAPX*, AT1G77490) and a potentially lumen localized APX (AT4G09010). The lumen-APX shows high sequence similarity to other APX proteins, but compromises no *in vitro* and *in vivo* APX activity (Granlund et al. 2009). Analysis of single and double mutants of the remaining two APX genes revealed a crucial role of tAPX and sAPX during short-term stress exposure (Kangasjärvi et al. 2008).

1.3 Acclimation to low temperature stress

The ability to tolerate low temperatures and freezing conditions is an important fitness trait for plants distribution and crop yield in the temperate climate zone. Plants endurance to low temperatures can be grouped into chilling tolerance (above 0 °C) and freezing tolerance (below 0 °C). Most plants of the temperate climate zones are chilling tolerant and accomplish higher freezing tolerance after exposure to chilling stress, a process that is called cold acclimation or cold hardening (Gilmour et al. 1988; Thomashow 1999). Plants of tropical and subtropical origins, like cassava and soybean are sensitive to chilling stress and are deficient for cold acclimation (Raison and Lyons 1986), which results in chlorosis, sterility and in the worst case to death of the plant upon cold temperatures (Knight 2012).

Already few degrees under the optimum temperature cause biochemical perturbations in the plant including membrane rigidification, secondary DNA structure formation and protein stability changes (Ensminger et al. 2006; Steponkus 1984). Several examples are described how plants sense these cellular perturbations and translate that into a cellular response (Fig. 2).

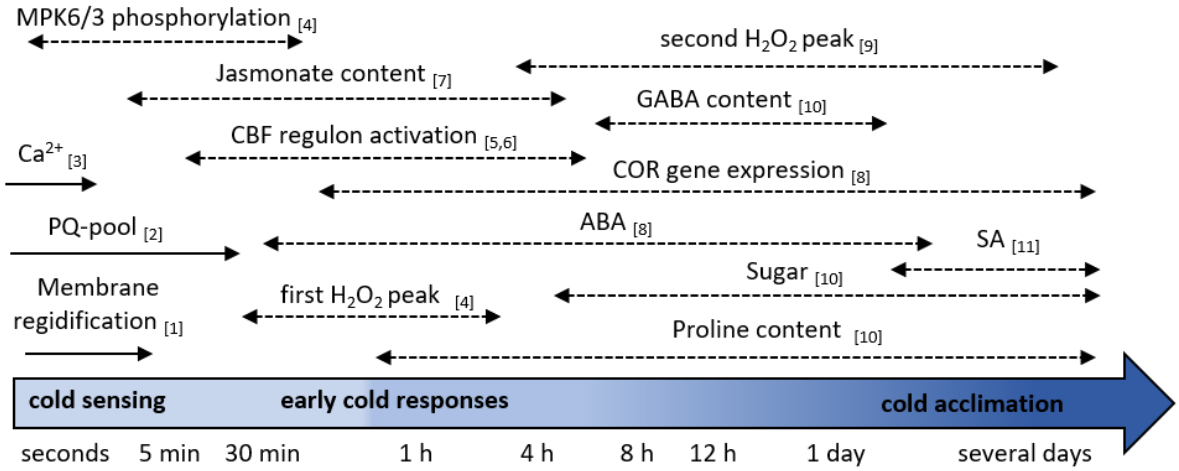

Figure 2: Timeline of sensing, signal transmission and acclimation processes in Arabidopsis thaliana upon 4 °C chilling stress: *The numbers refer to the citations and the arrows indicate the duration of each process: [1] Örvar et al. (2000); [2] Ensminger et al. (2006); [3] Knight et al. (1996); [4] Zhao et al. (2017); [5] Gilmour et al. (1988); [6] Thomashow et al. (2001); [7] Hu et al. (2013); [8] Thomashow et al. (1997); [9] Sin'kevich et al. (2016); [10] Kaplan et al. (2004) and [11] Scott et al. (2004). The figure was published in a slight modified form in Baier et al. (2019).*

For instance, treatments of plants with agents that cause rigidification of membranes results into an opening of Ca^{2+} channels. The Ca^{2+} influx induces expression of cold regulated (COR) genes although the plants never received chilling stress (Sangwan et al. 2001). A recent study, that used aquaporin based luminescence imaging, revealed distinct Ca^{2+} dynamics within the first seconds of chilling stress in Arabidopsis *(Zhu et al. 2013)*. The Ca^{2+} regulated receptor-like kinase *CRLK1* (AT5G54590) was reported to be a positive cold tolerance regulator. CRLK1 interacts with and phosphorylates MPKK1 (Furuya et al. 2013), which in turns activates *the MKK2-MPK6* cascade and downstream targets (Teige et al. 2004).

Besides the changes in membrane fluidity, cold disturbs protein stability and weakens the utilization of absorbed light energy (Ensminger et al. 2006). Light energy, which exceed dissipation and chemical quenching capacity can trigger the formation of ROS (Mittler 2002). Wanner and Junttila (1999) demonstrated that Arabidopsis plants which were grown in darkness or treated with photosynthesis inhibitors during chilling exposure were not able to enhance cold tolerance, showing the requirement of functional photosynthesis for cold acclimation. Sensing and transmitting of the ROS and Ca^{2+} signature result in a massive reprogramming of transcription, post-transcriptional modifications, metabolism and translation upon cold stress (Kaplan et al. 2004; Calixto et al. 2018; Kaplan et al. 2007; Fowler and Thomashow 2002).

1.3.1 Cold acclimation trough the Arabidopsis CBF-regulon

Research over the last decades has shown that most of the processes in cold acclimation are controlled by changes in gene expression and post-transcriptional modifications (Calixto et al. 2018). In Arabidopsis, a 24-hour treatment at 4 °C for instance leads to the transcriptional induction of 4000 COR genes (Zhao et al. 2016). Approximately 10 % of all COR genes are under the direct control of three well-described c-repeat binding factors (*CBF1/DREB1B/*AT4G25490, *CBF2/DREB1C/*AT4G25470 and *CBF3/DREB1A/*AT4G25480, Zhao et al. (2016)). Activation of the CBF regulon results in the accumulation of freezing protectants (Jaglo-Ottosen et al. 1998), like the increased abundance of the cold regulated *COR15A* (AT2G42540), which stabilizes cell membranes (Thalhammer et al. 2014).

Several positive and negative regulators of the CBF-regulon were identified: The inducer of CBF expression 1 (*ICE1*, AT3G26744) is an MYC-like transcription factor which binds to the *cis*-acting elements in the CBF promoter regions and is controlled by post transcriptional modifications (Chinnusamy et al. 2003). Recent results showed evidence that the MKK4/5–MPK3/6 cascade promotes degradation of *ICE1*, which prevents activation of CBF-regulon activity (Li et al. 2017). Further, the JA zinc-finger inflorescence meristem (ZIM) domain protein JAZ1 (AT1G19180) repress the function of *ICE1* (Hu et al. 2013). Chilling stress leads to an accumulation of JA in Arabidopsis seedlings. Subsequently, JA perception by the SCF^{COI1}complexe results into the degradation of *JAZ1* through the 26s-proteasome pathway (Thines et al. 2007) and by that *ICE1* is activated (Hu et al. 2013). Besides *ICE1*, the circadian clock associated gene (*CCA1*, AT2G46830) and the late elongated hypocotyl gene (LHY, AT1G01060) are involved in regulating CBF expression, indicating an important role of the photoperiod in the cold response (Dong et al. 2011). The CBF regulon is further responsive to several second messengers. For example activation of the CAMTA proteins (*CAMTA1–5*) by Ca^{2+} influx enhances the expression of *CBF1* and *CBF2* (Kidokoro et al. 2017). Genome wide transcription analysis after treatment with di-chlorophenyl-dimethylurea (DCMU) or dibrom-3-methyl-6-isopropyl-benzochinon (DBMIB), which both inhibit photosynthetic electron transport, revealed that *CBF3* and *CBF1* expression is elevated by DBMIB but not by DCMU (Bode et al. 2016). In detail, DCMU inhibits the electron transfer from PSII into the plastoquinone pool, whereas DBMIB inhibits the electron transfer after the plastoquinone pool. Therefore, DBMIB application results not only in an abolished PSII function like application of DCMU but also to an over-oxidation of plastoquinone. This links *CBF1* and *CBF3* expression to the redox state of the plastoquinone pool in the chloroplast (Przybyla et al. 2008). Transient silencing of *tAPX* results in a higher sensitivity to chilling stress and supress the cold response of *CBF1* as well as *CBF2*, which further hints to an redox dependency of the CBF regulon (Maruta et al. 2012).

Complexity in cold acclimation is increased by the fact, that not only the CBFs but also at least 25 % of the CBFs interactions with their target genes are regulated by at least two factors (Park et al. 2015). One example is binding of the CBF proteins to the *COR15A* promoter, which relies on a second factor – the *High expression of the Osmotic Sensitive gene 15* (*HOS15*, AT5G67320) mediated degradation of the deacetylase HD2C (AT5G03740). HD2C binds and deacetylate the CBF binding site in the promoter of the cold regulated gene *COR15A*. Chilling stress leads to a cold induced degradation of HD2C mediated by HOS15, which leads to a significant increase of H3-acetylation and by that promotes the binding of CBF proteins (Park et al. 2018).

1.3.2 Crosstalk and cross-tolerance caused by cold acclimation

The accumulation of osmolytes, antioxidants and protein stabilizing heat shock proteins upon cold acclimation can ensure higher resilience to other environmental perturbations, a process which is termed cross-tolerance or cross-acclimation (Hossain et al. 2018). One prominent example is the cross-tolerance between drought and chilling stress. Both perturbation can results into cellular dehydration and therefore it is not surprising that both share the activation of the CBFs, which deliver osmo-protectants and stabilize membranes (Stockinger et al. 1997).

Besides the obvious connection between stresses, also less self-explanatory cross-tolerance effects are described, like the higher chilling tolerance of tomato plants (*Solanum lycopersicum*) after mechanical stress (Keller and Steffen 1995) or higher pathogen resistance after chilling stress (Kim et al. 2017; Wu et al. 2019). A recent study revealed that chilling stress promotes the monomerization of the non-expresser of pathogenesis-related genes 1 (NPR1, AT1G64280) oligomers in the cytosol and to the accumulation of NPR1 monomers in the nucleus (Olate et al. 2018). Besides the known function of *NPR1* in pathogen resistance leads the activation of NPR1 monomerization to an increased freezing tolerance in Arabidopsis by promoting expression of the heat shock factor *HSFA1* (AT1G32330, Olate et al. (2018).

1.3.3 The similarity between cold and high light acclimation

According to genome wide expression data during different stress events, cold responsive transcription has the highest similarity to high light responsive transcription (Rasmussen et al. 2013). Fluctuating light conditions are common in nature (Ort 2001) and plants evolved several mechanisms to deal with fluctuating high light intensities to avoid photooxidative damage (Bailey et al. 2001). So far, acclimation to different light intensities ranging from 400 to 3000 µmol photons s^{-1} m^{-2} were described (Bailey et al. 2001; Galvez-Valdivieso et al. 2009; Vogel et al. 2014). Treatments with light intensities above 2000 µmol photons s^{-1} m^{-2} are referenced as excess light due to their irreversible damage (Karpinski et al. 1999). Due to the local perturbation at the photosynthetic electron transport by the excess electron pressure, early high light acclimation relies mainly on retrograde signalling pathways from the plastid to the nucleus (Estavillo et al. 2011; Exposito-Rodriguez et al. 2017), including already introduced ROS signalling pathways like the SAL1-PAP pathway (Estavillo et al. 2011).

Some processes are activated within seconds to counteract the excess light energy, such as the non-photochemical quenching (Demmig-Adams et al. 1990). Other processes requires minutes of high light exposure, such as the reprogramming of transcription (Vogel et al. 2014). One example is the rapid induction of *APX2* expression, mainly through the ZAT transcription factor ZAT12 (Fryer et al. 2003). During long-term high light acclimation developmental adjustments are involved in avoiding excess light energy, like changes in the leaf angle (Berry and Bjorkman 1980) and chloroplast avoidance movement (Kasahara et al. 2002).

The threat of ROS formation due to excess light energy is a common consequence of cold and high light. Thereby, both perturbations trigger common protective traits against the excess energy (Rasmussen et al. 2013). But also, distinct adjustments are known, due to the different nature of both perturbations (Ivanov et al. 2012). During high light for example, photosynthetic metabolism is still functional as electron sink and in contrast to cold, high light can naturally occur in local spots, which activates systemic signalling (Rossel et al. 2007). Furthermore, the response to cold and high light partially differs, because the response to cold stress not only requires adjustment against excess light energy, but also counteracting mechanism against membrane rigidification and protection against freezing injuries (Ensminger et al. 2006).

1.4 Stress recovery and priming

Acclimation to the cold is an expensive process (Browse and Lange 2004). Therefore, plants start immediately the reversion of acclimation, also called deacclimation, after the removal of the stressor. The timing of deacclimation is an important fitness trait: if the transition of deacclimation is made too late, the plants miss valuable growth time (Zuther et al. 2015). But premature loss of cold acclimation, for example during warm season in early spring can result in a major reduction in fitness as happened during a sudden cold spell in the spring season in 2007, which led to excessive yield losses in eastern USA (Gu et al. 2008).

Cold deacclimation is a rather fast process compared to cold acclimation. For example in *Solanum commersonii* deacclimation starts within two hours after the end of a chilling stress and the complete cold hardiness is lost within one day (Chen and Li 1980). In comparison, 15 days were required to establish the maximum freezing tolerance that was built up during cold acclimation. Full cold acclimation of the annual plant *Lolium multiflorum (Poaceae)* requires 22 days, which is already lost after 7 days at 14 °C (Gay and Eagles 1991). Gusta and Fowler (1976) suggested that the rate of deacclimation depends on the fortitude of the temperature shift, as they showed that *Secale cereale* plants loss cold hardiness faster at 20 °C then at 15 °C air temperature.

Arabidopsis thaliana requires at least 3 days of chilling stress to build up the maximum freezing tolerance by cold acclimation (Wanner and Junttila 1999). The extend of cold acclimated freezing tolerance as well as the extend of deacclimation depends strongly on natural variation of Arabidopsis accessions (Zuther et al. 2015). Oono et al. (2006) showed, that two hours under normal growth conditions already decreased the obtained freezing tolerance. Transcriptional reprogramming in response to cold acclimation is nearly converted after 24-hour deacclimation (Pagter et al. 2017). Besides transcriptional changes, also accumulated glucose, fructose and raffinose quickly decrease in pool size within the first 24-hour deacclimation (Pagter et al. 2017), indicating a fast reversion of the acclimation state.

Besides cold deacclimation, also recovery from other types of stress is described as tightly controlled and fast process. For example, Crisp et al. (2017) mentioned that 87 % of the stress upregulated mRNAs after high light (1000 µmol photons m^{-2} s^{-1}) exhibit a very fast recovery, with a transcript half-life below 60 min after stress removal. This leads to a complete reversion of 22 % of the high light deregulated genes after 60 minutes of stress recovery (Crisp et al. 2017).

1.4.1 The concept of priming

As introduced, acclimation is process that requires days of stress exposure and is erased quickly within hours after removal of stress. Under highly fluctuating conditions, it might be beneficial to maintain information on a temporally limited stress during a period of optimal conditions, to be prepared for a subsequently reoccurring stress (Trewavas 2003). Mathematical models suggested that a certain amount of costs for information storage, would be beneficial for the plant fitness under a certain probability of a second stress event (Douma et al. 2017). Such a process, where a temporally or locally limited first stress (priming stimulus) leads to an indication and thereby to a modification of the plant response to a future or distal stress (triggering stimulus) with temporal and/or spatial distance between both treatments (*lag-phase*), is termed priming. The investment into the priming memory during the lag-phase pays off during a future stress exposure by a faster, stronger, or more sensitized stress response (Fig. 3). Several examples prove this concept of priming with different kinds of priming and triggering stimuli (Ding et al. 2012; Zuther et al. 2019; Hilker et al. 2016; van Buer et al. 2016; Lämke and Bäurle 2017; Conrath et al. 2015; Byun et al. 2014).

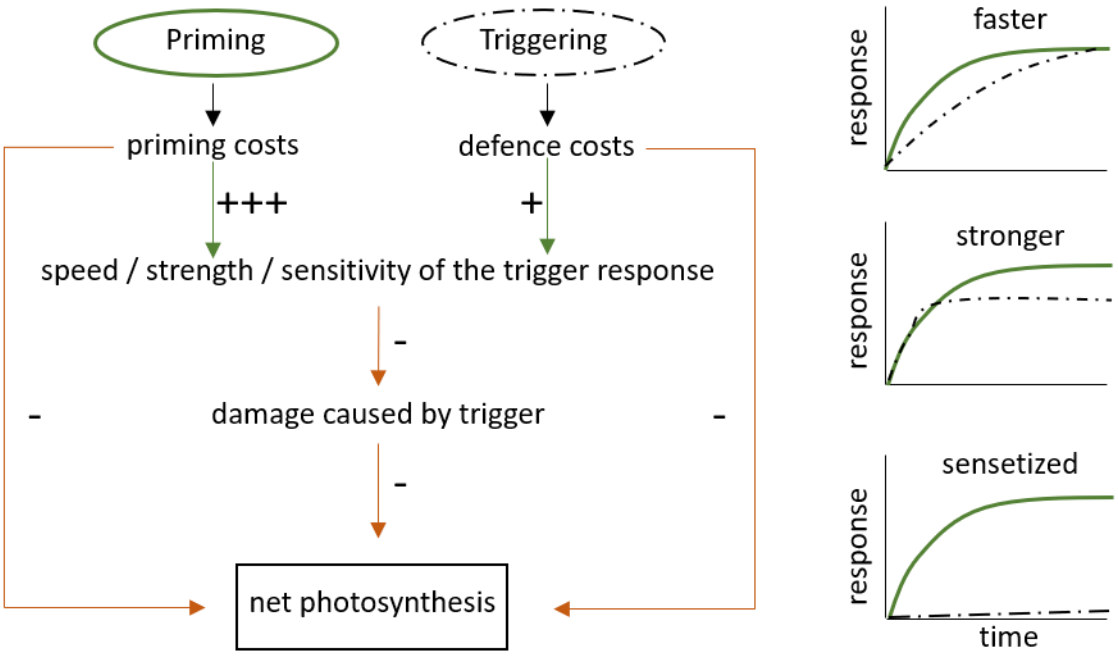

Figure 3: Concept of the costs and benefits of priming: *The left side shows a model of the negative (orange) and positive (green) connections between priming, defence and photosynthesis. The strength of the interactions is depicted by the number of + and - signs. The figure was modified from the mathematical model published from Douma et al. (2017). The diagrams on the right side depicts potential benefits of the primed response (green lines) compared to the naive response (dotted black lines) during a stress exposure and is a modified figure from Lämke and Bäurle (2017).*

Priming is an old practice in agriculture, especially in the context of seed conditioning. In the ancient Greece, Theophrastus (372-287 BC) recommend to pre-treatment cucumber (*Cucumis sativus*) seeds with milk before sowing, to increase robustness of the seedlings (Paparella et al. 2015). Conditioning of cress (*Lepidium sativum*) seeds by salt-water was studied by Darwin (1855), with the purpose to enhance the stress resistance of the seedlings. Nowadays several chemicals and environmental conditions are described for their properties to induce seed-priming for enhancing germination rate and resilience of the plants (Paparella et al. 2015).

Besides seed priming, also vegetative tissue can reach a primed state. In the beginning of the last century, studies reported immunity of plants towards a later re-infection of pathogens (Chester 1933). Older analyses with abiotic stressors were often conducted without an exact differentiation between acclimation, cross-tolerance and priming. Willemot and Pelletier (1979) for example reported higher freezing tolerance of winter wheat after drought priming

but did not consider a phase of recovery between both treatments, which would prove the maintenance of information. From the 1990s onward, priming has been specified in various contexts and was differentiated from acclimation or cross-tolerance by including a stress free lag-phase (Hilker et al. 2016; Conrath et al. 2015; Mauch-Mani et al. 2017). Additionally, stress-priming studies are differentiated in *cis*- and *trans*-priming experiments, where both stimuli are either of the same or of different nature, respectively.

1.4.2 Molecular mechanisms in priming formation and maintenance

One good example for priming is the dehydration memory of Arabidopsis: Plants that received several cycles of dehydration maintained a higher relative water content than plants, which received the dehydration treatment only once, a process which is called dehydration memory (Ding et al. 2012). Further experiments revealed that already a single dehydration stress alters the response to a second dehydration stress, also if both stresses are separated by a five days long recovery phase (Ding et al. 2012). A stress-free period longer than five days leads to a loss of dehydration memory, indicating a process of forgetting in plants. Genome wide transcript analysis upon reoccurring dehydration stress, showed that plants separates also on a transcriptional level between a first and a second stress exposure, illustrating a concept of a transcriptional stress memory (Ding et al. 2013).

The transcriptional regulation of dehydration memory is associated with higher levels of trimethylated histone H3 Lysine4 (H3K4me3) nucleosomes (Liu et al. 2014). Also, during other re-occurring stress events, like in heat memory, epigenetic modifications plays a role in memory maintenance (Friedrich et al. 2019; Espinas et al. 2016; Lämke and Bäurle 2017). For instance *forgetter1* (AT1G79350) support the hyper-induction of heat shock memory genes during reoccurring heat stress by modifying the nucleosome occupancy (Brzezinka et al. 2016).

1.4.3 Mechanism in cold priming

Besides heat and drought, plants are also able to memorize chilling stress (Byun et al. 2014; van Buer et al. 2016; Zuther et al. 2019). Byun et al. (2014) demonstrated, that a pre-treatment with 0 °C for 24 hours led to a higher survival rate of Arabidopsis during a subsequent freezing stress, also if both stimuli were separated by a three days long lag-phase. Global transcription analysis during the first and second application of cold stress revealed that cold priming cause transcriptional imprints in the cold response (Byun et al. 2014). Li et al. (2014) showed, that the decrease of the maximum quantum yield upon cold stress was stronger pronounced in two different wheat cultivars if they had faced the cold for the first time, compared to plants, which were cold primed 14 days before the second cold stimulus, which hints to a protective role of cold priming on photosynthesis.

Within this line, van Buer et al. (2016) showed that a first cold stimulus reduce the response of the plastid oxidative stress marker *BAP1 (AT3G61190)* and *ZAT10* (AT1G27730) upon a second cold stimulus, also both treatments were separated by five day long lag-phase. Both transcription-factors are under the control of the executer-mediated chloroplast-to-nucleus ROS signalling (Lee et al. 2007) and are responsive to chloroplast ascorbate peroxidase activity (Laloi et al. 2007). The reduced cold response of *BAP1* and *ZAT10* in cold primed plants indicates that priming counteracts oxidative stress or oxidative stress signalling during a subsequent stress exposure. Cold priming analyses of the antioxidant system in chloroplasts revealed that the promoter activity, expression and protein abundance of the thylakoid located ascorbate peroxidase (tAPX) is induced upon recovery after a first cold exposure (van Buer et al. 2016). Recent results demonstrate that a transient overexpression of *tAPX* can mimic the cold priming effect on oxidative stress marker gene expression in non-primed plants (van Buer et al. 2019). In addition, transient silencing of *tAPX* after cold priming abolished the reduced cold response of ROS marker genes, demonstrating that cold priming dependents on plastid antioxidant system. Interestingly, transient silencing of the stromal ascorbate peroxidase had no impact on cold priming regulation, thereby highlighting that cold priming is rather specifically controlled at the thylakoid membrane then by general ROS signals (van Buer et al. 2019). tAPX is an important scavenger of H_2O_2 in the vicinity of the photosystems, which

protects the plants against photoinhibition. For instance upon cold stress, transient silencing of *tAPX* cause higher protein oxidation rates and decreased the freezing tolerance of cold acclimated plants (Maruta et al. 2012). However, during other oxidative stress types, for example during exposure to high light intensities, silencing of *tAPX* cause antagonistic as well as synergistic effects in Arabidopsis (Maruta et al. 2012). Therefore, it is unclear to which extend cold priming interferes with the acclimation other oxidative stress exposures.

1.5 Aim of the study

The fact that common stress responsive genes, such as *ZAT10*, are affected by cold priming can lead to the hypothesis that a first cold exposure could have common effects on different oxidative stress types. But also, the opposite can be argued, due to the stress specific effects of *tAPX* silencing (Maruta et al. 2012). Aim of this study was to compare the impact of cold priming on the transcriptional and physiological response of *Arabidopsis thaliana* to high light and cold, to elucidate stress specific as well as common cold priming imprints upon both treatments.

For this purpose, a heat filtered high light treatment was developed and characterized. Genome-wide transcription analysis with full-factorial *cis*- and *trans*-priming was conducted, to provide a comprehensive overview of cold primed gene regulation upon cold and high light exposure. Key results of the transcriptional analysis were further investigated by genetic- and physiological- attempts, to extend the knowledge in the priming regulation pathway. The study will provide a benchmark of cold priming regulated transcript profiles, which will enable further analysis, how plants integrate past information's to specify their response to the current environment.

Results

2.1 The experimental design and defining of the stress conditions

To examine the stress specificity of cold priming in *Arabidopsis thaliana*, an experimental set up consisting of three treatment groups (control, *cis*-priming and *trans*-priming) were conducted (Fig. 4). All experiments were carried out with 4-weeks old plants consistent with the previous cold priming studies by van Buer et al. (2016) and Cvetkovic et al. (2017). Half of the plants were treated for 24 hours at 4 °C, referred here as cold priming. The other half, which received no cold pretreatment, was assigned as naive plants. After cold priming, plants were transferred back to normal growth conditions for 5 days, which is the maximal time for stable persistence of the cold priming effect on the plastid antioxidant system (van Buer et al. 2019). Part of the naïve and cold primed plants were harvested directly after the five days long lag-phase (control group) and the others were cold treated at 4 °C (*cis*-priming) or exposed to a high light intensity of 800 µmol photons s^{-1} m^{-2} (*trans*-priming).

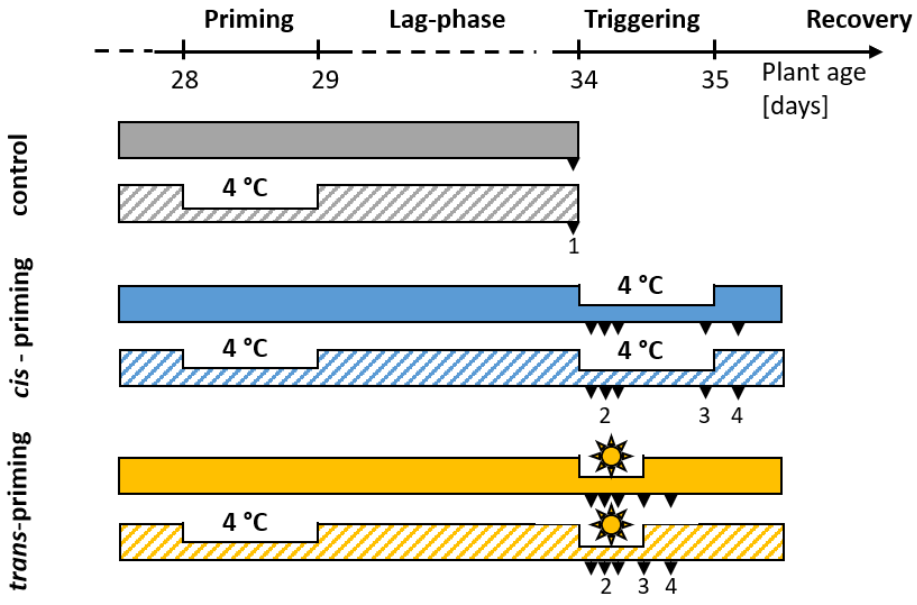

Figure 4: **Schematic representation of the different priming set-ups:** *28-days old plants were either grown under normal conditions (solid bars) or treated with 4 °C for 24 hours (cold priming, striped bars). Both groups were grown side by side for additional 5 days at normal growth conditions (lag-phase) and were either harvested directly after the lag-phase (1) as control group (grey) or were exposed to a second cold stimulus (cis-priming, blue) or to an light intensity of 800 µmol photons s^{-1} m^{-2}(trans-priming, yellow). Triggered plants were harvested during the onset of triggering (2), after triggering (3) and two hours after the end of the triggering stimulus (4).*

2.1.1 Validation of the high light treatment

High light applications are often accompanied with heat excitation (Yamamoto et al. 2008). The combination of high light and heat triggers unique responses, which are distinct from the acclimation to only high light (Balfagón et al. 2019). To avoid potential heat stress during high light application a heat filtering water layer was implemented (Material and methods, Fig. 42). The experimental set up was validated by measuring the leaf surface temperature over a period of two hours light treatment (Fig. 5A). The maximal recorded leaf temperature without any heat-counteracting filter was 37.5 °C. High light application combined with the heat filtering water layer caused a maximal leaf temperature of only 24.1 °C. Additionally, the transcript abundance of heat specific marker genes (Swindell et al. 2007), namely heat shock factor *HSFA7a* (AT3G51910) and *HSFA7b* (AT3G63350), were analyzed by quantitative real time PCR (Fig. 5B). The transcript levels of both heat responsive genes were strongly increase after two hours non-heat filtered high light. High light application in combination with the heat-filtering water layer had no impact on the transcript abundance of both heat marker genes, which indicates that the water-filtered high light was not sensed as heat stress.

