RAP2.4c and RAP2.4d in the regulation of cold stress and cold priming in *Arabidopsis thaliana*

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Summary

Cold stress is a major factor restricting plant performance and fitness. Depending on the length and intensity of the cold stimulus, plants respond to the stress event by alleviating immediately occurring adverse effects of cold stress and by inducing cold acclimation. In the case of a timely limited cold stimulus, *Arabidopsis thaliana* responds with the formation of a cold memory that modifies its response to a future cold stimulus, a phenomenon which is called priming.

Short-term cold priming was previously shown to attenuate chloroplast to nucleus signalling in the regulation of cold-induced *ZAT10* expression. In the present study, this effect was mimicked by transient overexpression of *tAPX*, but not of *sAPX* at 20 °C, while counteracting priming-induced *tAPX* accumulation during the lag-phase abolished the priming effect. This demonstrated that cold priming is mediated at the thylakoid membrane and is regulated by post-priming *tAPX* expression.

Electrolyte leakage assays demonstrated that the AP2/ERF-Ib transcription factors RAP2.4c and RAP2.4d, that have been proposed to regulate chloroplast *APX* gene expression, negatively regulate the direct cold response and cold acclimation. RNAseq analysis of *rap2.4c* and *rap2.4d* KO plants one hour after transfer to 4 °C revealed stronger induction of genes that are involved in JA/ET, JA and SA signalling pathways than in wild type Col-0 pants. Subsequent analyses did not show differences in hormone contents and in sensitivity to hormone signals in *rap2.4c* and *rap2.4d* plants, demonstrating that misregulation of gene expression in the knock-out lines is independent of hormone availability and sensing. Transient overexpression of *RAP2.4c* and *RAP2.4d* also did not influence hormone-related transcript levels. Additionally, the lack of *RAP2.4c* and *RAP2.4d* did not affect cold priming-dependent attenuation of *ZAT10* regulation.

The similarities of the expression patterns of *rap2.4c* and *rap2.4d* with that of an *npr1* mutant in the cold indicate an upstream function of both transcription factors in NPR1-mediated gene expression regulation. A putative target is *TRXH5*, which is involved in quaternary structure regulation and, consequently, nuclear translocation of NPR1. This gene was strongly upregulated in the *rap2.4d* line. Besides a DRE-motif, which is a known RAP2.4d binding site, the *TRXH5* promoter contains a RAP2.4d binding motif, which was identified in the present study based on a Yeast-One-Hybrid screen with RAP2.4d and random genomic DNA fragments of *Arabidopsis thaliana*. The present study highlights RAP2.4d, and to a lesser extent RAP2.4c, as cold-inducible inhibitors of *TRXH5* expression attenuating the NPR1-mediated induction of JA/ET, JA and SA responses in the early cold response.

Zusammenfassung

Kältestress ist ein wichtiger Faktor, der das pflanzliche Wachstum limitiert. Abhängig von der Intensität und Dauer des Kältestimulus, induzieren Pflanzen Reaktionen, die die direkten Auswirkungen der Kälte bekämpfen und die längerfristige Kältetoleranz aufbauen. Im Falle eines kurzen Kältestimulus können *Arabidopsis thaliana* Pflanzen ein Gedächtnis anlegen, mit dessen Hilfe sie im Falle eines zukünftigen Kälteeinbruchs angepasst reagieren. Dieses Phänomen wird Priming genannt.

Zuvor wurde gezeigt, dass Kältepriming Signale aus den Chloroplasten in den Zellkern unterdrückt, was zu einer modifizierten *ZAT10* Expression führt. In der vorliegenden Studie wurde gezeigt, dass dieser Effekt durch die Überexpression von *tAPX*, nicht aber von *sAPX* bei 20 °C simuliert werden kann. Wurde dagegen die kälteinduzierte Akkumulation von *tAPX* während der *lag-phase* unterdrückt, wurde das Gedächtnis an den ersten Stimulus gelöscht. Die Daten zeigen, dass das Gedächtnis durch die *tAPX* Expression während der *lag-phase* und durch ihre Funktion an der Thylakoidmembran reguliert wird.

Electrolyte leakage Versuche haben gezeigt, dass die AP2/ERF-Ib Transkriptionsfaktoren RAP2.4c und RAP2.4d, von denen angenommen wird, dass sie die Expression der plastidären *APX* Gene regulieren, die Kälteantwort und -akklimatisation negativ beeinflussen. RNAseq Analysen von *rap2.4c* und *rap2.4d* KO Linien eine Stunde nach dem Transfer in eine 4 °C Klimakammer, zeigten relativ zum Col-0 Wildtyp die verstärkte Expression von Genen, die in den JA/ET, JA und SA Systemen involviert sind. Die anschließenden Analysen der Hormonspiegel und Sensitivität gegenüber den Hormonen in *rap2.4c* und *rap2.4d* wiesen keine Unterschiede zum Wildtyp auf, was zeigte, dass die veränderte Genregulation unabhängig von Hormonspiegeln oder Hormonsensitivität war. Auch die transiente Überexpression von *RAP2.4c* und *RAP2.4d* hatte keinen Einfluss auf die Expression hormonassoziierter Gene. Außerdem hatte das Fehlen von *RAP2.4c* und *RAP2.4d* keinen Effekt auf die primingabhängige Regulation von *ZAT10*.

Ähnlichkeiten in den Expressionsmustern von *rap2.4c* und *rap2.4d* und denen von *npr1* Mutanten in der Kälte legen nahe, dass beide Transkriptionsfaktoren in der Signaltransduktion oberhalb von NPR1 fungieren. Ein mögliches direktes Ziel ist *TRXH5*, das die Quartärstruktur von NPR1 modifiziert und dadurch seine Translokation in den Zellkern steuert. Dieses Gen war in *rap2.4d* stark induziert. Neben einer für RAP2.4d bekannten DRE-Bindestelle, befindet sich ein weiteres Motiv in dem *TRXH5* Promotor, das in dieser Studie durch ein *Yeast-One-Hybrid* Experiment mit RAP2.4d und zufälligen Fragmenten aus dem *Arabidopsis thaliana* Genom identifizierte wurde. Diese Studie zeigt RAP2.4d, und in geringerem Maße RAP2.4c, als kälteinduzierte Inhibitoren der *TRXH5* Expression, was die NPR1-abhängige Induktion der JA/ET, JA und SA Reaktionen in der frühen Kälteantwort hemmt.

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List of abbreviations

1CP/2CP(A)	1/2-Cys peroxiredoxin (A)
3-AT	3-Amino-1,2,4-triazole
bp	Base pairs of DNA/RNA
ABA	Abscisic acid
ABF	ABRE binding proteins
ABI4	ABA insensitive 4
ABRE	Abscisic acid-Responsive Element
acc	Acclimated
ACT7	Actin 7
AP2	Apetala 2
APS	Ammonium persulfate
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
*att*L/*att*R	Attachment sites in LR-reactions
BAP1	BON-associated protein 1
bp	Base pairs on DNA
BSA	Bovine serum albumin
bZIP	Basic-domain leucine zipper
C	Control
CBF	C-repeat/DRE binding factor
CBL	Calcineurin B-like
cfu	Colony forming unit
CHIT	Chitinase
CPE	Core promoter elements
Col-0	*Arabidopsis thaliana* var. Col-0
COR	Cold regulated
CSD2	Copper/zinc superoxide dismutase 2
ct(-value)	Cycle threshold (value)
CUL3	Cullin 3
Cvi-1	*Arabidopsis thaliana* var. Cvi-1
dd water	Double-distilled water
DNA	Deoxyribonucleic acid
DO	Drop out media
DRE	Dehydration-Responsive Element
DREB	DRE-binding factor
DTT	Dithiothreitol
DYL1	Dormancy-associated protein-like 1
EDTA	Ethylenediaminetetraacetic acid
EF hand	Calcium binding protein domain
EL_{50}	Electrolyte leakage 50
EMSA	Electrophoretic mobility shift assay
ERF	Ethylene response factor
ERS	Ethylene response sensor
ET	Ethylene
flu mutant	*Fluorescent in blue light* mutant
FPKM	Fragments per kilobase million
GC	Gas chromatography

gDNA	Genomic DNA
GO	Gene ontology
GPX	Glutathione peroxidase
GUN1	Genomes uncoupled 1
H3K4me3	Trimethylated lysine 4 of histone H3
HIS3	Imidazole glycerol phosphate dehydratase
HOS	High expression of osmotically responsive gene
HPLC	High pressure liquid chromatography
HSFA1	Heat shock transcription factor A1
IAR3	IAA-alanine resistant 3
ICE1	Inducer of *CBF* expression 1
ICS1	Isochorismate synthase 1
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JA	Jasmonic acid
JAV1	Jasmonate-associated VQ motif gene 1
JAZ	Jasmonate-zim-domain protein
KO	Knock-out
LB	Lysogeny broth
LHC	Light harvesting complex
MDA	Mondehydroascorbate
MES	2-(N-morpholino) ethanesulfonic acid
MAPK(K)(K)	Mitogen activated protein kinase (kinase) (kinase)
MEKK1	MAPK/ERK kinase kinase 1
MEME	Multiple EM for Motif Elicitation
MKK2	MAP kinase kinase 2
MOPS	3-(N-morpholino) propanesulfonic acid
MPK	MAP kinase
MS	Murashige Skoog
MWCO	Molecular weight cut-off
MYC2	Basic helix-loop-helix (bHLH) DNA-binding family protein
$NAD(P)^+$	Oxidised nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Reduced nicotinamide adenine dinucleotide (phosphate)
NCBI	National Center for Biotechnology Information (website)
NPR1	Nonexpresser of *PR* genes 1
NTA	Nitrilotriacetic acid
$OD_{600/450}$	Optical density at wavelengths 600/450 nm
OE	Overexpression
ORA59	Octadecanoid-responsive AP2/ERF 59
OST1	Open stomata 1
P	Primed
PAGE	Polyacrylamide gel electrophoresis
PDF1.2	Plant defensin 1.2
PEG	Polyethylene glycol
PET	Photosynthetic electron transport chain
PIC	Preinitiation complex
PKS	Protein kinase SOS2-like
PR	Pathogen related
PT	Primed and triggered
PCR	Polymerase chain reaction
POLII	DNA-dependent RNA polymerase II
PP2C	Proteins phosphatase 2C
PRX	Peroxiredoxin

PSI / PSII	Photosystem I / photosystem II
Pst	*Pseudomonas syringae* pathovar tomato DC3000
PYL	PYR1-like
PYR1	Pyrabactin resistance 1
RAP2.4	Related to apetala 2A
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Rounds per minute
ROS	Reactive oxygen species
RT-qPCR	Quantitative real-time PCR
SA	Salicylic acid
sAPX/tAPX	stromal/thylakoid bound ascorbate peroxidase
Ser5P POLII	POLII that is phosphorylated at serine 5
SD	Standard deviation
SD medium	Single amino acid drop-out medium
SDS	Sodium dodecyl sulphate
SnRK	SNF1-related protein kinase
SOD	Superoxide dismutase
STN7	State transition 7
T	Triggered
T1, T2, T3	Generations of transformed plants
T-DNA	Transfer-DNA
Taq	Thermostable DNA-polymerase from *Thermus aquaticus*
TBP	TATA-binding protein
TBS(T)	Tris-buffered saline (with Tween-20)
TEMED	Tetramethylethylenediamine
TFIIA-H	Transcription factor TFII complex A-H
TGA	TGACG sequence-specific binding protein
TI	Tumour-inducing
Tris	Tris(hydroxymethyl)-aminomethan
TRX	Thioredoxin
TRXH	TRX H-type
VSP1	Vegetative storage protein 1
WHY1	WHRILY1
WRKY	WRKY DNA-binding protein
Ws-2	*Arabidopsis thaliana* var. Wassilewskija-2
XVE	Chimeric transcription factor
ZAT10	Salt tolerance zinc finger

Amino acids and nucleotides are abbreviated according the International Union of Pure and Applied Chemistry (IUPAC).

1 Introduction

Plants are constantly challenged by various stressful stimuli, e.g. pathogen attacks, herbivore feeding, competing plants, light fluctuations, nutrient limitations, water availability and variations in temperatures. They can sense adverse conditions and induce appropriate responses. These include the induction of defensive compounds upon pathogen attacks (Ausubel *et al*., 1995), closing of stomata upon drought (Raghavendra *et al*., 2010), modifications in photosystem composition and light usage efficiency in fluctuating light (Depège *et al*., 2003) and the production of compatible solutes at chilling temperatures (Hayashi *et al*., 1997). Single stimuli typically induce more than one response. First responses are induced within seconds. Typically, slower responses activate long term protection (Grant and Loake, 2000; Gilmour *et al*., 2004).

1.1 Cold stress in plants

1.1.1 Perception and signalling

Cold stress is one of the major abiotic factors limiting plant growth and crop production worldwide (Xin and Browse, 2000). A reduction of growth temperatures leads to an array of changes. An early and inevitable consequence of declining temperatures is a decrease in membrane fluidity (Murata and Los, 1997). On the long term, the reduction of membrane fluidity is compensated by the induction of fatty acid desaturases, which induce double bonds in the fatty acids, restoring membrane fluidity from pre-stress conditions (Murata and Wada, 1995).

Another almost immediately occurring consequence of cold stress is the influx of calcium ions (Ca^{2+}) into the cytosol. These ions originate from the apoplast and from the vacuole, which is a major Ca^{2+} storage in plant cells (Knight *et al*., 1996). This import of Ca^{2+} is a response to the relative change in temperature per time rather than a response to a certain temperature threshold (Plieth *et al*., 1999). The Ca^{2+} influx is required to induce cold acclimation and to induce gene expression of various *COLD REGULATED* (*COR*) genes (Tähtiharju *et al*., 1997). Treatment of alfalfa protoplasts with the membrane fluidizer benzyl alcohol prevents Ca^{2+} influx and expression of *COR* genes at 4 °C, whereas the treatment with the membrane rigidifier dimethyl sulfoxide induces calcium influx and *COR* gene expression at 25 °C (Orvar *et al*., 2000), demonstrating that calcium influx is a consequence of reduced membrane fluidity. Ca^{2+}-permeable mechanosensitive channels called MID1-COMPLEMENTING ACTIVITY (MCA) 1 and 2 to contribute to the cold-induced calcium influx (Mori *et al*., 2018). Calcium influx is apparently preceded by a destabilization of the cytoskeleton structures consisting of actin

filaments and microtubules, as inhibition of cold-induced destabilization of the cytoskeleton prevents calcium influx into the cytosol and subsequent induction of downstream gene expression in the cold (Sangwan *et al*., 2002).

Changes in cellular calcium concentrations are sensed by an array of calcium-binding proteins, such as CALCIUM-DEPENDENT PROTEIN KINASES (CDPK), CALCINEURIN B-LIKE (CBL) proteins and CAMODULIN (CaM) (Kudla *et al*., 1999; Chin and Means, 2000; Harmon *et al*., 2000). CaM is a well characterized calcium-binding protein which consists of two globular domains with EF hand motifs that are connected by a flexible α-helix. EF hand motifs, which are rich in glutamic acid (E) and phenylalanine (F) residues, bind single Ca^{2+} ions (Strynadka and James, 1989), which leads to a conformational change and enables interaction of CaM with target proteins (Crivici and Ikura, 1995). This induces conformational changes in the target proteins and modifies their activity enabling Ca^{2+}-dependent functions. Ca^{2+}-activated CaM can for example bind to the Ca^{2+}/CALMODULIN-REGULATED RECEPTOR-LIKE KINASE (CRLK1), which is anchored to the plasma membrane and is required for the induction of well-characterized cold marker genes, including *CBF1*, *RD29A*, *COR15a*, and *KIN1* (Yang *et al*., 2010a). Signalling is mediated by phosphorylation of the MAPK/ERK KINASE KINASE 1 (MEKK1) (Yang *et al*., 2010b). MEKK1 is a member of the MITOGEN-ACTIVATED PROTEIN (MAP) kinase pathway, which consists of a heavily interconnected three-step kinase cascade system that regulates various stress responses (reviewed in Xu and Zhang (2015)). In cold stress, activated MEKK1 phosphorylates MAP KINASE KINASE 2 (MKK2), which then phosphorylates the MAP KINASEs MPK4 and MPK6 (Teige *et al*., 2004). Plants overexpressing a constitutively active allele of *MKK2*, show higher expression of selected cold-responsive genes, among them the *C-REPEAT-BINDING FACTORS* (*CBF*) 2 and 3.