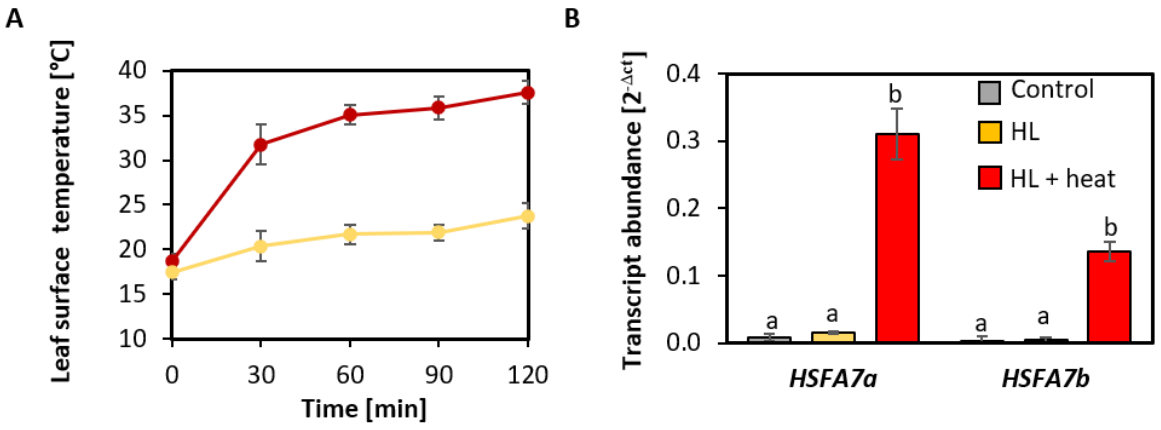

Figure 5: Leaf surface temperature and transcript levels of heat responsive genes during the light treatment: *Four-week old Arabidopsis plants were either treated with heat filtered high light (HL, yellow) or non-heat filtered high light (red).* ***A)*** *The leaf surface temperature was measured at eight different positions (mean ± SD) of the set up during a two-hour's time course.* ***B)*** *The transcript abundance of well-known heat responsive genes (HSFA7a - AT3G51910 and HSFA7b - AT3G63350) were evaluated by qPCR in three independent replicates (mean ± SD). The transcript abundances were normalized against the transcript abundances of the reference gene YLS8. Different letters represent significance according to Turkey´s post-hoc test (p-value < 0.05).*

After excluding potential heat stress, the heat filtered high light was defined by transcript analyses of well-known high light responsive genes (Fig. 6A). One of the best described high light marker is the *early light inducible gene 2* (*ELIP2*, AT4G14690), which controls the biogenesis of the chlorophyll binding complex (Hutin et al. 2003). Consistent with previous studies (Casazza et al. 2005), transcript analysis showed a 5-times higher *ELIP2* transcript abundance upon heat filtered high light compared to untreated plants. In addition to *ELIP2*, the *phenylalanine ammonia-lyase 1* (PAL1, AT2G37040) and the ROS scavenger *glutathione peroxidase 7* (*GPX7*, AT4G31870) were selected as high light regulated genes by public expression data (Waese et al. 2017). The transcript abundances of both genes were significantly increased after the application of heat filtered high light. In summary, the high light application (800 µmol photons s^{-1} m^{-2}) activates expression of high light responsive genes, despite the implemented water layer between the light source and the plants.

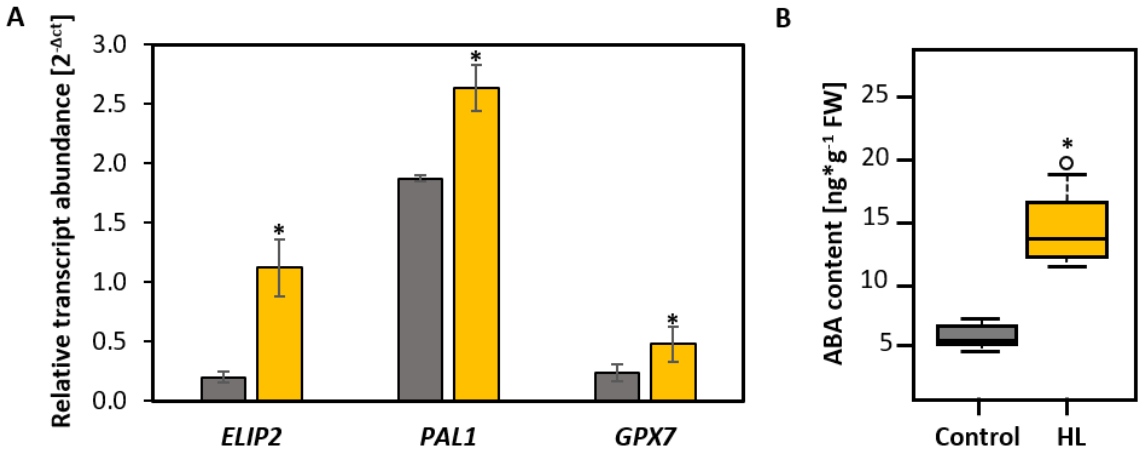

Figure 6: Transcript abundances of high light responsive genes and the effect of high light on the ABA content*: 4-week old plants were exposed to heat-filtered high light (yellow) for two hours and compared to untreated controls (grey).* ***A)*** *The transcript levels of the high light marker genes ELIP2 (AT4G14690), PAL1 (AT2G37040) and GPX7 (AT4G31870) were analyzed by qPCR in four independent replicates (mean ± SD) and normalized against the transcript levels of the reference gene YLS8.* ***B)*** *The abscisic acid levels were measured by HPLC-MS/MS in eight independent replicates. The hormone levels were normalized against deuterium labelled extraction standards and are shown relative to the fresh weight as boxplot (solid black line = median). Stars indicate significant differences according to Fischer's t-test (p - value < 0.05).*

Rossel et al. (2007) demonstrated that exposure to high light leads to the accumulation of the phytohormone abscisic acid (ABA), which is important in regulating stomata conductance and the response of several ROS scavenger to high light (Rossel et al. 2006). Galvez-Valdivieso et al. (2009) showed that the high light induced ABA response depends on the relative air humidity. To characterize if the heat-filtered light treatment results in an ABA-response, ABA contents were assessed with a high-performance liquid chromatography coupled with a tandem mass spectrometry analysis (Fig. 6B). Compared to control conditions, the accumulation of ABA was threefold higher after two hours of heat filtered high light treatment. This ABA increase was slightly higher than in other high light analysis (Rossel et al. 2007) and hints to an ABA dependent response upon heat-filtered high light.

2.1.2 Comparison of the high light and the cold treatment

A shift from standard growth conditions to stress conditions can disrupt the equilibrium between light energy absorption and light energy utilization, which promotes the formation of ROS (Mittler 2002). To validate, whether the heat filtered high light caused a similar oxidative threat like the 24 hours exposure to 4 °C, the total H_2O_2 content was measured in middle aged leaves after both stress treatments (Fig. 7A). The H_2O_2 level was 50 % higher in high light treated leaves compared to untreated ones and reached a similar level like after cold stress. The total H_2O_2 content in control and cold triggered plants was lower than described by other cold response studies, but the fold-change between untreated and stress treated plants was similar to previous reports (Sin'kevich et al. 2016).

Measuring the precise levels of organellar ROS is not possible (Noctor et al. 2016). To define the oxidative burden of the plastids after the cold and heat filtered high light treatment, oxidative damage of the photosystem was estimated by defining the maximum quantum yield of photosystem II (PSII) and by measuring the cold and high light response of the ROS marker gene *ZAT10* (Fig. 7b and Fig. 7c). The maximum quantum yield was assessed by imaging pulse amplitude modulated (PAM) chlorophyll-a fluorescence after 20 min dark acclimation. The cold as well as the high light treatments strongly reduced the maximum quantum yield of PSII. The decrease was similar for both stress treatments which hints to a comparative burden of

the PSII performance (Fig. 7B). The transcript abundance of the oxidative stress marker *ZAT10* (Mittler et al. 2006) was strongly increased after two hours of high light stress and 24 hours of cold stress (Fig. 7C). The induction was by trend slightly lower after the application of the heat filtered high light compared to the cold stressed plants. The comparable negative impact on PSII performance and the similar increase of the *ZAT10* transcript abundance after both stress applications indicate, that both stress treatments led to an oxidative threat for the chloroplast.

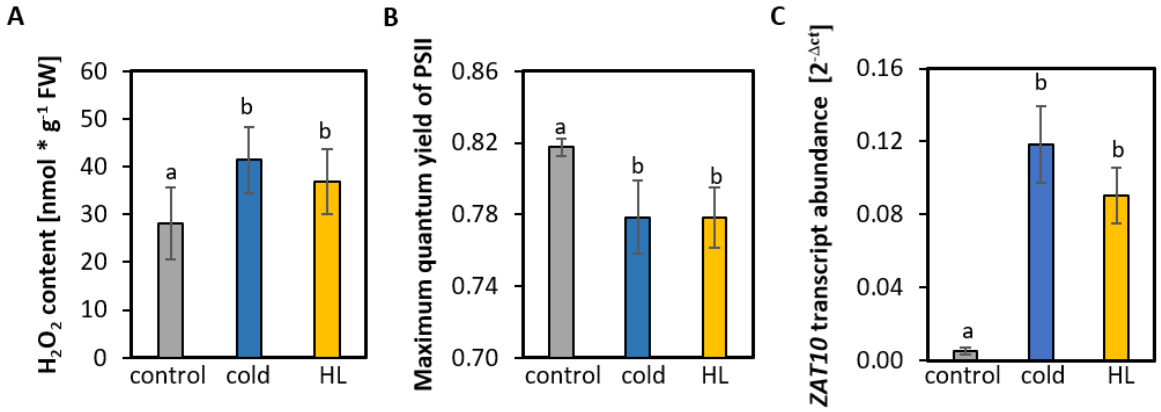

Figure 7: H_2O_2 content, maximum quantum yield of PSII and ZAT10 transcript abundances after cold and high light stress: *Oxidative stress parameter in middle aged leaves were compared after control conditions (grey), 24 hours of cold stress (blue) and two hours of heat filtered high light (yellow)* ***A)*** *The H_2O_2 contents were assessed photometrically in ten biological replicates performed in two independent experimental replicates (mean ± SD). The results were based on a H_2O_2 standard curve and normalized to the fresh weight of each sample.* ***B)*** *The maximum quantum yield of PSII was determined by chlorophyll-a fluorescence after 20 min long dark acclimation in five independent replicates (mean ± SD).* ***C)*** *Transcript abundances of ZAT10 were analyzed by qPCR in three independent replicates (mean ± SD) and normalized against the transcript abundances of reference gene YLS8. Different letters represent significant differences according to Tukey's post hoc test (p - value < 0.05).*

2.2 The impact of cold priming on cold and high light response

In previous priming analysis with a small group of oxidative stress marker genes, the cold response of *ZAT10* showed the strongest priming regulation during a subsequent cold stress (van Buer et al. 2016). The transcript abundances of *ZAT10* are elevated by cold and heat-filtered high light (Fig. 7). Therefore, the transcriptional response of *ZAT10* is the perfect starting point to compare the impact of cold priming on cold and high light regulation. First, a *in silico ZAT10* - co expression network analysis was conducted (Fig. 8) to predict additional cold priming targets for the comparison of *cis*- and *trans* - priming effects (Fig. 9-10).

The multi-association algorithm of the GeneMania platform (Warde-Farley et al. 2010) identified several zinc-finger transcription factors (*ZAT12* AT5G59820, *ZAT11* AT2G37430, *ZAT6* AT5G04340 and *ZAT5* AT2G28200), ethylene response factors (*ERF6* AT4G17490, *ERF13* AT2G44840 and *ERF104* AT5G61600) and two *WRKY* transcription factors (*WRKY40* AT1G80840 and *WRKY33* AT2G38470) as highly *ZAT10* co-expressed. Further highlighted the analysis the *bonsai associated protein BAP1* (AT3G61190) as *ZAT10* co-regulated. Previous analysis showed, that the transcriptional response of *BAP1* is, like the response of *ZAT10,* reduced during a second cold exposure (van Buer et al. 2016). Additional analysis on the String-platform (Roth et al. 2014) added *ACC synthase6* (AT4G11280), which encodes an enzyme of the ethylene metabolism (Yang and Hoffman 1984), to the *ZAT10* co-regulation network.

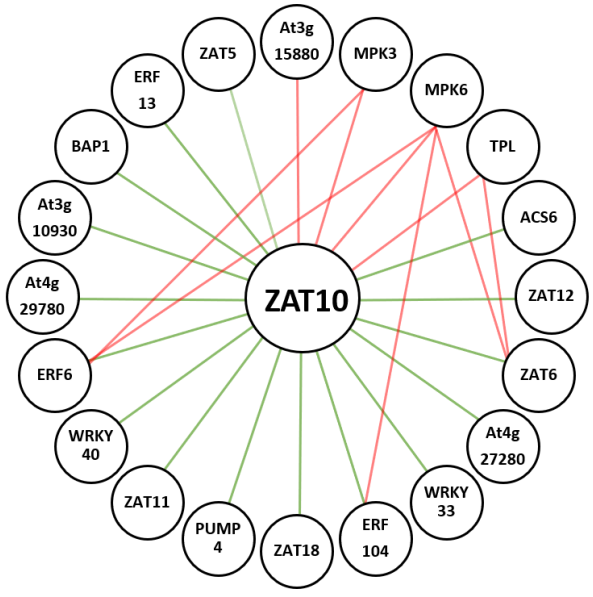

Figure 8: The interacting network of the transcription factor ZAT10: *Frequently ZAT10 co-expressed genes (green lines) and direct interaction partners of ZAT10 (red lines) were selected according to the multi-association algorithm of the GeneMania platform (Warde-Farley et al. 2010) and the combined score values of the string web tool (Roth et al. 2014).*

To identify common interaction partners within the *ZAT10* co-expression network, also protein-protein interaction was taken in account. *ZAT10* is a direct target of the mitogen activated protein kinases *MPK6* and *MPK3* (Nguyen et al. 2012). The *in silico* analysis further mentioned the topless gene (*TPL*; AT1G15750) as direct *ZAT10* interactor. Besides *ZAT10*, also *ZAT6* (Liu et al. 2013), *ERF6* (Wang et al. 2013), *ERF104* (Bethke et al. 2009), *WRKY40* (Lassowskat et al. 2014) and *ACS6* (Liu and Zhang 2004) are direct targets of *MPK6*.

2.2.1 *ZAT10* and *ZAT10* co-regulated gene expression during *cis*-priming

Most of the *ZAT10* co-regulated genes are under the control of MPK6 and responsive to several stress conditions. Such genes are designated as core stress responsive genes (Hahn et al. 2013). To evaluate the extend of the priming impact on the core stress response, the transcript levels of a subgroup of *ZAT10* co-expressed genes were analyzed under *cis*-priming conditions (Fig. 9). Additionally, the transcript abundance of the enolase *LOS2* (AT2G36530) was analyzed, because the transcriptional cold response of *ZAT10* is under the direct control of *LOS2* (Lee et al. 2002). For the analysis, cold primed and naïve plants were exposed to 4 °C for 24 hours consistent with previous experiments (van Buer et al. 2016).

The transcript levels of *ZAT10* were 22-fold higher in cold treated plants without priming in comparison to the untreated control (Fig. 9). This induction was significantly lower in cold primed plants as previously published (van Buer et al. 2016; van Buer et al. 2019). Besides *ZAT10*, also the transcript abundances of *ZAT12*, *ZAT6* and *LOS2* were increased upon cold triggering. The cold response of *ZAT6* and *ZAT12* was significantly reduced in cold primed plants, as described for the cold response of *ZAT10* (van Buer et al. 2016). However, the cold response of *LOS2,* which is mainly controlled at the transcriptional level (Lee et al. 2002)*,* was not altered in cold primed plants compared to only cold triggered plants. The transcript levels of *WRKY40* and *ACS6* were neither impacted by 24 hours cold stress nor altered in transcription by cold priming. In summary, the initial *cis*-priming analysis of *ZAT10* co-regulated genes indicates that only few specific genes of the core stress response network of Arabidopsis, are impacted by cold priming.

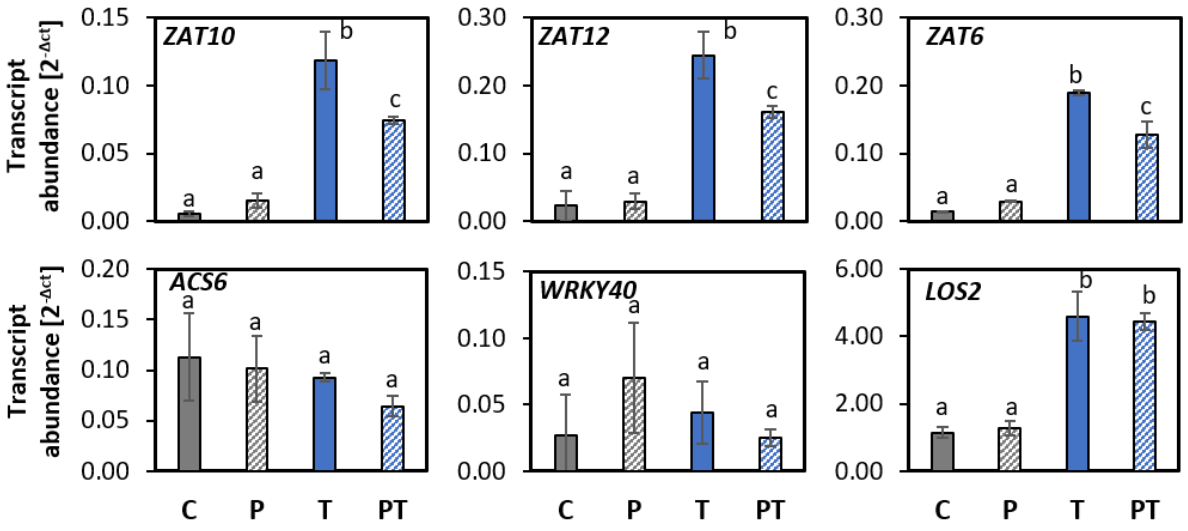

Figure 9: The transcript abundances of ZAT10 and ZAT10 co-regulated genes during cis-priming: *The transcript levels of ZAT10, of ZAT10 co-expressed genes and of the ZAT10 upstream regulator LOS2 were analyzed by qPCR in three independent replicates (mean ± SD). Naïve (solid bars, C,T) and cold primed (striped bars, P, PT) plants were harvested before (grey, C, P) and after 24 hours of cold stress (blue, T, PT). All values were normalized against the transcript abundances of the reference gene YLS8. Different letters represent significant differences according to Tukey's post-hoc test (p-value < 0.05).*

2.2.2 *ZAT10* and ZAT10 co-regulated gene expression during *trans*-priming

To examine, if cold priming effects also the high light response of *ZAT10*, *ZAT12* and *ZAT6,* cold primed and naïve plants were harvested after two hours high light exposure for transcript evaluation by quantitative PCR analysis (Fig. 10). The transcript abundance of *ZAT10*, *ZAT12* and *ZAT6* was strongly increased upon the heat filtered high light application, consistent with earlier high light studies (Davletova et al. 2005; Balfagón et al. 2019). The induction of the transcript abundance of *ZAT6* and *ZAT10* reached after high light stress a similar extent as in cold stressed plants (Fig. 9 vs. Fig. 10). The high light response of *ZAT12* was lower in comparison to the cold response. However, the high light induced expression of all three genes was not altered between cold primed and naïve plants. Only the high light response of *ZAT6* was by trend slightly reduced in cold primed plants. Taken together, all three genes showed a common regulation upon the first stress exposure but showed a cold and high light specific impact in cold primed plants, indicating stress type specific imprints by cold priming.

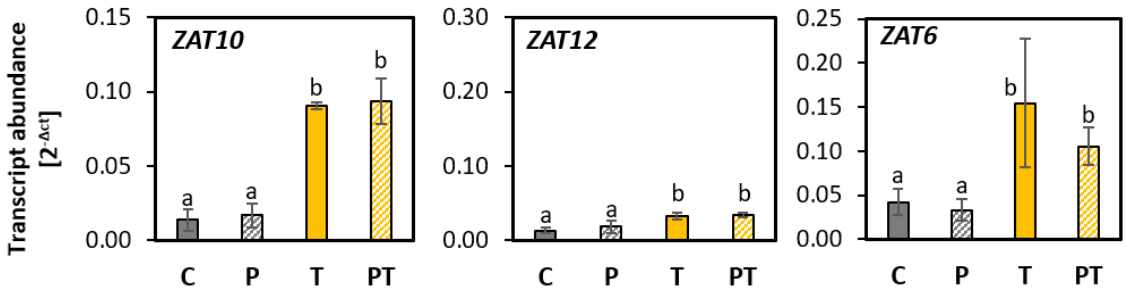

Figure 10: The effect of cold priming on the transcript abundances of ZAT10, ZAT12 and ZAT6 upon high light exposure: *Naïve (solid bars, C, T) and cold primed (striped bars, P, PT) plants were harvested before triggering (grey, C, P) and after two hours of high light stress (yellow, T, PT). The transcript abundances of the cis-priming responsive genes ZAT10, ZAT6 and ZAT12 were analyzed by qPCR in three independent replicates (mean ± SD). All values were normalized against the transcript abundances of the reference gene YLS8. Different letters represent significant differences according to Tukey's post hoc test (p-value < 0.05).*

2.2.3 Photosynthetic performance after *cis*- and *trans*- priming

A common consequence of cold and high light stress is the electron pressure at the photosystems, but the reason of this overexcitation differs between both perturbations (Ensminger et al. 2006). The stress type specific impact of cold priming on the high light and cold response of the ROS-marker genes *ZAT10*, *ZAT6* and *ZAT12* may result from effects on photosynthesis, which is differentially affected by both treatments. To investigate this assumption, pulse amplitude modulated (PAM) chlorophyll-a fluorescence was assessed after *cis*- and *trans*-priming (Fig. 11). The analysis was focused on middle-aged leaves, because they show the strongest impact of priming on the *ZAT10* cold response (van Buer et al. 2019).

To assess the maximum quantum yield of PSII, all plants were dark acclimated for 20 min before imaging chlorophyll-a fluorescence. Plants which receive either a 24-hour cold treatment or a two-hour high light treatment, showed a slightly lower maximum quantum yield of PSII (ΦPS-II, timepoint 0 min) in comparison to non-treated plants as shown before (Fig. 4). Upon illumination with 185 µmol quanta m^{-2} s^{-1} for 420 seconds, the effective quantum yield of PSII (ΦPS-II) was slightly elevated in triggered plants, which hints to a photoprotective acclimation after cold and high light. The photochemical quenching (qP) was by trend higher in cold and high light triggered plants, demonstrating that both stress treatments led to a

higher proportion of open PSII reaction centres, which underlines that both treatments led to slightly elevated photoprotection (Maxwell and Johnson 2000). The non-photochemical quenching was lower in stressed plants than compared to untreated plants. This effect was stronger during the heat-filtered high light application in comparison to the cold treated plants, which indicates a higher energy dissipation due to light acclimation processes (Maxwell and Johnson 2000). The analysed photosynthetic parameters were not affected by *cis*- and *trans*-priming (Fig. 11, T vs PT), which contradicts the hypothesis that stress specific priming regulation of *ZAT*10, *ZAT6* and *ZAT12* results from stress specific priming effects on the photosystem.

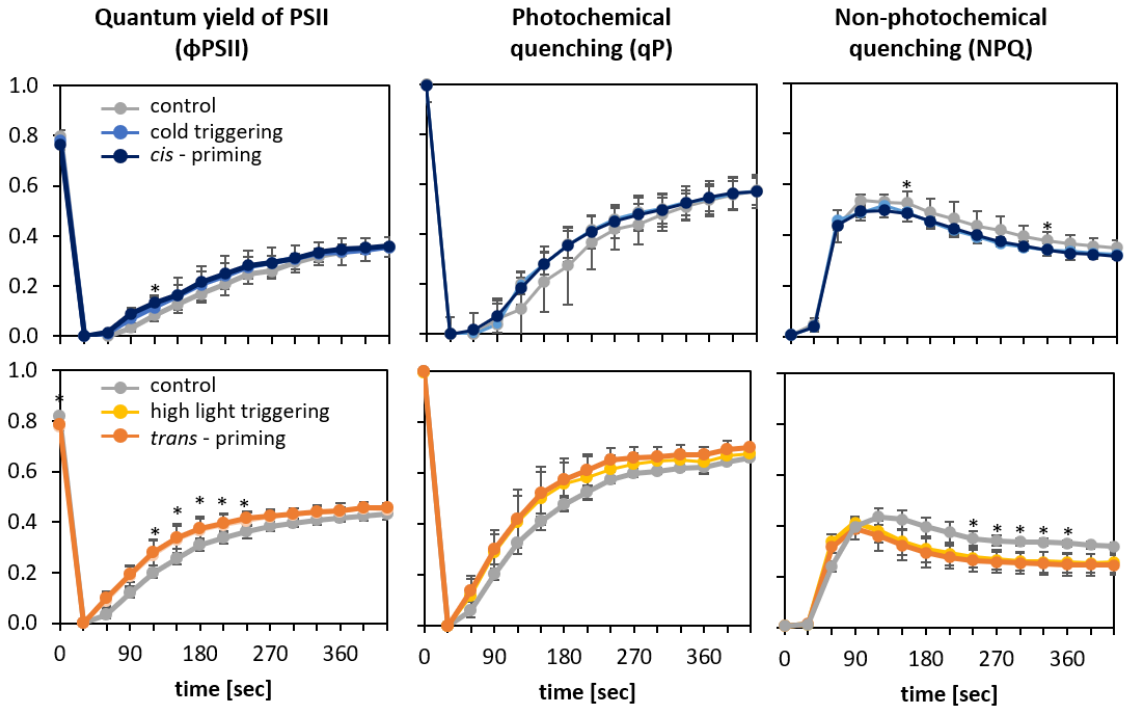

Figure 11: The impact of cold priming on the photosystem II performance after exposure to cold and high light : *The chlorophyll-a fluorescence was assessed in middle aged leaves after treatment of control conditions (grey), after 24 hours of cold stress (top) and after two hours of high light stress (bottom) in at least five independent replicates. All plants were dark acclimated for 20 min before measuring the chlorophyll-a fluorescence. The effective quantum yield, photochemical quench and non-photochemical quench was calculated as described in chapter 5.5. Stars represent significant differences according to Fischer's t-test (p-value < 0.05).*

2.3 The impact of cold priming on genome wide transcription upon stress recovery

To achieve a comprehensive understanding on gene regulation in response to cold and high light in cold primed plants, a genome wide transcript analysis was conducted. The samples were taken at the end of the lag phase (C and P), two hours after the end of cold triggering (T-C and PT-C) and two hours after the end of high light exposure (T-HL and PT-HL, timepoint 4 in fig. 4). For each treatment, RNA material of ten plants were pooled for the cDNA library construction. The sequencing of the six cDNA libraries resulted in average to 2.4 x 10^7 raw reads per sample (Table 1). By average, 99.9 % of the raw reads were assigned as clean reads (details material and methods, chapter 4.3) and 98.5 % of the filtered reads could be aligned to the reference genome TAIR10 with the help of the R-package Bowtie2 (Langmead and Salzberg 2012). In total, transcript profiles from 24,000 genes out of 33,602 TAIR10 annotated genes were identified.

Table 1: Read number and quality of the RNA-seq: *shown by the total number of sequenced reads, the ratio of clean reads as well as the ratio of aligned reads per sample. For the total number of aligned reads, the ratio for unique mapping is given.*

Sample	Read number [10^6]	Clean reads [%]	Aligned Reads [%]	Unique match [%]
C	24.14	99.91	98.61	88.65
P	23.76	99.94	98.63	89.14
T-C	23.83	99.93	98.72	90.10
PT-C	24.14	99.87	98.43	88.84
T-HL	24.14	99.91	98.49	88.09
PT-HL	23.84	99.92	98.77	89.56

2.3.1 Variance analysis of the genome wide transcription

As an initial step of the data analysis, a principal component analysis (PCA) was used to provide a global view on the main variance factors between the different treatments (Fig. 12). The overwhelming factor in the experimental set up (79.6 % of variance) was the triggering treatment. The first main variance component (50.6 % of the variance) separated the non-triggered samples (C and P) from the high light and cold treated ones. The second variance component (29 % variance) separated the high light response from the cold response. Cold

priming caused only a mild variation in genome wide transcription two hours after the end of triggering compared to the impact of the stress treatment only, but the variance between both triggering stimuli was slightly higher in cold primed plants compared to the only triggered samples (Distance between PT-C and PT-L compared to the distance between T-C and T-L).

Without any triggering stimulus, cold priming had only a minor impact on the variance of genome wide transcription, indicating transcriptional recovery from the first cold stress after five days of recovery, also called lag-phase. Due to the small variance without triggering, it can be suggested that transcriptional changes play only a minor role in cold memory maintenance.

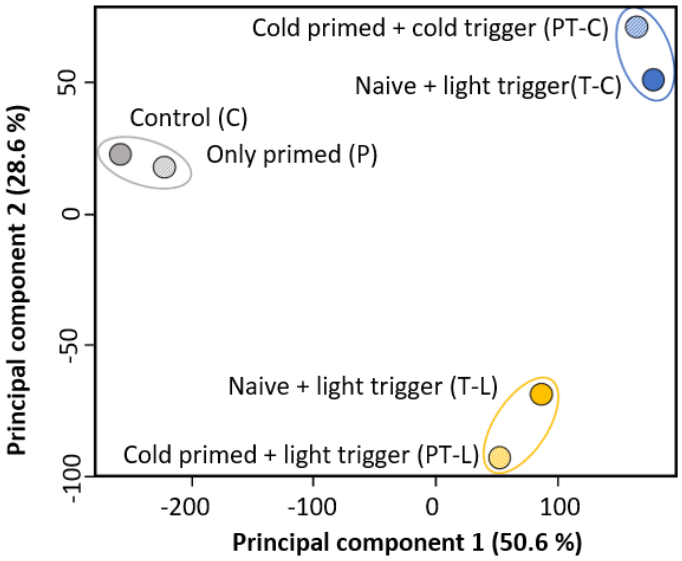

Figure 12: Principle variance components of the genome-wide transcription of cold primed and naïve plants: *The two main variance components of the cold primed (striped circle) and naïve (solid circle) samples of the RNA-seq were identified by principal component analysis (PCA) and are depicted in a score plot. Genome wide transcription was analyzed after lag-phase (grey), two hours after the end of cold stress (blue) and two hours after the end of high light stress (yellow).*

2.3.2 Gene regulation upon cold and high light triggering in non-primed plants

For gene regulation analysis, genes were considered with a transcript level higher than five FPKM in at least one treatment, to avoid overestimated gene regulation of weakly expressed genes. Using these criteria, 13775 genes were selected. From this selection, 1817 genes were at least two-fold regulated upon cold triggering ($\log_2$(T-C/C) ≥ |1|).

Previous transcriptome analysis revealed that the transcriptional response to cold is very similar to the transcriptional response to high light in Arabidopsis (Rasmussen et al. 2013). In my study, 61.7 % of the cold up-regulated genes and 32.8 % of the cold downregulated genes were also two-fold regulated in same direction upon the heat-filtered high light exposure. In contrast, only 5.5 % of the stress responsive genes were inversely regulated between both treatments (T-C/C compared to T-L/C). Therefore, it can be concluded that the heat-filtered high light treatment (T-L) and cold treatment (T-C) cause a similar transcriptional reprogramming in non-primed plants, as reported by others.

The transcript levels of many well-known cold and light-responsive transcription factors, like *CBF1* (AT4G25490), *ANAC078* (AT5G04410) and *ZAT10* were already strongly decreased two hours after the end of the triggering. Genes of the secondary cold and high light response such as the CBF3-regulated gene *COR15A* (Thomashow et al. 1997; Kimura et al. 2003) were still induced, indicating that the primary stress response was recovered, but targets of the primary stress response were still induced two hours after the end of both triggering stimuli. The transcript abundance of senescence regulated genes, such as *ORE9* (Balazadeh et al. 2010) or of programmed cell death responsive genes (Olvera-Carrillo et al. 2015) were not induced in any sample of the RNA-seq, indicating that cold and high light did not activate senescence .