1.1.2 The ICE-CBF-COR pathway

The three closely related genes *CBF1*, *CBF2* and *CBF3* encode transcription factors that are master regulators of the cold response. The CBFs induce both short-term cold responses and long-term cold acclimation by binding to the promoters of their target genes (among them *COR* genes) and induction of their expression (Gilmour *et al*., 1998). The DNA motif, which is recognised by the CBFs, the C-repeat motif, contains a 5-bp core, CCGAC, which is present in various genes that are induced during cold and dehydration stress (Baker *et al*., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger *et al*., 1997; Liu *et al*., 1998). CBF-regulated genes include *COR15a*, *COR47* and *COR6.6*, which are marker genes of cold stress events. *COR15a* encodes an intrinsically disordered protein that folds into amphipathic α-helices upon mild dehydration. Due to extracellular formation of ice crystals, freezing stress is accompanied by dehydration of the cytosol (Steponkus, 1984), which leads to the folding of COR15a (Thalhammer *et al*., 2014). Already above-zero chilling temperatures can lead to mild

dehydration of the cells, because water uptake is inhibited in the cold (Beck *et al*., 2007). After structural re-organisation, COR15a binds to the inner membrane of the chloroplast envelope resulting in its stabilization during freezing conditions (Navarro-Retamal *et al*., 2018). Thus, cold-induced CBF-dependent expression of *COR15a* leads to the acclimation of the plants to subsequent freezing temperatures.

Under standard conditions, expression of *CBF1*, *CBF2* and *CBF3* is hardly detectable. Expression is induced within 15 minutes in the cold and peaks after three hours before it declines back to control levels after approximately 24 hours (Gilmour *et al*., 1998; Fowler *et al*., 2005). Their expression in the cold is predominantly regulated by the constitutively expressed transcription factor INDUCER OF CBF EXPRESSION 1 (ICE1) (Chinnusamy *et al*., 2003). Constitutive expression of *ICE1* enables rapid regulation of its target *CBF* genes by posttranscriptional regulation of ICE1 activity. Phosphorylation by the cold-activated kinase OPEN STOMATA 1 (OST1) enhances ICE1 stability and its transcriptional activity (Ding *et al*., 2015). The ubiquitin ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1) counteracts ICE1 function by ubiquitinating ICE1, which marks it for degradation via the 26S-proteasome pathway (Kim *et al*., 2015b). Furthermore, ICE1 activity is inhibited by the interaction with the ZIM domain proteins JAZ1 and JAZ4, which are both degraded upon JA signalling (Hu *et al*., 2013). As such, ICE1 is a convergence point integrating cold responses with other signalling pathways.

Apart from the adjustment of membrane stability by desaturation and stabilization, plants cope with cold conditions by the increased production of compatible solutes which osmotically protect the cytoplasm from water loss (Steponkus, 1984). Compatible solutes comprise various classes of molecules including low molecular weight saccharides such as sucrose, raffinose and maltose as well as amino acids and their derivates such as proline and glycinebetaine (Hayashi *et al*., 1997; Guy *et al*., 2008). As a metabolomic study has demonstrated, cold-induced shifts in the metabolome are more pronounced in the Arabidopsis accession Wassilewskija-2 (Ws-2) than in the accession Cape Verde Islands-1 (Cvi-1), which correlated with their freezing tolerance (Cook *et al*., 2004). The same study has shown, that 256 (79 %) out of 325 metabolites that are upregulated in Ws-2 in the cold, also accumulate in *CBF3* overexpressing plants, indicating the CBF pathway to play a central role in the regulation of compatible solute levels. Accumulation of sugars is evident already after two hours in the cold which preceded changes in freezing tolerance (Wanner and Junttila, 1999). During acclimation, sugar levels further increase and contribute to enhanced freezing tolerance, but their levels sharply decline when transferred back to control conditions (Zuther *et al*., 2015). The ICE-CBF-COR pathway is by far the best studied pathway regulating the short-term and long-term response to cold stress in plants (reviewed in Liu *et al*. (2019)). However, Fowler and Thomashow (2002) have demonstrated that

only 12 % of all known cold-responsive genes are regulated by this pathway, suggesting the existence of CBF-independent pathways regulating the cold-response. One such pathway is regulated by the phytohormone abscisic acid (ABA).

1.1.3 The ABA-dependent pathway

ABA levels transiently and moderately increase during cold exposure (Lang *et al*., 1994) and enhance cold resistance (Xing and Rajashekar, 2001; Nayyar *et al*., 2005), whereas they strongly increase during drought (Xiong and Zhu, 2003). Mutations in ABA-related genes, such as *ABA INSENSITIVE 1* (*ABI1*) and *ABA DEFICIENT* (*ABA*) 1 and 3, impair cold acclimation in Arabidopsis (Gilmour and Thomashow, 1991; Mantyla *et al*., 1995; Xiong *et al*., 2001). The cold-responsive genes *CBF 1-3* are additionally induced by elevated ABA levels (Knight *et al*., 2004) and a fourth member of the family, CBF4, is induced by ABA upon drought, but not in the cold, and its overexpression leads to the activation of DRE-motif containing downstream genes, resembling the cold response (Haake *et al*., 2002). This way, cold and drought result in the activation of a similar set of genes whose promoters contain DRE-motifs.

The CBF-dependent and the ABA-dependent pathways are interconnected, as the *CBFs* and several *COR* genes are inducible by ABA (Gilmour and Thomashow, 1991; Nordin *et al*., 1991; Knight *et al*., 2004). However, as the ABA-signalling mutation *abi1* strongly impaired ABA-induced expression of such *COR* genes, which was not affected in cold-treated *abi1* plants, the ABA-pathway represents a separate cold-signalling pathway, next to the ICE-CBF-COR (Gilmour and Thomashow, 1991; Nordin *et al*., 1991).

Apart from the DRE-motif, the Abscisic acid-Responsive Element (ABRE)-motif is present in many cold-responsive promoters which are activated by the abscisic acid (ABA)-dependent pathway (Guiltinan *et al*., 1990). Various BASIC-DOMAIN LEUCINE ZIPPER (bZIP) transcription factors have been identified to bind to the ABRE-motif and to induce expression of ABA-responsive genes (Choi *et al*., 2000; Uno *et al*., 2000). Such bZIP transcription factors include the ABRE BINDING PROTEINS (ABF) and are differentially regulated in stress situations. *ABF1* is induced in the cold, *ABF2* and *ABF3* are induced at high salt concentrations and drought and *ABF4* is upregulated by cold, high salt and drought (Choi *et al*., 2000), again demonstrating an overlap in the responses to these three stimuli. Treatments of plants with exogenous ABA results in enhanced cold tolerance, which correlates with elevated levels of proline and soluble sugars, water retention and photosynthesis and reduced membrane peroxidation (Huang *et al*., 2015; Kim *et al*., 2016; Huang *et al*., 2017b; Huang *et al*., 2017a).

Park *et al*. (2009) have demonstrated that PYRABACTIN RESISTANCE 1 (PYR1) and 13 PYR1-LIKE (PYL) proteins are the ABA receptors that bind and inactivate PROTEIN PHOSPHATASE 2C (PP2C) proteins in

the presence of ABA. Such PP2Cs are encoded by *ABI1* and *ABI2* (Leung *et al*., 1994; Meyer *et al*., 1994; Leung *et al*., 1997). In the absence of ABA, active PP2Cs inactivate certain SNF1-RELATED PROTEIN KINASES (SnRK) by dephosphorylation and by the formation of a PP2C-SnRK complex (Yoshida *et al*., 2006). These SnRKs (SnRK2.2; SnRK2.4 and SnRK2.6) have been identified as positive regulators of the ABA response (Mustilli *et al*., 2002; Yoshida *et al*., 2002). At elevated ABA levels, PRY/PYL proteins bind ABA, undergo a conformational change and recruit PP2Cs leading to their inactivation (Park *et al*., 2009), releasing the SnRKs from the inhibitory complex (Ng *et al*., 2014). The SnRKs undergo autophosphorylation and induce ABA signalling by phosphorylating their target transcription factors, such as the ABFs (Belin *et al*., 2006; Furihata *et al*., 2006; Yoshida *et al*., 2006). The ABFs in turn induce the expression of various cold- and drought-responsive genes, such as *LOW-TEMPERATURE-INDUCED 78* (*LTI78*) (Xu *et al*., 2013). While the precise mode of action in the cold is not well described, *abf1* mutants were compromised in seedling establishment specifically in the cold (Sharma *et al*., 2011). Together, these findings establish the ABA-dependent pathway as a parallel mechanism for the formation of cold tolerance, apart from the CBF1-3 pathway.

1.1.4 The NPR1-pathway

NONEXPRESSER OF *PR* GENES (NPR1) is a well-described master regulator of the salicylic acid (SA)-dependent induction of defence responses against biotrophic pathogens (Spoel *et al*., 2003; Pieterse *et al*., 2012). In the absence of pathogens, it forms oligomers that are stabilized by intermolecular disulfide bonds. Upon pathogen-induced increases in SA levels, the oligomers disassemble into monomers (Mou *et al*., 2003), that translocate into the nucleus, where they interact with transcription factors and induce defence responses (Fan and Dong, 2002).

Recently, a function of NPR1 was observed in the cold response. *npr1* knock-out (KO) plants that were transferred to 4 °C for 24 hours failed to induce various cold-responsive genes compared with the wild type (Olate *et al*., 2018). In the wild type, *NPR1* transcripts increased in the cold, which was also observed in plants lacking the three cold-inducible CBFs or a central enzyme for the biosynthesis of ABA (*aba2*), indicating that this pathway is independent from the CBFs and from ABA. In the same study, it was shown, that two THIOREDOXINs (TRXH3 and TRXH5) that facilitate cold-induced NPR1 monomerization and the kinase SnRK2.8 that phosphorylates NPR1 monomers to induce nuclear import are necessary for the cold-induced translocation of NPR1 into the nucleus. In the nucleus, NPR1 interacts with the HEAT SHOCK TRANSCRIPTION FACTOR 1 (HSFA1) and induces expression of various HSFA1 target genes that encode chaperones. Apart from chaperones, several other cold-responsive genes were found to be NPR1 targets, while the NPR1-interacting transcription factor that leads to the expression of these genes remains elusive. Furthermore, *npr1* mutants exhibit reduced freezing

tolerance after seven days of cold acclimation at 4 °C (Olate *et al*., 2018). These findings establish NPR1 as a recently identified CBF- and ABA-independent pathway in the cold response.

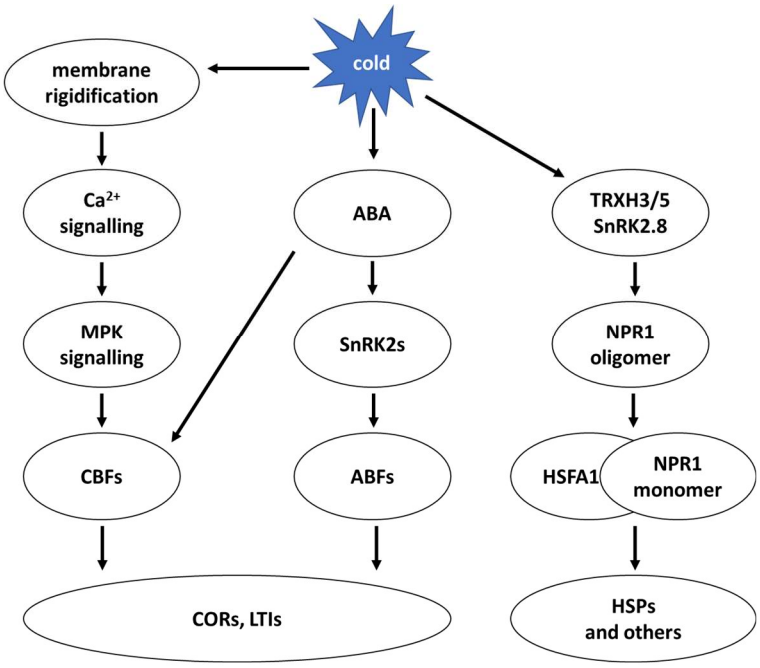


Figure 1: Major pathways regulating the cold response.
Cold stress leads to a reduction of the membrane fluidity and increased ABA levels. Membrane rigidification results in Ca^{2+} signalling which activates the MPK signalling pathway and ultimately in CBF-dependent expression of *COR* genes. Increases in ABA lead to the activation of CBF4 and three members of the SnRK2 family. SnRK2s phosphorylate ABFs and two of them regulate the expression of cold-responsive genes. In the cold, NPR1 oligomers monomerize in a TRXH/SnRK2.8-dependent manner. NPR1 monomers translocate into the nucleus and interact with HSFA1 which leads to the expression of *HSP* genes.

A simplified model describing the cold response is shown in **Figure 1**. The CBF regulon controls approximately 12 % of all cold-inducible genes (Fowler and Thomashow, 2002), which partly overlap with the ABA-dependent pathway (Haake *et al*., 2002). The recently identified NPR1-dependent pathway represents an independent node in the regulation of cold stress responses (Olate *et al*., 2018). While these pathways represent important regulatory mechanisms in the cold response, it is safe to predict the existence of further, to date unidentified mechanisms that facilitate cold tolerance and cold acclimation.

1.1.5 ROS formation in the cold

In addition to the rigidification of membranes and Ca^{2+} ion influx into the cytosol, the formation of reactive oxygen species (ROS) is another inevitable consequence of cold stress. ROS are highly reactive

oxygen containing molecules and are constantly formed in all aerobic cells, also in the absence of stressors (Apel and Hirt, 2004). Due to its two unpaired electrons in the π* orbitals, molecular oxygen is a potent acceptor for single electrons (Halliwell, 2006). Compartments as the mitochondria and chloroplasts harbour biochemical reactions that involve the transfer of electrons along a reduction potential gradient. In the case of chloroplasts, the transfer occurs from water, which is split using the energy of the sun light, via various carrier molecules such as plastoquinones to $NADP^+$. This circumstance makes ROS production in aerobic organisms unavoidable.

Once they are formed, ROS can spontaneously react with biomolecules including lipids, proteins and DNA, causing severe damage at higher concentrations which may lead to programmed cell death (Desikan *et al*., 1998). Also in widespread human diseases such as Parkinson's disease and Alzheimer's disease, increased oxidative damage to various biomolecules was detected in the brains of patients (Halliwell, 2001). Initially, ROS were assumed to exclusively be toxic by-products of various reactions. Over time it became clear, that they are also central stress signalling molecules (Mittler *et al*., 2004) taking part in the regulation of drought resistance (Pei *et al*., 2000), excess light responses (Mullineaux and Karpinski, 2002), growth regulation (Foreman *et al*., 2003) and other responses. Apart from their signalling capabilities, they fulfil functions in directly killing pathogens in a so-called ROS burst (Lamb and Dixon, 1997). Furthermore, ROS are also intentionally produced in order to facilitate developmental processes, such as cell wall extension and cross-linking (Schopfer, 2001; Passardi *et al*., 2004). The group of ROS comprises various molecules with different properties and functions.

These include singlet oxygen ($^1O_2$), which is produced at photosystem II (PSII) especially in high electron pressure situations (Macpherson *et al*., 1993). If oxygen takes up an electron, for example in the Mehler-reaction (Mehler, 1951), this electron enters one of the π* antibonding orbitals and a superoxide radical anion (O_2^-) is formed (Fridovich, 1995). During various kinds of stresses, this electron transfer occurs at elevated rates at the photosystem I (PSI) (Asada, 2006). Apart from this source of superoxide production that occurs passively as a consequence of limited availability of electron acceptors in the photosynthetic electron transport (PET) chain, it can also be actively produced by plasma membrane localised RESPIRATORY BURST OXIDASE PROTEINs (RBOHs) as an early response to pathogen attack (Grant *et al*., 2000; Torres *et al*., 2006). SUPEROXIDE DISMUTASEs (SODs) catalyse the first detoxification step in which superoxide is converted into hydrogen peroxide (H_2O_2) (Kliebenstein *et al*., 1998).

H_2O_2 is the type of ROS with the longest half-life of approximately 1 ms in living cells (Hossain *et al*., 2015). Since it is the product of spontaneous or enzymatic conversion of superoxide, it accumulates directly at the sites where superoxide is produced, e.g. at the PSI or at the plasma membrane (Grant *et al*., 2000; Asada, 2006). An array of enzymes detoxifying H_2O_2 to water is available in different plant

compartments, including CATALASES (CAT) in the peroxisomes, ASCORBATE PEROXIDASE 2 (APX2) in the cytosol and various enzymes in the plastids, that will be discussed in the following chapters (Tolbert, 1981; Fryer *et al*., 2003; Asada, 2006). H_2O_2, as well as the other ROS, oxidises other biomolecules and may lead to their inactivation (Imlay *et al*., 1988; Groen *et al*., 2005; Siddique *et al*., 2011).

As stated above, ROS formation in plants is ubiquitous and unavoidable even under optimal conditions and systems for their detoxification are active at all times to minimize toxic effects. However, various types of stressors lead to the elevated production of ROS which then function as signalling molecules. In the cold, ROS formation is strongly increased in the chloroplasts. During oxygenic photosynthesis, photons are absorbed by chlorophyll molecules and their energy is used to split water molecules and to extract electrons from them (Nelson and Ben-Shem, 2004). These electrons are subsequently fed into the photosynthetic electron transport chain (PET) and are finally transferred to oxidised $NADP^+$ molecules to form reduced NADPH which serves as a short-term storage for these electrons (Whatley *et al*., 1963). A proton gradient which is formed during this process is used to produce adenosine triphosphate (ATP), which is an energy storage molecule that enables various enzymatic reactions or acts as a donor of phosphate groups in other reactions (McCarty *et al*., 2000). The energy that is stored in ATP and NADPH is used for enzymatic reactions, e.g. in the Calvin-Benson cycle which fixes atmospheric carbon dioxide (CO_2) (Benson *et al*., 1950).

Enzyme reactions are highly temperature-sensitive. A decrease in temperature leads to a decrease in enzymatic reaction velocities, as less energy is available from the surrounding medium (Schmidt-Nielsen, 1990). Whereas the enzyme-dependent Calvin-Benson cycle is slowed down by low temperatures, the PET relies on physical transfer reactions that do not involve enzymes and as such, are only mildly affected by low temperatures (Hiller and Raison, 1980; Tardy and Havaux, 1997; Skupień *et al*., 2017). Thus, in the cold, the same amount of energy is trapped and stored as ATP and reduced NADPH, but this energy cannot be consumed in the slowed Calvin-Benson cycle. Pools of oxidised $NADP^+$ get depleted and electrons from the PET cannot be transferred to their designated acceptors, increasing the electron pressure. These excess electrons are then transferred to molecular oxygen at a higher rate, forming O_2^- which is subsequently dismutated to H_2O_2 (Kliebenstein *et al*., 1998; Asada, 2006). This pattern is very similar in high light stress, where more light is captured in the photosystems leading to a higher rate of electron transport in the PET which cannot be consumed in the Calvin-Benson cycle that is adjusted to normal light conditions (Huner *et al*., 1998). Cold and high light stress both lead to the elevated production of ROS in the chloroplasts via a similar mechanism, that leads to the activation of similar stress-related downstream regulatory pathways. Further signals need to be sensed that integrate the nature of the stress into the regulation of the stress response.

1.2 The plastid antioxidant system

Plants have evolved a sophisticated network of antioxidants that are necessary to keep ROS at non-toxic levels (Asada, 1999). Antioxidants can be divided into enzymatic and non-enzymatic low molecular weight compounds. The most important low molecular weight antioxidants are the tripeptide glutathione and ascorbate which can accumulate to millimolar concentrations (Noctor and Foyer, 1998). As the chloroplasts are one of the most important sources of ROS in plants, they contain 30-40 % of total cellular ascorbate (Foyer *et al*., 1983; Inzé *et al*., 2002). Glutathione and ascorbate have been shown to directly reduce and detoxify ROS (Njus and Kelley, 1991; Galano and Alvarez-Idaboy, 2011).

Additionally, ascorbate also functions as a co-enzyme of a group of ascorbate peroxidases (APX) converting H_2O_2 to water at the expense of their electron donor ascorbate (Asada, 1992; Ursini *et al*., 1995). Arabidopsis chloroplasts contain two APX isoforms (Jespersen *et al*., 1997; Pitsch *et al*., 2010). One of them is a soluble APX that is targeted to the stroma (sAPX) and the other one is bound to the thylakoid membrane (tAPX), in close proximity to the photosystems (Miyake *et al*., 1993; Dekker and Boekema, 2005). Both APX variants are susceptible to oxidative inactivation during low ascorbate availability (Miyake and Asada, 1996) which occurs during stress situations with elevated H_2O_2 levels and may work as a switch to allow for a ROS burst above the inactivation threshold, leading to another set of ROS responses (Kitajima, 2008). While both isoforms are similar in their molecular functions in mature leaves, they exhibit distinct phenotypes during stressful conditions (Kangasjärvi *et al*., 2008). In the same study, *sapx* KO seedlings were susceptible to photooxidative stress during germination, while *tapx* KO plants displayed only a mild phenotype. Transient silencing of *tAPX* resulted in elevated H_2O_2 levels in the plastids and subsequently to reduced *CBF1* expression and thus, to reduced cold resistance (Maruta *et al*., 2012). Knock-out of both genes in the *sapx/tapx* double KO line led to compensation by the elevated expression of members of the PEROXIREDOXIN (PRX) family after acclimation to high light intensities (Kangasjärvi *et al*., 2008). As in the case of APX, chloroplasts contain two GPX variants (GPX1 and GPX7) that take part in the regulation of the response to photooxidative stress and in biotic defence signalling (Chang *et al*., 2009). Additionally, chloroplasts contain the PRXQ, the type II PEROXIREDOXINS, the 1-CYS REOXIREDOXINS (1CP) and the 2- CYS REOXIREDOXINS (2CP) (Horling *et al*., 2002). The 2CPs are ubiquitously found in all organisms (Baier and Dietz, 1996; Baier *et al*., 2000). As opposed to APXs, PRXs and GPXs do not require co-enzymes, but reduce H_2O_2 via oxidation of conserved cysteine residues. PRXs need to be regenerated by reduction of their previously oxidised cysteines by either of various enzymes, including THIOREDOXINs (TRX) (Chae *et al*., 1994; Kwon *et al*., 1994).

Oxidation of ascorbate results in the formation of monodehydroascorbate (MDA) that is regenerated via reduction by either FERREDOXIN (FER) (Miyake and Asada, 1994) or by NAD(P)H catalysed by chloroplastic MDA REDUCTASE (Sano *et al*., 2005). If MDA is not reduced, it disproportionates into ascorbate and dehydroascorbate (DHA) which is reduced to ascorbate by DHA REDUCTASE using electrons from glutathione (Foyer and Halliwell, 1977; Shimaoka *et al*., 2003). Finally, oxidised glutathione is regenerated with electrons from NADPH which is catalysed by GLUTATHIONE REDUCTASE (Meister and Anderson, 1983; Mhamdi *et al*., 2010). This way, electrons that are extracted from water during the first steps of photosynthesis are transferred to oxygen, forming ROS, which are detoxified with electrons that also originate from photosynthesis, with water as the end product of this detoxification pathway (Asada, 1999). For this reason, this detoxifying and regenerating system is known as the water-water cycle (Asada, 1999). Hence, the formation of ROS represents an important sink for PET-derived electrons during stress situations, as the formation and detoxification of ROS both require electrons from the PET, reducing electron pressure and limiting over-excitation of the electron carrier pools. The redox states of electron carriers, such as plastoquinone, FER and TRX have been shown to elicit retrograde signalling under such stressful conditions, controlling the expression of nuclear genes (Karpinski *et al*., 1997; Baier *et al*., 2000; Piippo *et al*., 2006; Lepetit *et al*., 2013).

1.3 Priming

1.3.1 The concept of priming

Cold exposure elicits a multitude of different responses that were introduced above. After a cold stimulus is over, these cold responses quickly stop, and metabolic shifts and gene expression regulation are widely reverted within 24 hours, a process which is called deacclimation (Zuther *et al*., 2016). The timing of these deacclimation responses is individual for single compatible solutes and genes, but the majority of such cold responses declined very sharply after cold exposure (Zuther *et al*., 2015). After short-term exposure, single components may normalize more slowly and serve as an information storage that helps the plant to respond better to a future stress (Hilker *et al*., 2016; Zuther *et al*., 2019).

In several studies, it has been shown, that plants can store information on a certain first stress event (the priming stimulus) over a stress-free period and use this information to respond to a future stress (the triggering stimulus) in a more beneficial way (Hilker *et al*., 2016). This phenomenon is called priming. Priming capabilities have been shown in several contexts in plants. These include priming in pathogen attack, heat stress, herbivory and cold stress (Boyko *et al*., 2007; Charng *et al*., 2007; Helms *et al*., 2013; van Buer *et al*., 2016). In most cases, primed plants show an earlier, faster or stronger response to the second stress, as compared to naïve plants, which allows them to withstand the

adverse conditions more effectively (Conrath, 2009). Various mechanisms have been demonstrated and hypothesized to enable plants to store the information on the past stress despite the lack of a nervous system.

1.3.2 Mechanisms in memory formation and maintenance

Priming-induced accumulation of inactive signalling components that are quickly activated upon exposure to the triggering stimulus enables plants to mount defence responses more quickly than in unprimed (naïve) plants that do not accumulate these signalling components. Examples are the well-characterised signal transduction proteins MPK3 and MPK6, that accumulate as inactive proteins upon a chemical priming stimulus and are activated more strongly upon challenge with biotic and abiotic stressors (Beckers *et al*., 2009). In addition to inactive MPKs, accumulation of inactive transcription factors was hypothesized to be a mechanism that enables priming in a similar way (Conrath, 2006).

A number of genes have been shown to be inducible to a higher extent in drought stressed plants that previously had been primed by drought stress (Ding *et al*., 2012). These so-called "trainable genes" were specifically associated with Ser5P DNA-DEPENDENT RNA POLYMERASE II (POLII) at their transcription start sites during the recovery phases, which was not found at other genes. POLII which is phosphorylated at serine 5 (Ser5P) is found in promoter regions and its dephosphorylation is necessary for the induction of transcription (Komarnitsky *et al*., 2000; Buratowski, 2003). Stalled POLII at promoters of primed genes that respond more strongly upon a second challenge is in line with the finding that stalled POLII was found in promoters that quickly respond to environmental changes (Core *et al*., 2008; Nechaev and Adelman, 2011).

Apart from Ser5P POLII, high levels of trimethylation of lysine 4 of histone H3 (H3K4me3) were associated with trainable genes during recovery (Ding *et al*., 2012). Both Ser5P POLII and H3K4me3 marks were also found in other genes during drought treatments, but they only persisted in trainable genes during stress-free phases. H3K4me3 represents another mechanism that is involved in priming and that is strongly correlated with active gene expression (Ng *et al*., 2003). This way, a priming stimulus may induce H3K4me3 marks in histones specifically bound to primable genes and enable earlier or stronger expression of such genes upon a second challenge. Jaskiewicz *et al*. (2011) have demonstrated that salicylic acid (SA) treatments induced various histone modifications that are associated with active gene expression, including di- and trimethylation and acetylation on the promoters of several WRKY transcription factors. While these activating marks did not immediately result in elevated gene expression, they facilitated enhanced responsiveness upon pathogen infection.

Cold-primed Arabidopsis plants have been shown to exhibit a reduced expression of the plastid H_2O_2 marker gene *ZAT10* upon cold triggering, as compared with naïve plants that strongly induce *ZAT10* (van Buer *et al*., 2016). During the five-day lag-phase that separated both cold stimuli, tAPX protein and transcript levels increased and were still elevated at the beginning of the triggering stimulus. The *ZAT10* priming phenomenon was not observed in plants lackeding tAPX, which detoxifies H_2O_2 that is generated at the photosystems. It was hypothesized, that elevated tAPX levels protect cold-primed chloroplasts more effectively from triggering-induced H_2O_2 accumulation as compared to naïve chloroplasts, which would not lead to the induction of the chloroplast H_2O_2-responsive gene *ZAT10* (van Buer *et al*., 2016).

Whereas several mechanisms have been reported, that contribute to store information on a previous stress event, less is known about how this information is "forgotten" over time. Mechanisms that involve the accumulation of inactive signalling molecules (i.e. MPKs) or of protective enzymes (i.e. tAPX) may weaken over time and depend on the half-life of these molecules. However, this explanation may not explain all priming phenomena, as it does not take into account further regulatory pathways (Hilker *et al*., 2016). Particularly transgenerational priming, which is regulated via epigenetic marks, does not follow this explanation, as these marks are sometimes persistent over several generations and thus, countless cell divisions (Hauben *et al*., 2009; Whittle *et al*., 2009; Kathiria *et al*., 2010).

1.3.3 Relevance of cold priming in natural environments

Priming and acclimation are two different strategies of plants to cope with adverse conditions. They differ in their physiological outcomes in terms of fitness, in their relevance with respect to environmental conditions, in their molecular mechanisms and consequently in the costs that are connected with the establishment of an acclimated or primes state (reviewed in Hilker *et al*. (2016)). Cold acclimation represents a strategy that involves the enhanced production of proteins and compatible solutes, both at high levels, that confer enhanced tolerance to chilling and freezing temperatures (reviewed in Knight and Knight (2012)). The massive production of cold-responsive proteins is a costly response that pays off for example in autumn or winter, when it is more predictable that the cold stress persists for an extended period of time (Huner *et al*., 1993). Mounting full cold acclimation responses during a short cold spell would be a waste of energy and result in reduced resources that are available for growth and reproduction (Cook *et al*., 2004).

As opposed to cold acclimation, cold priming is a comparably cost-efficient strategy to increase fitness in the cold. Plants that have perceived a timely limited cold stimulus can avoid cold acclimation responses and store information on this stimulus in the form of epigenetic marks, elevated levels of a

single or of a few signalling (e.g. MPKs) or defence components (e.g. tAPX), that allow the plant to respond to a future stress in a way that is beneficial to its fitness in the cold (van Buer *et al*., 2016). This strategy relies on comparably few alterations to the cellular activity as compared to the massive transcriptional reprogramming and protein biosynthesis that is induced by cold acclimation (Lee *et al*., 2005).

A field study with six natural Arabidopsis accessions that differed in their potential to acquire freezing tolerance by cold acclimation, demonstrated that selectively those accessions with lower cold acclimation capacities (WS, Col-0 and Van-0) showed positive priming effects on photosystem II performance and on reproductive fitness (Cvetkovic *et al*., 2017). This indicated that cold acclimation and cold priming may be two strategies that counteract each other and that natural selection for one of both traits opposes selection for the other one, respectively.

1.4 Transcription factors in stress responses

1.4.1 Transcriptional regulation

Transcriptional reprogramming enables strong cold responses (Lee *et al*., 2005). Gene expression (the synthesis of a primary RNA) is regulated by a complex system of transcription factors that may have a positive or negative influence on RNA synthesis. Apart from transcription factors, various further factors such as transcriptional co-activators (Fan and Dong, 2002), chromatin structure (Hu and Tee, 2017), DNA methylation (Bemer, 2018) and occupancy by histone proteins (Hu *et al*., 2011) take part in the regulation of gene expression. However, the relative importance of transcription factors was demonstrated, as 1968 genes in the Arabidopsis genome were identified as transcription factors, which represents 7.4 % of all genes (Iida *et al*., 2005).

Transcription factors are usually composed of at least two protein domains: A DNA binding domain and a protein-protein interaction domain. The DNA binding domain is responsible for the recognition of specific DNA sequences (Schlotmann and Beyreuther, 1979). In the most cases, these DNA sequences are located within a few hundred base pairs (bp) upstream of the gene, in the promoter region. Upon binding to their recognition site, transcription factors serve as a binding platform for other regulatory proteins to form a protein complex in the promoter region (Kim *et al*., 1994; Koleske and Young, 1994). This kind of interaction requires the protein-protein interaction domain of the transcription factor. Promoters contain several such target sequences to allow for a more complex regulation rather than regulation by only a single transcription factor (Raikwar *et al*., 2015). So called

enhancer sites are recognition sites, that may be thousands of bp away from their target gene. In these cases, specific DNA 3D structures lead to a close proximity of the promoter and the enhancer sites facilitating the required protein-protein interactions which are necessary for the regulation of gene expression (Yang *et al*., 1994).

Gene expression regulation is a complicated multi step process. Promoters contain core promoter elements (CPE) that are common to all genes. CPEs are bound by the so-called general transcription factors. They form the preinitiation complex (PIC), which recruits the POLII and regulates POLII activity (Thomas and Chiang, 2006). Additionally, promoters contain *cis*-acting elements that are recognised by transcription factors conferring context specificity to gene expression and that differ between genes.

Gene-specific transcription factors that bind to *cis*-acting elements modulate gene expression, e.g. by recruitment of further components of the PIC and of co-activators (Ptashne and Gann, 1997), modification of chromatin structure and expression enhancing functions that occur after the formation of the PIC, such as activating stalled POLII (Hahn and Young, 2011; Adelman and Lis, 2012). The latter was predominantly observed for genes that quickly respond to environmental cues and that require fast activation in eukaryotes (Core *et al*., 2008; Nechaev and Adelman, 2011).

The regulation of transcription factors takes place at several levels. One such regulatory mechanism is the regulation of the abundance of transcription factors that in many cases strongly increases upon a certain stress or developmental cue. An example for such an inducible transcription factor is CBF1, that strongly accumulates in the cold at the transcript and protein levels and leads to the expression of genes that have functions in the cold (Fowler *et al*., 2005).