2.3.3 Cold priming responsive gene regulation after cold and high light triggering

For priming dependent gene regulation, genes with at least two-fold higher or two-fold lower transcript abundance in primed plants compared to the respective non-primed sample were selected. Thereby, three priming regulation groups were assigned: lag-phase- (P/C), *cis*-priming - (PT-C/T-C), and *trans*-priming (PT-L/T-L) regulated transcript profiles. The selection yielded to 16 lag-phase regulated genes, 15 *cis*-priming regulated genes and 44 *trans*-priming regulated genes (Fig. 13). Only one transcript (AT4G22510), which encodes a putative transmembrane protein, was altered by *cis*- and *trans*-priming in the same direction. The remaining genes were only after high light or after cold stress differential expressed in response to priming, which hints to rather stress specific effects of cold priming on cold and high light responsive transcription, as supposed by the initial transcript analysis of *ZAT10*.

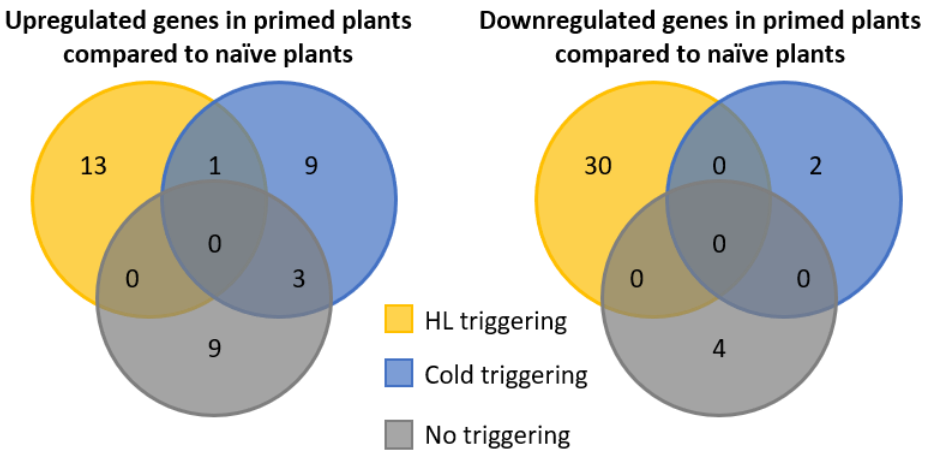

Figure 13: Overlap of stringent priming regulated genes after cold and high light triggering: *The Venn diagram represents the overlap priming regulated genes without triggering (grey), after cold triggering (blue) and heat-filtered high light triggering (yellow). Genes were assigned as priming regulated, if they showed a log_2-fold higher or lower transcript abundance in cold primed plants*

Long term persisting priming regulation at the end of the lag-phase

According to the stringent priming regulation criteria, only four genes were identified as priming downregulated at the end of the lag-phase, independent of the high light and cold triggering (Fig. 13, right side). The four genes encoding a lipid transfer protein (*LTP4*, AT5G59310) and three transcripts with unknown function (AT1G04800, AT1G53890 and AT4G29660).

Twelve genes were upregulated five days after cold priming (Fig. 13, left side). Three out of the twelve genes were also increased after cold triggering, including a nitrilase (*NIT2*, AT3G44300), which is involved in the metabolism of auxin (Hillebrand et al. 1998), a trypsin inhibitor (AT2G43510) and a senescence associated gene (*SAG13*, AT2G29350). The remaining priming regulated genes at the end of the lag-phase encoding two defense related lipid transfer proteins (AT4G12490 and AT4G12480) and two trypsin inhibitors (AT1G73260 and AT2G43510). Based on the stringent selection of priming targets, transcriptional regulation might play only a minor role at the end of the lag-phase if no triggering was applied, as the annotation of the deregulated genes (Fig. 13, grey area) showed no enrichment of a specific biological process.

***Cis*-priming specific gene regulation**

Nine genes were identified as priming upregulated specific after cold triggering (Fig. 13, left side). None of the nine genes are associated with a known function, but six of them are annotated to the same genomic region (Chr4: 11,840,000 -11,860,000) and encode short overlapping transcripts (Fig. 14A) with repetitive sequence elements. Thereby, single cDNA reads could multiple mapped to these transcripts, resulting in an overestimation of the actual transcript abundance. Exemplarily, the transcript abundance for three of the six genes were analyzed by qPCR. Exon-exon junction primers or oligomers, which bind the more specific 3´-UTR, were used to guarantee specificity of the amplificated transcript fragments. The transcript abundance of one gene (AT4G22485) was below the detection limit of the method. Transcript analysis of the remaining two genes (AT4G22490 and AT4G22495) recorded no difference between cold primed and naïve plants (Fig. 14B), which indicates a misleading transcript abundance obtained by multi-alignment of single reads.

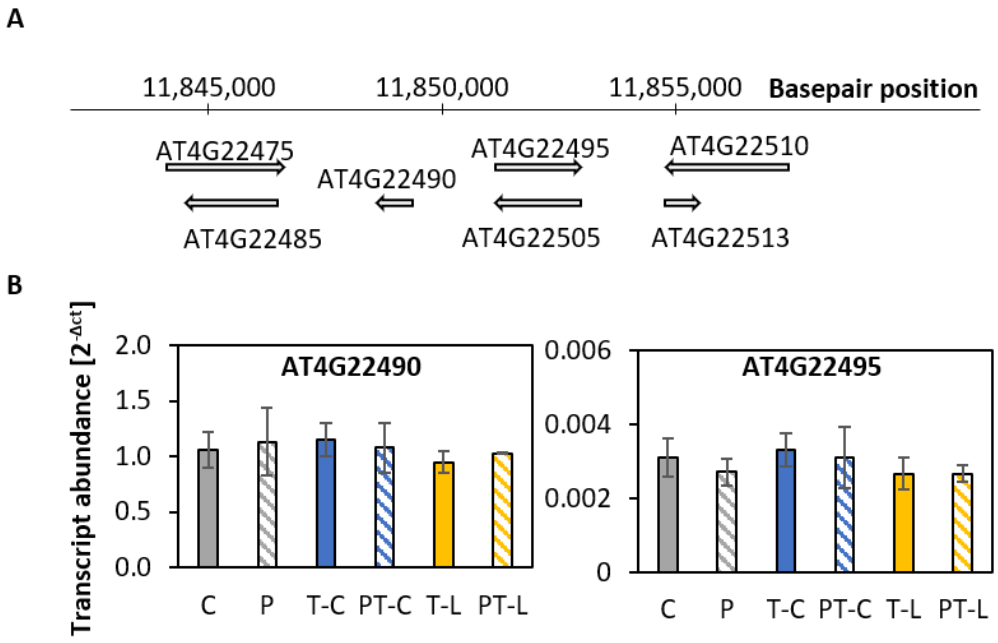

Figure 14: Genomic region of positive cis-priming regulated transcripts and validation of transcript abundance: A) Gene annotation of the genome region Chr4:11,840,000 -11,860,000 according to annotation of TAIR10. **B)** The transcript levels of AT4G22490 and AT4G22495 were analyzed by qPCR in two independent replicates (mean ± SD) before triggering (grey), after cold triggering (blue) and after high light triggering (yellow).

Only two genes, namely expansin A8 (AT2G40610) and AT2G05440, were identified as stringent priming downregulated two hours after cold triggering (Fig. 13, right side). The transcript abundance of e*xpansin A8* was 8-fold lower in response to cold triggering compared to control plants. This cold stress reduced transcript abundance was stronger pronounced in cold primed plants (20-fold reduction). AT2G05440 encodes a glycine rich protein and showed an elevated transcript abundance in response to cold stress. This cold responsive upregulation was blocked in cold primed plants.

Trans-priming specific gene regulation

In the group of *trans*-priming regulated targets, 14 genes were at least two-fold upregulated in cold primed plants after high light stress compared to the non-primed high light response (Fig. 13, left side). Among these were three heat shock proteins (AT2G29500, AT5G59720 and AT5G12020), one methyl esterase inhibitor (AT5G62360), which is a negative regulator of cold acclimation (Chen et al. 2018), a protein involved in the chloroplast avoidance movement (AT5G38150) and a 1,3-beta-D-glucan synthase subunit (AT2G31960). Only the transcript levels of one gene, the heat shock protein HSP18.2 (AT5G59720), were increased upon high light in non-primed plants. The remaining genes were uniquely high light responsive in cold primed plants, which indicates priming specific activation of the high light response.

At the same time, 30 genes were identified as cold priming downregulated after the high light application (Fig. 13, right side). To get a comprehensive overview of the function of these genes, a gene ontology enrichment analysis based on hypergeometric distribution was performed (Ge et al. 2019). 17 out of the 30 *trans*-priming downregulated transcripts are annotated as pathogen responsive genes according to the nomenclature of TAIR 10 (Fig. 15). All 17 biotic stress related genes showed an increased transcription in response to high light triggering in non-primed plants. This high light response was blocked in cold primed plants. It is well known that high light stress supports plant tolerance to pathogen infection (Mühlenbock et al. 2008; Szechyńska-Hebda et al. 2010). Based on the RNA-seq, it can be considered that cold priming weakens the cross-tolerance between high light and biotic stress.

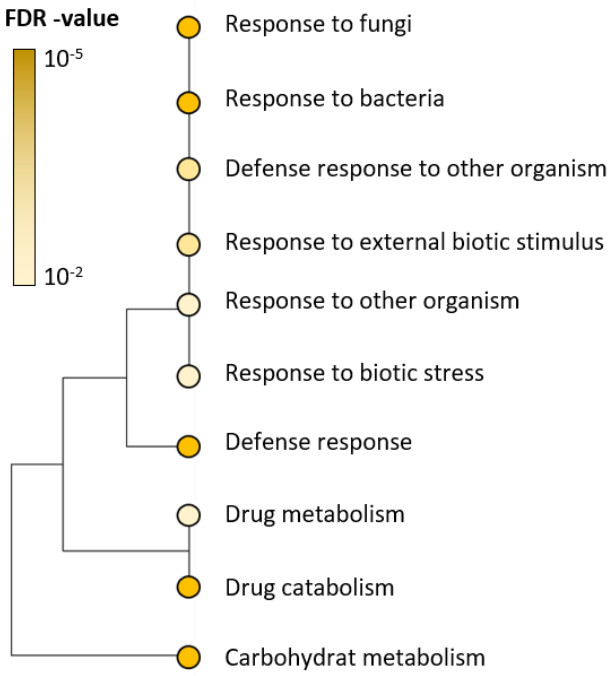

Figure 15: Functional characterization of stringent trans-priming downregulated genes: *Genes that were identified as cold priming upregulated after high light triggering were functionally characterized based on gene ontology annotation. Significant enriched GO-terms were selected according to hypergeometric distribution (FDR value < 0.05) and hierarchical clustered with the help of ShinyGO (Ge et al. 2019)*

2.3.4 Validation of cold priming responsive gene regulation

To evaluate the gene regulation, observed by the single RNA-seq experiment, transcript levels of 15 RNA-seq selected priming targets were assessed by qPCR in at least four independent replicates (Fig. 16). For each treatment group, three priming upregulated and two priming downregulated genes were selected with respect to a high basal transcript abundance.

Thirteen, out of the fifteen selected genes, showed a significant priming-dependent regulation (one-sided t-test, $p < 0.05$) consistent with the RNA-seq results**.** The remaining two genes were by average at least two-fold priming regulated, which reflects the regulation pattern observed by RNA-seq. It can be concluded that the priming impact on the transcript abundance is at least for the 15 RNA-seq selected genes in the range of independent experimental replicates.

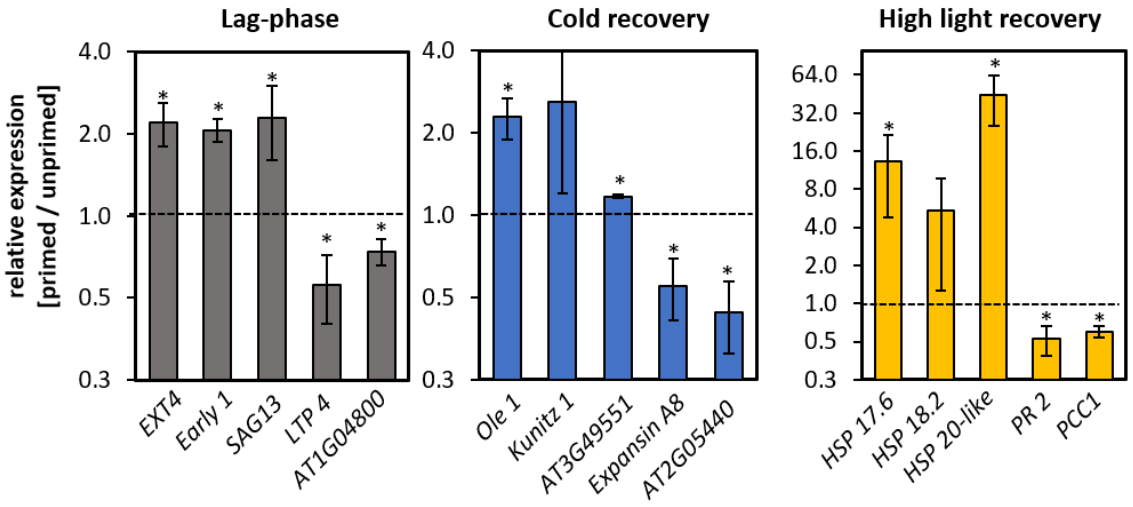

Figure 16: The transcript abundances of 15 priming regulated genes: *The priming regulation of five genes per treatment group (lag-phase = grey, cold recovery = blue, light recovery = yellow) out of the 71 identified cold priming targets were analyzed by gene specific primer and quantitative qPCR in at least four independent replicates. The transcript levels were normalized against the transcript abundances of the reference gene YLS8 and are shown relative to the respective naïve value ($2^{-\Delta\Delta ct} \pm SD$). Significant difference to a priming regulation (PT/T) of one according to a one-sided t-test (p-value < 0.05) is depicted by stars.*

2.3.5 Wider comparison of *cis*- and *trans*-priming regulated transcription

After the validation of highly stringent selected priming regulation (log_2-fold change >|1|), the transcriptome data were less stringently filtered for widening the comparison of cold priming impacted transcript profiles after cold and high light triggering (log_2-fold change ≥ |0.5|, FPKM ≥ 5). The less stringent selection increased the target group to 992 *trans*-priming regulated genes, 324 *cis*-priming regulated genes and 365 priming regulated genes without any triggering (Fig. 17). Only two genes, namely AT5G23411 and a LURP1-like protein (AT1G53870), were upregulated in cold primed plants in all three treatment groups. Additionally, one gene (hypothetical protein, AT1G13470) were commonly downregulated in cold primed plants, before triggering, after cold triggering and after high light triggering. If comparing the impact of priming after five days lag-phase without any triggering with the priming impact after cold and high light triggering, less than 5 % of the selected genes showed consistent priming regulation, which indicates an active transcriptional reprogramming of long lasting priming effects due to triggering.

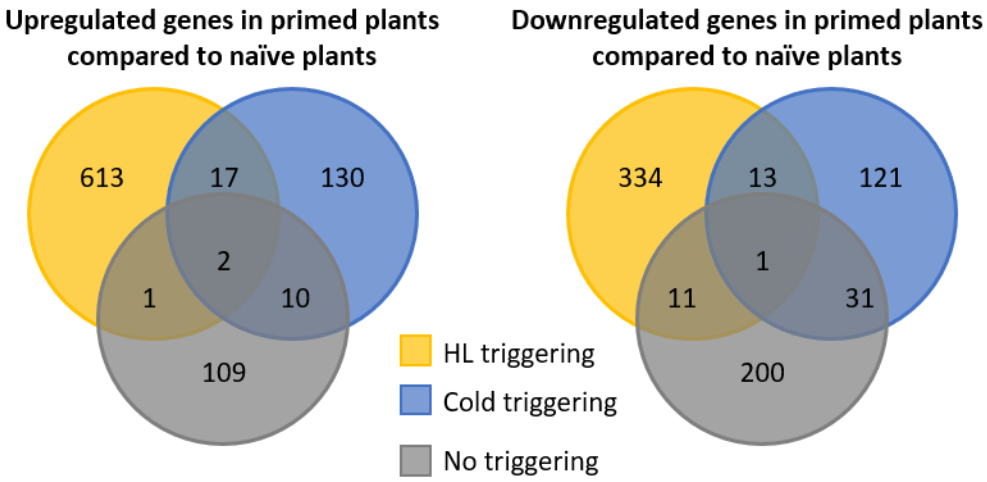

Figure 17: Comparison of priming regulated genes selected by less stringently criteria: *Venn diagram depicting the overlap of less stringent cold priming regulated genes (log_2-fold-change > |0.5|) according to RNA-seq. The transcriptome of cold primed and naïve plants was analyzed after 5 days of lag phase without any triggering (grey), two hours after the cold stress (blue) and two hours after the high light stress (yellow).*

Besides the small group of priming targets, which were commonly priming regulated before and after triggering, only 33 genes showed the same transcript pattern after *trans*- and *cis*-priming (Fig. 17). 19 out of the 33 genes were commonly upregulated after *cis*- and *trans*-priming. One third of these genes are annotated to a small genomic region (AT4G22490, AT4G22495, AT4G22505, AT4G22510, AT4G22513 and AT4G22520), which was already mentioned as potential overstated misinterpretation due to multi-alignment of single reads within these genomic region (Fig. 14). The remaining common priming upregulated genes encoding a diverse group of proteins, including a photosystem subunit (ATCG00220), a putative kinase (AT2G28870) and a plastid encoded ribosome subunit (ATCG01020).

In the group of cold priming downregulated genes (Fig. 17, right side), 14 genes showed a common regulation after cold and high light triggering. Three genes encoding proteins with heavy metal binding capacity (AT5G24580, AT5G22580 and AT2G35850) and the remaining genes encoding putative proteins with unknown function. The low number of common priming downregulated genes after cold and highlight supports the observation, that cold priming caused distinct imprints in the cold and high light response of Arabidopsis.

***Cis*-priming targeted processes**

The large number of cold and high light specific targets within the less stringently filtered cold priming targets raised questions about their functions. To address these questions, an enrichment analysis of annotated gene ontology was performed. Functional annotation of the 130 *cis*-priming upregulated genes showed an overrepresentation of ten biological processes (false discovery rate ≤ 0.01, Fig. 18). Eight of the ten enriched processes were related to biotic stress, which points to an upregulation of biotic defense traits in cold primed plants in response to cold triggering. By an additional functional characterization by AgriGO (Du et al. 2010) *cis*-priming upregulated genes were associated with phenylpropanoid-, flavonoid- and anthocyanin metabolism. Previous priming analysis showed that cold priming strongly activates the transcriptional cold response of the *phenylalanine ammonia-lyase* (AT2G37040) and the *chalcone synthase (AT5G13930),* which are both involved in the early steps of the phenylpropanoid biosynthesis (van Buer et al. 2016). The transcript abundance of *PAL1* and *CHS* was also increased in cold primed plants two hours after cold triggering, although they did not pass the selection criteria of priming regulation, according to the RNA-seq data.

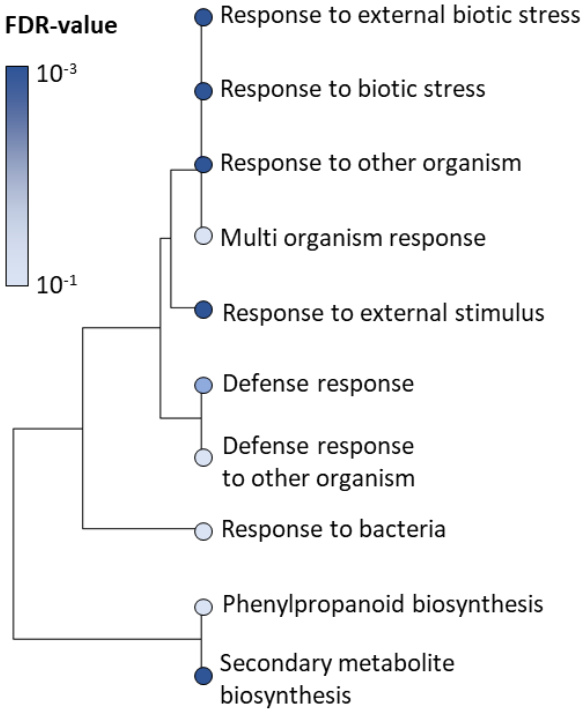

Figure 18: Functional characterization of cold priming upregulated genes during cold stress recovery: *Genes that were identified as cold priming upregulated after cold triggering (Log_2-(PT-C/T-C) < -0.5) were functionally characterized based on gene ontology annotation. Significant enriched GO-terms were selected according to hypergeometric distribution (FDR value < 0.05) and hierarchical clustered with the help of ShinyGO (Ge et al. 2019).*

In parallel to the priming supported cold response of genes involved in phenylpropanoid metabolism and pathogen defense, cold priming decreased the transcript levels of 121 genes after cold triggering. According to ShinyGo based enrichment analysis (Ge et al. 2019), no particular biological process was overrepresented within these downregulated genes. Additional analysis by the singular enrichment algorithm of AgriGo (Tian et al. 2017) indicated that *cis*-priming had a negative impact on genes, which are involved in growth (GO:0040007, FDR = 0.04), single organism transport (GO:0044765, FDR = 0.04) and single organism localization (GO:1902578, FDR = 0.04).

***Trans*-priming regulated processes**

To examine, if cold priming preferentially affects the same biological processes after high light like after cold stress, the 992 *trans*-priming regulated genes were functional characterized by enrichment analysis of TAIR10 annotated gene ontology. Genes identified as *trans*-priming upregulated genes were annotated to 42 enriched biological processes. The ten biological processes with the highest enrichment probability are all related to development and cellular organization (Fig. 19). In addition, gene ontology annotation indicates a strong support of growth- and propagation-related gene expression in cold primed plants after high light triggering compared to only high light triggered plants.

Within the group of cold-priming downregulated genes after high light triggering were genes with functions in pathogen defense and biotic stress response overrepresented (Fig. 19). The functional annotation of trans-priming down-regulated genes points further to a negative priming impact on JA related gene expression.

In summary, functional annotation points to a cold priming downregulation of the biotic stress involved genes after high light and cold priming upregulation of biotic defense traits after cold triggering, which indicates an inverse priming regulation of biotic stress responsive genes in a trigger specific manner.

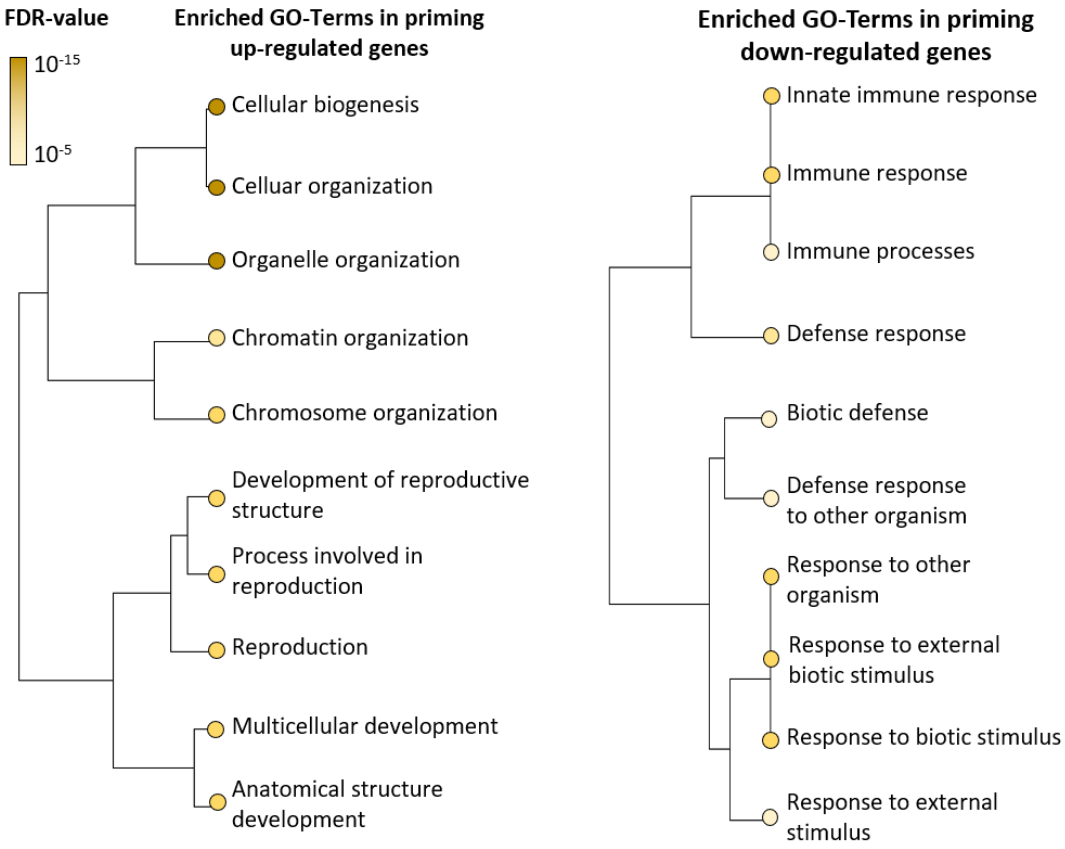

Figure 19: Functional characterization of cold priming regulated genes during high light recovery: *Genes that were identified as cold priming upregulated (left side) and cold priming downregulated (right side) after high light triggering were functional characterized based on gene ontology annotation. The ten most enriched biological processes were selected according to hypergeometric distribution (FDR value) and hierarchical clustered with the help of ShinyGO (Ge et al. 2019).*

2.3.6 Analysis of inverse regulated transcription between *cis*- and *trans*-priming

To investigate inverse regulation between cold and high light in cold primed plants in more detail, *cis*-priming upregulated genes were compared with *trans*-priming downregulated genes and *vice versa* (FPKM > 5, $\log_2$-fold change > |1|). The comparison resulted in a selection of 29 priming inverse regulated genes. 17 genes were increased after *cis*-priming and reduced after *trans*-priming. These 17 genes are mainly annotated as biotic responsive genes, which supports the suggestion of a cold priming inverse regulation of biotic stress responsive genes in a stress type specific manner assumed by functional annotation (Fig. 18-19). The remaining 12 inverse priming regulated genes are not related to any biological process.

To confirm the trigger specific impact of priming on the transcript abundance of biotic stress related genes, the transcript levels of three pathogen defense genes, selected from the transcriptome data, were exemplarily analyzed in independent replicates (Fig. 20). The pathogen resistance gene *PR4* (AT3G04720) encodes an antifungal chitin binding protein and showed in naïve plants no alteration in the transcript abundance after cold and high light stress. The transcript abundance of *PR4* was three-fold higher after cold stress in cold primed plants compared to only cold triggered ones. At the same time, *trans*-priming reduced the transcript levels of *PR4*, as expected from the RNA-seq. The transcript abundance of the pathogen and circadian controlled (*PCC1*, AT3G22231) gene were more than seven-fold upregulated in non-primed plants in response to high light. This induction was two-fold lower in cold primed plants. In contrast, *cis*-priming increased the transcript abundance of *PCC1*. The transcript abundance of the trypsin inhibitor *kunitz1* (AT1G73260) showed by average the same trend as *PCC1*. In conclusion, the qPCR analysis confirmed the RNA-seq observed regulation pattern for three out of 29 priming inverse regulated genes. The inverse regulation of the genes between *cis*- and *trans*-priming, indicates an active reprograming of the cold priming memory during each triggering application.

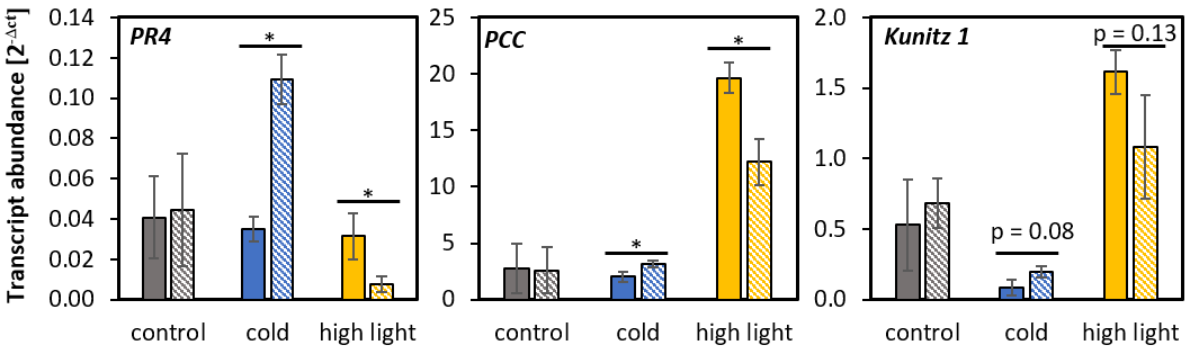

Figure 20: The effect of cis- and trans-priming on the transcript abundance of three biotic stress related genes: *The transcript levels of three cold-priming-inverse supported genes were analyzed before triggering (grey), 2 hours after the end of high light triggering (yellow) and 2 hours after the end of cold triggering (blue) by qPCR in four independent replicates (mean ± SD). Stars depicting significant differences between naïve and cold primed plants as determined by Fischer's t-Test (p-value < 0.05).*

2.4 The impact of cold priming on the early cold and high light response

Exposure to perturbations, such as high light and cold, can activate transcriptional changes within the first minutes of stress exposure (Vogel et al. 2014) and appropriate acclimation is determined during the first hour of stress exposure by fast acting transcriptional responses. One well explained example is the CBF regulon, which is strongly expressed within the first 30 min of cold exposure (Gilmour et al 1998). To examine, if cold priming has an impact on the dynamics of early stress response and to investigate, if cold priming interferes with the early high light response and early cold response to the same extend, time serial analysis during the first two hours of triggering were carried out.

2.4.1 The early cold response of *ZAT10*, *ZAT6* and *ZAT12* in cold primed plants

Previous analysis revealed the reduced induction of *ZAT10*, *ZAT12* and *ZAT6* in cold primed plants compared to non-primed plants after 24 hours at 4 °C (Fig. 9). All three transcription factors belonging to the "first wave" of early oxidative stress inducible genes (Sakamoto et al. 2004; Mittler et al. 2006; Park et al. 2015). The transcript abundance of *ZAT10* is for instance strongly elevated after 15 min at 4 °C (Sakamoto et al. 2004). To obtain a first impression, if cold priming impacts the onset of cold response, the transcript abundance of the already known *cis*-priming targets *ZAT10*, *ZAT12* and *ZAT6* were analyzed every 30 minutes within a two hours long cold exposure (Fig. 21). Additional, the transcript abundance of *CBF2* (AT4G25470) was assessed as a marker gene for the early cold stress response (Thomashow et al. 2001).