Many transcription factors are present in an inactive form and require activation. One such example is the WRKY DNA-BINDING PROTEIN 33 (WRKY33) that is phosphorylated by MPK3 and MPK6 upon infection with *Botrytis cinerea* (Mao *et al*., 2011). This study demonstrated that MPK3- and MPK6-dependentp activation of WRKY33 leads to the elevated expression of its target genes and enhanced resistance against pathogens.

The expression of certain genes is modified depending on the reductive state of the cell. Several transcription factors are directly modulated in their activity by redox signals, others by proteins sensing such conditions, e.g. MPKs. RAP2.4a is such a redox-sensitive transcription factor. It changes its quaternary structure in response to redox signals and activates *2CPA* expression *in vivo* under ambient and slightly oxidising conditions, but not under strongly reducing and oxidising conditions (Shaikhali *et al*., 2008).

While the recognition of specific DNA sequences (motifs) in the promoters/enhancers of certain genes confers some degree of regulatory specificity, other factors are equally important to guarantee appropriate transcriptional regulation. For example, two transcription factors may recognise the same DNA motif, but they may be expressed in different tissues or at different times of the day or at different temperatures. Even though two transcription factors could potentially bind to the same promoters, context-dependent availability of other transcriptional regulators that may be essential for the induction or suppression of target gene expression may lead to vastly different transcriptional responses (Cheatle Jarvela and Hinman, 2015).

Often, transcription factors have very specific functions and are only activated or expressed in very specific situations, regulating the expression of only a few genes, as hypothesized for the redox sensor protein WHIRLY1 (Foyer *et al*., 2014). Contrary, there are transcription factors that play roles under several conditions and that are activated accordingly. One example is the basic helix-loop-helix (bHLH) DNA-binding protein MYC2, the master regulator of the phytohormone jasmonic acid (JA). During JA signalling, *MYC2* expression increases and the gene product binds to several promoters of JA-responsive genes, leading to their expression, while it has inhibitory effects on other genes involved in indole glucosinolate biosynthesis (Dombrecht *et al*., 2007). Furthermore, *MYC2* not only responds to JA, but also to ABA, another phytohormone, leading to a distinct transcriptional response (Kazan and Manners, 2013). As JA is mainly involved in the response to insect feeding and some developmental cues and ABA mainly regulates responses to abiotic stresses, such as drought and cold, it becomes clear how widely spread and context-specific MYC2 functions are.

Transcription factors can be grouped depending on homologies in their DNA-binding domains. Prominent groups of transcription factors include the HELIX-TURN-HELIX, the BASIC REGION/LEUCIN ZIPPER MOTIF (bZIP), the ZINC FINGER and the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) families (Takatsuji, 1999; Jakoby *et al*., 2002; Aravind *et al*., 2005; Nakano *et al*., 2006). The AP2/ERF family, which contains 147 members in Arabidopsis, can be further sub-divided based on their AP2/ERF domain which is involved in DNA binding. The members of the AP2 group contain two AP2/ERF domains, proteins of the ERF family contain a single copy of the domain and the RAV family members contain a B3 domain in addition to a single AP2/ERF domain (Nakano *et al*., 2006). Homologs of AP2 domain containing proteins that function as endonucleases have been identified in cyanobacteria and viruses and it was hypothesized, that the AP2/ERF family was acquired by plants via lateral gene transfer (Magnani *et al*., 2004). The ERF family comprises several well-studied transcription factors that contribute to the regulation of biotic and abiotic stress responses, among them the CBFs that have been introduced above (Nakano *et al*., 2006). Other members include the central regulators of the JA and ethylene (ET) pathway, ERF1, ERF2 and ORA59 which are involved in the regulation of the

resistance against necrotrophic pathogens by inducing the expression of target genes such as *PDF1.2* (Berrocal-Lobo *et al*., 2002; Lorenzo *et al*., 2003).

1.4.2 RAP2.4 transcription factors

The ERF family is divided into several sub-groups. The AP2/ERF-Ib group contains eight members that are also known as the RELATED TO APETALA 2 (RAP2) group number 4 (RAP2.4), numbered a-h (Nakano *et al*., 2006; Rudnik *et al*., 2017). Members of this group have reported functions in abiotic stress responses. For example, RAP2.4a regulates the expression of the *2-CYS-PEROXIREDOXIN A* (*2CPA*) in a redox-dependent manner by directly binding to its promoter (Shaikhali *et al*., 2008). Constitutive overexpression of *RAP2.4a*, *RAP2.4b*, *RAP2.4d* and *RAP2.4e* results in strong cell proliferation and callus formation, while other RAP2.4 members exhibited no such function, suggesting partly redundant and partly independent functions (Iwase *et al*., 2011). *RAP2.4b* and *RAP2.4d* are among the most closely related members of the family and double knock-out (KO) lines have a reported function in the regulation of six aquaporin genes in drought stress (Rae *et al*., 2011). According to publicly available micro array data from the eFP browser, genes of the *RAP2.4* family are induced upon various abiotic stresses, such as drought, salt, UV-B, mechanical wounding and high and low temperature (Kilian *et al*., 2007; Winter *et al*., 2007; Rudnik *et al*., 2017).

RAP2.4c and RAP2.4d have reported functions in the transcriptional regulation of plastidic H_2O_2 degrading ascorbate peroxidases (APX). KO lines of the two *RAP2.4* genes resulted in strongly reduced expression of the thylakoid bound version of the *APX* (*tAPX*) in *rap2.4c* and of the *tAPX* and the stromal version (*sAPX*) in *rap2.4d* under standard conditions (Bulcha, 2013). This is in line with Yeast-One-Hybrid results, that confirmed direct binding of all members of the family to a *tAPX* promoter fragment (Rudnik *et al*., 2017). Both RAP2.4c and RAP2.4d additionally act as transcriptional repressors of several *COR* genes, as their constitutive overexpressing lines exhibited reduced *COR* gene induction in the cold, compared to the wild type (Bulcha, 2013). As both transcription factors are induced transiently in the cold and reach their peak after around one hour at 4 °C (as during several other abiotic stresses, according to the eFP browser data (Kilian *et al*., 2007; Winter *et al*., 2007)), it was hypothesized, that both genes act to suppress premature induction of a full cold response during transient cold snaps. In addition to all RAP2.4 transcription factors binding to the *tAPX* promoter, it has been demonstrated that RAP2.4a and RAP2.4d bind to the DRE-motif *in vivo* and *in vitro*, respectively and that RAP2.4c binds to G(A/T/C)GGCG and AGGC(C/G) motifs *in vivo* (Rae *et al*., 2011; Bulcha, 2013).

1.5 Aim of the study

The two AP2/ERF-Ib transcription factors RAP2.4c and RAP2.4d have been demonstrated to be negative regulators of the cold response (Bulcha, 2013) and to positively regulate the expression of the antioxidant enzymes sAPX and tAPX (Rudnik *et al*., 2017), which take part in the formation of the memory in cold priming in Arabidopsis (van Buer *et al*., 2016).

The first part of this study aimed to further characterise functions of RAP2.4c and RAP2.4d in the cold response. In that, the study focused on the identification of regulatory pathways that are involved in during the early cold response. To achieve this, a transcriptomics approach that compared gene regulation at 4 °C in *rap2.4c* and *rap2.4d* KO plants compared with Col-0 wild type plants was used. A Yeast-One-Hybrid assay was used to identify the RAP2.4d *in vivo* DNA binding motif to integrate promoter analyses into the transcriptomic expression data of putative RAP2.4d target genes. Additionally, both KO lines were analysed for their involvement in the regulation of cold tolerance and cold acclimation.

In the second part of this study, sAPX and tAPX, which are implicated in cold priming, were further analysed for their capabilities to establish the cold-induced memory, using 17β-estradiol-inducible overexpression and silencing lines in cold priming experiments. The transient overexpression lines were used to mimic a cold priming event at ambient temperatures and a *tAPX* silencing line was used to counteract a cold priming event in order to discriminate between sAPX and tAPX in terms of memory formation in cold priming.

2 Material and methods

2.1 Growth conditions for plants and bacteria

2.1.1 Plant growth on soil

Plants were grown on soil composed of 70 volumes “Topferde“ (Einheitserde, Sinntal-Altengronau, Germany), 70 volumes “Pikiererde“ (Einheitserde, Sinntal-Altengronau, Germany), 25 volumes Perligran Classic (Knauf, Iphofen, Germany) supplemented with 0.5 gl^{-1} dolomite lime (Deutsche Raiffeisen-Warenzentrale, Frankfurt am Main, Germany). Seeds were sown on wet soil and stratified at 4 °C for three days in darkness. Pots were subsequently transferred to a growth chamber with a constant air temperature of 18 °C, 60 ± 5 % air humidity, ten hours of light (120 – 130 µmol photons m^{-2} s^{-1}, L36W/840 Lumilux Cool White fluorescent stripes (Osram, Munich, Germany)) and 14 hours of darkness. After five to eight days, plants were transferred to single pots of 6 cm of diameter and the soil was kept moist. If not stated otherwise, plants were grown for 28 days before experiments started.

Experiments used *Arabidopsis thaliana* var. Col-0 as the reference wild type, since the used T-DNA insertion lines *rap2.4c* and *rap2.4d* and transgenic inducible lines had a Col-0 background. Two T-DNA KO lines were used in this study: *rap2.4c* (At2g22200, SALK_108879) and *rap2.4d* (At1g22190, SALK_139727).

For cold treatments, 28-day old plants were transferred to the 4 °C growth chamber that, apart from the temperature, had the same conditions as for regular growth. Experiments were always started two and a half hours after the onset of light in the morning to eliminate circadian effects. Harvesting of plant material, shifting plants to 4 °C and chemical treatments were always carried out at this time.

2.1.2 Sterile plant growth on MS agar

For selection of transformed plants, seedlings were grown on Murashige Skoog (MS) medium (Duchefa Biochemie B.V, Haarlem, The Netherlands) under sterile conditions. Approximately 200 seeds were sterilized in 750 µl of 70 % ethanol for ten minutes. The ethanol was removed, and the seeds were incubated in 750 µl of 100 % ethanol for five minutes. The seeds were then spread on Whatman paper (Schleicher&Schuell, Düren, Germany) that had previously been soaked in 70 % ethanol and air dried under sterile conditions. After the seeds had dried, they were dispensed on MS-agar plates containing 15 µg ml^{-1} Hygromycin B with approximately 50 – 70 seeds per plate. Seeds were stratified at 4 °C for three days in the darkness. The plates were subsequently transferred to a Cu-22L Percival growth

cabinet (CLF Plant Climatics, Wertingen, Germany) at 22 °C and 130 µmol photons m^{-2} s^{-1}. After six hours, the light was switched off for 48 hours and switched back on for further 24 hours. Transgenic plants exhibited enhanced hypocotyl growth compared with wild type plants and were transferred to soil for normal growth (Harrison *et al*., 2006).

MS-agar

0.43 % (w/v)	MS basal medium
0.5 % (w/v)	Saccharose
5 mM	MES
0.5 % (w/v)	Phytagel

The pH was adjusted to 5.7 with KOH.

2.1.3 Growth of *Escherichia coli* cells

E. coli cells (either Top10 or BL21(DE3)pLysS) were grown at 37 °C in Lysogeny broth (LB) medium. Liquid cultures were shaken at 180 rpm and bacteria on LB plates containing agar were incubated with no agitation.

Top10 genotype: F^- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G. (Thermo Fisher Scientific, Waltham, USA)

BL21(DE3)pLysS genotype: F^- *omp*T *hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3) pLysS (Cam^R). (Thermo Fisher Scientific, Waltham, USA)

LB medium (1.5 % (w/v) agar was added for solid LB plates)

1.0 % (w/v)	NaCl
1.0 % (w/v)	Peptone
0.5 % (w/v)	Yeast extract

Transgenic cultures were selected for cells containing specific plasmids on plates or in liquid media containing appropriate antibiotics:

Antibiotics concentrations

100 µg/ml	Ampicillin
50 µg/ml	Kanamycin
34 µg/ml	Chloramphenicol

2.1.4 Growth of *Agrobacterium tumefaciens* cells

A. tumefaciens GV3101 (pMP90) cells were grown at 28 °C in YEP medium containing 150 µg/ml rifampicin and 25 µg/ml gentamycin. If the cells were transformed with pMDC7 plasmids, 100 µg/ml

spectinomycin were added to the medium for selection. Liquid cultures were shaken at 180 rpm and bacteria on YEP plates containing agar were incubated with no agitation.

GV3101 (pMP90) genotype: C58 (Rif^R) Ti pMP90 (pTiC58DT-DNA) ($Gent^R$/$Strep^R$) Nopaline

YEP medium (agar was added for solid YEP plates)

0.5 % (w/v)	NaCl
1.0 % (w/v)	Peptone
1.0 % (w/v)	Yeast extract
1.5 % (w/v)	Agar

2.1.5 Growth of *Pseudomonas syringae* cells

Pseudomonas syringae (*Pst*) var. Tomato DC3000 cells were grown for two days at 28 °C on NYGA medium plates containing 100 µg/ml rifampicin.

Solid NYGA medium

0.5 % (w/v)	Peptone
0.3 % (w/v)	Yeast extract
0.2 % (v/v)	Glycerol
1.5 % (w/v)	Agar

2.1.6 Growth of *Saccharomyces cerevisiae* cells

S. cerevisiae Y187 cells were grown at 30 °C in YPAD medium. The cells were grown in different selective Minimal SD Base media lacking specific amino acids, depending on the plasmids they had been transformed with. Cells transformed with pACT2 plasmids were grown in media lacking leucin, cells containing pHIS2 plasmids were grown in media lacking tryptophan, cells containing both plasmids were grown in media lacking leucine and tryptophan. The final assays were performed on Minimal SD Agar Base plates lacking leucine, tryptophan and histidine. The media were prepared according to the manufacturer's instructions (Clontech Laboratories, Inc., Mountain View, USA)

Y187 genotype: MATα, *ura3*-52, *his3*-200, *ade2*-101, *trp1*-901, *leu2*-3, 112, *gal4*Δ, *met*–, *gal80*Δ, *MEL1*, URA3::GAL1UAS -GAL1TATA-*lacZ*

YPAD medium (agar was added for solid YPAD plates)

1.0 % (w/v)	Yeast extract
2.0 % (w/v)	Peptone
80 mg l^{-1}	Adenine hemisulfate
1.8 % (w/v)	Agar

2.2 Manipulation of nucleic acids

2.2.1 Extraction of genomic DNA from plant material

For the extraction of genomic DNA (gDNA) from plant material, a single leaf of an approximately three-week old plant was transferred to a reaction tube, supplemented with 700 µl gDNA-Ex buffer and ground with a micro pestle. Insoluble cell debris was pelleted at 16,000 x g for two minutes and 500 µl of the supernatant were precipitated with 500 µl of isopropanol in a new tube. After 2 minutes of incubation at room temperature, the DNA was pelleted at 16,000 x g and 4 °C for 15 minutes. The pellet was washed with 500 µl of 70 % ethanol and pelleted again at 16,000 x g and 4 °C for 4 minutes. After the supernatant was removed, the pellet was air dried and re-dissolved in 100 µl of water. For up to one month, the DNA was stored at 4 °C, for long term storage it was frozen at -20 °C.

gDNA-Ex buffer

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

2.2.2 Polymerase chain reaction (PCR)

PCRs amplifying DNA fragments for cloning purposes were always conducted with the OptiTaq polymerase enzyme (Roboklon, Berlin, Germany) and with a home-made Taq (*Thermus aquaticus*) polymerase to test for the presence of certain sequences in biological samples. All primers were ordered from Sigma-Aldrich (St. Louis, USA) and sequences can be found in the appendix.

20 µl OptiTaq reaction

2 µl	10x PCR buffer B/C
0.8 µl	dNTPs (5 mM, each)
0.5 µl	Primer forward (5 mM)
0.5 µl	Primer reverse (5 mM)
0.2 µl	OptiTaq (5 U/µl)
2 µl	Template (1 – 20 ng / µl)

20 µl home-made Taq reaction

2 µl	10x PCR buffer
1 µl	dNTPs (10 mM, each)
1 µl	Primer forward (10 mM)
1 µl	Primer reverse (10 mM)
2 µl	Home-made Taq
2 µl	Template (1 – 20 ng / µl)
1 µl	50 mM $MgCl_2$

PCR program

1.	Pre-heating	2 min	94 °C
2.	Strand separation	30 sec	94 °C
3.	Primer annealing	30 sec	variable
4.	Strand elongation	1 min / kb	72 °C
5.	Final elongation	5 min	72 °C

Steps 2-5 were run in 30-40 cycles

2.2.3 Hydrolysis of nucleic acids via endonucleases

For restriction site-based cloning, fragments were digested with conventional restriction enzymes (Thermo Fisher Scientific, Waltham, USA). For analytical purposes, for example to identify DNA fragments based on their fragmentation pattern after hydrolysis, FastDigest enzymes from the same company were used. The following reaction mixture was used irrespective of the enzymes used. Only the reaction time was adjusted to ten minutes for FastDigest enzymes or at least three hours or overnight for conventional enzymes. The 10x buffers depended on the enzymes used in the reaction.

20 µl DNA hydrolysis reaction	
2 µl	10x buffer (appropriate buffer, supplied with the enzyme)
1 µl	per restriction enzyme
2-10 µl	DNA (200 – 1000 µg)

2.2.4 Nucleic acid separation in agarose gels

In order to separate DNA fragments according to their molecular weight, 20 µl DNA samples were supplemented with 4 µl of 6x DNA loading buffer and mixed. 18 µl of this mix were loaded on an agarose gel and run at 100 V for 20 – 30 minutes. Agarose gels were composed of 0.5 – 2 % of agarose, depending on the size of the nucleic acids that should be separated, melted in TAE buffer, cooled to approximately 50 °C and supplemented with ~0.0001 % (w/v) ethidium bromide. After the run, the gels were subjected to 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)	Bromphenol blue

2.2.5 Extraction of DNA fragments from agarose gels

DNA fragments were recovered from agarose gels by cutting them out with a clean scalpel with a minimum amount of surrounding agarose. The DNA extraction from the gel fragment was carried out with the Invisorb Spin DNA Extraction Kit (Stratec Biomedical AG, Birkenfeld, Germany) and according to the manufacturer's instructions.

2.2.6 Ligation of DNA fragments

DNA fragments can be covalently recombined with the help of DNA Ligase enzymes. All ligation reactions were carried out overnight at 22 °C with the T4 DNA Ligase from Thermo Fisher Scientific (Waltham, USA) kit in a volume of 20 µl.

20 µl ligation reaction	
2 µl	10x T4 DNA Ligase Buffer
100 ng	Linear vector DNA
var.	Insert DNA (5:1 molar ratio over vector)
1 µl	T4 DNA Ligase (5 Weiss Units)

2.2.7 Gateway cloning

Gateway cloning is a sequence specific recombination system (Katzen, 2007) which is used to exchange DNA fragments between two plasmids. The two DNA fragments are located between inverse attachment sites (*att*L1 and *att*L2 in the entry plasmid, *att*R1 and *att*R2 in the destination plasmid). During the Gateway cloning process, the *att*L1 and *att*R1 and the *att*L2 and *att*R2 sites of both plasmids are attached. The LR-Clonase reaction exchanges the DNA fragments flanked by these attachment sites between the two plasmids. As a result, the insert from the entry plasmid is transferred into the destination plasmid. All LR-Clonase reactions were carried out with the Gateway LR-Clonase II Enzyme mix from Thermo Fisher Scientific (Waltham, USA) at 25 °C overnight.

6 µl LR-Clonase reaction	
50 ng	Entry vector
75 ng	Destination vector
1 µl	LR-Clonase
ad 6 µl	TE buffer, pH 8.0

2.2.8 Precipitation of nucleic acids

To increase DNA concentration, remove proteins or to replace buffers, RNA or DNA solutions were mixed with 0.1 vol of 3 M sodium acetate (pH 5.2) and 2.5 vol of 100 % ethanol and incubated at -80 °C for at least one hour (or ideally overnight). The nucleic acids were precipitated at 16.000 x g and 4 °C for 15 minutes. The supernatant was discarded, and the precipitate was washed in 700 µl of 70 % (v/v) ethanol. After centrifugation at 16.000 x g and 4 °C for ten minutes, the supernatant was removed. The pellet was air dried and then dissolved in an appropriate amount of water or buffer.

2.2.9 Transformation of plasmid DNA into bacterial cells

2.2.9.1 Transformation of plasmid DNA into *E. coli* cells

For the production of chemically competent *E. coli* cells, 100 ml of LB medium were inoculated with a fresh overnight culture of the appropriate strain and grown to an OD_{450}=0.6. The suspension was cooled on ice for 45 minutes and then pelleted at 5,000 x g and 4 °C for ten minutes. The cells were washed in 20 ml of ice cold 0.1 M $MgCl_2$ and pelleted again for five minutes. The cells were resuspended in 20 ml of ice cold 0.1M $CaCl_2$ and gently shaken horizontally on ice for 30 minutes. Finally, the cells were pelleted again, dissolved in a volume of 10 ml (0.086 M $CaCl_2$ and 14 % (v/v) ml glycerol) and aliquots of 100 µl were frozen in liquid nitrogen and stored at -80 °C until use.

For cloning, plasmids were transformed into Top10 cells (Thermo Fisher Scientific, Waltham, USA). Overexpression of genes was conducted in BL21(DE3)pLysS cells (Thermo Fisher Scientific, Waltham, USA). The transformation was prepared by the addition of 2 ng of clean plasmid or of 10 µl of a fresh ligation reaction to 100 µl of chemically competent cells on ice. The mix was incubated on ice for 30 minutes and subsequently heat shocked at 42 °C for 45 seconds. After a 2-minute incubation on ice, 400 µl of SOC medium were added and the cells were shaken at 180 rpm and 37 °C in a rocking incubator. After a brief centrifugation at 5,000 x g for one minute, all but approximately 100 µl of the supernatant was removed. The cells were re-suspended in the remaining 100 µl and 10 µl were plated on one plate and 90 µl were plated on another plate. Plates contained appropriate antibiotics for selection.

SOC medium

0.6 % (w/v)	NaCl
2.0 % (w/v)	Peptone
0.5 % (w/v)	Yeast extract
2.7 mM	KCl
10 mM	$MgSO_4$
10 mM	$MgCl_2$
20 mM	Glucose

The pH was adjusted to 7.0 with KOH

2.2.9.2 Transformation of plasmid DNA into *Agrobacterium tumefaciens* cells

100 µl aliquots of chemically competent cells were thawed on ice and 1 µg of plasmid DNA was added. After gently mixing, the cells were incubated on ice, shock-frozen in liquid nitrogen and thawed at 37 °C. Each step was carried out for five minutes. 800 µl of LB medium were added and the cells were shaken at 180 rpm and 28 °C for four hours. After sedimentation of the cells at 3,000 x g for two minutes the pellet was re-suspended in 100 µl of LB medium and plated on YEP plates containing

25 µg/ml gentamycin, 25 µg/ml rifampicin and 34 µg/ml chloramphenicol. Clones were picked after two days of incubation at 28 °C.

2.2.10 Transformation of plasmid DNA into *Arabidopsis thaliana*

A. thaliana plants were transformed with the help of previously transformed *Agrobacterium tumefaciens* cultures. For each transformation, several pots with 4 Arabidopsis Col-0 seedlings per pot were grown until the inflorescences had reached a height of approximately 10 cm and the plants had formed plenty of not yet opened buds.

A. tumefaciens GV3101 (pMP90) cells that had been transformed with appropriate plasmids were grown over night at 28 °C in 5 ml of LB medium containing 25 µg/ml gentamycin, 25 µg/ml rifampicin and 34 µg/ml chloramphenicol. 1 ml of overnight culture was used to inoculate 250 ml of YEP medium and cells were cultivated under the same conditions for 16-24 hours. The cells were harvested at 5,500 x g for ten minutes. The pellet was re-suspended in 5 % (w/v) sucrose and adjusted to a final OD_{600} of 0.8. Additionally, 0.02 % (v/v) of Silvet L-77 were added to the cell suspension to facilitate infection. Developing siliques were cut from the plants and inflorescences were dipped into the agrobacteria solution for one minute with gentle agitation. Dipped plants were laid horizontally into plant growth trays that were bedded with wet paper towels and covered with a lid. After incubation on the bench overnight, plants were put in an upright position and normally grown to produce seeds for harvesting.

2.3 Transcript analysis

2.3.1 Extraction of total RNA from plant material

For the extraction of RNA from plant material, plant samples were ground to a fine powder in liquid nitrogen. A small portion of plant powder (~50 – 100 mg) was transferred to a 2 ml tube. Until the first contact of the sample with the first extraction buffer of the kit, the plant material was always kept frozen to prevent RNA degradation. The extraction was carried out with the Universal RNA / miRNA Purification Kit from roboklon (Berlin, Germany) according to the manufacturer's instructions, including the optional DNaseI digestion. Freshly extracted RNA was immediately used for quality control and cDNA synthesis and subsequently stored at -80 °C.

2.3.2 Quantification of transcript abundances

2.3.2.1 Transcriptome analysis via RNA sequencing (RNAseq)

For transcriptome analysis, total RNA was extracted from frozen ground plant material omitting the on-column DNase digestion. RNA integrity was tested on a 1 % (w/v) 3-(N-morpholino) propanesulfonic acid (MOPS) agarose gel. RNA samples were treated with 2 U of DNase I (Thermo Fisher Scientific, Waltham, USA) for 20 minutes and precipitated overnight. Re-dissolved RNA was analysed on an agarose gel again and intact samples were stored at -80 °C until they were shipped with dry ice.

The synthesis of the cDNA library, the RNAseq, the transcript annotation and the basic bioinformatic analysis were carried out by BGI (Beijing, China) with a BGISEQ-500 system. For the synthesis of the cDNA library, BGI followed a protocol in which messenger RNA was enriched, and reverse transcribed with random N6 primers. The second strand was synthesized, and adaptors were ligated to end-repaired double-stranded cDNAs that enabled cyclization of heat-denatured single-stranded cDNAs, representing the cDNA library. Per sample, 24,136,983 raw reads or 4,057,386 clean reads were generated on average. For data evaluation, only genes with a raw count of at least 20 reads were considered. Genes were considered differentially regulated if they were up- or down regulated at least two-fold relative to a reference sample (for example the wild type).

MOPS buffer

200 mM	MOPS
10 mM	Sodium acetate
10 mM	EDTA

The pH was adjusted to 7.0

2.3.2.2 Quantitative real-time polymerase chain reaction (RT-qPCR)

Transcript abundances were determined using RT-qPCR and the $2^{-\Delta ct}$ or $2^{-\Delta\Delta ct}$ evaluation methods (Livak and Schmittgen, 2001). All transcript data determined by RT-qPCR are relative to the geometric mean of transcript levels of the reference genes *ACTIN 7* (*ACT7*, AT5G09810) and *YELLOW-LEAF-SPECIFIC GENE 8* (*YLS8*, AT5G08290). RT-qPCR experiments were conducted with the C1000 Thermal Cycler equipped with a CFX96 Real-Time System (Bio-Rad, Hercules, USA) and the CFX Manager 3.1 software (Bio-Rad, Hercules, USA).

PCR program

1.	Pre-heating	5 min	94 °C
2.	Strand separation	15 sec	94 °C
3.	Primer annealing	30 sec	60 °C
4.	Strand elongation	30 sec	72 °C

Steps 2-4 were run in 40 cycles followed by the melting curve

qRT-PCR master mix

2.00 µl	10x qRT-PCR buffer
0.80 µl	50 mM $MgCl_2$
0.40 µl	dNTPs (5 mM, each)
0.20 µl	1x SYBR green (Sigma-Aldrich, St. Louis, USA)
0.04 µl	Opti-Taq polymerase (5 U/µl)
0.12 µl	Primer mix (50 µM, each)
13.44 µl	Water

qRT-PCR 10x buffer

160 mM	Ammonium sulfate
1 M	Tris HCl, pH 8.3
0.10 % (v/v)	Tween-20

2.4 Protein analysis

2.4.1 Protein extraction from plant material

For extraction of proteins from plant material, plant samples were ground to a fine powder in liquid nitrogen. Some of the powder was filled into a 2 ml tube until it reached the 0.5 ml mark. 500 µl of protein extraction buffer were added and thoroughly vortexed. Samples were heated at 95 °C for ten minutes and allowed to cool back down to room temperate. After centrifugation at 16,000 x g for ten minutes, the supernatant containing the proteins was transferred to a new tube. Protein samples were placed on ice for subsequent use or stored at -20 °C.

Protein extraction buffer

62.5 mM	Tris HCl, pH 6.8
10 % (v/v)	Glycerol
1 % (w/v)	SDS
5 % (v/v)	β-mercaptoethanol (freshly added every time)

2.4.2 Extraction of nuclei from plant material

Following a protocol modified after Kinkema *et al*. (2000), approximately 1.5 g of plant material were ground to a fine powder in liquid nitrogen and subsequently mixed with 3 ml of Honda buffer. The mix was filtered through a nylon mesh with 62 µm pores. The samples were supplemented with triton X-100 to a final concentration of 0.5 % (v/v), briefly vortexed and incubated on ice for 15 minutes. After centrifugation at 1,500 x g and 4 °C for five minutes, an aliquot of the supernatant was saved and stored as a nuclei depleted sample and the remaining supernatant was discarded. The pellet was re-suspended in 2.5 ml of Honda buffer containing 0.5 % (v/v) of triton X-100. After the previous centrifugation step was repeated, the pellet was re-suspended in 2.5 ml of Honda buffer without triton X-100. During a centrifugation step at 100 x g and 4 °C for five minutes, remaining cell debris was

pelleted. The nuclei containing supernatant was transferred to a new tube and centrifuged at 2,000 x g and 4 °C for five minutes to pellet the nuclei. Nuclei were re-suspended and frozen at -20 °C in 100 µl of Honda buffer.

Honda buffer	
2.5 % (w/v)	Ficoll 400
5.0 % (w/v)	Dextran T40
0.4 M	Sucrose
25 mM	Tris HCl, pH 7.4
10 mM	$MgCl_2$
5 mM	DTT
1x	Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, USA)

2.4.3 Determination of protein concentration

Concentration of protein samples was determined using the Protein Assay (Bio-Rad, Hercules, USA) which is based on Bradford (1976). For every sample of interest, two reactions were measured containing either 2 µl or 4 µl of the sample. Water was added to the proteins to a volume of 800 µl. Then, all samples were mixed with 200 µl of Bradford reagent and incubated in the dark for 20 minutes. The absorption of the samples was determined at 595 nm and standardized based on a calibration curve with 0-11 mg BSA ml^{-1}.

2.4.4 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

For the separation of denatured proteins on SDS-polyacrylamide gels, 20 µg of proteins were mixed 5x loading buffer and heated at 95 °C for ten minutes. The samples were cooled down on ice, briefly spun down and proteins were then loaded on a 12 % polyacrylamide gel (Rotiphorese gel 30 (37.5:1), Carl Roth, Karlsruhe, Germany) overlaid with a 5 % stacking gel. For separation, the gel was run in SDS running buffer at 40 V until the samples had passed the stacking gel. Then the voltage was increased to 100 V. The run was stopped when the bromophenol front had passed the gel.

Stacking gel		Separating gel	
5 % (v/v)	Polyacrylamide	12 % (v/v)	Polyacrylamide
120 mM	Tris HCl, pH 6.8	375 mM	Tris HCl, pH 8.8
0.1 % (w/v)	SDS	0.1 % (w/v)	SDS
0.1 % (w/v)	APS	0.1 % (w/v)	APS
0.2 % (v/v)	TEMED	0.1 % (v/v)	TEMED

5x Protein loading buffer	
625 mM	Tris HCl, pH 6.8
50 % (v/v)	Glycerol
10 % (w/v)	SDS
0.025 % (w/v)	Bromphenol blue
20 % (v/v)	β-mercaptoethanol

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

2.4.5 Immunological detection of proteins (Western Blot)

The protein samples were transferred electrophoretically onto a nitrocellulose membrane (Carl Roth, Karlsruhe, Germany) according to Kyhse-Andersen (1984). The transfer was conducted for 40 minutes with the current set to 2x the surface area of the membrane in cm^2 in mA.

To control the transfer, the proteins were stained on the membrane in 0.2 % (w/v) Ponceau S in 3 % (v/v) acetic acid for five minutes. Afterwards, the membrane was rinsed several times in water until clear protein bands were visible with a weak background. After documentation, the membrane was then de-stained in water until the staining was removed.

The membrane was then blocked for at least 2 hours in 5 % (w/v) milk powder in TBST at room temperature. Subsequently, the blocking solution was replaced by the primary antibody (1:1,000 in 5 % (w/v) milk powder in TBST) and incubated at 4 °C overnight, while gently shaking. The membrane was then washed three times for five to ten minutes in TBST, shaken with the secondary antibody (1:10,000 in 5 % (w/v) milk powder in TBST) and washed as stated before. Agrisera ECL SuperBright solutions were mixed 1:1 or 1:1:2 with water. The detection solution was incubated in the dark for 20 minutes before it was evenly applied onto the membrane in a clear film. After five minutes, the membrane was drained from excess solution with a lint-free tissue and then exposed for detection in a LAS4000 CCD camera system (GE Healthcare, Chicago, USA) for one to 60 seconds until clear bands were visible with low background.