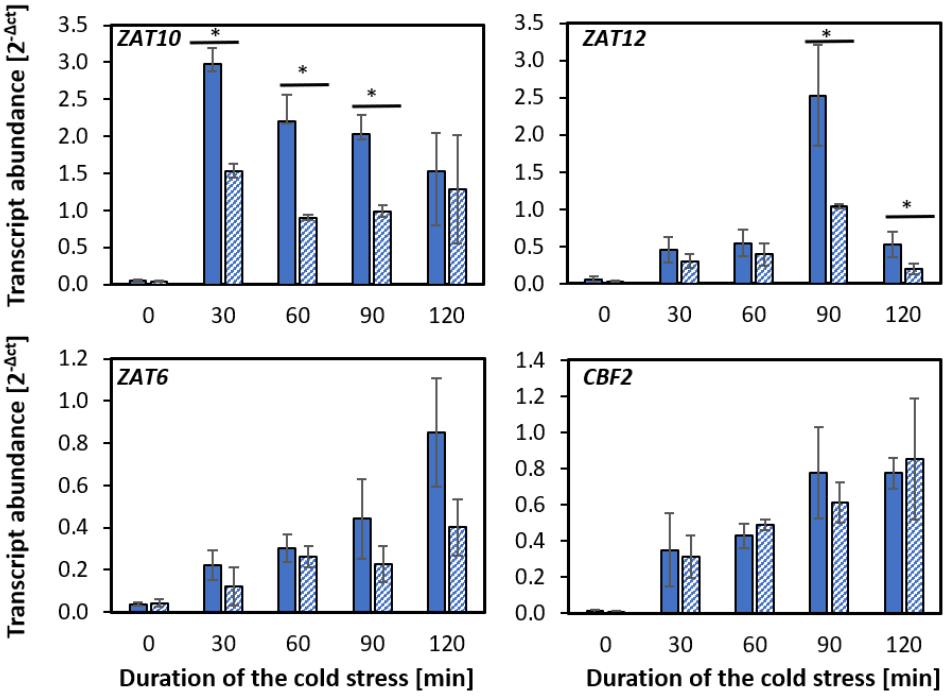

Figure 21: The effect of cold priming on the transcript levels of early cold responsive genes: *Naïve (solid bars) and cold primed plants (striped bars) were harvested every 30 min within a two hours cold stress. The transcript abundances of the priming marker ZAT10, ZAT6, ZAT12 and of the early cold stress marker CBF2 were evaluated by qPCR in four replicates (mean ± SD). All values were normalized against the reference transcript levels of YLS8 and stars represent significant differences (t-test, p-value < 0.05).*

The transcript levels of *ZAT10* accumulated almost immediately after the transfer into cold (Fig. 21), which is consistent with previous transcript analysis (Mittler et al. 2006). The induction of *ZAT10* transcription declined with the duration of the cold stress but was still 35-fold higher after two hours of cold triggering than in non-treated plants. In comparison to *ZAT10,* the induction of *ZAT12* was slower and reached the maximum value after 90 min of cold exposure. The transcript abundances of *ZAT6* and *CBF2* steadily increased with the duration of the cold stress.

In cold primed plants, the cold induced transcript abundance of *ZAT10* was 2-fold lower. Furthermore, the cold induction of *ZAT6* and *ZAT12* was reduced in cold primed plants. On the contrary the cold induced transcript levels of *CBF2* were not cold priming effected. The results show that priming regulation is already manifested during the onset of cold response. However as shown for *CBF2*, cold priming affects not all aspects of the early cold stress response.

2.4.2 The early high light response of *ZAT10*, *ZAT12* and *ZAT6* in cold primed plants

Previous transcript analysis revealed stress type specific effects of cold priming after 24 hours cold triggering and two hours of high light triggering (Fig. 9-10 and Fig. 17), despite similar consequences of both treatments in non-primed plants (Fig. 8). To elucidate, if cold priming led also to triggering specific effects upon the onset of the stress response, the transcript levels of *ZAT10*, *ZAT6* and *ZAT12* were analyzed every 30 minutes of high light exposure (Fig. 22).

The transcript abundance of *ZAT10*, *ZAT12* and *ZAT6* showed a high variation in response to high light between the six independent replicates, but the transcript levels of all three transcription factors reached after two hours high light application a similar level as shown in the initial high light analysis (Fig. 10 in comparison to Fig. 22). Exposure to early cold stress resulted in stronger transcript accumulation of all three genes than early high light stress, although the maximum transcript abundance during high light was like the transcript abundance after 24 hours of cold stress. Neither the high light response nor the variation of the high light response was altered in cold primed plants as compared to non-primed ones. Thereby, it can be proposed that cold priming only interferes with the early cold response, but not the early high light response of *ZAT10*, *ZAT6* and *ZAT12*.

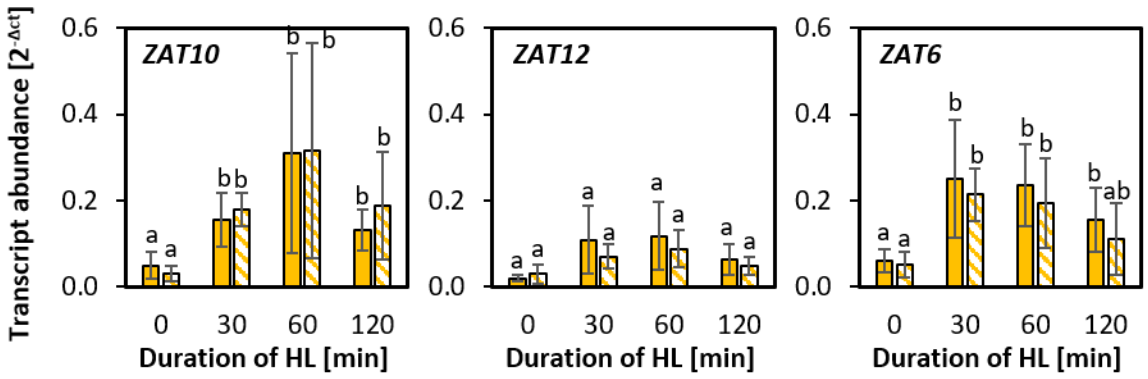

Figure 22: The effect of cold priming on the early high light response of ZAT10, ZAT6 and ZAT12: *Naïve (solid) and cold primed (striped) plants were harvested after 30, 60 and 120 min of high light stress. The transcript abundances of the priming marker ZAT10, ZAT12 and ZAT6 were assessed by qPCR in six independent replicates (mean ± SD). The transcript levels were normalized against the transcript abundances of the reference gene YLS8 and letters represent significant differences according to Tukey´s post hoc test (p-value < 0.05).*

2.4.3 RNA-seq analysis of the early cold and high light response in cold primed plants

RNA-seq time courses are strong tools to investigate the dynamic of transcriptional stress responses, as shown recently in Arabidopsis upon chilling stress (Calixto et al. 2018). In my study, genome wide transcription was assessed in cold primed and non-primed plants after 0, 30, 60 and 120 min of the cold and high light triggering, to unravel the impact of cold priming on the dynamics of the high light and cold response of Arabidopsis.

The RNA-seq experiment gain an average number of 70 ± 8 million reads per sample (Tab. 2), 98.8 % of the reads passed the quality threshold (for details see chapter 4.3) and were aligned against the TAIR10 reference genome using Bowtie2 (Langmead and Salzberg 2012). In total, 27,860 different transcripts were identified. The number of aligned reads for each transcript were normalized to the total read number of each sample and to the length of the gene to enable comparative regulation analysis (FPKM value).

Table 2: Quality of each RNA-seq sample during the early stress response: *shown by the total number of sequenced reads, the percentage of clean reads as well as the percentage of TAIR10 aligned reads. For the total number of aligned reads, the ratio of unique alignments is given.*

Sample	Raw Reads (M)	Clean Reads [%]	Aligned Reads [%]	Uniquely aligned [%]
C	62.57	96.65	96.41	82.98
P	85.19	96.30	96.32	82.51
T-C 30 min	67.14	96.51	96.50	82.63
PT-C 30 min	80.50	96.55	96.43	82.81
T-C 60 min	64.15	96.51	96.32	83.16
PT-C 60 min	64.76	96.38	96.57	82.96
T-C 120 min	72.08	96.46	96.27	82.67
PT-C 120 min	58.26	96.41	96.21	83.02
T-HL 30 min	72.18	96.88	96.27	82.78
PT-HL 30 min	89.86	96.41	96.21	82.33
T-HL 60 min	80.28	96.78	96.52	83.49
PT-HL 60 min	74.18	96.82	96.36	83.02
T-HL 120 min	63.29	96.34	96.10	83.28
PT-HL 120 min	71.48	97.11	96.61	83.53

2.4.4 Variance analysis of the early cold and high light response after cold priming

For a first global overview, main variance components within the cold primed and naïve cold- and high light response were defined by a principal component analysis (Fig. 23). Only the 10,000 most abundant transcripts (average FPKM value) were used for the analysis, to avoid an overrepresentation of low expressed genes in the variance.

Within the cold stressed samples (Fig. 23, left side), the two main components explained more than half of the variance (58 %) of the genome wide transcription. The main driving factor in transcription was the duration of the cold stress, which cause 34 % of the variance. The second component (22 % of the variance) clearly separated the naïve samples from the cold primed samples, indicating a strong impact of cold priming on the early cold response. The PCA analysis showed further, that the variance between naïve and cold primed plants depends strongly on the duration of the cold stress. For instance, there was nearly no variance between primed and naïve plants before the triggering started, as shown in the previous transcriptome dataset (Fig. 23 vs. Fig. 12). In contrast, there was a high variance between primed and naïve samples after 60 min of cold stress, pointing to strong impact of cold priming after 60 min of cold exposure.

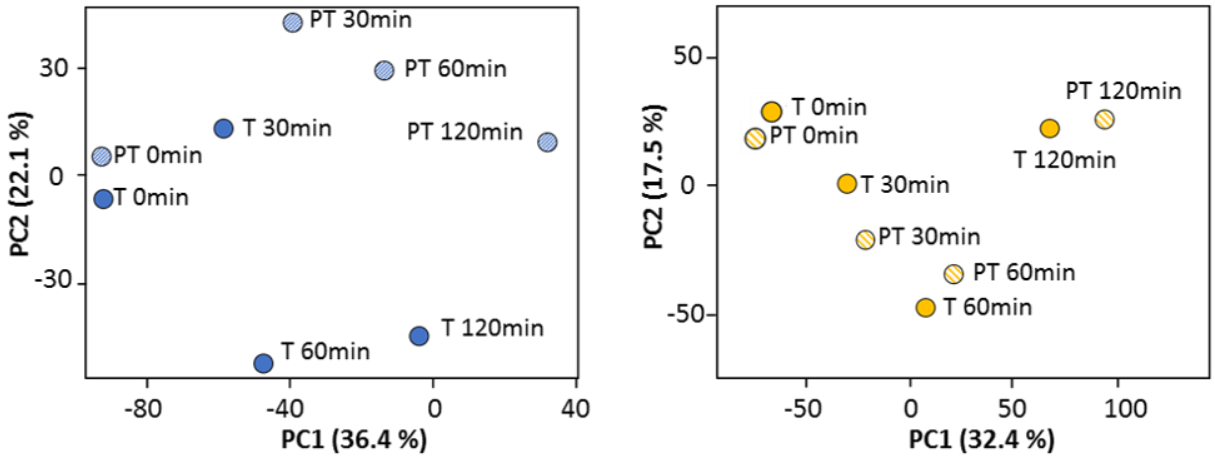

Figure 23: Cold priming effect on the transcriptome wide variation during early stress response: Variance components of the transcriptome of cold primed (striped circle) and naïve plants (filled circle) *upon early cold stress (left site, blue) and early high light stress (right site, yellow) were defined by a principal component analysis (PCA). The PCA was based on single value decomposition (see chapter 4.3.2) and the two main variance components are depicted as a score-plot.*

The two main variance components of the high light triggered samples (Fig. 23, right side) explained 50 % of the variation, which is less than the two main variance components of the cold triggered samples. Neither the duration of the high light stress nor a priming dependent pattern in the high light response could be shown by the two main variance components. One reason for the lower explanation of the variance of high light treated samples by the two main variance components compared to the cold treated samples could be a higher variation within high light stressed plants. To proof this assumption, the overall average standard derivation of transcription in the cold treated samples were calculated and compared to the average standard derivation of the high light triggered samples. The comparison revealed a higher mean standard derivation in high light treated samples (± 19 FPKM) than in cold treated ones (± 11 FPKM), indicating stronger fluctuations in the transcription upon high light exposure.

2.4.5 Comparison of *cis*- and *trans*-primed transcription during early stress

For cold priming dependent transcript analysis, only genes with a transcript value of at least 5 FPKM in at least one sample were selected, to avoid overestimated gene regulation due to low expression values. Genes with at least two-fold priming regulation in at least one timepoint (T 30 min vs PT 30 min, T 60 min vs PT 60 min and T 120 min vs PT 120 min) were assigned as priming targets, which led to a selection of 560 priming responsive genes. Half of these genes were identified as only down-regulated targets and 34 % of the genes were identified as only cold priming upregulated. The remaining 16 % showed priming up- and down-regulation in dependency of the duration and of the type of triggering. One example is the inverse regulation between the onset of triggering (e.g. 30 min) and late triggering (e.g. 120 min) in cold primed plants.

In total were 275 genes identified as priming upregulated within the stress response. More than 50 % of the priming targets were already regulated after 30 min of cold and high light triggering, which indicates a strong impact of cold priming on the onset of the early high light and cold response.

Only 19 genes were identified as commonly priming up-regulated upon high light and cold triggering (Fig. 24, left side). Most of them, like *PCC1* and *PR2*, were annotated as biotic stress responsive genes and showed only common priming regulation after 30 min of triggering. Cold priming increased the transcript abundance of these genes after longer high light stress. During *cis*-priming, most of the biotic related genes were sustained up-regulated in cold primed plants, regardless of the duration of the cold triggering. The result is a common regulation of pathogen response related genes during the onset of both responses (30 min), which turns to a stress type specific priming regulation in later timepoints, as shown before (Chapter 2.3).

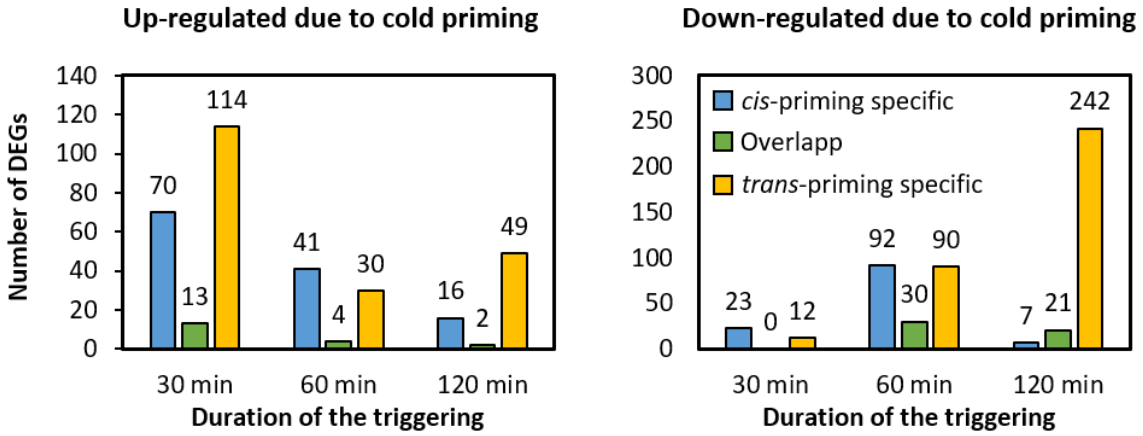

Figure 24: Number of cold priming regulated genes upon the early stress phase: *Differential expressed genes (DEGs) were identified according to the log_2-fold change between naïve and cold primed plants (log_2 (PT/T ≥ \|1\|). The number of only cis-priming regulated genes (blue), only trans-priming regulated genes (yellow) and common priming targets (green) were depicted as a bar-plot for each timepoint.*

Further, 369 genes were identified as cold priming downregulated in at least one timepoint of the early high light and/or early cold response. More than 50 % of these genes were downregulated after 120 min of high light triggering. In case of cold triggering, cold priming led to the strongest deregulation after 60 min of triggering. Consequently, the peak of priming caused downregulation was later than the peak of priming caused upregulation (shift between 30 min and 60 min of triggering), which would explain the increase of variance between primed and non-primed plants at this timepoint in the previous principal component analysis (Fig. 23).

Despite the cold and high light specific effects in the group of cold priming up-regulated genes, there was a strong overlap between down-regulated genes after 60 min (25 % of *cis*-priming regulated genes) and 120 min of cold and high light triggering (95 % of *cis*-priming regulated genes). The functions of these common cold priming downregulated genes were assessed by the annotation according to gene ontology terms (Fig. 25) and showed a strong over-representation of genes, which are involved in JA metabolism and in the response to JA response were strongly overrepresented. The preference of JA related genes within the common priming downregulated genes indicates a strong impact of cold priming on the JA pathway upon early cold and high light triggering.

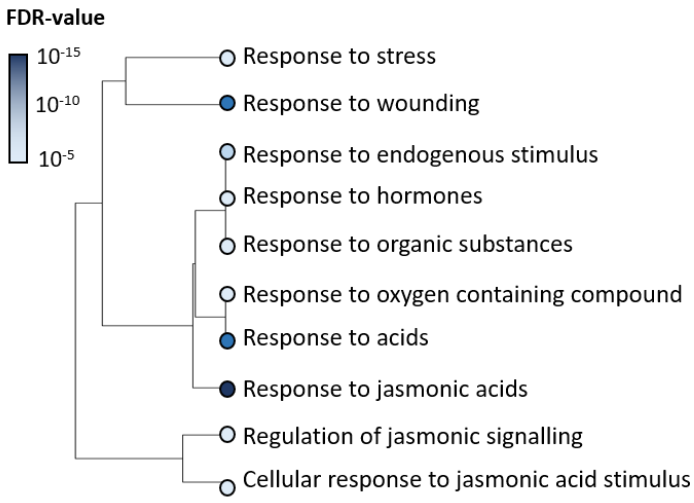

Figure 25: Functional annotation of common cold priming downregulated genes: *The 51 Genes that were identified as common cold priming downregulated were functionally annotated based on gene ontology. Significant enriched GO-terms were selected according to hypergeometric distribution (FDR value < 0.05) and hierarchical clustered with the help of ShinyGO (Ge et al. 2019).*

2.4.6 Cold priming responsive the gene regulation upon early high light

To further investigate the impact of cold priming on the dynamics of transcription, Pearson correlation and Euclidian distance calculation were used to cluster genes according to their cold primed regulation pattern (log_2-fold change PT/T). The correlation analysis was first focused on the 607 cold priming deregulated genes during early high light exposure and resulted into five regulatory gene cluster (Cluster I-V, Fig. 26).

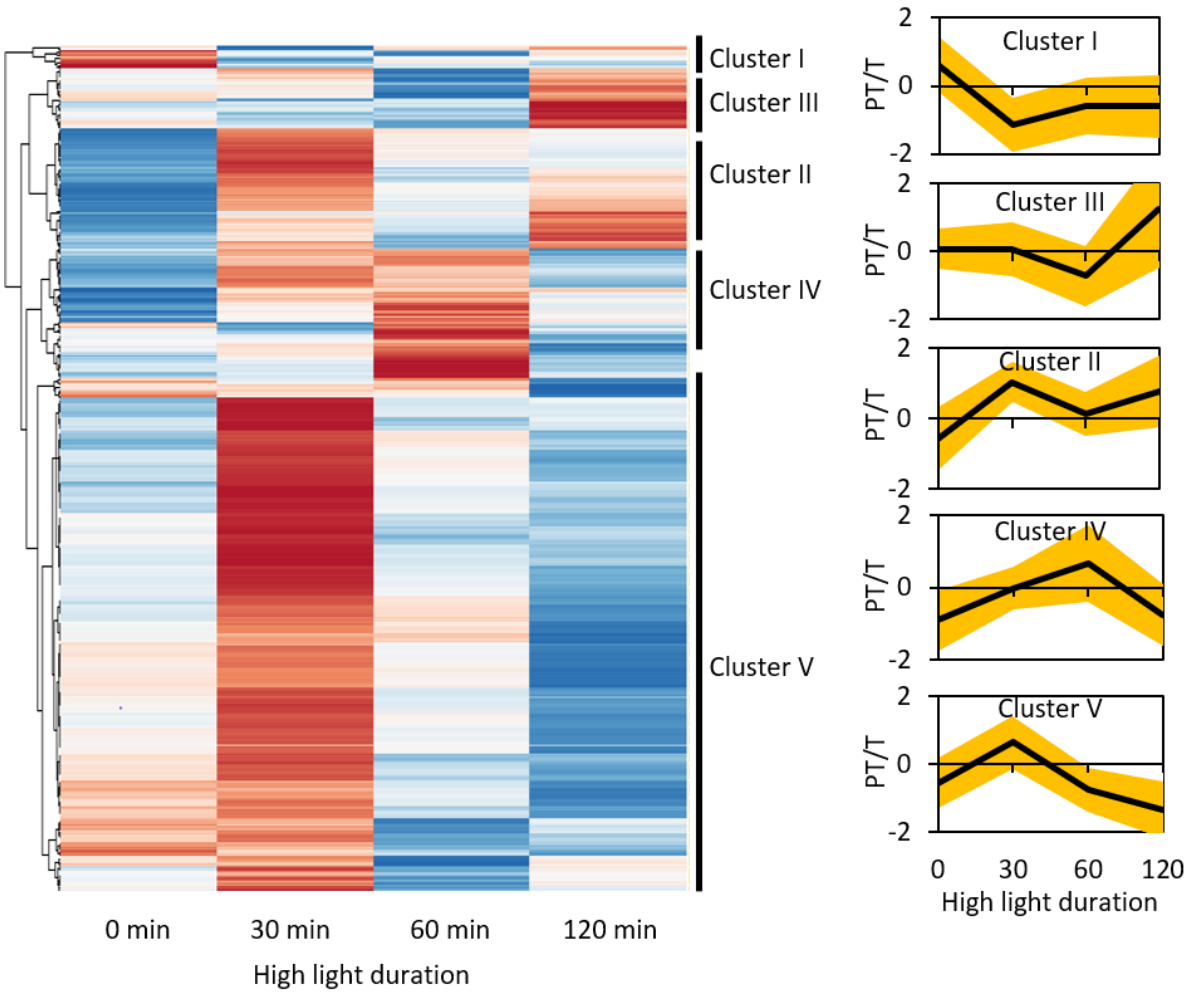

Figure 26: Hierarchical clustering of trans-priming regulated genes: *The transcript abundances of the 607 genes that were identified as cold priming regulated genes within two hours high light stress were normalized by unit variance scaling and clustered according to their priming regulation using Euclidean distance metrics.* ***Left side:*** *The normalized fold change between primed and naïve plants was depicted by a red-blue color scale for each timepoint and gene.* ***Right side:*** *The average priming log_2-fold-change of each identified gene cluster was plotted as solid black line and the yellow area depicting standard derivation of the priming fold-change within the cluster (right site).*

Cluster I identified twelve genes with a reduced high light response in cold primed plants compared to non-primed plants. Four of the twelve genes are assigned as long non-coding RNAs according to annotation of TAIR10. Further encodes on gene the isopentenyl transferase 3 (*IPT3*, AT3G63110), which is involved in the first step of cytokinin synthesis (Hirose et al. 2007).

Cluster II showed a high variance of the priming log_2-fold change but were in average priming upregulated after 120 min of high light triggering. The 36 genes of the Cluster II encode a diverse group of proteins, that includes one ROS scavenger (APX2, AT3G09640), the 3-ketoacyl-CoA synthase (AT2G28630) and a glucosinolate S-oxygenase (AT1G62570).

Cluster III displayed a group of 73 genes with priming upregulated transcript profiles after 30 and 120 min of heat-filtered high light exposure. Functional annotation identified that 43 % of the genes of Cluster III are responsive to heat-, high light- and oxidative stress (Fig. 27). Within this gene group is the shock factor *HSFA2* (AT2G26510), which is an important factor in the heat and high light response of Arabidopsis (Jung et al. 2013). The transcript levels of *HSFA2* were induced by the high light stress which is stronger pronounced in cold primed plants according to the RNA-seq data. A direct comparison of *HSFA2* controlled gene regulation (Nishizawa et al. 2006) with Cluster III, reveals that on fifth of the 73 cold priming upregulated genes are potential controlled by *HSFA2*. Lämke et al. (2016) showed a hyper-induced transcription of *HSFA2* upon reoccurring heat stress similar to the cold priming induced high light response in my study, which indicates a certain overlap between heat memory and cold primed high light response.

Cluster IV represents genes with an inverse priming regulation pattern compared to the second cluster. 10 % of 79 genes are involved in the flavonoid and anthocyanin biosynthesis, indicating a positive cold priming impact on the phenylpropanoid pathway after 60 min high light triggering

Cluster V compromised with 315 genes, the highest number of *trans*-priming targets and showed in average a reduced high light response in cold primed plants. One quarter of the genes of Cluster V are involved in the biotic stress response according to gene ontology annotation (Fig. 27). Within these genes are also *PR2*, *PR1* and *PCC1*, which were already proven as cold priming downregulated genes upon high light triggering (Fig. 20). Beside the overrepresentation of biotic stress responsive genes, compromised Cluster V preferentially JA responsive genes. 25 % of the annotated genes of the JA metabolism were present in Cluster V (Fig. 27), suggesting a strong negative impact of cold priming on JA related processes during early high light stress as mentioned earlier in figure 25.

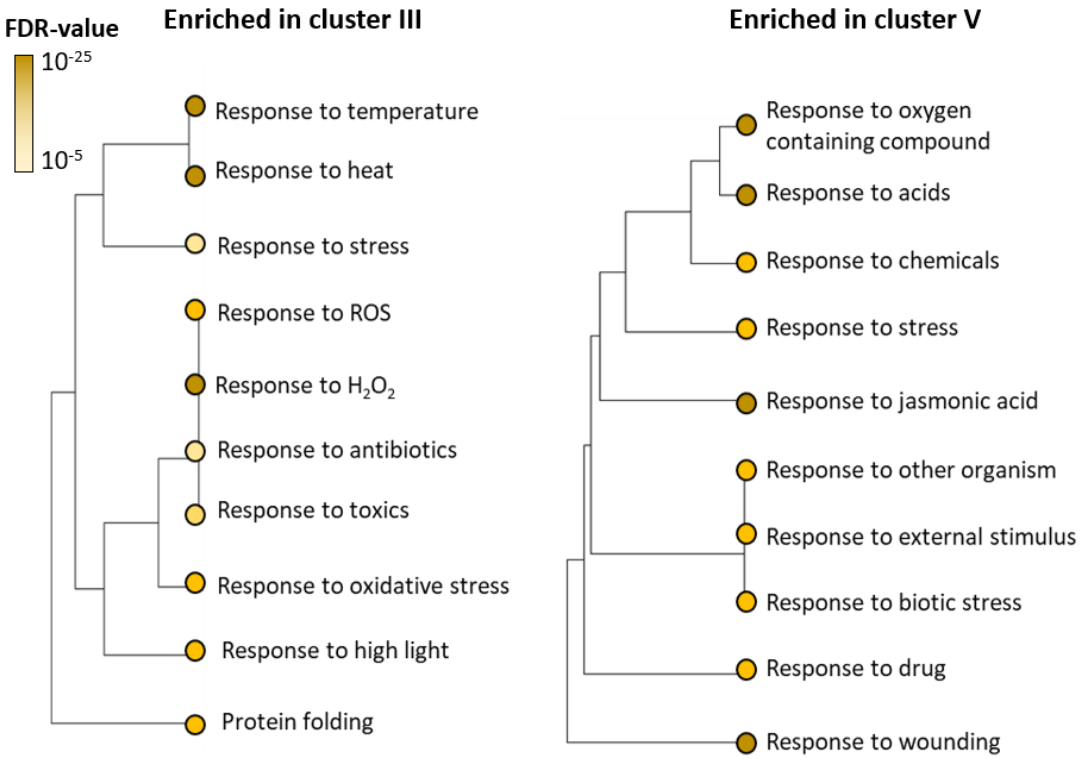

Figure 27: Functional annotation of trans-priming regulated gene cluster: *Genes of the trans-priming regulated Cluster III and Cluster V were functionally annotated based on hypergeometric distribution and gene ontology terms of TAIR10. The ten biological processes with the lowest FDR - value were hierarchically clustered according to correlation between the processes.*

2.4.7 Validation of the cold priming responsive gene regulation upon early high light

The principal component analysis of the transcriptional variation within the high light treated samples, indicating a strong variance in the high light response (Fig. 23). Furthermore, was the high light induction of *ZAT10*, *ZAT12* and *ZAT6* analyzed by qPCR highly variable (Fig. 22). To examine, if the cold priming regulation of the high light response is despite the high variance of high light regulated transcription confirmable, RNA-seq selected gene expression was analyzed by qPCR in six independent replicates (Fig. 28).

The Cluster I contained only one gene with known function, namely *IPT3* (AT3G63110). RNA-seq observed reduction of IPT3 transcript abundance in cold primed plants could be not confirmed by qPCR in five independent replicates. Cluster II and III compromised genes with priming increased transcript levels upon 30- and 120 min of heat filtered high light exposure. The transcript abundance of the selected genes 3-ketoacyl-CoA synthase 12 (*KCS12*, AT2G28630) and *HSFA1* showed in average the same regulation as observed by RNA-seq. But the cold priming regulated high light response of both genes was high variable within the five independent replicates. Cluster IV and V compromised downregulated *trans*-priming targets and showed a strong enrichment of JA metabolism involved genes. The leuco-anthocyanidin dioxygenase (LDOX, AT4G22880) and the lipid oxygenase (LOX3, AT1G17420), which were exemplarily selected to represent Cluster IV and Cluster V, showed both a significant decreased high light response in cold primed plants compared to non-primed ones, similar to RNA-seq. Taken together, genes identified as *trans*-priming upregulated showed a strong variability within independent replicates but by trend, most RNA-seq observed priming regulation patterns were also observed by qPCR. Only the first Cluster could not be confirmed by quantitative PCR analysis.

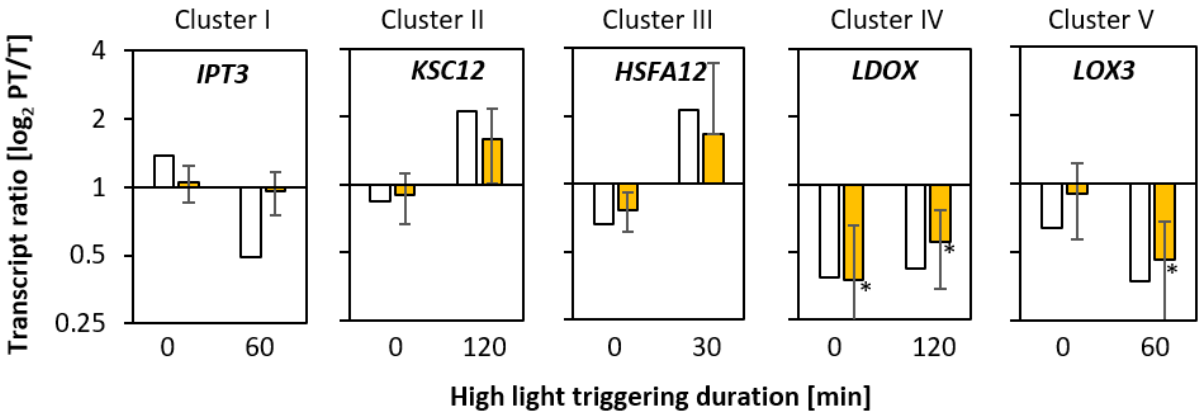

Figure 28: Validation of cold-priming regulated genes upon early high light stress*: The transcript levels of five trans-priming regulated genes from the RNA-seq (white bars) was analyzed by qPCR in five independent replicates (mean ± SD, yellow bars).The transcript levels were normalized to the transcript abundance of the reference gene YLS8 and was divided by the respective non-primed transcript abundance (PT/T). The priming regulation was depicted as log_2-fold and significant differences according to an on-sided t-test (p-value < 0.05) is depicted by stars.*

2.4.8 Cold priming responsive gene regulation upon early cold stress

After analyzing the impact of cold priming on high light response, priming regulation pattern in the transcriptional response to early cold stress were defined, based on correlation analysis. 318 genes were at least two-fold cold priming regulated in at least one timepoint of the early cold response and are clustered four distinct *cis*-priming regulation pattern (Fig. 29).

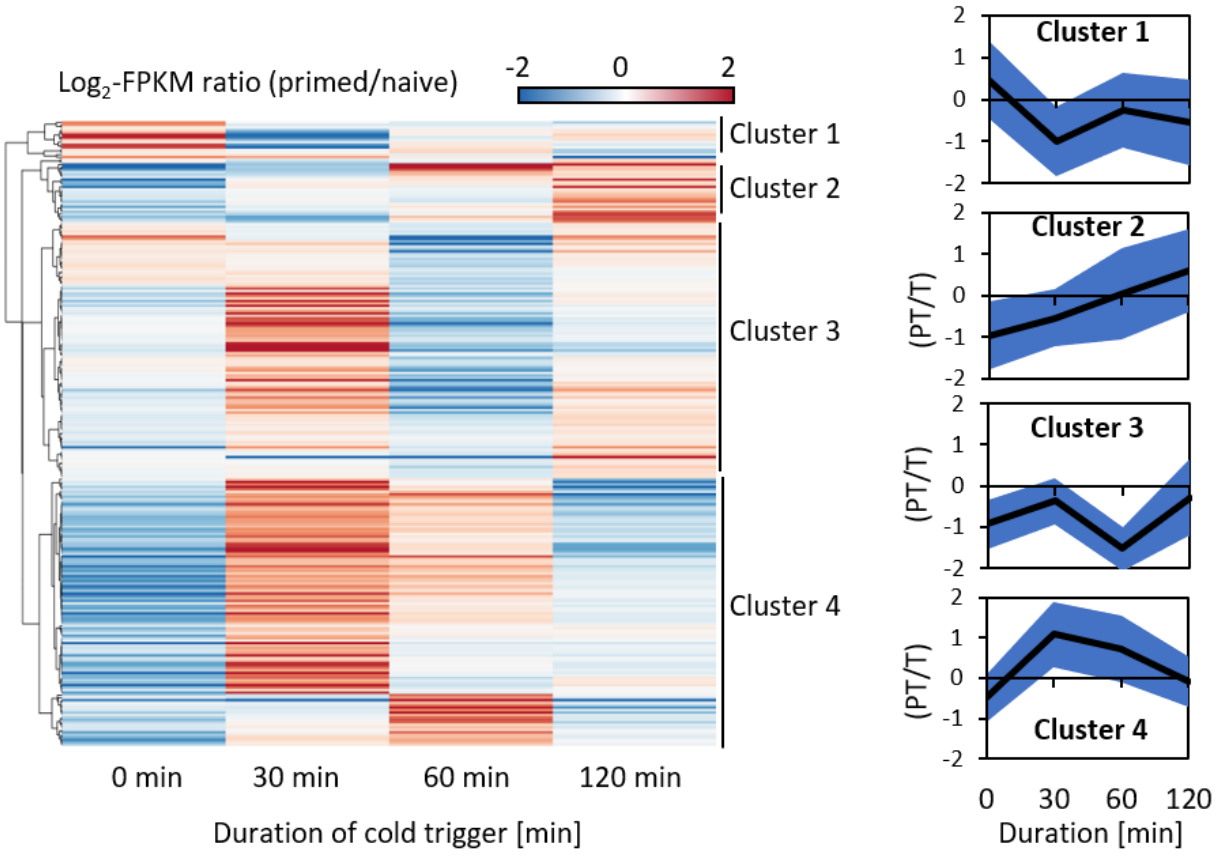

Figure 29: Hierarchical clustering of cis-priming regulated genes during early cold stress: *The 318 cis-priming regulated genes (log_2-fold change > |1|) were normalized by unit variance scaled and clustered according to priming regulation pattern using Euclidean distance metrics.* ***Left side:*** *The normalized fold change between primed and naïve plants was depicted by a red-blue color scale for each timepoint and gene.* ***Right side:*** *The mean priming log_2-fold change of each selected genes cluster was plotted as solid black line and the blue are represents the standard derivation of priming fold-change within the cluster.*

Cluster 1 represents 16 genes and showed in average a decreased transcript abundance in cold primed plants upon 30 min of cold stress. Cluster 1 compromised a diverse group of proteins, including two plastid encoded ribosome subunits (ATCG00920, ATCG01210), one transcription factor (*ddf1*, AT1G12610) and one cytochrome (AT2G27690), which is involved in the catabolic turnover of JA-isoleucine (Heitz et al. 2012).

Cluster 2 showed a gradual increase in the transcript abundances during cold triggering in cold primed plants. Six out of the 26 genes in in the cluster are annotated as wounding responsive genes (GO:0009611) and two genes encode transcription factors (RAP2.6, AT1G21910 and ERF2, AT5G47220).

Cluster 3 defined 106 genes with a common priming pattern and showed a reduced cold response in cold primed plants, especially after 60 min of triggering. One fifth of the 106 genes are annotated as JA responsive genes (GO:0009753) and 10 % of the genes are directly involved in JA biosynthesis (GO:0009694), which points to a downregulation of JA related processes upon early cold stress in cold primed plants, similar to the cold priming impact on the high light response of JA related processes (Fig. 27). Based on this results and the annotation of common *cis*- and *trans*-priming regulated genes (Fig. 25), it can be considered, that cold priming has a common impact on JA related gene expression independent of the type of triggering.

Cluster 4 compromised 105 genes. In average, compromised this cluster genes with a higher transcript abundance in cold primed plants upon cold triggering compared to only cold triggered ones. Based on gene ontology annotation, most of the genes are important in pathogen defense, acquired resistance and biotic stress response. Transcriptome analysis of the cold priming impact on the response to 24 hours cold triggering pointed already to an upregulation of pathogen related genes in the *cis*-priming experimental set-up (Fig. 18). The gene annotation of Cluster 4 is in line with this observation, indicating cold priming supported transcription of biotic stress responsive genes within the first min of cold stress exposure.

2.5 JA signalling and JA biosynthesis in the context of *cis*-priming

Genome wide transcript analysis upon the first hours of cold and high light exposure revealed that cold priming impacts the onset of both stress responses. Most of the priming responsive genes were specifically regulated either by cold or by high light application (Fig. 24). One striking exception was the negative priming regulation of JA related genes in both priming set-ups (Fig. 25). Therefore, further analyses were focused on the priming impact on the JA related stress responses. Variance analysis indicated a higher variability in the cold priming regulation of the high light response. Also, the impact of cold priming on JA related genes was stronger pronounced upon cold triggering (Fig. 27-29). Therefore, further investigations were focused on the impact of cold priming on the cold response and not on the high light response of JA related gene expression.

2.5.1 JA responsive gene regulation upon *cis*-priming

To examine the impact of cold priming on the early cold response of JA related processes, transcript abundance of four JA responsive genes were analyzed under *cis*-priming conditions (Fig. 30). The transcript abundance of *ZAT10* was used as positive control for the cold priming impact on the cold response. The analysis was focused on 60 minutes of cold stress because RNA-seq indicates the strongest priming regulation of JA related transcription at this timepoint.

The transcript levels of all four JA responsive genes were strongly induced in naïve plants after 60-min cold stress, as reported in an analysis of Arabidopsis seedlings upon chilling stress (Hu et al. 2013). In cold primed plants was the cold induction of *ZAT10* strongly reduced as report in previous *cis*-priming analysis (Fig. 9 and Fig. 21). The cold response of the basic helix-loop-helix transcription factor MYC2 (AT1G32640), which controls two-thirds of all JA responsive genes (Zander et al. 2020), was inhibited in plants, which received the first cold stress five days earlier (Fig. 30). Beside the cold response of MYC2, also the cold induction of the JA responsive gene 2 (*JR2*, AT4G23600) was significantly lower in cold primed plants. The transcript abundances of the other two JA target genes, namely vegetative storage protein 2 (*VSP2*,

AT5G24770) and JA responsive gene 1 (*JR1*, AT3G16470), were by average strongly decreased in cold primed plants compared to only cold triggered plants. The cold induced transcript abundance of *VSP2* was in all four replicates at least three-fold higher in non-primed plants than in cold primed plants and the cold induced transcript abundance of JR1 was in three out of four replicates reduced in cold primed plants. The transcript analyses, especially of the JA master regulator MYC2 (Fig. 30), indicate a strong reduction of the cold induced transcript abundance of JA responsive gene in cold primed plants.

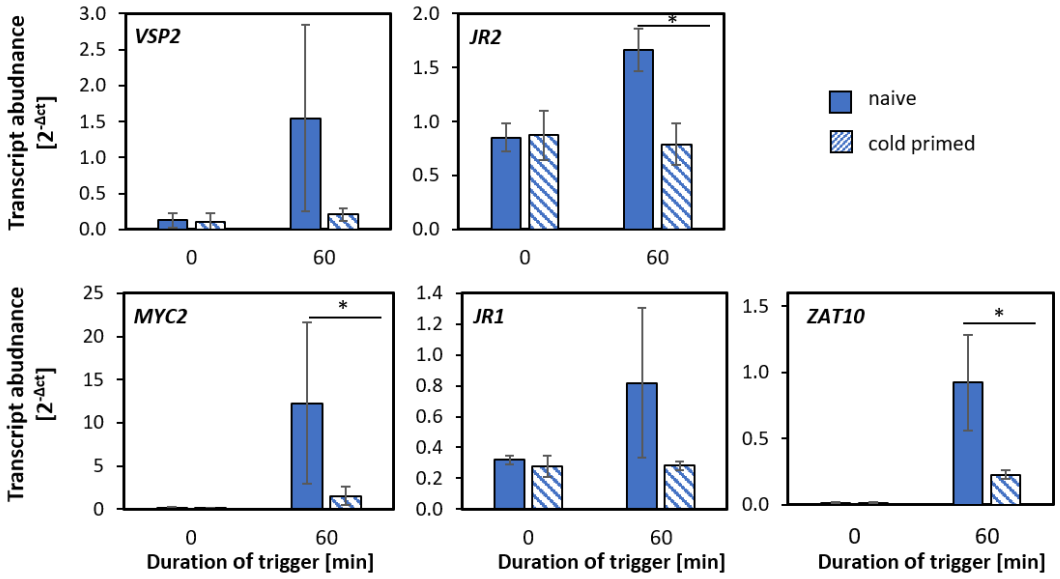

Figure 30: The cold priming effect on JA responsive gene expression: *The transcript abundance of four JA responsive genes and of ZAT10 was analyzed in naïve plants (solid bars) and cold primed (striped bars) before (0 min) and after 60 min of cold triggering by qPCR in four independent replicates (mean ± SD). All values were normalized to the transcript levels of the reference gene YLS8 and stars represent significant differences according to Fischer's t-test (p-value < 0.05).*

The transcription factor *MYC2* controls not only JA responsive genes, but also the JA metabolism itself via an positive autoregulatory loop (Zander et al. 2020). Transcript analyses demonstrate a strong reduction of the cold response of *MYC2* in cold primed plants (Fig. 30). This led to the suggestion, that cold priming also impacts on cold responsive transcription of genes involved in JA metabolism, as indicated by the RNA-seq (Fig. 29).

To proof this suggestion and to confirm priming regulation observed by the RNA-seq, the transcript abundance of four JA metabolism involved genes were assessed in four independent replicates (Fig. 31). The allene oxide cyclase 2 (*AOC2*, AT3G25770), the allene oxide synthase (AOS, AT5G42650), the lipid oxygenase 2 (*LOX2*, AT3G45140) and lipid oxygenase 3 (*LOX3*, AT1G17420) were chosen for transcript analysis, due to the high basal transcript abundance and the strong priming regulation in the RNA-seq experiment (Fig. 29). The transcript levels of all four genes were slightly reduced five days after cold priming without any triggering treatment. The application of cold stress for 60 min strongly increased the transcription levels of all four JA biosynthesis involved genes in non-primed plants. This cold induced gene regulation of *AOC2*, *AOS*, *LOX2* and *LOX3* was two-fold lower in cold primed plants (Fig. 31), as supposed from the transcriptome data (Fig. 29, Cluster 3)

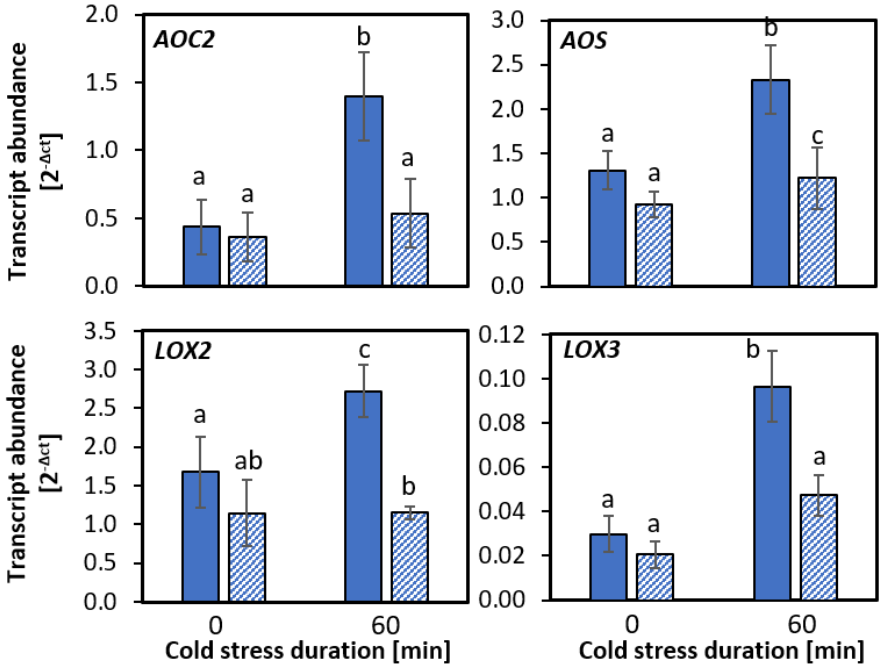

Figure 31: The cold priming effect on transcript abundance of JA biosynthesis involved genes: *Transcript levels of four genes, which are involved in JA biosynthesis, were analyzed in cold primed (striped bars) and naïve (solid bars) plants before (0 min) and after 60 min cold triggering by qPCR in four independent grown replicates (mean ± SD). All values were normalized against the transcript abundances of the reference gene YLS8 and letters depicting significant differences according to Tukey's post hoc test, p-value < 0.05).*

2.5.2 Lipid peroxidation upon *cis*-priming

The abundance of AOS is a critical factor in JA metabolism, because the function of AOS cannot be substituted by others (Stenzel et al. 2003). Transcript analysis showed that cold priming suppresses the accumulation of AOS in response to cold stress (Fig. 31), which typically activates the biosynthesis of JA upon cold stress (Hu et al. 2013). This rise the question if cold priming affects the cold responsive metabolism of JA. The first step in JA metabolism relays on the peroxidation of tri-unsaturated lipids (Wasternack and Hause 2013). Primary lipid peroxide products are often highly reactive and thereby unstable. Therefore, stable secondary byproducts are often used to estimate lipid peroxidation (Morales and Munné-Bosch 2019). For a first impression, if cold priming effects overall lipid peroxidation and, thereby, JA metabolism, the content of secondary lipid peroxidation byproducts was estimated by the reaction electrophilic compounds with thio-barbituric acid (TBA, Fig. 32). The content of TBA reactive substances (TBRAS) was elevated but not significantly (p-value < 0.05) increased upon 60 minutes of cold stress. Interestingly, cold priming reduced this elevation of the TBRAS content upon early cold exposure (Fig. 32).

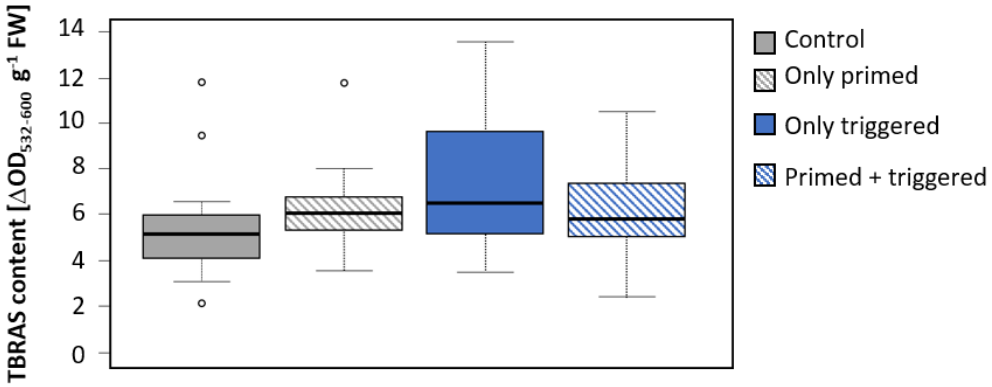

Figure 32: The effect of cold priming on the TBRAS content upon cold stress: *The TBRAS content was assessed photometrical at an absorbance of 532 nm in extracts of middle aged leaves of naïve (solid boxes) and cold primed plants (striped boxes). The plant material was harvested before (grey) and after 60 min of cold triggering (blue) in 15 replicates (n = 3 * 5). The OD_{532} was normalized against the background absorbance at 600 nm and divided by the fresh weight.*

The analysis of TBA reactive substances indicated a slightly negative impact on lipid peroxidation during early cold stress (Fig. 32), but the method is rather unspecific for distinct lipid peroxidation byproducts. The metabolism of JA relays specifically on the peroxidation of linolenic acid catalyzed by C13- lipoxygenases (in Arabidopsis: *LOX2*, *LOX3*, *LOX4* and *LOX6*). To get more specific insights in JA related lipid peroxidation, the *in vitro* activity of C13-LOX was assessed in extracts of cold primed and naïve plants (Fig. 33). The analysis was conducted according to the protocol of Axelrod et al. (1981) and was focused on the LOX activity after 120 min cold triggering to take the time into account, which is required to proceed the cold priming regulated transcription into enzyme activity.

The LOX activity was by trend elevated after 120 min of cold stress. Cold priming significantly reduced the LOX activity upon cold triggering in comparison to only cold triggered plants. The effect of cold priming on LOX activity (Fig. 33) mirrored the priming regulation on the cold triggered induced transcription of JA related genes (Fig. 31). The results indicate, that cold priming suppress the early steps of JA metabolism upon the first hour of cold response of Arabidopsis.

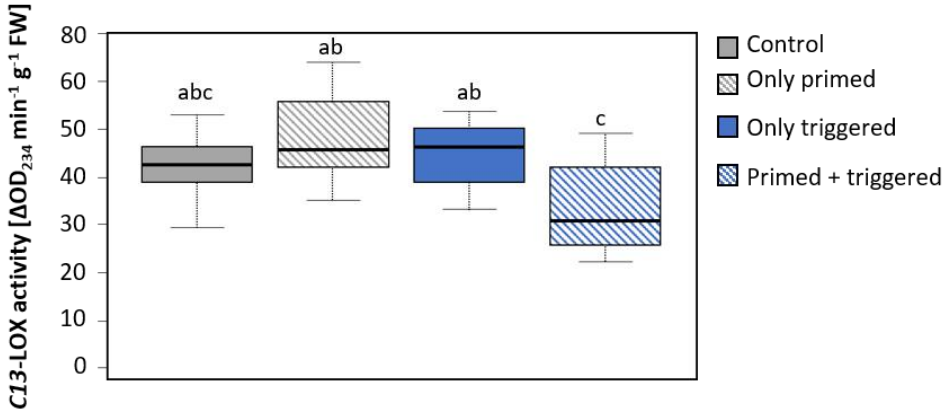

Figure 33: The effect of cold priming on C13-Lipoxygenase activity: *Proteins of cold primed (striped boxes) and naïve plants (solid boxes) were isolated before (grey) and after 120 min cold stress. The isolates were used for measuring in vitro the LOX activity measured at an absorbance of 234 nm after 10 min of incubation with linolenic acid as substrate. The OD_{234} was normalized against the incubation time and the fresh weight used for protein isolation. C13-LOX activity was measured in three independent replicates with five individual plants per sample and significant differences according to tukey's post-hoc test (p-value < 0.05) are depicted as letters-*

2.5.3 Hormone content analysis upon *cis*-priming

Based on the reduction of the LOX activity in cold primed plants upon cold triggering (Fig. 33) it can be suggested that cold priming dampens the activation of JA accumulation during early cold stress. To investigate this suggestion, the leave content of JA-, of JA-isoleucine (JA-ile) and of the JA precursor cis-(+)-12-oxo-phytodienoic acid (OPDA) were analyzed every 30 min within two hours exposure at 4°C (Fig. 34). Extracts from middle aged leaves were used for the analysis, because they show the strongest cold priming regulation of *ZAT10* cold response in previous analysis (van Buer et al. 2019). The HPLC-tandem mass spectrometry analysis of JA, JA-Ile and OPDA was conducted by Bettina Hause (Martin-Luther Universität, Halle, Germany).

The steady state levels of JA and JA-Ile were close to the detection limit of the method. Exposure to cold triggering had no significant impact on the JA and JA-Ile content, which differs from the observations by Hu et al. (2013), who reported in two-week-old Arabidopsis seedlings an strong increase of the JA content upon exposure to 4 °C. Beside JA, also the JA precursor OPDA was analyzed and was slightly elevated in non-primed plants after 30 and 60 min of cold triggering in comparison to untreated plants (Fig. 34). At the same time, the OPDA content was by trend reduced in cold primed plants compared to naïve plants at all timepoints of the analysis. in combination with the LOX-activity measurement (Fig. 33), the OPDA measurement hints to a negative impact of cold priming on the first steps of JA metabolism, which is not converted to a negative priming impact on the JA steady state level (Fig. 34).

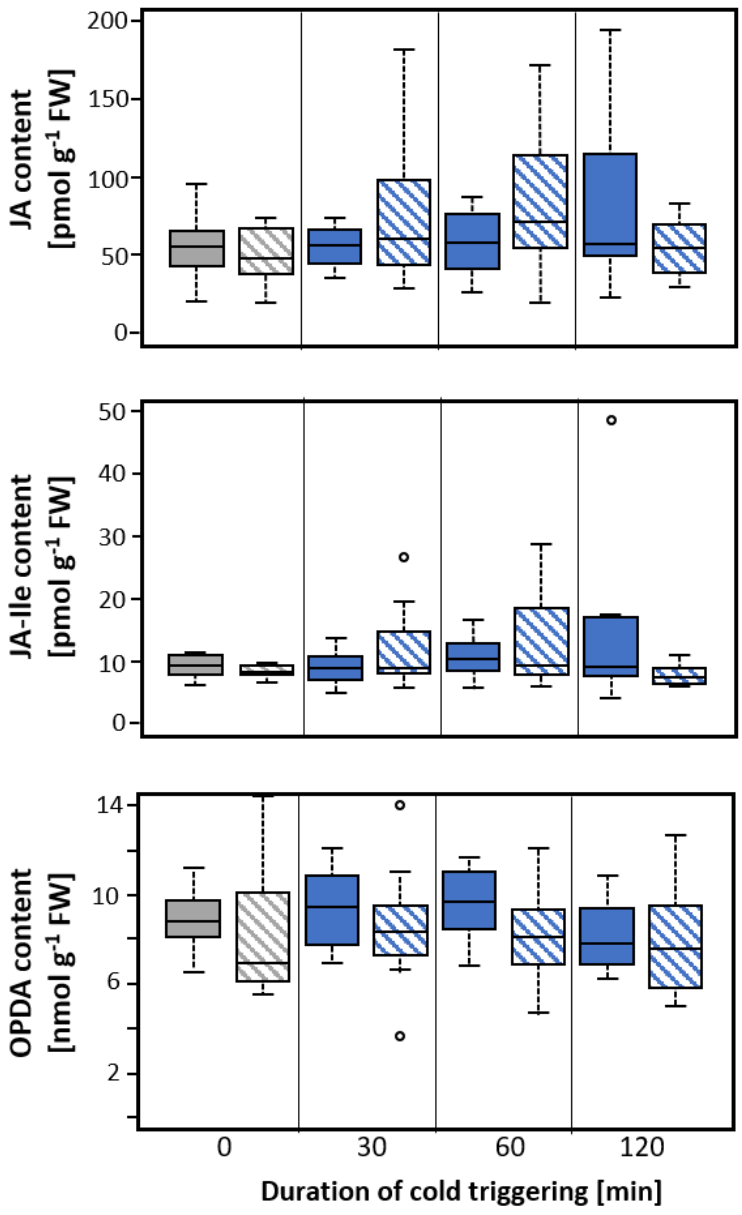

Figure 34: The effect of cold-priming on the oxylipid content during early cold stress: *The contents of JA, JA-Ile and OPDA were analyzed in middle aged leaf material of naïve (solid boxplots) and cold primed plants (striped boxplots) before (grey) and after 30 , 60 and 120 min of cold stress (blue) by HPLC-tandem mass spectrometry. JA and JA-ile levels are given as pmol per gram fresh weight (FW) and OPDA as nmol per gram fresh weight from twelve plants per treatment, which were grown in two independent replicates (n = 2* 6). All hormone contents were normalized against deuterium labelled extraction standards and the fresh weight of the samples.*

2.5.4 Comparison of the hormone contents with the gene regulation upon *cis*-priming

To compare the priming regulated transcript abundance of JA related genes with the priming regulation of the oxylipid content, a Pearson correlation analysis was conducted and visualized as correlation matrix (Fig. 35). Prior to the analysis, the transcript abundance of JA related genes and the hormone data were normalized to the respective untreated control value. The analysis revealed a strong positive correlation between the priming regulated pattern of the OPDA content and the priming regulated transcript abundance of JA responsive genes. The transcript abundance of the JA master regulator *MYC2* (AT1G32640) was even significantly (p-value < 0.05, FDR < 0.01) correlated with the OPDA content. In contrast, the priming regulation of the JA content was slightly negative correlated with the cold priming regulation of several JA responsive genes. Further analysis addressed the ratios between OPDA and JA levels, demonstrating a positive correlation of the OPDA/JA ration with *cis*-priming regulated transcript abundance of JA related genes (Fig. 35). This correlation analysis proposed that the cold priming regulated transcription of JA related genes is rather linked to the OPDA content than to the JA or JA-Ile content.

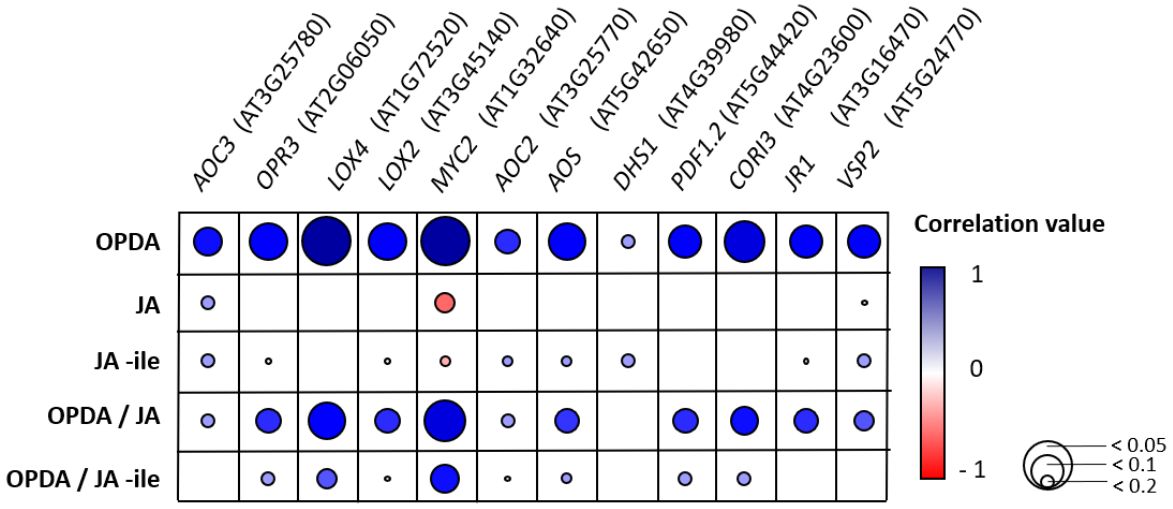

Figure 35: Correlation analysis between the oxylipid content and the transcript abundances of JA responsive genes: *The content of the oxylipids JA, OPDA and JA-ile and the transcript levels of several JA related genes were normalized against the respective control (C0) for each replicate. Correlation between the priming regulated transcript levels and the priming regulation of oxylipids is represented by colors (Pearson correlation) and the size of the circle decrease with value of probability (p-value).*

2.5.5 *Cis*-priming regulated gene expression in JA deficient mutant lines

To dissect the role of JA and OPDA in *cis*-priming regulated transcription, the literature and public t-DNA line collections were screened for mutants which tagging the coding region of the OPDA reductase *OPR3* (AT2GG06050) and of the allene oxide synthase AOS (AT5G42650). *AOS* catalyze the last step in OPDA metabolism and crucial for JA biosynthesis (Wasternack and Hause 2013). OPR3 is essential for the conversion of OPDA to JA. Thereby, mutation in *OPR3* only effects JA biosynthesis but not the metabolism of OPDA (Stintzi and Browse 2000).