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

TBST	
10 mM	Tris HCl, pH 7.5
150 mM	NaCl
0.05 % (v/v)	Tween-20

Antibody combinations:

Primary: α-HIS-tag antibody (mouse) from Sigma-Aldrich (St. Louis, USA)
Secondary: α-mouse-HRP conjugate from Sigma-Aldrich (St. Louis, USA)

Primary: α-tAPX serum from rabbit detecting both sAPX and tAPX (described in van Buer *et al*. (2016))
Primary: α-RAP2.4c serum from rabbit immunized against heterologous expressed RAP2.4c
Primary: α-RAP2.4d serum from rabbit immunized against heterologous expressed RAP2.4d
Secondary: α-rabbit-HRP conjugate from Sigma-Aldrich (St. Louis, USA)

2.4.6 Protein overexpression in *E. coli* cells

For the overexpression of plant proteins in *E. coli*, 4 ml of LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol were inoculated with *E. coli* BL21(DE3)pLysS cells carrying the pOPINF plasmid encoding the desired protein. After incubation at 37 °C and 180 rpm overnight, this culture was used to inoculate 100 ml of pre-warmed LB medium without antibiotics, diluting the cells approximately 1:50. The cells were further cultivated under the same conditions until they had reached an OD_{600} = 0.6. To induce overexpression, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. This lactose analogue binds to the LacI repressor protein and leads to its dissociation from the operator upstream of the cDNA that was previously cloned into the pOPINF vector and thus, to its expression. After three hours of protein overexpression, the cells were harvested by centrifugation at 3,200 x g for 15 minutes. The pellets were immediately used for protein extraction.

2.4.7 Extraction of native protein from *E. coli* cells

Cell pellets of bacteria that were generated after protein overexpression in *E. coli*, were re-suspended in lysis buffer (4 ml g^{-1} cells). Lysozyme was added to a final concentration of 1 mg ml^{-1} and the cells were incubated on ice for 30 minutes. The suspension was sonicated on ice with a Sonopuls sonicator (Bandelin, Berlin, Germany) (settings: cycle 9, 80 % energy level) for six times, each time for one minute with breaks of 30 seconds in between. The lysate was centrifuged at 10,000 x g and 4 °C for 30 minutes to separate soluble and insoluble fractions.

Lysis buffer

50 mM	NaH_2PO_4
300 mM	NaCl
10 mM	Imidazole

The pH was adjusted to 8.0 with KOH

2.4.8 Re-solubilisation of inclusion bodies

Inclusion bodies were solubilized in 10 ml of IB wash buffer and centrifuged at 8,000 x g for 30 minutes. The pellet was re-dissolved in 10 ml of sterile water and centrifuged again the same way. The pellet

was re-dissolved in 5 ml of 50 mM Tris HCl (pH 8.5). This solution contained the re-solubilised overexpressed proteins and was used for His-tag purification in the next step.

IB wash buffer	
50 mM	Tris HCl, pH 8.0
5 mM	EDTA
2 % (w/v)	Sodium deoxycholate

2.4.9 His-tag purification

All proteins that were overexpressed with the pOPINF vector carried an N-terminal 6x His-tag that can be used for affinity purification of the proteins. His-tagged and re-solubilised proteins were purified with Ni-NTA-agarose (Qiagen, Venlo, The Netherlands). 500 µl of Ni-NTA-agarose were sedimented for a few seconds, the supernatant was removed, 1 ml of lysis buffer was added and briefly centrifuged again. The supernatant was removed, and the protein solution was gently agitated with the Ni-NTA-agarose at 4 °C in a 15 ml tube for one hour to facilitate binding of the proteins to the Ni ions in the matrix. The suspension was loaded on a column at 4 °C and allowed to run through by gravity. The agarose matrix was washed twice with 2 ml of wash buffer. Purified proteins were eluted four times with 250 µl of elution buffer. Highest protein concentrations are obtained in elution fractions 2 and 3.

Lysis buffer	Wash buffer	Elution buffer	
50 mM	50 mM	50 mM	NaH_2PO_4
300 mM	300 mM	300 mM	NaCl
10 mM	20 mM	250 mM	Imidazole
pH 8.0	pH 8.0	pH 8.0	Adjusted with KOH

2.4.10 De-salting of proteins

Kinase enzymes usually rely on bivalent cations such as Mg^{2+} and Mn^{2+}. High concentrations of imidazole in the elution buffer from the previous step may bind these cations and render them unavailable for enzyme reactions. Thus, the imidazole was removed from the protein solutions using the Zeba Spin Desalting Columns (7K MWCO) from Thermo Fisher Scientific (Waltham, USA) that deplete the loaded solution from molecules that are smaller than 7 kDa. The columns were loaded with the maximum volume of 130 µl of fraction 3 of the His-tag purified proteins. The de-salting was carried out according to the manufacturer's instructions.

2.4.11 Kinase assay

In a kinase assay, radioactively labelled ATP is used as a co-substrate. Radioactivity that is incorporated into the target proteins can be subsequently detected, confirming or disproving the protein as a target of the kinase.

In each reaction, 300 ng of a kinase were mixed with 300 ng of a target protein, filled to 10 µl with water and then supplemented with 10 µl of the 2x kinase reaction buffer (Lumba *et al*., 2014). All proteins were previously His-tag purified and de-salted under native conditions. Reactions were incubated at 22 °C for 20 minutes and then stopped by the addition of 5x protein loading dye and heating the samples at 95 °C for ten minutes. Samples were then separated via SDS-PAGE and gels were dried in a Model 583 Gel Dryer (Bio-Rad, Hercules, USA) in a vacuum over night while the gels were heated at 80 °C for the first 2 hours. Dried gels were exposed to BAS-MS phosphoimaging films (Fujifilm Holdings Corp., Tokyo, Japan) and visualized by autoradiography with in a Typhoon FLA 9500 phosphoimager (GE Healthcare, Chicago, USA) at maximum intensity.

2x Kinase buffer	
20 mM	$MgCl_2$
20 mM	$MnCl_2$
40 mM	Tris HCl, pH 7.5
20 µM	ATP
1 µCi	[γ^{32}P]ATP (Hartmann Analytic GmbH, Braunschweig, Germany)

2.5 Phytohormone analysis

2.5.1 Phytohormone extraction from plant material

Plant material was ground to a fine powder in liquid nitrogen. Approximately 100 mg of powder were transferred to a new tube and the precise weight was noted. Four Zirconox beads with a diameter of 2.8–3.3 mm (Mühlmeier Mahltechnik, Bärnau, Germany) were added per sample. 1 ml of extraction buffer 1 (998 µl ethyl acetate and 2 µl of the internal standard composed of deuterated hormone molecules: 10 ng μl^{-1} of salicylic acid, abscisic acid and jasmonic acid-isoleucine and 30.2 ng μl^{-1} of jasmonic acid) was added to the frozen samples and to an empty tube as a blank. All samples were thoroughly mixed in a FastPrep-24 Classic grinder (Mp Biomedicals, Santa Ana, USA) twice for 30 seconds. The samples were centrifuged at 13,000 x g and 4 °C for ten minutes and the supernatant was transferred to a new tube. A new tube was filled with extraction buffer 1 as another blank control. All samples were concentrated in a vacuum concentrator 5301 (Eppendorf, Hamburg, Germany). During the concentration of the samples, the plant material that already had been extracted once was used for a second identical extraction procedure, only this time with pure ethyl acetate lacking the

internal standard. The supernatant from the second extraction was added to the concentrated solution from the first extraction and the combined extract was further concentrated until only roughly 50 µl of a highly viscous solution were left. All samples and a new re-eluation blank tube were supplemented with 400 µl of re-eluation buffer (70 % methanol and 0.1 % formic acid) and vortexed for ten minutes. After centrifugation at 13,000 x g for ten minutes, 200 µl of the supernatant were transferred without any suspended solids into GC/HPLC vials and stored at -20 °C until analysis.

2.5.2 Phytohormone quantification

Hormone extracts were analysed using a UPLC-ESI-MS/MS Synapt G2-S HDMS (Waters, Milford, Massachusetts, USA). Hormone concentrations were calculated relative to the fresh weight that was used for hormone extraction and to the internal standards that were added to the samples during the extraction procedure (Nguyen *et al*., 2016).

2.6 Electrolyte leakage assay

Plant cells that are exposed to adverse freezing temperatures lose electrolytes from the cytosol to the surrounding medium. The extent of this loss can be interpreted as a means of freezing tolerance. The less electrolytes a cell loses at a given sub-zero temperature, the more freezing tolerant it is. Measuring the electrolyte leakage over a temperature gradient in different genotypes allows for comparison of freezing tolerance between these genotypes.

Four-week-old plants were used for the assessment of the electrolyte leakage. These plants were either used directly (naïve), or they were acclimated at 4 °C for three days (acc). Per genotype, measurements were carried out at temperatures between 0 and -18 °C (in 2 °C intervals). Each of these treatments was measured in triplicates and each of the single samples consisted of three leaves taken from three different plants. These leaves were harvested and put into glass tubes that contained 200 µl of ice-cold double distilled water (dd water) with the cut petioles covered in water. All, but the 0 °C samples were put into a “Huber P100 Process” oil bath (Peter Huber Kältemaschinenbau GmbH, Offenburg, Germany) that was pre-cooled to -1 °C and samples were incubated for 45 minutes. A bit of ice was ground to a fine powder in liquid nitrogen and small amounts of this snow were added to every sample to facilitate nucleation of the water surrounding the leaves. After 45 minutes of incubation, the oil bath was set to start a temperature gradient, reducing the temperature by 4 °C per hour. Triplicates of all genotypes were transferred from the oil bath to an ice bath at the temperatures mentioned above. All samples were left on ice in the cold room overnight. On the next day, 5 ml of dd

water were added to all samples and they were vigorously shaken at 4 °C until the next day. Avoiding organic material, 1 ml of all samples was added to 15 ml tubes containing 5 ml of dd water. The remaining water containing the leaves was heated in a boiling water bath at 100 °C for 45 minutes to destroy all cell membranes and release all electrolytes as a 100 % reference. After the samples were cooled down to room temperature, 1 ml of every sample was again transferred to 15 ml tubes containing 5 ml of dd water. Conductivity of all samples was measured with a Seven Excellence Multiparameter conductometer (Mettler-Toledo, Columbus, USA) resulting in two values per sample (leaked electrolytes and 100 % electrolytes). Values were analysed with GraphPad.

2.7 *Pseudomonas syringae* DC3000 infection assays

Infection assays allow to assess the resistance of Arabidopsis plants towards various bacteria. In this assay, cells of the coronatine secreting pathogenic bacterium *Pseudomonas syringae* (*Pst*) var. Tomato DC3000 were plated on a NYGA agar plate containing 100 µg/ml rifampicin and incubated over night at 28 °C to form a dense layer of cells. On the next day, 5 ml of 10 mM $MgCl_2$ were added to the plate and it was gently shaken for five minutes to re-suspend the cells. The same was done with an identical but sterile plate as a blank solution for a photometric measurement. Both samples were transferred to a 50 ml tube and filled to 50 ml with 10 mM $MgCl_2$. Adding more $MgCl_2$ solution, the bacteria containing sample was adjusted to an OD_{600} = 0.001 relative to the blank sample. Three leaves of similar developmental age of each plant were marked with a permanent marker and infected by infiltrating the leaves with a syringe through the stomata. The syringe was placed on the abaxial side of the leaves while applying gentle counter pressure with a finger from the top. The leaves were infiltrated until the apoplast was infiltrated with the bacterial suspension. The plants were then grown under standard conditions in a separate chamber for three days. Leaf discs with a radius of 0.25 cm were cut out of the infected leaves with a cork borer and all three discs from a single plant were transferred into 1 ml of 10 mM $MgCl_2$ containing 0.01 % (v/v) Silvet L-77. The discs were then shaken at 300 rpm and 28 °C for two hours to extract the bacteria from the leaves. Every sample was then serially diluted 1:10 for six times in 10 mM $MgCl_2$. 15 µl of each dilution were plated on a NYGA plate containing 100 µg/ml rifampicin. The plates were dried and subsequently incubated at 28 °C for two days. After growth, cfu were counted and cfu/cm² leaf were calculated.

NYGA agar

5 g	Peptone
3 g	Yeast extract
2 % (v/v)	Glycerol
1.5 % (w/v)	Agar

2.8 Yeast methods

2.8.1 Transformation of plasmid DNA into Saccharomyces cerevisiae

The transformation protocol was adopted from Gietz and Schiestl (2007a). *S. cerevisiae* Y187 cells were plated on YPAD agar plates and grown at 28 °C for two to three days until colonies were visible. A single colony was picked, re-suspended in 5 ml of liquid YPAD medium and shaken overnight at 180 rpm and 28 °C. The OD_{600} was determined in a 1:100 dilution ($1x10^6$ cells ml^{-1} give an OD_{600} = 0.1 in the dilution), 2.5 x 10^8 cells were added to 50 ml of pre-warmed YPAD medium and incubated for approximately four hours until the suspension contained at least 2 x 10^7 cells ml^{-1}. The cells were harvested at 3,000 x g for five minutes and resuspended in 25 ml of sterile water. Centrifugation was repeated, the cells were resuspended in 25 ml of sterile water again, sedimented again and resuspended in 1 ml of sterile water. The cells were transferred into a fresh 1.5 ml tube, centrifuged at 13,000 x g for 30 seconds and divided into a number of aliquots equal to the number of planned transformations (max. 10). The aliquots were pelleted again at 13,000 x g for 30 seconds and the supernatants were removed. During the washing steps, carrier DNA (2 mg ml^{-1} salmon sperm DNA) was heated at 100 °C for five minutes and then stored on ice. 360 µl of transformation mix containing the plasmid DNA (see below) were added to the cells and carefully mixed. The cells were incubated at 42 °C for 40 minutes and pelleted at 13,000 x g for 30 seconds. The pellets were re-suspended in 1 ml of sterile water and aliquots of 20 µl and 200 µl were plated on SD plates lacking specific amino acids.

Transformation mix

33 % (w/v)	PEG 3350
0.1 M	LiAc
0.1 mg	Salmon sperm DNA
500 µg	Plasmid DNA

2.8.2 Yeast-Two-Hybrid assay

Single colonies of *Saccharomyces cerevisiae* Y187 cells that contained two plasmids, each one encoding one of the putative interaction partner proteins fused to either of the two fragments of the yeast GAL4 transcriptional activator and a selective marker gene, were dissolved in 100 µl of sterile water. 15 µl of these suspensions were dripped on SD-LTH plates containing 0, 1, 2.5, 5 or 10 mM 3-AT. Successful interaction of both fusion proteins brings the two GAL4 fragments in close proximity, which enables it to induce expression of the *HIS3* gene that allows the yeast cells to survive on selective medium lacking histidine (H). 3-AT was added to the medium to suppress leaky *HIS3* expression discriminating cell growth due to leaky expression from protein-protein interaction-triggered expression. These plates

were incubated at 30 °C for several days until clear growth of the yeast cells could be observed that allowed for comparison of the different protein combinations.

2.8.3 Inverse Yeast-One-Hybrid assay

The inverse Yeast-One-Hybrid assay was conducted following the protocol established by (Bulcha, 2013).

2.8.3.1 Transformation of library scale plasmid DNA into Saccharomyces cerevisiae

For the library scale plasmid transformation into yeast cells, the protocol developed by (Gietz and Schiestl, 2007b) was applied. Yeast cells that had previously been transformed with the RAP2.4d_pACT2 construct were grown overnight in SD-L medium. Cell density was determined spectrophotometrically as described in chapter 2.8.1 and the volume containing 6.25 x 10^8 cells was harvested at 3,000 x g for five minutes. The pellet was re-suspended in an appropriate volume of warm SD-L medium, resulting in a cell density of x 10^6 cells per ml. The cells were incubated for four to five hours and harvested by centrifugation. The cells were re-suspended in 360 µl transformation mix and incubated at 42 °C for two hours. After another centrifugation step, the cells were re-suspended in 20 ml of sterile water and plated on SD-LT plates of 150 mm in diameter.

Transformation mix

33.3 % (w/v)	PEG 3350
0.1 mM	LiAc
0.28 mg	Salmon sperm DNA
60 µg	Plasmid DNA

2.8.3.2 Analysis of the interacting clones

The interaction assay was carried out by plating yeast clones that contained a prey and a bait plasmid on selective SD-LTH plates containing 0 mM, 60 mM, 100 mM, 160 mM, 200 mM, 250 mM and 300 mM 3-amino-1,2,4-triazole (3-AT). The cells were incubated at 30 °C for five days to allow the cells to grow. Colonies that had grown on agar plates at higher 3-AT concentrations indicated positive interactions. Plasmid DNA was extracted from these clones with the Easy Yeast Plamsid Isolation Kit (Clontech, Mountain View, USA) according to the manufacturer's instructions and sent to GATC Biotech (Konstanz, Germany) for sequencing of the inserts, to identify interacting DNA fragments.