For the coding region of *AOS* are at least 42 different mutant lines available. In this study, SALK_017756 was selected for priming analysis, because the t-DNA insertion in SALK_107756 is near to the 3´ end of the *AOS* coding region and the mutant line is routinely used in JA transduction analysis (Matschi et al. 2015; Lee et al. 2018). Homozygosity of the insertion was validated by DNA amplification with insertion specific primer. In addition, the plants were screened for the lack of trichome formation because the development of trichomes depends on JA (not shown).

The function of OPR3 was identified in a JA mutant screen in the Arabidopsis accession *Wassilewskija* called *dde1-1* (Stintzi and Browse 2000). *dde1-1* is frequently used to dissect the function between OPDA and JA (Stenzel et al. 2003), but natural variation has a strong impact on cold priming (Cvetkovic et al. 2017), which makes *dde1-1* due to the *Wassilewskija* background not suitable for priming regulation analysis. According to public seed collections, only one germplasm line with Col-0 background carries a mutation in the coding region of *OPR3*, namely SALK_201355. The T-DNA in SALK_201355 is located in the second intron of *OPR3* (Fig. 36A). DNA amplification with insertion specific bordering primer demonstrated that all plants, which were tested, carried a homozygote insertion of the T-DNA in the *OPR3* coding region (Fig. 36B). To check if the T-DNA insertion in the second intron abolishes *OPR3* expression, transcript abundance was analyzed by qPCR with primer, which binds downstream of the insertion. The qPCR analysis showed that SALK_201355 reach only 2 % of the wildtype *OPR3* transcript fragment abundance (Fig. 36C), which hints to a strong knock down of *OPR3* due to the homozygote insertion in the second intron.

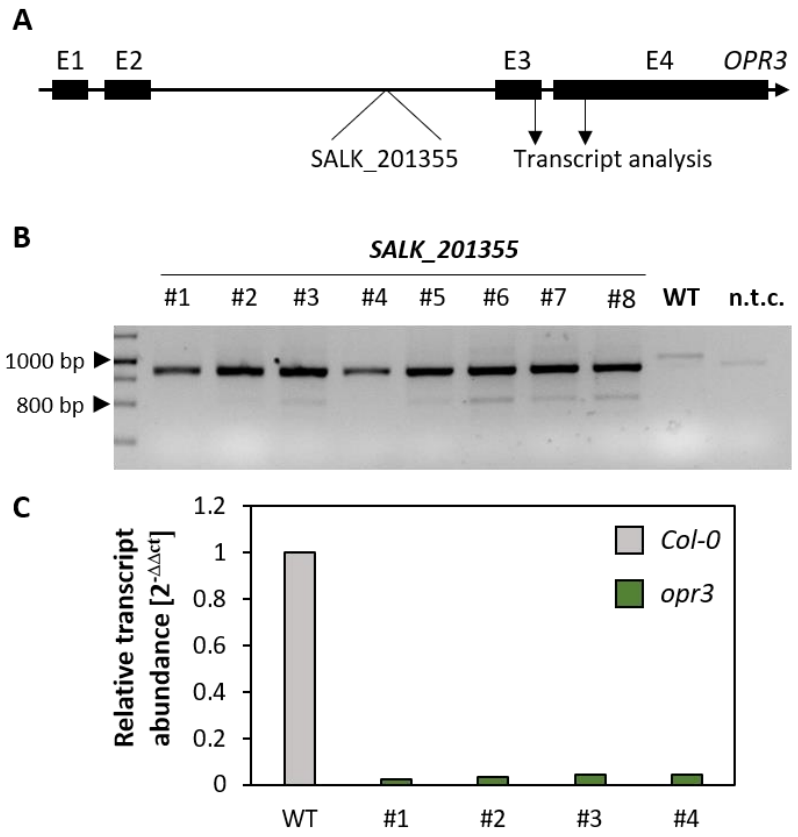

Figure 36: Schematic map and characterization of SALK_201355: A) *The open triangle represents the t-DNA position in SALK_201355 and the black arrows represent the primer position for OPR3 transcript analysis. The four exons (E1-E4) of the OPR3 gene locus were designated as black boxes according to the annotation in TAIR10* ***B)*** *Insertion specific and gene specific sequences were amplified from the genomic DNA of 8 independent SALK_201355 plants and one wild type Col-0 with a triple primer mix (LP+ RP + LBb) and visualized under UV – light. The mutant allele led to expected product with a size of 900 base pairs (bp) and the wild type allele led to an expected product with a size of 1000 bp.* ***C)*** *The transcript abundance of OPR3 was analyzed in Col-0 (grey) and four homozygote SALK_201355 plants (green) by quantitative qPCR analysis. The transcript levels were normalized against the transcript abundance of the reference gene YLS8.*

To examine the role of OPDA and JA in the cold priming reduced cold response, SALK_017756 and SALK_201355 were exposed to the full factorial *cis*-priming set up and the transcript abundance of *ZAT10* was assessed as a read out for successful priming (Fig. 37). The transcript abundance of *ZAT10* was induced in response to cold stress in non-primed wildtype plants, which was blocked in cold primed ones, like shown in previous analysis (Fig. 21). The *ZAT10* transcript levels were also strongly induced after 60 min of cold stress in SALK_201355 (*opr3*) like the cold response in wildtype plants. The cold response of *ZAT10* was reduced in

SALK_201355 after cold priming, which indicates that conversion of OPDA to JA is not important for the cold response and the priming impact on *ZAT10* transcription. The basal transcript abundance of *ZAT10* was slightly higher in SALK_201355 in comparison to untreated Col-0 plants. SALK_017756, which is abolished in OPDA biosynthesis, showed a two-fold lower *ZAT10* induction upon cold triggering, which implement that OPDA metabolism is important for the cold response of the priming marker. The reduced cold response of *ZAT10* in SALK_017756 resembles the cold response of cold primed plants. The results indicate that the cold priming reduced OPDA content could be linked with cold priming regulated cold response of *ZAT10*, but the conversion from OPDA to JA has no impact in the cold response.

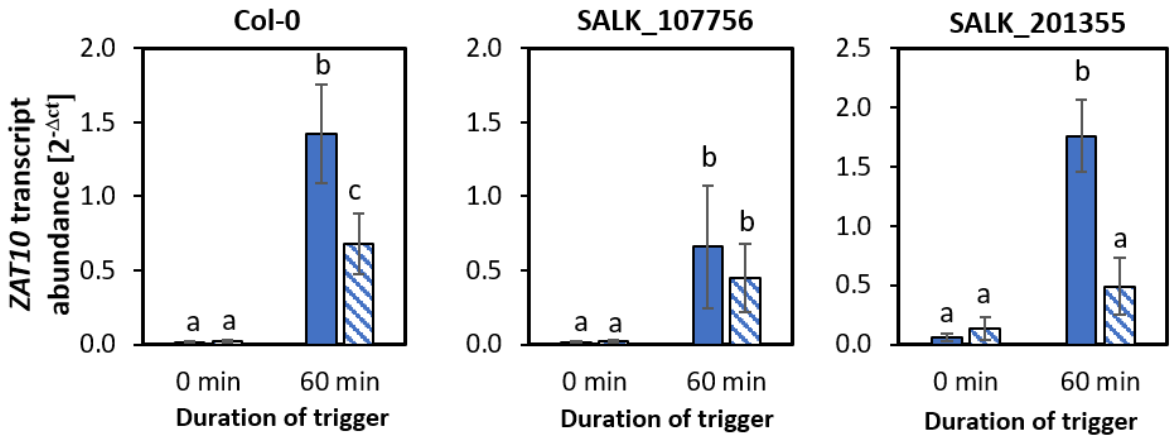

Figure 37: The impact of cold priming on transcript abundances of ZAT10 in JA deficient mutant lines: The transcript abundance of *ZAT10* in wildtype plants (Col-0), *SALK_017756 (aos) and SALK_201355 (opr3)* was analyzed in three independent replicates by qPCR (mean ± SD). The cold primed (striped bars) and naïve plants (solid bars) were harvested before (0 min) and 60 min after cold triggering. Letters depicting significant differences according to Tukey's post hoc test (p-value < 0.05).

2.5.6 *Cis*-priming regulated gene expression after JA application

To investigate the potential impact of cold priming on oxylipid perception, cold primed and naïve plants were treated with oxylipids. The oxylipids were applied three hours before harvesting, to enable the uptake of the phytohormones before applying the triggering. The transcription of *ZAT10* is responsive to OPDA (Taki et al. 2005) and was used as a read out for oxylipid perception (Fig. 38).

A mock treatment (0.01 % Ethanol (v/v)), used as control, had no impact on the increased transcript abundance of *ZAT10* upon cold triggering and did not impact on the priming reduced cold regulation of *ZAT10* in comparison to previous priming analysis (Fig. 21). Treatment with 100 µM JA resulted into an elevated transcript abundance of *ZAT10* already without any triggering. The JA induction of *ZAT10* was 5-fold higher upon cold triggering. Cold priming had no impact on the cold induced JA response of *ZAT10*, demonstrating that cold priming does not affect the JA perception. Taki et al. (2005) reported that besides JA also OPDA can strongly activate the expression of *ZAT10*. Due to delivery problems of OPDA within the last six months of these study, OPDA treatment was not applicable, to proof if cold priming impacts the perception and response upon OPDA application

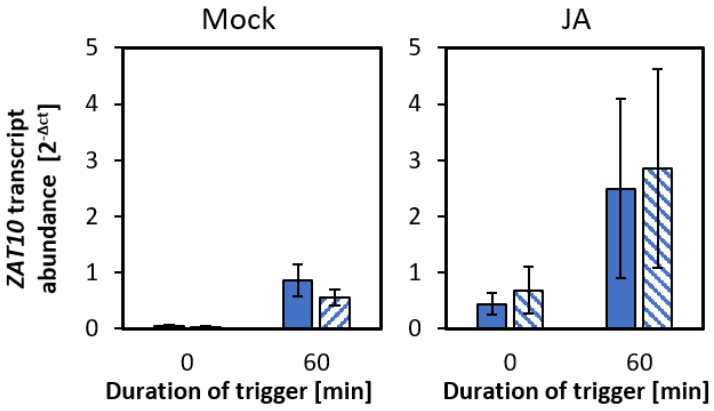

Figure 38: The effect of exogenous JA and OPDA application on the priming regulation of ZAT10: *The transcript abundances of ZAT10 were analyzed by qPCR in four independent replicates (mean ± SD). Cold primed and naïve plants were either treated with mock solution or 100 µM JA. Different letters represent significant differences according to Tukey's post hoc test (p-value < 0.05).*

2.5.7 The *tAPX* dependency of the cold priming regulation of JA related gene expression

Previous analysis showed, that cold priming activates the accumulation of tAPX during the lag-phase and that this regulation is causal for the reduced cold response of *ZAT10* during a second cold exposure (van Buer et al. 2019). To investigate, if the cold priming regulation of genes of the OPDA metabolism depends also on the accumulation of tAPX, transiently *tAPX* over-expression and transiently *tAPX* silencing system Arabidopsis lines (van Buer et al. 2019) to manipulate the tAPX abundance. The transcript abundance of *AOS*, *AOC2* and *ZAT10* were assessed as a read out for the impact of cold priming on the early cold response after manipulating *tAPX* expression (Fig. 39).

As initial analysis, a homozygote *tapx* T-DNA insertion lines (Kangasjärvi et al. 2008) was used. Abolishment of *tAPX* transcription diminished the cold priming impact on the early cold response of *ZAT10 (Fig. 39, left side)*. The cold induced transcript abundance of *ZAT10* in non-primed plants was as the cold response in wild type plants, demonstrating that tAPX is only defining the cold priming effect but not the cold response itself. Neither the cold induced transcript abundance nor the cold priming reduction of the early cold response of *AOS* and *AOC2* was altered in the *tapx* mutant line, which indicating that the cold priming effect on JA related gene transcript levels is independent of the absence tAPX.

Additionally, an estradiol inducible *tAPX* silencing system were used, to investigate the impact of transient *tAPX* changes without the acclamatory effects due to constitutive mutation of tAPX. Plants without any estradiol treatment received a mock solution as a control treatment. Estradiol inducible *tAPX* silencing during the lag-phase led to an abolishment of the cold-priming effect on the early cold response of *ZAT10*, as seen in the constitutive *tapx* mutant line. In contrast, transient *tAPX* silencing had no impact on the priming effect on the cold induced transcript abundance of *AOS* and *AOC2*. It can be concluded, that transient as well as constitutive abolishment of *tAPX* expression impacted only the priming regulation of *ZAT10* but not the regulation of *AOS* and *AOC2*.

Inducible *tAPX* overexpression lines, which received an estradiol treatment instead of cold priming, was used to test if transient tAPX accumulation can mimic the effects of cold priming. The estradiol induced tAPX abundance reduced the cold response of *ZAT10* after 60 min of triggering, demonstrating that transient tAPX accumulation can mimic the impact of cold priming on the early cold response. The estradiol treatment as well as the mock treatment reduced the cold response of *AOS* and *AOC2*. Analysis in the *tAPX* silencing line, which were conducted in parallel, showed no impact of the mock treatment itself, indicating that transient *tAPX* overexpression background is causal for the reduced *AOS* and *AOC2* transcript abundance. van Buer et al. (2019) reported a leakiness of the estradiol inducible *tAPX* overexpression system, which led to a slightly elevated tAPX protein abundance without any treatment. Therefore, it can be assumed that the elevated abundance of tAPX through the leakiness of the system, has a negative impact on the cold response of *AOS* and *AOC2*.

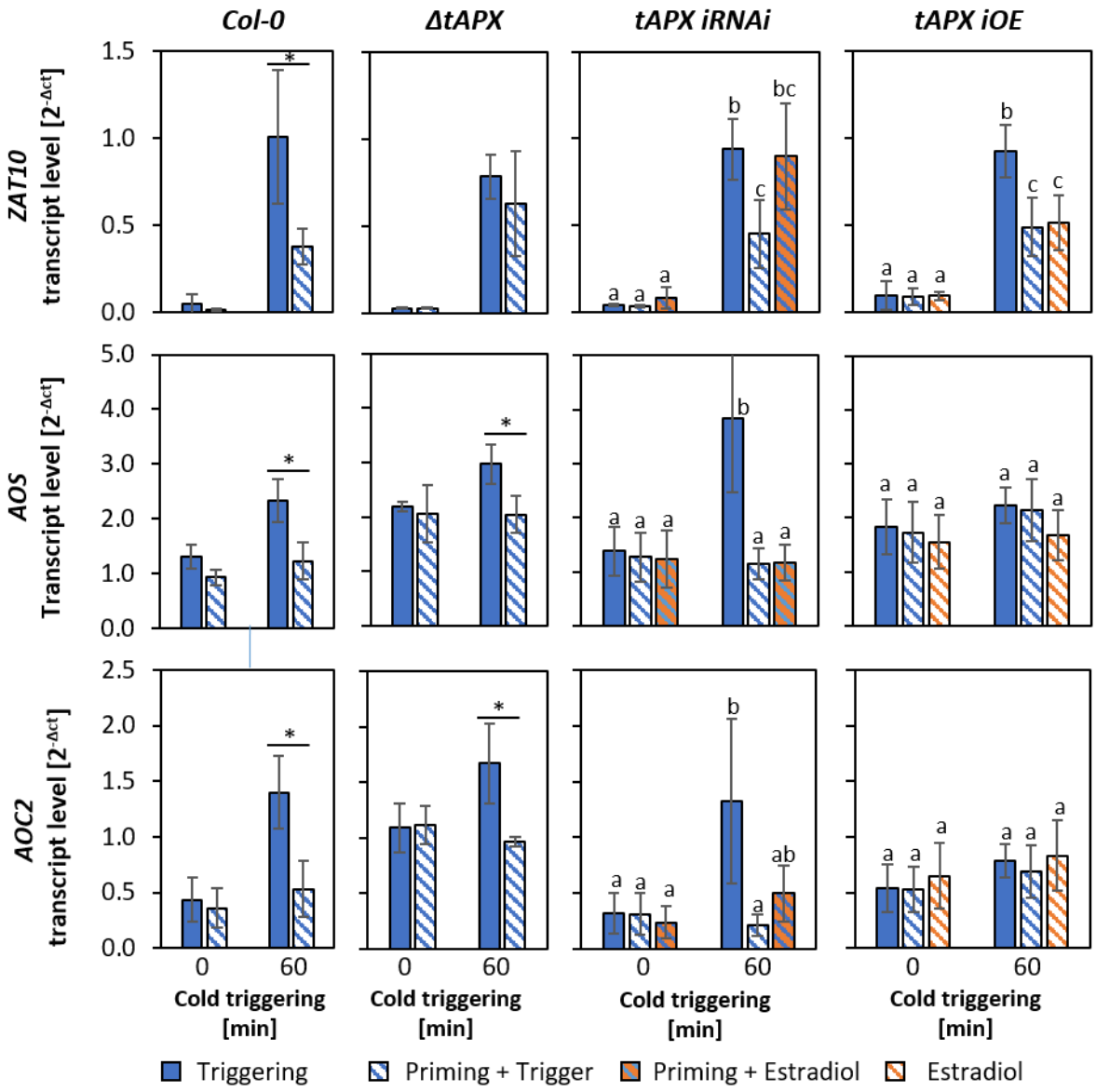

Figure 39: The effect of cold priming in combination with deregulation of *tAPX* on the early cold response: The transcript abundances of *ZAT10*, *AOS* and *AOC2* were assessed in naive (solid bars) and cold primed plants (striped bars) by qPCR in at least four independent replicates (mean ± SD) in different genetic backgrounds. **Left side)** Wildtype plants and tAPX knock out mutants were treated with mock solution and were harvested before and after cold stress (60 min). Asterisk represents significant differences according to Fischer's t-test (p-value < 0.05). **Middle)** Mock treated (blue) and estradiol treated plants (orange + blue), which carried an estradiol inducible *tAPX* silencing system were harvested after cold triggering. Letters represents significant differences according to post-hoc test (p-value < 0.05). **Right side)** Naïve and cold primed plants which received a mock treatment (blue) and plants which received an estradiol treatment (orange) instead of cold priming were harvested after cold triggering. The experiment was carried out in plants with an estradiol inducible *tAPX* overexpression construct. Letters represent significance according to post hoc test (p-value < 0.05).

Discussion

In case of a first transient cold stress, plants adjust their plastid antioxidant system during a stress-free lag-phase by increasing the APX abundance at the thylakoid membrane (van Buer et al. 2019). The exact mechanism which promotes formation, maintenance, and loss of the cold priming induced *tAPX* abundance is unknown, but first consequences during a subsequent second cold exposure are described (van Buer et al. 2019). tAPX prevents protein oxidation during cold stress exposure and is a key factor in cold acclimation, shown by analysis with transient tAPX silencing lines (Maruta et al. 2012). However, the role of *tAPX* during other oxidative stress events than cold stress is not so clear. For instance, transient *tAPX* silencing results in synergistic as well as in antagonistic effects in the high light response of Arabidopsis (Maruta et al. 2012). This raises the question how and if cold priming interferes with other oxidative stress types. On the one hand, several different stress conditions can induce an excessive electron pressure at the thylakoid membrane, which could be counteracted by higher tAPX abundance. On the other hand, *tAPX* silencing led to stress specific effects, indicating a function in 'fine-tuning' stress specificity (Maruta et al. 2012). This study here investigated the impact of cold priming (4°C for 24 hours) on the cold and high light response to compare potential mechanism of cold priming upon both stress treatments and revealed mainly stress specific imprints in the cold and high light response(Fig. 24). One of the few common priming effects were the reduced responsiveness of JA related genes (Fig. 25), which is, at least upon cold stress, connected with the elevated tAPX abundance (Fig. 39).

3.1 Cold priming impacts common stress response genes in a trigger-type specific manner

The comparison of the cold priming impact on both stress treatments was started by transcript analysis of a subgroup of core stress response genes (Fig. 8). Cold as well as the high light application were sufficient to activate the transcription of the zinc-finger transcription factors

ZAT10, *ZAT6* and *ZAT12* in non-primed plants (Fig. 9-10). However, cold priming impacted only on the cold response but not on the high light response of these genes, which gave first indication for stress specific imprints of cold priming, despite the common stress response of the analyzed genes in non-primed plants (Fig. 9-10).

Previous analysis during reoccurring dehydration, high light and cold (0 °C) exposure revealed that priming adjusts genome wide transcription in response to a second stress exposure (Ding et al. 2013; Byun et al. 2014; Crisp et al. 2017). Thereby, four transcriptional memory types were proposed: the hyper-induced (PT > T > C), the hyper-reduced (PT < T < C), the positive revised (PT > T ≤ C) and the negative revised (PT < T ≥ C) memory response (Ding et al. 2013). In case of dehydration memory, hyper-induced priming regulation supported transcription of drought responsive genes. At the same time, common stress responses, such as the JA pathway, showed a negative revised memory response (Ding et al. 2013). Therefore priming, at least for reoccurring dehydration stress, can be considered as a kind of filter, which prevents stress unspecific 'panicky' and supports stress specific adjustments (Avramova 2017). The transcript levels of *ZAT10*, *ZAT12* and *ZAT6* show high responsiveness to different abiotic and biotic stress conditions (Hahn et al. 2013). Such broad stress responsive genes are prone to serve as candidates for a revised memory response according to the concept of 'filtering by priming'. Reoccurring cold stress (Fig. 9), and to a weaker extend also dehydration memory (Ding et al. 2013), showed a revised memory response of *ZAT10*, *ZAT6* and *ZAT12*. In contrast, *trans*-priming had no impact on the enhanced transcript abundance of the three transcription factors during triggering (Fig. 10), which contrasting the concept of reduced responsiveness of common stress targets upon subsequent stress exposures.

While cold and drought usually affects the whole plant, high light exposure can occur in local spots, which activates a systemic acclimation response (Rossel et al. 2007). *ZAT10* plays an important role in this systemic acquired acclimation to high light and controls approximately 25 % of the high light responsive genes in distal leaves during systemic high light acclimation (Rossel et al. 2007). Consequently, plants could adjust the cold priming impact on *ZAT10* transcription in a stress specific manner (Fig. 9-10) to enable systemic acquired acclimation.

3.2 High light and cold triggering stimuli had similar consequences in non-primed plants

Cold stress can promote excess energy effects and, thereby, ROS formation, due to the slowdown of the photosynthetic metabolism (Ensminger et al. 2006). A sudden increase of light intensity under optimal growth temperature can promote a similar amount of excess light energy at the thylakoid membrane. But in contrast to cold stress, light energy consumption is still available as electron sink (Ensminger et al. 2006; Li et al. 2009b). The trigger specificity of cold priming results either by the different nature of cold and high light perturbations or by a different 'strength' of both triggering stimuli. Therefore, it is important to mention that cold and high light triggering had a similar negative impact on the maximum quantum yield of PSII (Fig. 7). Further, both treatments resulted in a comparable increase of the total H_2O_2 content (Fig. 7), suggesting that both triggering stimuli caused a similar photosynthetic disbalance.

Severe excess light energy, for example upon a light intensities above 1500 µmol photons s^{-1} m^{-2}, can activate secondary processes, such as programmed cell death (Mühlenbock et al. 2008). In my study, 'mild' stress conditions were selected for the priming regulation analysis to detect stress insult specific effects (Fig. 7). Neither cold triggering nor high light triggering caused visual stress symptoms, such as chlorosis. Further, the heat filtered high light and the cold treatment on the transcript abundance of senescence marker genes (Balazadeh et al. 2010) and of programmed cell death marker genes (Olvera-Carrillo et al. 2015) in non-primed plants according to RNA-seq (Chapter 2.3.2). At the same time, more than 50 % of the cold triggering regulated genes were regulated in the same direction by the heat filtered high light application (Chapter 2.3.2).

The similar reduction of the maximum quantum yield of PSII (Fig. 7) in addition to the comparable amount of accumulated H_2O_2 (Fig. 7) and overlap in transcriptional response in non-primed plants (Chapter 2.3.2), indicated that both triggering stimuli led to similar consequences in non-primed plants. Therefore, triggering specific effects in cold priming regulation must occur beside this common consequences, for example due to different reasons of photosynthetic reduction in response to cold and high (Ensminger et al. 2006).

3.3 Most of the priming regulated transcript profiles are trigger type specific

To elucidate the extend of cold priming dependent gene regulation during the two different stress treatments, transcriptome analyses of cold primed and naïve plants during high light and cold trigger were performed (Chapter 2.3 and Chapter 2.4). The results provide a benchmarked set of cold priming regulated transcript profiles during two different stress treatments (Fig. 13 and Fig. 24). The high number of cold priming regulated genes during cold and high light triggering, especially within the first hour of stress exposure (Fig. 24), demonstrated that the cold priming memory has a strong impact on the plants response to both stress types.

Only less than 5 % of the priming effected transcripts were commonly regulated in the *cis*- and *trans*-priming set-up (average overlap of both datasets), demonstrating that cold priming led mainly to triggering specific footprints in the transcriptome, despite the high transcriptional similarity of both treatments in naïve plants (Fig. 14 and Fig. 21). The fact that already 30 min of triggering is enough to cause priming specific gene regulation, indicates that the perception of cold and high light interacts with the plant memory (Fig. 21). Common stress hubs, such as different MAPK pathways, ROS transmitting signals and hormonal crosstalk are involved in stress perception (Chapter 1.2). Interference of cold priming with common stress response hubs could explain the high number of cold priming regulated genes within the first hour of stress exposure (Fig. 23-24).

Cold priming without any triggering stimulus had only a minor impact on the genome-wide transcription after five days of cold recovery (Fig. 12-13, Fig. 23-24). Stress recovery is in general a fast process (chapter 1.5). For instance, Crisp et al. (2017) showed a strong increase of the RNA decay of stress responsive genes after stress removal, which reverts the stress responsive transcript accumulation during stress recovery. The minor impact of cold priming on transcription after a five-day long lag-phase is in line with previous stress recovery analysis and suggests that the memory is maintained by other processes than storage of specific transcripts (Fig. 13 and Fig. 24). In case of short cold stress, increased tAPX abundance is the only known memory mechanism (van Buer et al. 2019). Other reoccurring stress treatments

postulated chromatin modifications, metabolite pools or other metastable processes as prone candidates for memory maintenance (Friedrich et al. 2019; Liu et al. 2014; Baier et al. 2019).

One example for memory maintenance by chromatin modifications is the H3K4 tri-methylation (H3K4me3). Reoccurring dehydration and heat events causing hyper-induced transcript profiles upon a second stress exposure. This hyper-induced memory genes are associated with a higher H3K4me3 abundance (Lämke et al. 2016; Liu et al. 2014). Exposure to cold and other abiotic stresses enhance the total H3K4me3 abundance, which increase the chromatin accessibility of stress responsive genes (van Dijk et al. 2010; Zeng et al. 2019). In case of dehydration stress is this higher H3K4me3 abundance maintained for at least five days, which led to priming effects upon the second dehydration stress (Liu et al. 2014).

Some genes, such as *ZAT10*, showed trigger-specific imprints of cold priming (Fig. 9-10 and Fig. 21-22), despite the common impact of high light and cold triggering on these genes in non-primed plants. 29 genes were even inversely regulated by cold priming after cold and high light triggering (Chapter 2.3.4). These results indicating an active reprograming of the priming memory during each triggering application. Therefore, priming upregulation of genes, which are only responsive to cold or high light exposure, by H3K4me3 can explain only partially the consequences of cold priming.

3.4 *Trans*-priming promote the transcript abundance of high light responsive genes

In total 845 genes showed elevated transcript levels during high light exposure in cold primed plants compared to naïve high light triggered ones (Fig. 17 and Fig. 24). Only 4 % of these *trans*-priming up-regulated genes were also positively affected by *cis*-priming, demonstrating strong trigger specificity of *trans*-priming supported transcription (Fig. 17 and Fig. 24). Functional annotation of cold priming upregulated genes indicates a stronger support of heat and high light responsive genes in cold primed plants than in naïve plants during high light triggering (Fig. 19 and Fig. 26-27).

One of the cold priming upregulated genes of early high light response encodes the heat shock transcription factor *HSFA2* (Fig. 28). Comparison of *HSF2A* supported transcript profiles (Nishizawa et al. 2006) with *trans*-priming upregulated targets, showed that 15 % of the cold priming supported genes during high light are potential under the control of *HSFA2* and indicated a strong impact of the transcription factor on the high light response in cold primed plants. The transcript abundance of *HSFA2* accumulates after H_2O_2 treatment and high light exposure but not during cold exposure (Nishizawa et al. 2006). Transient silencing of the H_2O_2 scavenger tAPX suppress the high light response of *HSFA2*, which supposes either an antagonistic effect of plastid H_2O_2 or a specific signalling role of *tAPX* beside his function as H_2O_2 scavenger (Maruta et al. 2012). Genetic analysis showed that transient *tAPX* overexpression, but not *sAPX* overexpression can replace cold priming (van Buer et al. 2019), which hints to an involvement of a thylakoid located signal and not to an general impact of plastid ROS plant memory.

The hyper-induced memory response of *HSFA2* during high light could be also given by chromatin modifications. Subsequent heat treatments result in a hyper-induction of *HSFA2* and HSFA2 target genes, which is linked with a higher abundance of H3K4me3 within these gene regions (Lämke et al. 2016; Charng et al. 2007). Additional, *HSFA2* activates the H3K27me3 demethylase *RELATIVE OF EARLY FLOWERING 6* (*REF6*) which promotes a positive autoregulatory activation of *HSFA2* transcription during heat exposure (Liu 2019). Analysis of the leaf temperature and of the transcript abundance of heat specific genes demonstrated that the high light application was not sensed as heat (Fig. 5). To which extend chromatin modification are involved in the high light response of *HSFA2* is unknown, but due to the strong evidence of the involvement of H3K4me3 in hyper-induced memory genes (Liu et al. 2014; Lämke et al. 2016), it can be hypothesized that H3 methylation/demethylation may also play a supportive role in *trans*-priming regulation of *HSFA2*.

3.5 Cold priming supports the response of phenylpropanoid biosynthesis involve genes and of plant pathogen defense related genes

During cold triggering, cold priming led to an elevated transcript abundance of genes annotated for anthocyanin biosynthesis and flavonoid biosynthesis (Fig. 18). Cold led further to elevated transcript levels of biotic responsive genes upon cold triggering (Fig. 18, Fig. 20 and Chapter 2.4.8). The majority of these biotic defense genes showed a negative priming regulation during high light triggering, demonstrating inverse priming regulation of these genes in response to high light compared to cold triggered plants (Chapter 3.3.6).