3 Results and discussion

3.1 Functions of RAP2.4c and RAP2.4d in the cold

Previous studies have indicated various functions of the two transcription factors RAP2.4c and RAP2.4d. Under standard growth conditions, both *rap2.4c* and *rap2.4d* KO plants exhibit reduced transcript levels of the plastid localised *ASCORBATE PEROXIDASE* (*APX*) *sAPX* (stromal version) and *tAPX* (thylakoid-bound version) (Rudnik *et al*., 2017). In other experiments using *RAP2.4c* and *RAP2.4d* overexpressing lines that had been transferred to 4 °C, it was shown that the cold-induced upregulation of *COLD REGULATED* (*COR*) genes, such as *COR47* and *COR15a* was reduced compared to the response in wild type plants, indicating a role as negative regulators of the cold response. These results were the basis to further investigate the functions of RAP2.4c and RAP2.4d in the cold and in cold priming.

3.1.1 Analysis of the freezing tolerance of *rap2.4c* and *rap2.4d*

Previous studies have focused on effects on *COR* transcript levels in the *rap2.4c* and *rap2.4d* KO lines in the cold (Bulcha, 2013). Additionally, both transcription factors have been demonstrated to regulate expression of plastid *APX* genes that are involved in the cold response (Prasad *et al*., 1994; Thomashow, 1999; Rudnik *et al*., 2017), but their impact on the cold tolerance has not been tested. To fill this gap and to analyse the capacity of both T-DNA KO lines to tolerate freezing stress before and after acclimation to 4 °C, an electrolyte leakage assay was carried out (**Figure 2**). Exposition of leaves from naïve (non-acclimated) plants to a freezing stimulus typically leads to electrolyte leakage from the leaves. Cold-acclimated plants have an altered composition of membranes resulting in adapted membrane fluidity and in reduced electrolyte leakage. Electrolyte leakage data of naïve and acclimated plants show to what extent freezing tolerance is activated and how efficient they have acclimated to 4 °C during three days of acclimation.

Compared with Col-0, *rap2.4c* plants exhibited improved cold acclimation with an unchanged direct cold response and *rap2.4d* plants had an enhanced direct cold response, while cold acclimation was wild type-like. These results indicate that RAP2.4c is involved in the regulation of cold acclimation. On the contrary, RAP2.4d affects the direct cold response. The results are consistent with the previous studies in which both RAP transcription factors were described as negative regulators of the cold response (Bulcha, 2013; Rudnik *et al*., 2017).

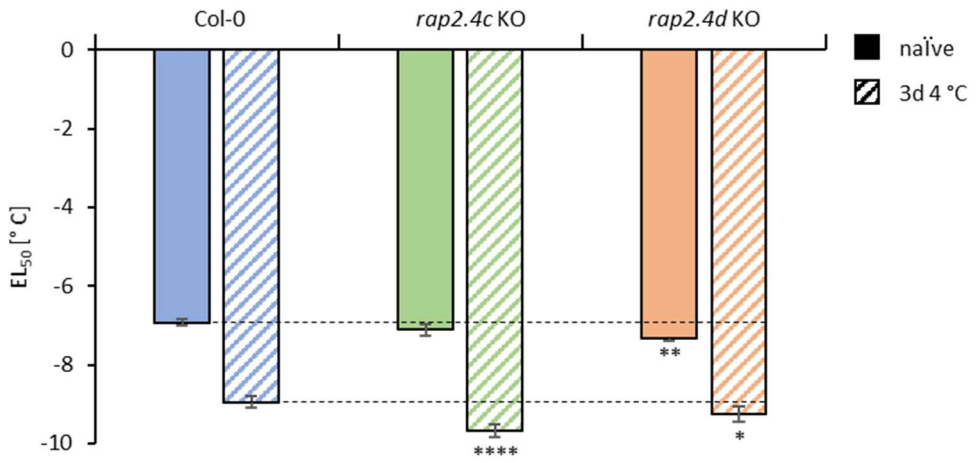


Figure 2: Freezing tolerance of Col-0, *rap2.4c* and *rap2.4d* determined by electrolyte leakage.
Four-week-old plants were either used for electrolyte leakage directly (naïve) or after a three-day cold acclimation pre-treatment at 4 °C. The EL_{50} represents the temperature at which 50 % of the electrolytes are released from the leaves. n=3±DS, Tukey's multiple comparisons test KO line vs. Col-0 in the same treatment, $p<0.05$.

The electrolyte leakage results indicated that RAP2.4c and RAP2.4d function in cold tolerance regulation and/or inhibition of regulatory processes leading to freezing tolerance. Many *COR* genes are regulated via the ICE1-CBF-COR pathway and facilitate direct cold responses and long-term cold acclimatory responses (reviewed by Knight and Knight (2012)). INDUCER OF CBF EXPRESSION 1 (ICE1) is a constitutively expressed transcription factor that is activated at low temperatures and leads to the expression of the cold master regulator genes *C-REPEAT BINDING FACTOR* (*CBF*) 1-3. Expression of these genes peaks after three hours in the cold and induces the expression of their *COLD REGULATED* (*COR*) target genes (Fowler *et al.*, 2005). To test if modulations of the ICE1-CBF-COR pathway are responsible for the altered electrolyte leakage, the expression of selected *COR* genes and of their upstream regulators, the *CBF* transcription factors, was analysed in the cold in four-week-old Col-0, *rap2.4c* KO and *rap2.4d* KO plants. Samples were taken before the start of acclimation and after two and three days to observe differences in *CBF* and *COR* gene expression during acclimation (**Figure 3**).

COR15a, *COR47*, *CBF1* and *CBF3* showed a strong induction of gene expression in the cold with variable absolute expression between the experiments. No significant differences in gene expression could be observed between Col-0 and both KO lines. Expression of central regulatory genes (*CBF*) and of genes directly affecting the cold response and cold acclimation (*COR*) were very similar between the genotypes and do not obviously explain the significant differences in freezing tolerance and cold acclimation that were shown in the electrolyte leakage experiment (**Figure 2**).

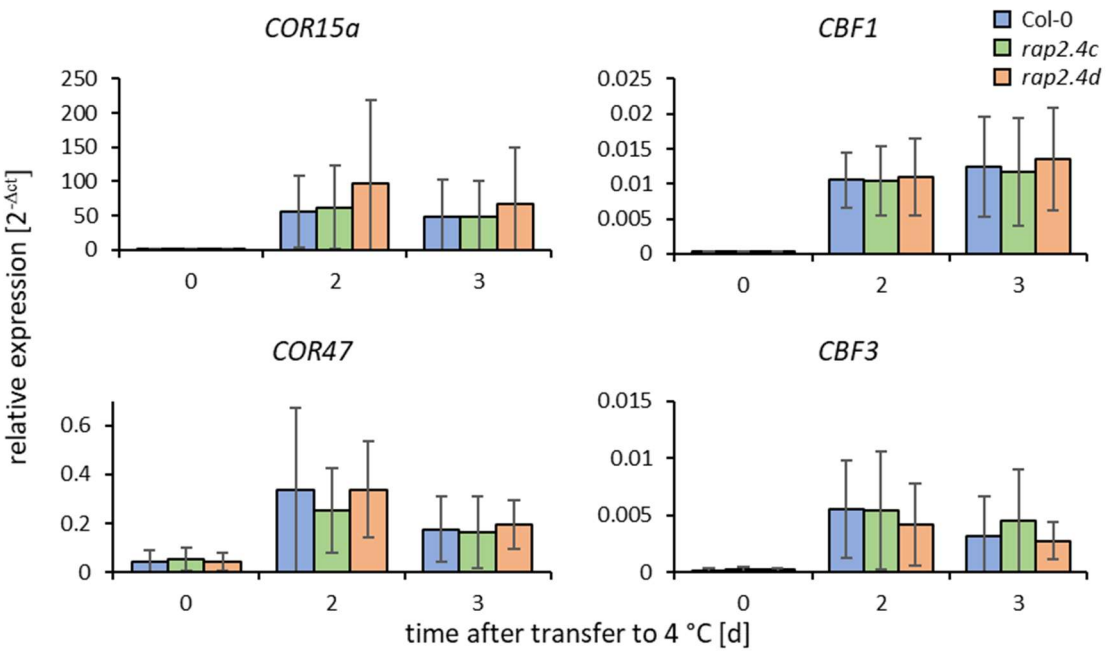


Figure 3: Relative expression of cold related genes determined by RT-qPCR.
Four-week-old Col-0, *rap2.4c* and *rap2.4d* KO plants were transferred to 4 °C and harvested before the transfer and after two and three days of acclimation to 4 °C. n=3±SD, student's t-test KO line vs. Col-0 at the same time, $p<0.05$.

3.1.2 Analysis of the RAP2.4d DNA binding motif

As demonstrated by Rae *et al*. (2011), RAP2.4d (there assigned as RAP2.4B) binds to the Dehydration-Responsive Element (DRE) sequence (CCGAC) in an electrophoretic mobility shift assay (EMSA). This is a motif typical for transcription factors from the DREB group within the AP2/ERF super family of transcription factors (Sakuma *et al*., 2002). RAP2.4c and RAP2.4d belong to the sub-family AP2/ERF-Ib (Nakano *et al*., 2006). *In vitro* analysis of DNA binding properties of transcription factors are useful tools, but *in vivo* binding is more complex, as interacting proteins may alter binding affinities. Jote Tafese Bulcha (Bulcha, 2013) conducted assays with RAP2.4a and RAP2.4c (there called RAP2.4d) and proposed CCG(A/G)C for RAP2.4a and G(A/T/C)GGCG and AGGC(C/G) for RAP2.4c as target motifs based on an inverse Yeast-One-Hybrid assay approach. The proposed RAP2.4a motif matched the DRE-motif, while the RAP2.4c motif was similarly GC-rich but deviated from the DRE-sequence. The same protocol was used to determine the DNA-binding motif of RAP2.4d in the present study.

For this purpose, the library of sheared Arabidopsis gDNA fragments of approximately 200 – 300 bp that was generated by Bulcha (2013), was used in the present study, representing the prey DNA library that was tested for interaction with RAP2.4d. The bait plasmid was generated by the insertion of the full-length coding sequence of *RAP2.4d* into the pACT2 vector. The *RAP2.4d* coding sequence was amplified by PCR using gene specific primers (forward: GAATTCCTAATTTACAAGACTCGAACACTGAAG

and reverse: GGATCCGAACAACTTCTATGGATTTTTACAGTAACAA), that added the restriction enzyme recognition sites of EcoRI and BamHI, respectively. The *RAP2.4d* PCR product was ligated into the pACT2 vector and transformed into *E. coli* Top10 cells. Sequencing confirmed the accuracy of the resulting construct.

A

B

Name	Start	*p*-value	Sites
AT1G35490	1230	1.22e-11	AATAAATCGT CCCGCGGTATACCGC GGATTAAAAT
AT1G32420	1007	1.22e-11	TAGATTTTAA CCCGCGGTATACCGC GGAACGATTT
AT1G60980	1007	1.22e-11	TAGATTTTAA CCCGCGGTATACCGC GGGACGATTT
AT1G44960	271	2.44e-11	TAGGATTTAA CCCGCGGTTTACCGC GGGCAATCGG
AT1G75000	944	3.37e-10	AATAAATAGT CCCGCGGTATATCGC AGGTTAAAAT
AT1G62240	250	6.38e-10	TAGATATTAA CCTGCAGTATACCGC AAGTACCCTT
AT1G22930	46	6.38e-10	TAGATATTAA CCTGCAGTATACCGC ATGTAGGCTT
AT1G61330	220	2.08e-9	TATAAGCCTA CCTGCGGTATACTGC AAGTTAATAT
AT2G31280	266	2.08e-9	TATAAGCCTA CCTGCGGTATACTGC AAATTAATCT
AT1G51990	175	2.64e-9	TAGATATTAA CCTGCTGTATACCGC AGGTAGACTT
AT1G69580	391	6.67e-9	TAGATATTAA CCTGTAGTATACCGC ATGTAAGCTT
AT1G35400	194	5.30e-8	TATAAGCATA CCTACGGTATACTGC AGATTAATCT
AT2G01023	1040	6.28e-8	TCCGTGAACA CCTGCACTTTCCCGC CACAAAGATA
AT3G41762	687	2.19e-7	TACACCCATG CCCGCGGCTTCTCCT TCGTGGGGAA
AT2G01010	827	2.19e-7	TACACCTTTG CCCGCGGCTTCTCCT TCGTGGGGAA
AT1G53590	1387	2.37e-7	TCGTATTTGT ACCGCGGTATTTCCC ACCATATTAT
AT3G46710	1018	5.45e-7	AAAAGCCTTT CCTACTGTATACCAC AAACATGAGG
AT3G52420	87	5.80e-7	AAAACATAAA CCCGTGATTTTCCCG CCAAAAACGT
AT3G29810	134	5.80e-7	AAAACGTAAA CCCGTGATTTTCCCG CCAAAAACGT
AT1G32460	1151	6.17e-7	AAGAGTTTCA CCTGTAGTATACCAT TTTGTATCAA
AT4G13930	154	1.74e-6	GGAGGACAAA TCCGTAATTTACCCC ATTTTCTACT

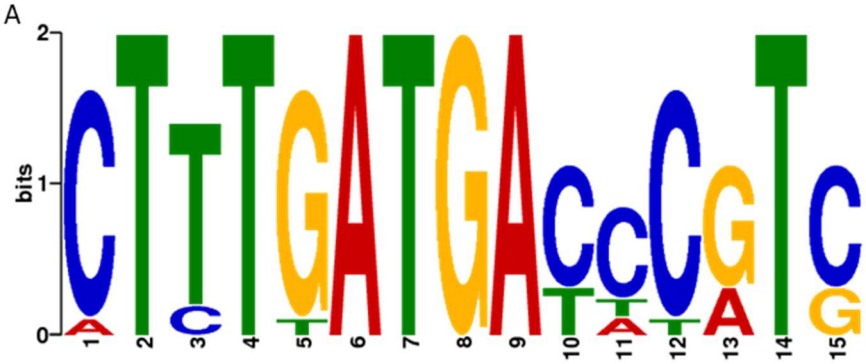
Figure 4: First putative DNA binding motif of RAP2.4d.
Sequenced interacting clones from an inverse Yeast-One-Hybrid assay were analysed for a common motif with MEME2.0. **A:** The highest scoring result is shown which had an E-value of 9.8E-25. **B:** The list names the genes, promoter positions and sequence hits that share the common motif.

The resulting sequences were blasted against the *Arabidopsis thaliana* genome using the NCBI Nucleotide BLAST function and only the 56 sequences that were within 3000 bp upstream of a coding gene were collected. These 56 sequences were subjected to the MEME motif finder (Multiple EM for Motif Elicitation) searching for overrepresented motifs. Searching for motifs with a length of only six

to ten bp yielded no motifs with an E-value below 0.05. Thus, the search was stepwise extended to 15 bp and two candidate motifs were identified (**Figure 4** and **Figure 5**).

The first motif (**Figure 4**) was found in 21 out of the 56 interacting promoter sequences and the second motif was identified in 14 sequences. Both motifs contain sequences that resemble the classical CCGAC DRE-motif with some variation in single base pairs, slightly varying between the target genes. The first motif starts with a GC-rich region that resembles the DRE-element. The TATA sequence at bases eight to eleven is flanked by two regions that have reverse-complement sequences to each other, that might form loops under certain conditions and that may modify binding of RAP2.4d. These reverse-complement sequences extend into the next two bp (GG) of the top four target sequences (**Figure 4B**). No such structure was found in the second motif. However, it contains a DRE-like motif at the 3' end which reads CCGTC instead of CCGAC.

A



B

Name	Start	*p*-value	Sites
AT1G44960	210	1.59e-10	TTTAGGAAAT CTTTGATGACCCGTC TTTAAATCAA
AT1G35400	141	1.59e-10	TTTAGGAAAT CTTTGATGACCCGTC TTTAGATCCA
AT1G22930	96	1.59e-10	TTTAGGAAAT CTTTGATGACCCGTC TTTAGATCCA
AT1G69580	341	1.59e-10	TTTAGGAAAT CTTTGATGACCCGTC TTTAGATCCA
AT2G31280	213	1.59e-10	TTTAGGAAAT CTTTGATGACCCGTC TTTAGATCCA
AT1G32460	911	1.14e-9	TTTAAAAAAT CTTTGATGATCCGTC TTTAGATCCA
AT1G35490	150	1.14e-9	TTTAGGAAAT CTTTGATGACCCATC TCTAGATCCA
AT1G53590	357	1.14e-9	TTTAAAAAAT CTTTGATGATCCGTC TTTAGATCCA
AT1G34520	222	5.22e-9	TGGCATTCTA CTTTGATGACACGTG TATTTATCAT
AT1G51990	125	2.35e-8	TTTAGGAAAT ATTTGATGACCCATC TTTAGATCCA
AT1G33940	1061	2.35e-8	TGGCACTATA CTTTGATGACACATG TACTTATCAT
AT1G62240	200	2.97e-8	TTTAGAAAAT CTTTGATGACTTGTC TTTAGATCCA
AT2G01023	1059	6.76e-8	ATACTTCAAA CTCTTATGATCCGTG AACACCTGCA
AT3G41762	172	1.09e-7	CTGCCCGTTG CTCTGATGATTCATG ATAACTCGAC

Figure 5: Second putative DNA binding motif of RAP2.4d.
Sequenced interacting clones from an inverse Yeast-One-Hybrid assay were analysed for a common motif with MEME2.0. **A:** The second highest scoring result is shown which had an E-value of 9.7E-17. **B:** The list names the genes, promoter positions and sequence hits that share the common motif.