3.5.1 The cold priming regulation of phenylpropanoid biosynthesis involved genes requires long-term cold triggering

Several secondary metabolites, including flavonoids and anthocyanins, are metabolized via the phenylpropanoid pathway (Emiliani et al. 2009). The transition from primary metabolism to the phenylpropanoid pathway is controlled by the phenylalanine ammonia-lyase (*PAL1*, AT2G37040, Rohde et al. (2004)). The flavonoid branch of the phenylalanine pathway is run by the chalcone synthase (*CHS*, AT5G13930, Kreuzaler and Hahlbrock (1972)). Previous priming analysis showed an induced transcript abundance of *PAL1* and *CHS* in cold primed plants upon cold triggering (van Buer et al. 2016). In line with these results, the cold induction of several phenylpropanoid pathway involved genes was upregulated in cold primed plants after 24 hours of cold stress (Fig. 18).

During early cold triggering, no induction and cold priming regulation of the transcript abundance of these genes could be observed. Leyva et al. (1995) described, that at least ten hours at 4 °C are required to activate *PAL1* as well as *CHS* transcript accumulation. At least three hours at 1800 µmol photons s^{-1} m^{-2} are required to elevate transcript levels of both phenylpropanoid biosynthesis involved genes (Vanderauwera et al. 2005). Consequently, the high light triggering was not long enough or strong enough to activated transcription of *PAL1* and *CHS.*

Evidence that both genes could be involved in different priming-triggering combinations are given by priming of the pathogen resistance (Conrath et al. 2015). Different treatments such as wounding, SA application or a first pathogen infection can support resistance against a subsequent pathogen infection (Beckers et al. 2009). Priming enhanced the transcript abundance of *PAL1* during the subsequent pathogen infection, which is considered to mediate stronger pathogen resistance in primed plants (Beckers et al. 2009; Kohler et al. 2002). Priming of the pathogen response of *PAL1* requires two major stress hubs, the MPK6/MPK3 cascade and transcriptional activation by NPR1 (non expressor of pathogen gene 1), which are integrating SA and oxylipid signalling (Kohler et al. 2002).

Some MPK6/MPK3 regulated genes, such as *ZAT12* and *ZAT10* (Fig. 8-9), were negatively regulated by cold priming during cold triggering. Other MPK3/MPK6 targeted genes, such as *ACS6* showed no alteration in the transcript abundance due to cold priming (Fig. 9) and the MPK6/MPK3 targeted gene *PAL1* showed elevated transcript levels in cold primed plants during cold response (Chapter 2.3.5). These contrasting results in cold primed plants hint to a distinct fine-tuned regulation which uncouple common stress hubs and their targets.

3.5.2 Pathogen defense related genes are inverse regulated after *trans*- and *cis*-priming

During early cold exposure, transcript levels of biotic stress response genes, such as *PR4* and *PCC1*, were strongly supported in cold primed plants (Chapter 3.4.7). This positive priming regulation of biotic defense related transcription was still observable two hours after the end of cold triggering (Chapter 2.3). In contrast, transcript levels of biotic defense related genes were strongly decreased in cold primed plants upon high light triggering and two hours after the end of high light triggering (Fig. 15, Fig. 19, and Fig. 27).

Several abiotic perturbations, like excess light intensity (Mühlenbock et al. 2008; Szechyńska-Hebda et al. 2010) and cold stress (Kim et al. 2017; Wu et al. 2019), can activate the plant defense against pathogens. Wu et al. (2019) observed a cold stress activated pathogen defense response and postulated positive effects of the cold response on the salicylic acid content which lowers plant susceptibility to *Pseudomonas syringae*. Here, only one gene, out of the

group of cold priming inverse regulated genes, was annotated as SA responsive (PCC1, supplement table). Other cold priming inverse regulated genes, such as *PR4* and *PR3,* are known as SA independent pathogen response genes (Thomma et al. 1998). Several hours of cold exposure are required to activate SA accumulation (Kim et al. 2013). However, triggering specific imprints of cold priming were observed within the onset of stress exposure (Chapter 2.4.5). Thus, SA signalling alone may have a minor role in inverse cold priming regulation of the transcript abundance of the biotic responsive genes.

3.6 Cold priming has a negative effect on oxylipid biosynthesis independent of the type of triggering

Several of the inversely priming regulated genes, such as *PR4*, *PR3*, *PDF1.2* and *ELI3* show elevated transcript levels in response to electrophilic carbonyl compounds (Alméras et al. 2003; Stintzi and Browse 2000; Mata-Pérez et al. 2015). Lipid-peroxidation products often contain electrophilic carbonyl group (Mata-Pérez et al. 2015) and Alméras et al. (2003) showed that several oxylipids can trigger the transcript accumulation of inversely priming regulated genes, indicating a potential involvement of oxylipids in trigger specific priming regulation.

Intense research has been focused on the biosynthesis of oxylipid JA and its function as signalling component (Wasternack and Hause 2013). Several abiotic and biotic treatments, such as drought, wounding and cold, enhance JA levels (Hu et al. 2013; Liu et al. 2016). During reoccurring dehydration stress, JA content accumulates only during the first stress exposure, but not during subsequent stress exposures (Liu et al. 2016). At the same time, dehydration primed plants had a higher resistance against dehydration during the second stress exposure. The authors suggested that the JA response is a common ‘panic’ response during first stress exposure which suppressed during reoccurring stress exposures (Avramova 2017).

The here presented transcript analyses showed a strong negative impact of cold priming on the early cold response of JA responsive genes (Fig. 30). Further, cold priming dampened the cold response of genes involved in JA metabolism (Fig. 31). Like the impact of cold priming on the cold response of JA related genes, RNA seq analysis indicated also a decreased high light

response of JA related genes in cold primed plants compared to non-primed ones (Fig. 27). The direct comparison of *cis*- and *trans*-priming regulated targets genes (Chapter 3.4.5) supported this indication, that cold priming dampens genes of JA biosynthesis and of the JA response upon both triggering stimuli (Fig. 25).

A direct comparison of cold *cis*-priming revised memory genes (PT ≤ C < T) with dehydration revised memory genes (Ding et al. 2013) and *cis*- high light revised memory genes (Crisp et al. 2017), showed that each priming-triggering combination has mainly specific priming targets (suppl. fig. 1) One exception is the common impact on JA processes in all priming analysis (suppl. fig. 1), indicating a stress type independent impact of priming on JA related processes. Further, the dehydration response of JA responsive genes also reduced in the monocot *Zea mays* during a second dehydration exposure (Ding et al. 2014), indicating that the negative impact of priming on the JA pathway is evolutionary conserved.

3.6.1 *Cis*-priming impacts on the OPDA metabolism

The basic helix-loop-helix transcription factor MYC2 is known as master regulator of JA signalling. (Breeze 2019). Transcript analysis of JA responsive genes revealed a strong transcript accumulation of the JA master regulator *MYC2* after 60 min cold triggering (Fig. 30), consistent with Hu et al. (2013). This cold response of *MYC2* was blocked in cold primed plants (Fig. 30), indicating negative priming regulation of JA signalling during early cold triggering. MYC2 controls not only JA responsive genes but also the JA biosynthesis itself, which promote a positive JA autoregulatory loop (Zander et al. 2020).

The early steps of JA biosynthesis take place in the chloroplasts, where linolenic acid is converted to 12-oxo-phytodienoic acid (OPDA) by three reactions, catalyzed by 13C-lipoxygenase´s (*LOX2*, *LOX3*, *LOX4* and *LOX6*), the allene oxide synthase (*AOS1*) and allene oxide cyclase's (*AOC1, AOC2, AOC3 and AOC4*). The OPDA gets transported to the peroxisomes where the OPDA reductase (*OPR3*) and subsequent β-oxidation converts OPDA to JA (Wasternack and Hause 2013). The transcript abundance of two *LOX* genes, *AOS* and *AOC2* was analyzed in detail (Fig. 31). All four genes showed an increased transcript abundance in

response to cold triggering, which was blocked in cold primed plants, as considered from RNA-seq data and priming regulated *MYC2* transcript abundance (Fig. 28-29). Elevated LOX activity in non-primed plants, which was not present in cold primed ones, indicates lipid oxidation rate beside the transcriptional impact of cold priming on JA metabolism (Fig. 32-33).

Hu et al. (2013) considered that the exposure to 4 °C strongly induces JA accumulation, based on analysis with 2-week-old Arabidopsis plants. But in contrast to Wu et al. (2019) and in contrast to the observed transcript abundance of JA responsive genes (Fig. 31), cold triggering had no impact on the JA and JA-Ile content, in my study (Fig. 34). One reason for the lack of JA accumulation could be the plant age. Munné-Bosch et al. (2007) showed an age dependent accumulation of JA in response cold stress in combination with high light. Under this combinatorial stress treatment, two-week-old plants had a higher JA accumulation than 4 week old plants (Munné-Bosch et al. 2007).

Interestingly, the level of the JA precursor OPDA was slightly higher in non-primed plants upon cold stress, but not in cold primed plants (Fig. 34). Regulation of OPDA strongly correlated with the transcript abundance of JA responsive genes during *cis*-priming (Fig. 35), suggesting a connection between cold primed reduced OPDA levels and revised memory transcript profiles. In addition, the transcript abundance of the glutathione transferase 6 (GST6) was increased in cold primed plants during cold triggering (Chapter 2.4.8). The enzyme conjugates glutathione to OPDA, which is considered to converts OPDA into the inactive *iso*-OPDA (Mueller et al. 2008) and indicates that priming inhibits OPDA signalling actively.

Several studies reported that OPDA or the recently discovered OPDA conjugate OPDA-isoleucine can provide a function independent of JA (Floková et al. 2016). First evidence, that OPDA is active component was given by analysis in mosses. *Physcomitrella patens*, *Merchantia polymorpha* and *Selaginella martensii* are able to produce OPDA but they are unable to convert OPDA into JA (Stumpe et al. 2010). Interestingly, OPDA takes over the function of the missing JA and plays a predominant role in the wounding response of mosses (Ponce De León et al. 2012). Savchenko et al. (2014) showed that OPDA functions also in higher plants. Exposure to drought led to a strong increase of the OPDA content in Arabidopsis. At the same time JA levels

were not altered (Savchenko et al. 2014). The application of OPDA during drought exposure was highly beneficial, indicating a role in abiotic stress response (Savchenko et al 2014).

Taki et al. (2005) demonstrated that 150 genes response more strongly to OPDA treatments then to JA treatments. Within this group of OPDA responsive genes are the cold *cis*-priming regulated transcription-factors *BAP1*, *ZAT10* and *ZAT12*. Transcript analysis during the onset of stress response showed that the cold induction of *ZAT10* is reduced in cold primed plants (Fig. 21). Transcript analysis with oxylipid mutants were performed to untangle the function of OPDA and JA in the regulation of ZAT10. Diminishing OPDA biosynthesis resulted in a reduced activation of the *ZAT10* cold response (*aos*, Fig. 36-37). However, abolishment of OPDA to JA conversion by a T-DNA insertion in *opr3*, had no impact on the cold response of *ZAT10* (Fig. 36-37). Further, priming was not altered in *opr3*, suggesting that the lower OPDA content is the reason for lower cold response of ZAT10 in cold primed plants.

3.6.2 The cold priming reduced OPDA response upon cold stress depends on the accumulation of *tAPX*

Several genetic analyses linked distinct biochemical processes at the thylakoid membrane with accumulation of OPDA. For example, high $^1O_2$ production by the shift of the conditional *flu* mutant from darkness into light (op den Camp et al. 2003), promote a rapid increase of the leaf OPDA content (Ochsenbein et al. 2006). Crossing of the *flu* mutant with *executer 1*, which plays an important role in retrograde signalling, demonstrate that not the $^1O_2$ itself, but retrograde signalling of $^1O_2$ is required for elevated OPDA biosynthesis (Przybyla et al. 2008).

The cold priming reduced cold response of the plastid H_2O_2 marker gene *ZAT10* depends on tAPX (van Buer et al. 2016). The cold response of *ZAT10* is linked with the OPDA biosynthesis, which is reduced in cold primed plants during stress exposure (Fig. 34). This brings up the question if tAPX also controls OPDA biosynthesis, and thereby provides a putative link between tAPX accumulation and the reduced cold response of *ZAT10*. Estradiol inducible *tAPX* overexpression showed a reduced activation of the *AOS* and *AOC2* transcript abundance during cold triggering without any cold priming (Fig. 39). Surprisingly, the *AOC2* and *AOS* transcript

abundances were also reduced in mock treated plants of the inducible *tAPX* overexpression line, but the mock treatment itself had no impact on the *AOS* and *AOC2* transcript abundance in other plant lines (Fig. 39). Previous analysis showed a slight leakiness of the estradiol inducible system, which led to an slightly elevated tAPX abundance without any treatment (van Buer et al. 2019). This indicates that a slight increase of tAPX abundance can reduce the cold response of *AOC2* and *AOS*.

Further evidence of a redox-dependent regulation of OPDA biosynthesis is given by Gollan et al. (2017), who showed, that the high light activated OPDA accumulation depends on the thylakoid located *proton gradient gene 5* (*PGR5*, AT2G05620). *PGR5* provide a cyclic electron transport around PSI. Therefore, it can be assumed that OPDA biosynthesis relates to the thylakoid pH status or directly with cyclic electron transport. The high *tAPX* abundance in cold primed plants provides an additional electron sink, which could impact cyclic electron transport and the thylakoid pH status and thereby the regulation of OPDA biosynthesis.

3.6.3 Potential mechanism to sense and transmit OPDA

Beside altered OPDA biosynthesis also altered perception of oxylipids in cold primed plants could explain the block of JA responsive gene expression during a second stress exposure. How plants sense OPDA and OPDA isoforms is still under investigation (Wasternack and Hause 2016). It is known that the JA receptor complex SCF^{COI1}-*JAZ* is unable to bind OPDA, dinor-OPDA and OPDA-Ile, hinting to an OPDA specific receptor (Thines et al. 2007; Floková et al. 2016). Affinity chromatography followed by mass-spectrometry revealed that the stromal located *Cyclophilin20-3* (*CYP20-3*) can bind OPDA, which promotes an interaction between CYP20-3 and the serine-acetyltransferase 5 (Park et al. 2013). However, abolished *CYP20-3* transcription affects only partially the OPDA dependent response (Park et al. 2013), indicating additional factors in OPDA sensing. Due to delivery difficulties of OPDA for several months, it was not possible to test the impact of cold priming on the perception of OPDA application, but *in silico* analysis of the cold priming targets from the transcriptome analysis allow some conclusions:

Promoter analysis of OPDA responsive genes, revealed a strong over-representation of the binding motif of TGA transcription factors (Mueller et al. 2008). Further analysis showed that 70 % of the OPDA responsive genes showed no OPDA responsiveness in the *tga2/tga5/tga6* triple mutant, which led to the consideration that class II TGA transcription factors are important for OPDA perception or/and signalling (Stotz et al. 2013). Analysis of the 500 bp-upstream promoter region of *cis*-priming regulated genes revealed that 30 % of the priming up-regulated genes and 18 % of the priming downregulated genes carrying the putative TGA binding motif TGACGA (suppl. tab. 3-4), which is a significant overrepresentation compared to genome wide distribution of the motif. The results suggest a strong potential of TGA mediated regulation of cold stress in cold primed plants. TGA transcription factors are interacting with the redox sensor NPR1, which could provide an integration point for stress specific redox-and phytohormone signalling.

3.7 A refined model for *cis*-cold priming

Cold triggering activates accumulation of *ZAT10*, *ZAT12*, *ZAT6* and other common stress response genes by several retrograde signals, for example by the *SAL1*-PAP pathway (Estavillo et al. 2011) and OPDA signalling (Taki et al. 2005). In cold primed plants, a first exposure to cold stress activates accumulation of tAPX, which is a ROS scavenger in close proximity to the photosystem (van Buer et al. 2019). This cold priming induced tAPX accumulation weakens the accumulation of *ZAT10* transcripts during a second cold stress. The fact that *tAPX*-, but not *sAPX*-overexpression can mimic the cold priming effect suggests *ZAT10* regulation by a thylakoid specific signal and not a general plastid ROS signal (van Buer et al. 2019).

This study here showed that cold priming dampens not only the *ZAT10* transcript abundance but impacts on a wide range of genes. Functional annotation highlighted a strong reduction of the transcript abundance of OPDA biosynthesis related genes during a subsequent second cold stress (Fig. 29-31). In addition, cold priming reduced enzymatic lipid oxidation and by trend also the OPDA content during early cold exposure. Genetic analysis showed that the transcription of OPDA biosynthesis involved genes is under the negative control of *tAPX*

abundance. The cold activation of *ZAT10* transcription depends on OPDA biosynthesis. TGA transcription factor are important transmitter of oxylipid signalling shown by analysis in TGA triple mutants (Mueller et al. 2008). *In silico* analysis indicated a strong over-representation of TGA binding motifs in *cis*-priming regulated genes (suppl. tab. 3-4). Taken together, cold priming led to a higher tAPX abundance that weakens the cold stress induced lipid oxidation rate and prevents cold response of common stress responsive genes.

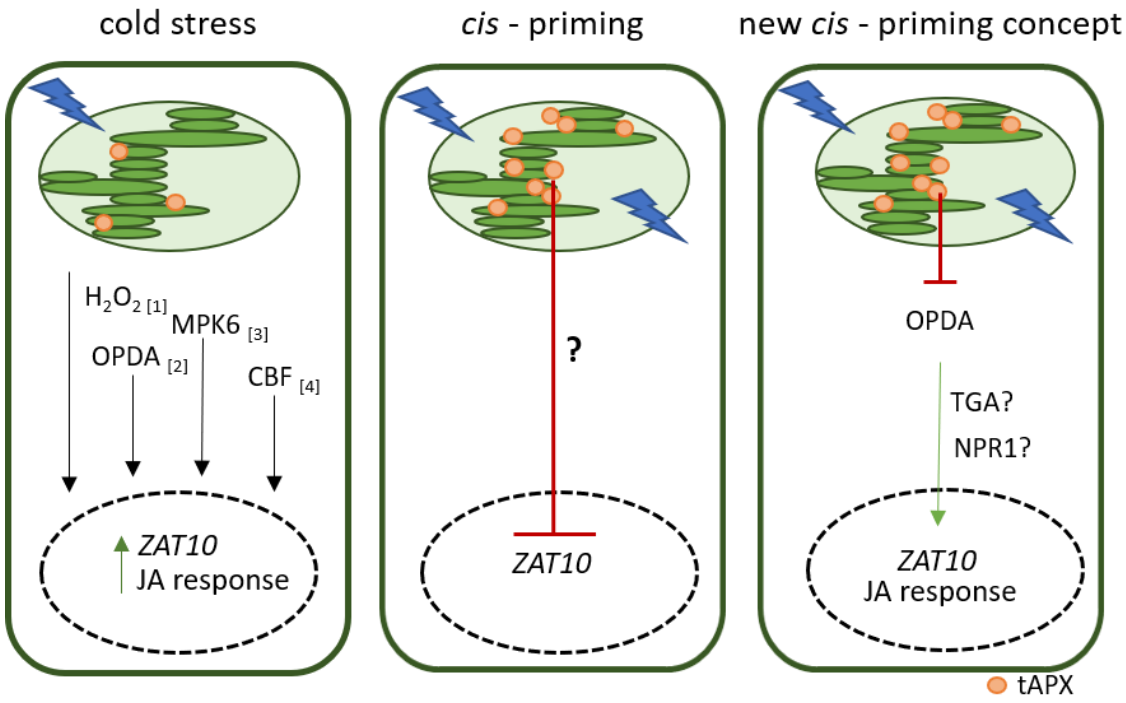

Figure 40: Model of the role of OPDA in cis-priming signalling: *Cold triggering activates ROS formation in the chloroplast. Several retrograde signals are described to be involved to transmit the plastid redox state in expression of common redox responsive genes such as ZAT10 (left side). Previous priming analysis showed activation of tAPX accumulation after cold priming, which dampens cold response of ZAT10 during second cold stress exposure through an unknown pathway (plant cell in the middle). The work here implements OPDA and probably OPDA signalling by TGA transcription factors as a link between tAPX accumulation and the negative priming regulation of common stress responsive genes. blue thunder = 4 °C, green circle = chloroplast, black dotted circle = nucleus*

3.8 The reduced lipid oxidation – candidate for a trigger specific regulation mechanism in cold primed plants

Comparative transcriptome analyses indicated that cold priming reduce not only the cold response, but also the high light response of genes, which are involved in OPDA metabolism (Fig. 25-27). However, priming-sensitive and OPDA regulated genes, such as *ZAT10*, *ZAT12*, *HSFA2, BAP1*, *PR3* and PR4 (Taki et al. 2005; Alméras et al. 2003) showed stress type specific regulation in cold primed Arabidopsis plants (Fig 9-10, Fig. 20 and Fig. 28). This raises the question, whether the redefined model of the cold primed cold response (Fig. 40) fits also to the cold primed high light response of Arabidopsis. Based on the knowledge about OPDA signalling, two main hypothesis can be postulated, how the common priming impact on genes of the OPDA metabolism can be translated to cold and high light specific cold priming imprints in the group of OPDA responsive genes (Fig. 41).

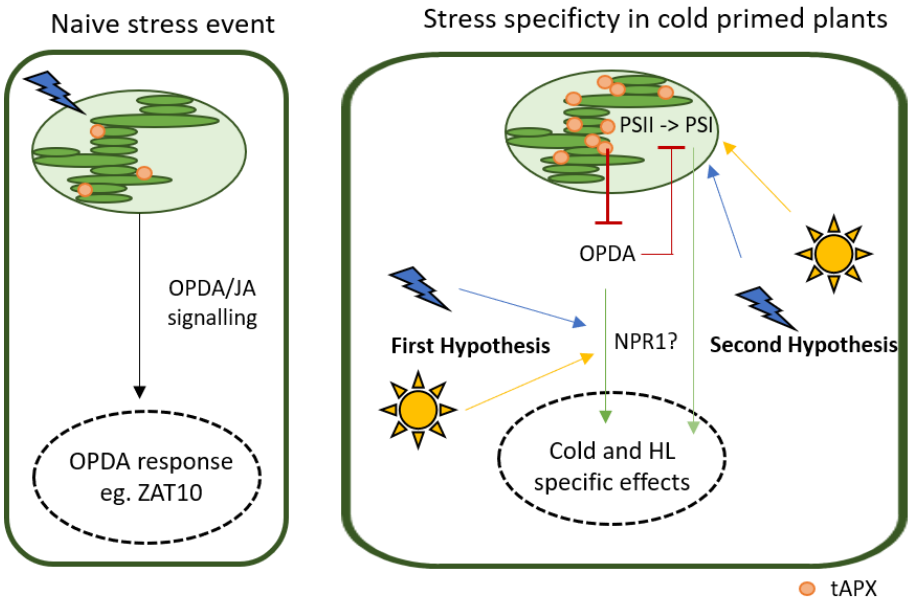

Figure 41: Model of stress type specificity in cold primed plants: *Oxidative stress in non-primed plants led to an higher rate of lipid peroxidation. Oxidized lipids inhibiting photosystem II (PSII) and activating reprogramming via NPR1, which is integrated in a complex stress response network. Cold primed plants exhibit a high abundance of APX (orange circles) in the thylakoid membrane (green circle), which prevents lipid peroxidation during perturbation. Trigger specific effects of cold priming are either caused by the cold priming reduced impact of OPDA in the stress hub network (hypothesis 1) or by the missing threat of oxylipids on photosynthesis (hypothesis 2).*

The first hypothesis is based on the high light and cold specific integration of OPDA signalling in the network of central stress hubs. On example of high light and cold specific effects on a integration point of oxylipid signalling within the network of central stress hubs is the redox sensor NPR1 (Spoel et al. 2003; Tada et al. 2008). After the exposure of a local leaf to high light, *NPR1* is required to activate the systemic high light response of *ZAT10* and other oxidative stress marker (Rossel et al. 2007). On important factor in the systemic high light response is the volatile reactive electrophile species β-cyclocitral, which enhance photoprotection under oxidative stress (Ramel et al. 2012). Lv et al. (2015) demonstrated, that SA signalling via NPR1 and TGA transcription factors is required for the β-cyclocitral induced tolerance against excess light. It is unknown if OPDA plays a role in high light, but the structural similarity between β-cyclocitral and OPDA as well as the involvment of the NPR1 interacting TGA transcription factors in OPDA signalling, may indicate a *NPR1* dependent OPDA signalling under high light in Arabidopsis (Stotz et al. 2013).

Recent results showed, that *NPR1* is also involved in cold acclimation (Olate et al. 2018). In contrast to the high light response, cold stress mediated NPR1 signalling is independent of SA signalling and not requires TGA transcriptionfactors (Olate et al. 2018). Therefore, stress type specific functions of NPR1 are considered. The high number of TGA motifs within the promoter region of priming regulated genes (suppl. tab. 3), indicates a potential interference between cold priming and the stress specificty of *NPR1* signalling, which could cause stress specific imprints by cold priming.

The second hypothesis is based on the impact of OPDA on the photosynthetic efficiency. Alméras et al. (2003), showed that OPDA strongly diminish the activity of PSII, due to the electrophilic carbonyl group of OPDA. Oxidative stress, such as cold and high light, cause accumulation of OPDA and, thereby, threaten photosynthesis (Ochsenbein et al. 2006). The reduction of lipid oxidation in cold primed plants upon triggering (Fig. 32-35 and Fig. 39) would consequently safeguard the photosynthetic electron transport. The prevented impact of OPDA and other RES on photosynthesis would allow a higher electron pressure in cold primed plants compared to naive ones during both triggering stimuli. Several alternative electron sinks, such

as thylakoid K^+ transporter and cyclic electron transport around PSI are described to manage excess electron pressures in a stress specific manner (Alric and Johnson 2017). For instance, Ivanov et al. (2012) showed that cold acclimated plants use other alternative electron sinks than non-acclimated plants in response high light. Mutant lines of the proton gradient regulation gene PGR5, which provide a cyclic electron flow around PSI, are vulnerable to fluctuating light conditions but not to high light intensity (Suorsa 2015; Suorsa et al. 2012). How exactly alternative electron sinks provide stress specificity requires further investigation, but one reason for stress specific impacts of alternative electron sinks could be the stress specific nature of electron pressure upon cold and high light exposure (Chapter 1.2.2).

In summary, the benchmark of stress specific gene regulation in cold primed Arabidopsis plants, obtained in this study, is a strong tool to further investigate crosstalk, integration, and specification of the cold and high light response in Arabidopsis. The high number of OPDA responsive genes within the group of cold priming targets upon cold and high light (Fig. 9-10, Fig. 20 and Fig. 28), indicates a function of oxylipid biosynthesis and oxylipid signalling in cold primed stress response that should be give attention in future studies.

Material and Methods

4.1 Plant Material and experimental conditions

4.1.1 Plant material

Arabidopsis thaliana variance *Col-0* and transgenic plants (Table 3) were cultivated on soil (70 volumes *Topferde*, 70 volumes *Pikiererde*, 25 volume *Perligran* and 0.5 g l^{-1} dolomite lime). Germination was synchronized by seed stratification for two days at 4 °C in the darkness. After seed stratification, plants were grown under a photoperiod of 10 hours with a light intensity of 100 ± 10 µmol photons*m^{-2}*s^{-1} (L36W/840 Lumilux white fluorescent stripes, Osram, Germany), a day/night temperature of 20/18 ± 2 °C and 60 ± 10 % relative air humidity. The seedlings were separated to individual pots (6 cm diameter) at an age of 7-9 days.

Table 3: *Labelling, kind of gene modification and source of all plant lines used in this study.*

Name	Kind of gene modification	Reference
Col-0	Wildtype	INRA Centre
tapx	T-DNA insertion in AT1G77490 (SALK_027804)	Kangasjärvi et al. (2008)
iOE tAPX	estradiol inducible over expression of AT1G77490	van Buer et al. (2019)
iOE sAPX	estradiol inducible over expression of AT4G08390	van Buer et al. (2019)
iRNAi tAPX	estradiol inducible silencing of AT1G77490	Maruta et al. (2012)
aos	T-DNA insertion in AT5G42650 (SALK_017756)	Matschi et al. (2015)
opr3	T-DNA insertion in AT2G06050 (SALK_201355)	SALK-institute

4.1.2 Abiotic stress treatments

For cold treatments, plants were transferred to a cold chamber equilibrated to 4 ± 1 °C. In the cold chamber was the same illumination rate, air humidity and air circulation as in the control growth chamber. Temperature was recorded during each cold treatment (Datalogger 210, Conrad AG, Wollerau, Germany). Plants were shifted to the cold chamber two hours after the onset of the light period.

For high light treatments a photon flux density of 700 ± 60 µmol photons $m^{-2} \cdot s^{-1}$ was applied with halogen lamps (R7s - 500 W lamps, Emil Lux GmbH, Wermelskirchen, Germany). A mild ventilation and a water layer between plants and light source was used to avoid heat dissipation during the experiment (Fig. 42). The leaf surface temperature was controlled by infrared thermometer during the high light treatment.

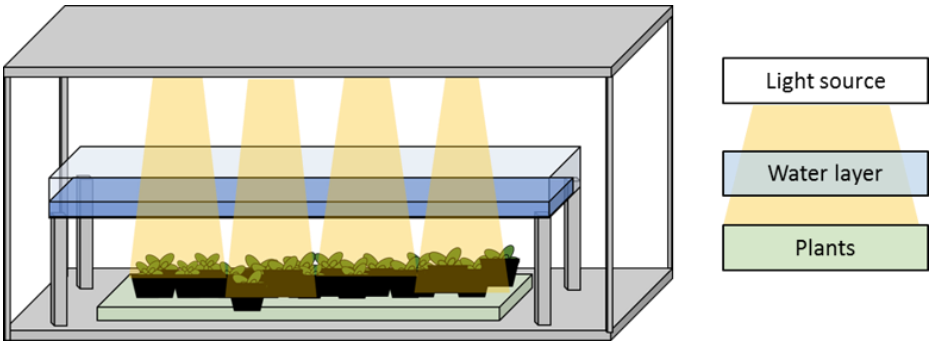

***Figure 42: The high light set up**: Up to 40 plants (green area) were treated under four halogen lamps (700 ± 60 µmol photons $m^{-2} \cdot s^{-1}$, yellow area). A water layer and mild ventilation (blue layer) between the light source and the plants was used for counteracting heat dissipation.*

4.1.3 Estradiol treatment

The open reading frame of the *tAPX* (van Buer et al. 2019) and a short *tAPX* cDNA fragment (Maruta et al. 2012) were cloned downstream of the XVE promoter. Transcription of the transgenes was induced with 100 µM Estradiol (Roth, Karlsruhe, Germany) dissolved in 0.8 % (v/v) DMSO and supplemented with 0.01 % (v/v) Tween as described in van Buer et al. (2019). Control plants were sprayed with a mock solution containing 0.8 % (v/v) DMSO supplemented with 0.01 % (v/v) Tween to exclude background effects by the treatment. All solutions were prepared few min before treatment to avoid precipitation of the estradiol.

4.1.4 Jasmonic acid treatment

The JA solution (100 µM, Duchefa, Haarlem, Netherlands) was dissolved in 1 % ethanol and sprayed on plant leaves three hours before harvesting. Control plants were treated with 1 % ethanol solution (v/v) to exclude background effects by the treatment.

4.2 cDNA library preparation and sequencing

4.2.1 RNA Isolation

For RNA isolation, complete rosette tissue of at least five plants per treatment were pooled and immediately frozen in liquid nitrogen. The material was ground in pre-cooled mortar. RNA from 100 mg plant material was isolated using the Gene Matrix Universal RNA Purification Kit (Roboklon, Berlin, Germany), which is based on the high binding efficiency of nucleic acids to a silica matrix. DNA was removed during RNA isolation with 1 U DNase added to 50 µl DNase buffer, as recommended from the kit manual.

4.2.2 RNA gel electrophoresis

After RNA isolation, RNA quantity was assessed with a Nano-Photometer P300 (Implen, München, Germany) based on the absorption at 260 nm. Calculated A260/A230 and A260/A280 ratios were used to evaluate the samples for contamination with sugars, phenol compounds and proteins. Samples with a A260/230 ratio below 2.0 and a A260/A280 ratio below 2.2 were not considered for further analysis.

RNA integrity was analyzed with electrophoresis on 1.5 % agaroses gel, based on the ribosomal RNA pattern of the sample. 500 ng isolated RNA was adjusted to a volume of 10 µl by the supplement of distilled H_2O and mixed with 2 µl 6x RNA loading buffer and 2 µl 6x loading dye. All samples were incubated at 65 °C for 5 min and immediately transferred on ice to avoid reformation of secondary structures. The RNA-gel was prepared by melting 2 % agarose in 3-(N-morpholino) propane-sulfonic acid buffer (MOPS buffer), supplementing Formaldehyde (2 % (v/v)) and casting the gel. Immediately after loading, the linearized RNA was separated for 25 min at an electricity of 120 V. Ethidium bromide stained RNA bands were documented under UV light (312 nm) with a Gel Imager (Intas science imaging GmbH, Göttingen, Germany).

MOPS buffer	**6x RNA Loading dye**	**6x RNA loading buffer**
200 mM MOPS	10 mM Tris-HCl (pH 7.6)	18 % Formaldehyde
10 mM Sodium acetate	60 % Glycerol (v/v)	200 mM MOPS
10 mM EDTA	60 mM EDTA	1 % Ethidium bromide
pH was adjusted to 7.0	0.03 % Bromphenol blue (w/v)	

4.2.3 cDNA library construction

The cDNA libraries were constructed according to the standard procedures of the Beijing Genomic Institute (BGI, Beijing, China). The mRNA was enriched using oligo (dT) magnetic beads and rRNA was depleted by DNA/rRNA hybridization. Random hexamer primer was used for first strand cDNA synthesis from the purified mRNA. After second strand synthesis, the double stranded cDNA was 5´-end repaired, 3´-end poly-A-tailed and fused to oligo-dT-adapter. Adapter binding oligomers were used to amplify adapter linked DNA based on a polymerase chain reaction.

4.2.4 cDNA library sequencing

The cDNA library obtained from stress recovery experiment (n = 1) was single end sequenced by the Illumina Hi-Seq 4000 platform at the Beijing Genomics institute (BGI, Beijing, China). The sequencing led to an average of 24 x 10^6 reads per sample with a read length of 50 base pairs. The probes, obtained from early stress response experiment (n = 1), were analyzed using the recently advanced pair-end sequenced platform (BGI-Seq-500). Pair-end sequencing led in average to 70 x 10^6 reads per sample with a read length of 100 base pairs.

4.3 Statistical and bioinformatic analysis of cDNA sequencing data

4.3.1 Read quality filtering and alignment

All raw reads obtained by cDNA library sequencing were quality controlled with the help of the SOAPnuke software (Chen et al. 2017). Residual adaptor sequences and reads with more than 5 % unclear base pairs were assigned as unclean reads. The remaining clean reads were aligned to the Arabidopsis reference genome TAIR10 (Lamesch et al. 2011) with a quality threshold of 64 (also called phred-value) using HISAT v. 2.1.0 (Kim et al. 2015).

$$Alignment\ error\ probality = 10^{\frac{-Quality\ treshold}{10}}$$

4.3.2 Normalization of the read number and transcriptome variance analysis

For gene regulation analysis, the number of reads for each transcript were normalized against the transcript length and the total number of reads per treatment (FPKM-value).

$$FPKM = \frac{read\ number\ per\ transcript * 10^6}{read\ number\ per\ sample * transcript\ lenght *\ 10^{-3}}$$

A principal component analysis based on the FPKM values of the 10,000 transcripts with the highest mean FPKM value was conducted to compare the transcription variance between different samples. The analysis was based on single value decomposition after unit variance scaling, as described in Metsalu and Vilo (2015). Differential expressed genes between two treatments were selected according to the log_2-fold change between two samples.

$$Regulation\ of\ transcript\ abundance\ = \log_2 \frac{FPKM\ of\ interest}{FPKM\ of\ control}$$

4.3.3 Gene cluster analysis

The log_2-fold change of gene regulation was normalized by unit variance scaling, before calculating hierarchical gene cluster based on Pearson correlation confidents and pairwise distance comparison. The hierarchical cluster and the unit variance scaled gene regulation was calculated and visualized with the help of the Clustvis web-tool (Metsalu and Vilo 2015). Venn diagrams, conducted by the Venn web tool (University of Ghent, http://bioinformatics.psb.ugent.be/webtools/Venn), was used to visualize the overlap between different gene groups.

4.3.4 Gene ontology analysis

Functional annotation of selected gene groups was conducted according to the gene ontology assignments of the Arabidopsis Information Resource (Lamesch et al. 2011). The single enrichment algorithm of the gene ontology platform AgriGOv2 was used to define enriched GO-terms within a group of selected genes (Tian et al. 2017). Significant enriched GO-Terms were selected by a p-value < 0.05 according to Fisher´s test and a false discovery rate (FDR) < 0.01 according to calculations based on Benjamini and Yekutieli (2001). To visualize relatedness between the enriched biological processes and to independently confirm gene ontology enrichments, ShinyGO v0.61 was used as described in Ge et al. (2019).

4.3.5 Co-expression analysis

The GeneMania database was used to define co-regulation networks based on the linear regression algorithm (Warde-Farley et al. 2010). Probabilities of functional associations between proteins within a network were calculated by combining interaction scores with the help of the String web-tool from the European Molecular Biology Laboratory (Szklarczyk et al. 2015).

4.3.6 Promoter motif analysis

Statistical motif enrichment analyses were performed with the 500 base-pair upstream sequences of a gene group of interest with the help of the TAIR motif analysis tool (https://www.arabidopsis.org/tools/bulk/motiffinder). The probability of the motif enrichment was calculated by the binomial distribution between the motif frequency in the query gene group and the motif frequency in the TAIR10 genome release. The ten highest enriched motifs are listed in the supplement table 3.

4.4 Single gene transcript abundance analysis

Transcript abundance for selected genes were assessed by quantitative real time polymerase chain reaction (qPCR). For each analysis, complete rosette tissue of at least five plants per treatment were collected and immediately frozen in liquid nitrogen. RNA isolation, RNA integrity test and RNA quantification were performed as described for RNA-seq (chapter 2.2).

4.4.1 cDNA library construction for quantitative polymerase chain reaction

For quantitative PCR analysis, RNA was converted to complementary DNA (cDNA) using the High Capacity Reverse Transcription Kit (Applied Biosystems, Carlsbad, USA) following manufacturer's instructions. 2 µg RNA was used as cDNA template and adjusted to an end volume of 10 µl by adding distilled H_2O. After adjusting the volume, 10 µl cDNA reaction mix was added to each sample and reverse transcription was carried out for 2 hours at 37 °C. The reaction was stopped by heat inactivation at 85 °C for 5 min. Before qPCR analysis, the cDNA library was diluted with distilled H_2O to a concentration of 15 ng template per µl.

cDNA master mix

1.0 x cDNA buffer
2.5 mM $MgCl_2$
4.0 mM dNTPs
2.5 µM oligo N_6 primer
2.5 µM oligo dt primer
2.5 U reverse transcriptase

4.4.2 Oligonucleotide design for quantitative PCR

All oligo nucleotides used for qPCR were designed to span exon-intron border, if applicable with the help of the QUANTPRIME software (Arvidsson et al. 2008). Melting curves at the end of each quantitative PCR was used to determine product specificity. All primers were ordered from Sigma-Aldrich (St. Louis, USA) and stored at -20 °C (100 µM). A list of oligonucleotides, used in this study, is outlined in the appendix (supplement table 1)

4.4.3 Quantitative real-time PCR

cDNA generated from 50 ng RNA was mixed with the qRT-PCR master mix The qRT-PCR were initiated with a 3 min long cDNA denaturation at 95 °C followed by 40 cycles 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. The amplification of PCR-products was fluorometrically monitored with SYBR-green (Sigma-Aldrich, St-Louis, USA), which intercalate with double stranded DNA and emits green light (λ_{max} = 520 nm). The qRT-PCR was performed in transparent 96 well plates (Bio-Rad, Hercules, USA) in the C1000 Thermal-cycler equipped with a CFX96 real time system (Bio-Rad, Hercules, USA). The cycle threshold value (Ct-value) was determined using the single threshold method of the Bio-Rad CFX software.

qRT-PCR master mix

1.0	x	qRT-PCR buffer (160 mM $(NH_4)_2SO_4$ and 1 M Tris-HCl, pH 8.3)
2.0	mM	$MgCl_2$
0.1	mM	dNTPs (Bio-Budget technologies, Germany)
0.001	% (v/v)	SYBR green (Sigma-Aldrich, USA)
0.01	$U\mu l^{-1}$	Opti-Taq polymerase (Roboklon, Germany)
0.3	µM	Primer mix (forward + reverse 1:1)

4.4.4 Transcript level calculation for single genes and statistical validation

Each transcript abundance analysis was performed with at least four independent experimental replicates and three technical replicates per experimental replicate. The CFX Manager 3.1 software application was used for melting curve validation, for setting the cycle threshold and for calculating the relative transcript abundance as reported in Pfaffl (2001). The transcript level of each gene was normalized against the transcript abundance of the reference gene *YLS8* (AT5G08290), which is stable expressed through out development and a wide range of treatments, including high light and cold stress treatments (Czechowski et al. 2005). The transcript abundance was shown as $2^{-\Delta ct}$.

$$Transcript\ abundance = 2^{-(ct\ of\ gene\ of\ interest - ct\ of\ reference\ gene)}$$

For testing significance between two values, Fischer's - Test was performed (p-value < 0.05). To test significant differences between several genes and treatments, an analysis of variance (ANOVA) was combined with Tukey´s post hoc test (p-value < 0.05). Correlation between different regulation patterns were calculated according to the Pearson correlation coefficient.

4.5 Physiological methods

4.5.1 H_2O_2 Quantification

The H_2O_2 content was assessed in 50 mg plant material of middle aged leaves, based on the photometrical measurement on the ferric-xylenol orange complex formation, as described in Gay et al. (1999). H_2O_2 was extracted by crushing the leaf material by a plastic pistil after adding 200 µl 5 mM KCN. Insoluble plant material was sedimented for 15 min at 4 °C at 13.000 x g and the supernatant was used for the photometrical assay. 990 µl of the working solution (100 volumes reagent B and 1 volume reagent A) were mixed with 10 µl of the sample and the absorbance at 560 nm was measured after 15 min incubation at room temperature in the darkness. A H_2O_2 standard curve in the range of 0 - 150 µM was generated for calculating the H_2O_2 content. The H_2O_2 contents were normalized to the fresh weight of leaf material.

Reagent A: 25 mM Ammonium ferrous (II) sulfate
2.5 M H_2SO_4
Reagent B: 100 mM Sorbitol
125 µM Xylenol orange

4.5.2 Chlorophyll – a fluorescent analysis

Light energy absorption by chlorophyll can led to a re-emit of light by chlorophyll, known as chlorophyll fluorescence (Kautsky and Hirsch 1931). The measurement of chlorophyll fluorescence by light pulse amplitude modulation (PAM) enables the analysis of the photosynthetic performance in a non-invasive matter (Schreiber et al. 1986). Here the Imaging PAM IMAG-K4B (Heinz Walz GmbH, Effeltrich, Germany) was used to determine the maximal

chlorophyll efficiency of PSII ($\phi PSII_{max} = \frac{Fv}{Fm}$), effective quantum yield of PSII ($\phi PSII = \frac{Fm-F}{Fm}$), photochemical quenching ($qP = \frac{Fm'-F}{Fm-F0}$) and the non-photochemical quenching ($NPQ = \frac{Fm}{Fm'}$). All values were determined within imaging middle aged leaves after 20 min dark acclimatization. A saturating light flash (1000 µmol photons m^{-2} s^{-1}) was applied to generate the maximum chlorophyll-a fluorescence. The ϕPSII, NPQ and qP were measured at an actinic light intensity of 185 µmol photon m^{-2} s^{-1} upon 14 saturating light pulses, spaced by 20 s gaps.

4.5.3 Photometrical malondialdehyde (MDA) measurement

The content of electrophilic lipid peroxidation side products was photometrical quantified based on the orange color change upon a interaction between thiobarbituric acid and electrophilic compounds (Heath and Packer 1968). 100 mg leaf material was harvested and transferred into 1.5 ml cold TBRAS extraction buffer (600 mM tri-chloracetic acid supplemented with 15 mM thiobarbituric acid). The plant material was ground with glass beads (Ø = 4 mm, Roth, Karlsruhe, Germany) for 1 min in the mixer mill MM400 (Retsch GmbH, Haan, Germany) at a frequency of four rounds per second. After 30 min incubation at 95 °C for 30 min, the solid material was sedimented by 10 min centrifugation at 10.000 x g in a precooled centrifuge. Absorbance of the supernatant was determined photometrical at an absorbance of 532 nm against the absorbance of the extraction buffer. The background absorbance at 600 nm was determined as correction value.

$$TBRAS\ content = \frac{OD_{532} - OD_{600}}{Fresh\ weight}$$

4.5.4 Measurement of C13-LOX activity

The C13-LOX-activity was measured photometrically at 234 nm according to the protocol of Axelrod et al. (1981) and modified by the report of Aanangi et al. (2016). For protein isolation, 150 mg leaf material was harvested and transferred in 1 ml precooled phosphate buffer (200 mM, pH 7.5), supplemented with 0.1 % Triton (v/v) as detergent. The plant material was homogenized with glass beads (Ø = 4 mm, Roth, Karlsruhe, Germany) for 1 min in the mixer

mill MM400 (Retsch GmbH, Haan, Germany) at a frequency of four rounds per second. After 10 min centrifugation at 12.000 x g at 4 °C, 20 µl of sample supernatant was mixed with 955 µl phosphate buffer (200 mM, pH 6.5). The enzymatic reaction was started by adding 25 µl substrate (400 µM linoleic acid dissolved in ethanol, Sigma-Aldrich, St.-Louis, USA). The absorption at 234 nm against the phosphate buffer was determined after 5 min incubation at 25 °C. The absorbance was normalized against the incubation time and the fresh weight of the leaf material.

4.5.5 UPLC-MS/MS measurement of OPDA, JA and JA-Ile

Contents of OPDA, JA and JA-Ile were quantified by ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) according to the protocol of Balcke et al. (2012). Approximately 50 mg of frozen leaf material was homogenized in 500 µl pure methanol supplemented with 50 ng [$^2H_5$]-OPDA, 50 ng [$^2H_6$]-JA, and 50 ng [$^2H_2$]-JA-Ile as standards. Insoluble plant material was sedimented by centrifugation. The supernatant was diluted with 9 volumes of water and subjected to a HR-XC columns (Macherey-Nagel, Düren, Germany) for solid phase extraction. The samples were eluted by 900 µl acetonitrile and 10 µl of the eluate were subjected to UPLC-MS/MS according to Balcke et al. (2012). The contents of OPDA, JA, and JA-Ile were calculated by the ratio between the metabolite of interest and the respective standard.

$$Hormon\ content = \frac{peak\ area\ of\ interest}{peak\ area\ of\ standard * freshweight}$$

4.5.6 UPLC MS/MS measurement of ABA

Approximately 100 mg of frozen leaf material was transferred into 1 ml of extraction buffer (ethyl acetate supplemented with 5 ng deuterated abscisic acid) and ground by the FastPrep-24 Classic grinder (Biomedicals, Santa Ana, USA) for 60 seconds. Insoluble plant material was sedimented by centrifugation at 13,000 x for 10 min at 4 °C. The supernatant was transferred to a new tube and concentrated in a vacuum concentrator 5301 (Eppendorf, Hamburg,

Germany). The sedimented plant material that already had been extracted once was used for a second identical extraction procedure with pure ethyl acetate. The supernatant from the second extraction was added to the solution from the first extraction and the combined extract was further concentrated until a viscous solution were left. The viscous solution was diluted with 400 µl methanol (70 % (v/v)) supplemented with 0.1 % (v/v) formic acid. After centrifugation at 13,000 x g for 10 min, 200 µl of the supernatant were transferred into GC/HPLC vials and the ABA content was analyzed using a UPLC-MS/MS Synapt G2-HDMS (Waters, Milford, USA).

4.6 DNA amplification for genotyping

4.6.1 Extraction of genomic DNA

For genomic DNA extraction, 100 mg leaf material was ground with a micro pistil in 600 µl extraction buffer. Insoluble plant material was sedimented by centrifugation at 16.000 g for 2 min. The supernatant was transferred to a new tube and 500 µl isopropanol were added to precipitate DNA for 10 min at room temperature followed by centrifugation at 16.000 g for 15 min. The supernatant was discarded, and the DNA pellet was washed in 70 % ethanol (v/v). The DNA pellet was dried in a speed vacuum concentrator (Eppendorf AG, Hamburg, Germany) to remove residual ethanol. The dry DNA pellet was eluted in 100 µl distilled water.

gDNA-Ex buffer

200	mM	Tris HCl, pH 7.5
250	mM	NaCl
25	mM	EDTA
0.5	%	SDS (w/v)

4.6.2 Polymerase chain reaction for genotyping

Homozygous T-DNA insertions in mutant lines were assessed by insertion specific DNA amplification according to the genotyping pipeline of O'Malley et al. (2015). Gene specific primers that border the predicted T-DNA insertion site were designed with the help of the isect-primer website (http://signal.salk.edu/tdnaprimers). The gene specific primer-pair was

mixed with a T-DNA insertion binding primer. For each sample, 2 µl genomic DNA was mixed with 18 µl ice-cold Taq (*Thermus aquaticus*) -polymerase reaction mix. The DNA amplification was conducted in a Flex Thermocycler (Analytic Jena, Germany) according to the genotyping PCR program.

Home-made Taq polymerase reaction

20 mM Tris-HCl (pH 8.4)
0.5 mM dNTPs
1.5 mM Primer mix
2.5 mM MgCl2
1.0 U μl^{-1} Taq polymerase

Genotyping PCR program

1. Pre-heating for 2 min at 94 °C
2. Strand separation for 30 sec at 94 °C
3. Primer annealing for 30 sec at 60 °C
4. Strand elongation for 1 min kb^{-1} at 72 °C

Step 2-4 were run for 35 cycles

4.6.3 DNA gel electrophoresis

To separate DNA fragments according to their molecular weight, 20 µl PCR product were supplemented with 4 µl of 6x DNA loading buffer. 1 - 2 % (w/v) Agarose, depending on the size of the products, was melted in TAE buffer. The gel material was cooled to 60 °C, supplemented with 1 mg l^{-1} ethidium bromide and casted in a gel tray. DNA was separated for 30-40 min at 100 V. After the run, PCR-product were detected under UV light and documented with a digital camera system from Intas (Göttingen, Germany).

TAE buffer

40 mM Tris HCl, pH 8.0
20 mM Acetic acid
1 mM EDTA

6x DNA loading buffer

50 % (v/v) Glycerol
0.025 % (w/v) Bromophenol blue

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Appendix

***Supplement table 1**: List of oligomers used for quantitative PCR analysis.*

Gene	Gene name	Forward primer	reverse primer
AT5G08290	YLS8	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT
AT1G27730	ZAT10	TCACAAGGCAAGCCACCGTAAG	TTGTCGCCGACGAGGTTGAATG
AT5G59820	ZAT12	TTGGTTACACGCGCTTTGTTGC	ACAAGCCACTCTCTTCCCACTG
AT5G04340	ZAT6	TCTACAAGCCACGTCAGCAGTG	TTCCGGTATCGGCGGTATGTTG
AT4G11280	ACS6	ACGGCGAGAATTCCTCTTATTTCG	ACGCATCAAATCTCCACAAAGCTG
AT1G80840	WRKY40	AGCTTCTGACACTACCCTCGTTG	TTGACAGAACAGCTTGGAGCAC
AT2G36530	LOS2	TGGAACCGAGGTTCAGATTGTCG	GCGATTGCCTTAGCAACTCTCTTG
AT2G37040	PAL1	GCAGTGCTACCGAAAGAAGTGG	TGTTCGGGATAGCCGATGTTCC
AT4G14690	ELIP2	CCACCAGTTAGCAAGCCTAAGGTG	TGGACCGCTAAACGCTAGCAAATC
AT4G31870	GPX7	CGTTAACGTTGCGTCAAGATGTGG	TGACCTCCAAATTGATTGCAAGGG
AT3G51910	HSFA7a	ACCACCACCACAACCAATGGAG	TCTTGGTCAGAAATGGAGGTGGAG
AT3G63350	HSFA7b	ATGGAGGGATTGCAGGAAGCAG	TGGATCACCAACCATCTCGAACG
AT2G40610	ExpansinA8	TGGTGCAATCCTCCTCTTCAGC	TGGTACTCTTCGGAAAGAGACAGG
AT3G22231	PCC1	CGTATGCTCCAGCCTCTGTACATC	GGTTTGGGCAACGACTTCTGTC
AT2G14560	LURP1	TGATAACGAGTGCGGACGGTAAG	TGCATGGTCATCATCTTCCCTCTC
AT3G57260	PR2	AGCTTCCTTCTTCAACCACACAGC	TGGCAAGGTATCGCCTAGCATC
AT3G04720	PR4	GCGGCAAGTGTTTAAGGGTGAAG	CGTTGCTGCATTGGTCCACTATTC
AT3G16670	OLE1	TTTCGCTAATGCGGTCGTCCAG	ATCGTTGAGGAGTGTGGGAAGG
AT5G59310	LTP4	AGTGTTCATCGTTGCATCAGTGG	AGACATGGACTCAAGCTACTTGCC
AT4G12480	EARLI1	AGACTCGGTGTATGTGCGAACG	ACATGGTTGAGCTGATGGCTGAC
AT1G73260	Kunitz1	TGGAGGCTTAAGGTTGCGTTCG	CCGACGTCTGTTTCGATGTTGAGG
AT1G04800	GRP9	ACTTAGGTGGTGGTGGTGGTATC	AACACTGCCTCCAATCCAACCG
AT2G29350	SAG13	AGGGAGCATCGTGCTCATATCC	CCAGCTGATTCATGGCTCCTTTG
AT2G29500	HSP20I	GGATCAGGTTAAGGCTGCGATGG	TCAGCCTTAGGCACCGTAACAGTC
AT5G59720	HSP18.2	TTCACGCCATCTTCTGCGTTGG	TGTAAACGCTGCCACATCACGAG
AT5G12020	HSP17.6	AAGACCCGCAACAACCCTTCAC	GTGTAGCAGCCATTGCCTTAGC
AT3G45140	LOX2	TGGAGGGCATAACTTGGTCGAG	TGCGTAGTCTTCTACCGTAATCCG
AT5G42650	AOS	GGTGGCGAGGTTGTTTGTGATTG	TTCCTAACGGCGACGTACCAAC
AT3G25770	AOC2	GCCAAGAAGAACCTCACTGCTTC	TCTTGAACTTTGCTTGGTCTAGGG
AT1G72520	LOX4	GGAAGACCACATCATCGGTCAAC	AAACGGTTCGTCTCTAACGCTTG
AT5G24770	VSP2	GGACTTGCCCTAAAGAACGACAC	TCGGTCTTCTCTGTTCCGTATCC
AT2G26150	HSFA2	AACAGCTTTGTGGTGTGGGA	TGCTTGAAGTAACGTGGAAGGA
AT3G16470	JR1	CCTGTCCTTGGAAGTGATCATGG	TCATCTGGTCCAAGCACAAACTCC
AT4G23600	JR2	CGAAGCCTGCACCTTCTTATGGAC	TCGTCTTCGATGTCCACAAAGCTC
AT3G63110	IPT3	GGTCTATTCAGAGAGTGGATGCG	AGCATCCATCTTGGACCTTCGC
AT2G28630	KCS12	TAGCAAGGGCTCCACGAATCTC	TGTTCGATCCCGGTCTTGAAGTTG
AT4G22880	LDOX	TTGGCTAACAACGCGAGTGGAC	GCGTACTCACTCGTTGCTTCTATG
AT1G32640	MYC2	TGAAGATAATTGGTTGGGACG	CAACTCCAAATCCATCAACG
AT4G25470	CBF2	GAATCCCGGAATCAACCTGT	CCCAACATCGCCTCTTCATC

Supplement table 2: *List the primer used for genotyping.*

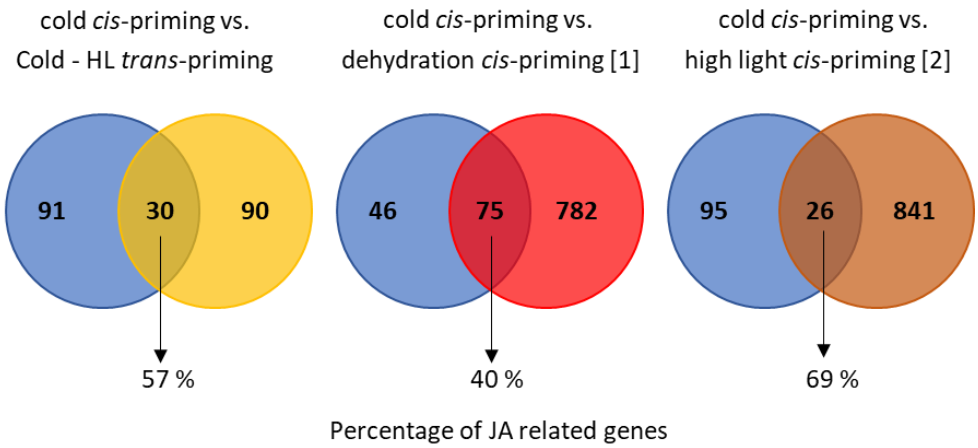
Primer ID	Mutant line	Sequence
LB_opr3	SALK_201355	TCCTCCGTATTGGTCAGTACG
RB_opr3	SALK_201355	AATAAAAATGATTGAATACCATTGG
LB_aos	SALK_017756	CGAGAAATTAACGGAGCTTCC
RB_aos	SALK_017756	CTAACCGGAGGCTACCGTATC

Supplement table 3: *Top ten enriched motifs within 500 bp region upstream of the transcription start of cold priming upregulated genes upon early cold triggering exposure. The table list the ration of genes with the motif in the query group and in the reference genome TAIR10.*

Motif	Query ratio	Genome ratio	p-value
TGACGT	30/121	4136/34292	5.20E-05
TAACGT	35/121	5394/34292	1.01E-04
GAACCA	44/121	7572/34292	1.30E-04
AAGTCT	48/121	8639/34292	1.67E-04
AAACCG	11/121	7245/34292	2.19E-04
ATGGGC	7/121	5647/34292	2.56E-04
ACGTTG	28/121	4122/34292	2.67E-04
ACGTAA	36/121	5992/34292	3.34E-04
CTAAAC	22/121	10731/34292	4.47E-04
AAATGG	62/121	12891/34292	6.97E-04

Supplement table 4: *Top ten enriched motifs within 500 bp region upstream of the transcription start of cold priming down-regulated genes upon early cold triggering exposure. The table list the ration of genes with the motif in the query group and in the reference genome TAIR10.*

Motif	Query ratio	Genome ratio	p-value
CACGTG	37/134	2899/34292	5.72E-11
ACGTGA	47/134	4581/34292	1.14E-10
AACGTG	42/134	4438/34292	1.67E-08
ACACGT	46/134	5350/34292	4.94E-08
ATGTAA	81/134	14794/34292	2.17E-05
ACGTGG	32/134	4000/34292	3.48E-05
CACATG	48/134	7270/34292	3.86E-05
CTCAGA	5/134	4799/34292	6.42E-05
ATATTT	114/134	24395/34292	7.15E-05
TTCCTC	17/134	8648/34292	1.63E-04



Supplemented figure 1: Overlap between revised memory genes (T > C ≥ PT) of different priming set-ups: *The overlap between cold priming down-regulated genes after 60 min of cold triggering (blue) and cold priming down-regulated genes after high light triggering (yellow), revised dehydration memory genes (red) and revised high light memory genes were depicted as Venn diagram. The overlapping gene groups were functional annotated according to ontology terms of TAIR10 and proportion of JA related genes is shown. Data for revised dehydration memory and revised high light memory were obtained from [1] Ding et al. (2013) and [2] Crisp et al. (2017)*

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List of publications

Article contributions

Baier M., **Bittner A.**, Prescher A., van Buer, J. (2019), Preparing plants for improved cold tolerance by priming. *Plant Cell Environ* 42: 782– 800

Bittner A., van Buer J. and Baier M. (2020), Cold priming uncouples light- and cold-regulation of gene expression in *Arabidopsis thaliana*. Preprint under revision at BMC plant science

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Conference contributions

Bittner A., Berking B., van Buer J., Lortzing T., Steppuhn A. and Baier M. (2017), Abiotic trans-priming in *Arabidopsis thaliana.* **Botanikertagung 2017**, Kiel, Germany (poster presentation)

Bittner A., Berking B., van Buer J., Lortzing T., Steppuhn A. and Baier M. (2018), Comparing abiotic *cis*- and *trans*- priming in *Arabidopsis thaliana. International Conference of Arabidopsis Research*, Turku, Finland (poster presentation)

Bittner A., Berking B., van Buer J., Lortzing T., Steppuhn A. and Baier M. (2018), Comparing abiotic *cis*- and *trans*- priming in *Arabidopsis thaliana*, **Oxidative and Abiotic stress symposium,** Potsdam, Germany (poster presentation)

Bittner A., Berking B., van Buer J., Lortzing T., Steppuhn A. and Baier M. (2018), Comparing abiotic *cis*- and *trans*- priming in *Arabidopsis thaliana*, **International meeting of the SPP 1710**, Berlin, Germany (poster presentation)

Bittner A., van Buer J., Prescher A. and Baier M. (2019), Short mild stress primes chloroplast stress signalling for several days in *Arabidopsis thaliana.* **Conference of Plant Molecular Biology**, Dabringhausen, Germany (poster presentation

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