Notably, the two lists of genes that contained the two putative motifs had an overlap of 12 genes, which left only nine promoters that were unique to the first motif and two promoters that were unique to the second motif. As both motifs contained a CCCG sequence at the 5' end of motif 1 and at the 3' end of motif 2, it was possible, that this CCCG sequence represented an overlap between both motifs.

The two motifs that were found in the 12 promoters that contained both motifs were compared for their location within the promoters. In all of the 12 cases, two distinct sequence fragments were assigned to the two motifs, establishing that both motifs represent separate genetic positions within the promoters.

The AP2 DNA binding domain that is common to all members of the RAP2.4 family is highly conserved and consist of 58 amino acids (Nakano *et al*., 2006). In these 58 amino acids, RAP2.4a and RAP2.4d have a high homology and share all but three amino acids. With nine mismatches, RAP2.4c and RAP2.4d are more divers in their AP2 domains (Rudnik *et al*., 2017). This is also reflected in their predicted DNA binding motifs that were all determined with the same method. RAP2.4a was predicted to bind to a DRE motif (CCG(A/G)C) (Bulcha, 2013) that is highly similar to the 3' end of the second motif (CCGTC) that was determined for RAP2.4d in the present study (**Figure 5**). The DNA binding motifs that were determined for RAP2.4c (G(A/T/C)GGCG and AGGC(C/G)) deviate more strongly from the classical DRE motif (Bulcha, 2013). This is in line with the calotte models that compare the 3D-structures of the AP2 domains of all RAP2.4 members (Rudnik *et al*., 2017). The loop 2 that connects the β-sheets 2 and 3 is in direct contact with the DNA (Allen *et al*., 1998). This loop is almost identical in RAP2.4a and RAP2.4d but differs more strongly in RAP2.4c, supporting its deviating DNA binding motif. Particularly the R → K substitution in RAP2.4c (K90) that lies in the loop 2 may influence DNA binding capabilities, as lysine (K) it is less bulky than the arginine (R) and has less potential for the formation of hydrogen bonds (Rudnik *et al*., 2017).

The promoter sequences of the cold-regulated genes *COR15a* and *COR47* that were shown to be inhibited by RAP2.4c and RAP2.4d (Bulcha, 2013) both contain the predicted DNA binding motifs of RAP2.4c and of RAP2.4d. The CCGTC (RAP2.4d, -505 bp) and several variants of the predicted RAP2.4c motifs are located in the *COR15a* promoter and the CCCGT (RAP2.4d, -990 bp) and variants of the GGCC motif (RAP2.4c) can be found in the *COR47* promoter. Furthermore, the promoters of *sAPX* and *tAPX* also contain these motifs. The *tAPX* promoter, to which RAP2.4c and RAP2.4d bind in a Yeast-one-Hybrid assay (Rudnik *et al*., 2017), has two CCCGT (-692 and -199 bp) and an AGGCG sequence (-23 bp) that were predicted for RAP2.4d and RAP2.4c, respectively. The *sAPX* promoter contains a CCCGTC

(RAP2.4d, -597 bp) and a GAGGCG (RAP2.4c, -14 bp) sequence that may facilitate binding of the two transcription factors.

3.1.3 Transcriptome analysis of Col-0, *rap2.4c* and *rap2.4d* in the early cold response

RAP2.4c and RAP2.4d appear to take part in the regulation of the cold response (**Figure 2**). Furthermore, an *in vitro* DNA binding motif was published for RAP2.4d (Rae *et al*., 2011) and putative *in vivo* motifs for RAP2.4c and RAP2.4d were determined by Bulcha (2013) and in the present study (**Figure 4** and **Figure 5**), but little was known about their target genes and pathways they are involved in.

Publicly available gene expression data (eFP browser) show that expression of *RAP2.4c* and *RAP2.4d* is upregulated during the first 30 minutes or one hour of cold stress, respectively, and then declines below initial levels (**Figure 6A**) (Kilian *et al*., 2007). These data were obtained with 18-day-old Col-0 plants that were grown on MS-agar under long day conditions. To test expression of both genes under the conditions used in the present study, four-week-old Col-0 plants that were grown on soil in a short day regime, were transferred to 4 °C and expression was analysed by RT-qPCR (**Figure 6B**). Both genes were upregulated quickly in the cold, while the effect was less intense compared with the eFP browser data. Especially, *RAP2.4c* expression was induced only 1.3-fold after 15 minutes and then declined below control levels, demonstrating rapid upregulation and degradation of the transcript. The expression of *RAP2.4d* peaked already after 30 minutes at a two-fold induction instead of after one hour and revealed another, stronger peak after twelve hours, which was absent in the eFP browser data. While induction intensities were lower under the conditions used in the present study, both data sets revealed induction of both genes quickly upon transfer to 4 °C and a subsequent decline below control levels.

To gain deeper insights into the regulatory function of RAP2.4c and RAP2.4d in cold stress, a transcriptome analysis by RNA sequencing (RNAseq) was performed. As both genes were induced within the first hour during cold stress exposure and the transcripts were rapidly degraded thereafter, both genes are likely to exert their function during this time. For this reason, the early cold response was analysed in the RNAseq experiment.

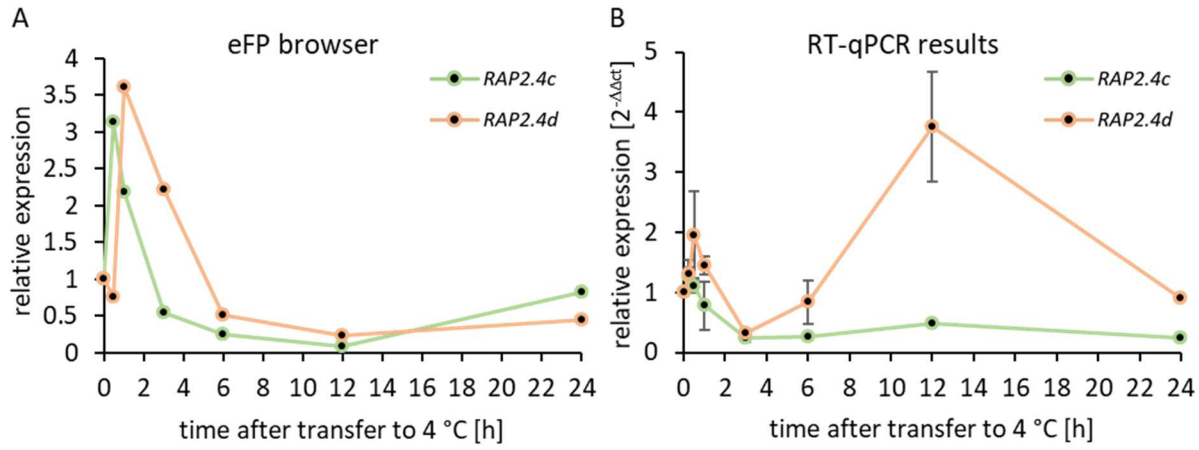


Figure 6: Relative expression of *RAP2.4c* and *RAP2.4d* at 4 °C in Col-0.
A: Publicly available data obtained from the eFP browser (Winter *et al*., 2007), data from Kilian *et al*. (2007). **B:** Four-week-old Col-0 plants were transferred to 4 °C and samples were taken according to the eFP browser data with additional 15 minutes samples. RNA was extracted and reverse-transcribed into cDNA which was analysed by RT-qPCR. n=3. All expression data are relative to the zero-hour control before the transfer to 4 °C.

For the RNAseq analysis, the wild type accession Col-0 and *rap2.4c* KO and *rap2.4d* KO plants were grown for four weeks under standard conditions and then transferred to 4 °C. Samples were taken before the transfer and after one and three hours to analyse the influence of both transcription factors on transcription shortly after the peak of their expression and two hours later when their levels are low.

3.1.3.1 Transcriptome data in unstressed plants

In the plants grown under control conditions, several genes were already differentially regulated in both KO lines relative to Col-0 (**Figure 7B**). These differentially regulated genes revealed only moderate overlap between both KO lines, indicating only partly redundant and more individual functions of both transcription factors.

Gene ontology (GO) term analysis of genes that were either up- or downregulated in either of the two KO lines supported more different functions of both transcription factors. Genes that were upregulated, revealed no significant GO terms for both KO lines. The same was true for genes that were downregulated in *rap2.4c*. However, genes that belong to several GO terms were significantly overrepresented among genes that were downregulated in unstressed *rap2.4d* plants, including the GO terms “defence response” (p-value 1.23×10^{-5}), “response to external biotic stimulus” (p-value 4.1×10^{-4}), “response to other organism” (p-value 4.1×10^{-4}), “response to biotic stimulus” (p-value 4.2×10^{-4}) and “multi-organism process” (p-value 1.31×10^{-3}). All these GO terms resemble each other

and partly contain the same genes. Taken together, a significant number of genes that are involved in the response to biotic interactions were downregulated in unstressed *rap2.4d* plants, while no such class of genes was identified for genes that were differentially regulated in unstressed *rap2.4c* plants.

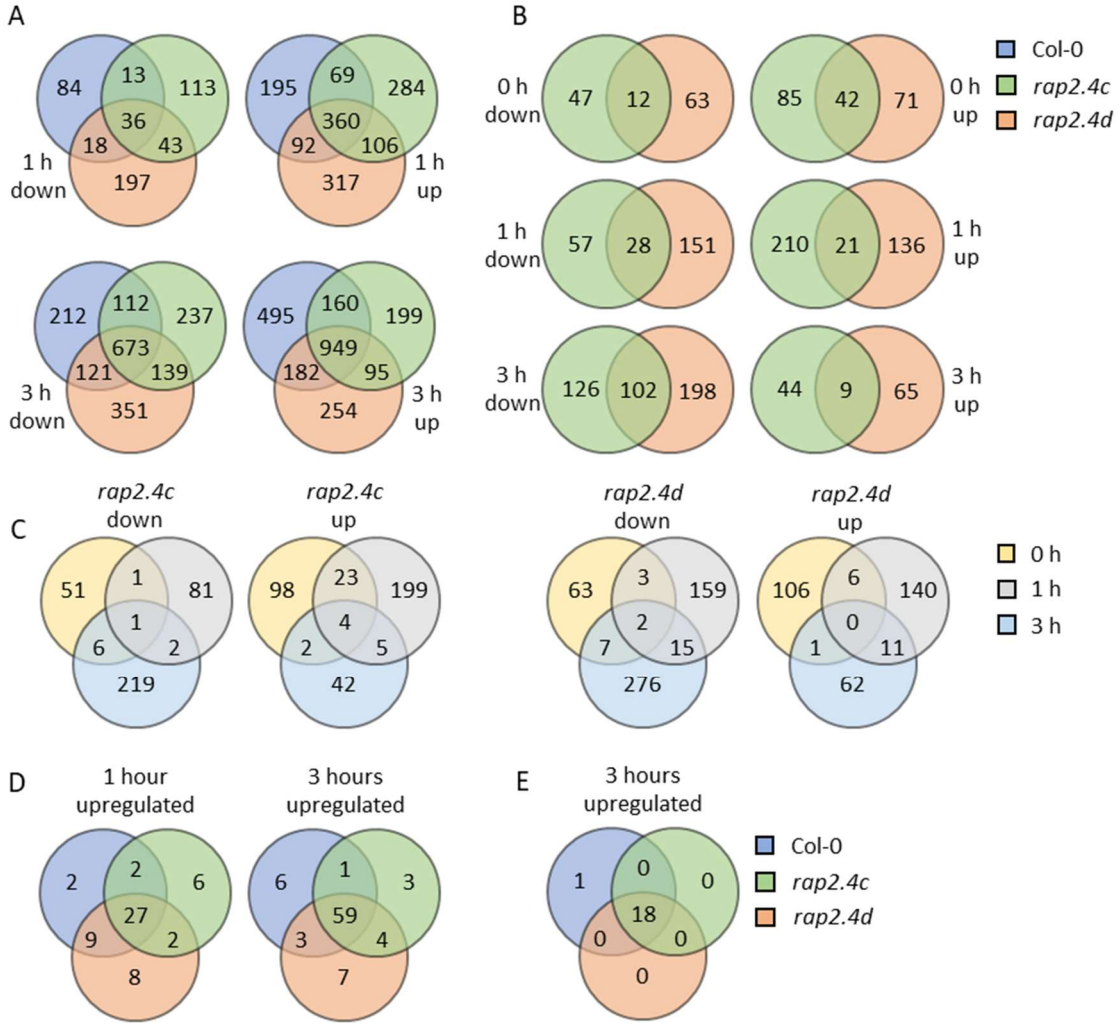


Figure 7: Venn diagrams showing differentially regulated genes in the RNAseq data.
Four-week-old Col-0, *rap2.4c* KO and *rap2.4d* KO plants were transferred to 4 °C and samples were taken before the transfer and after one and three hours. RNA was extracted and subjected to an RNAseq-based transcriptome analysis. Genes were considered differentially expressed if they had a raw count of at least 20 in one of the two compared samples and were up- or downregulated at least two-fold relative to the reference sample (Col-0 or zero hours). **A:** Comparison of genes that are differentially regulated after one or three hours at 4 °C relative to the untreated control sample within the same genotype. **B:** Comparison of genes that are differentially regulated in either of the *rap2.4* KO lines relative to the wild type before (0 h) and after one or three hours at 4 °C. **C:** Comparison of genes that are differentially regulated in either of the KO lines relative to the wild type at the different sampling times. **D:** Comparison of genes that were cold-regulated after one or three hours at 4 °C relative to untreated plants of the same genotype and that belong to the GO term response to cold. **E:** As **D**, but genes are shown that belong to the GO term cold acclimation. Genes that were downregulated were not significantly overrepresented in the GO terms response to cold (**D**) or cold acclimation (**E**).

3.1.3.2 Transcriptome data of plants after one hour at 4 °C

After one hour at 4 °C, more genes were up- than downregulated relative to unstressed plants in all three genotypes (**Figure 7A**). In the one-hour samples, the downregulated genes showed more differences than similarities between Col-0 and the KO lines, while in all other cold-treated samples (one and three hours), the similarities outweighed the differences. Genes that had responded to the cold in either of the genotypes after one hour in the cold (**Figure 7A**), were analysed for overrepresented GO terms and the top five GO terms of each genotype are shown in **Table 1**. After one hour at 4 °C, in Col-0, GO terms were overrepresented that involved responses to oxygen-containing compounds, to chemicals, to organic substances, to stimuli and to chitin. The transcriptional cold response in *rap2.4c* was very similar and only the response to chitin was missing in the top five GO terms, whereas they contained the GO term "response to stress". The top five hits in *rap2.4d* deviated more strongly from Col-0. They included "response to stress", "defence response" and "response to external biotic stimulus". Here again, the GO terms found in *rap2.4d* revolved more around biotic stimuli than in the wild type.

These differences after one hour in the cold were investigated more closely. Genes, that were differentially regulated in the KO lines relative to Col-0 (**Figure 7B**), were also screened for overrepresented GO terms (**Table 2**). Among the genes that were downregulated in either of the two KO lines, no significant GO terms were found, whereas the upregulated genes revealed significant GO terms. These GO terms belonged to a rather heterogenous group in *rap2.4c* ("response to stress", "response to acid chemical" and to "water deprivation", "multi-organism" and "defence" responses), while they were very homogenous in genes that were upregulated *rap2.4d* after one hour at 4 °C. The top five GO terms were identical to those that were downregulated in *rap2.4d* in unstressed plants, also in their order of significance, except for a single one which is also involved in biotic interactions ("defence response to other organism").

Differences in the cold response were of particular interest in this experiment, as significant phenotypes in the cold resistance were observed in the electrolyte leakage experiment (**Figure 2**). Genes that were differentially regulated after one hour at 4 °C within the genotypes relative to the unstressed plants, were analysed for cold-responsive genes. Genes that belong to the GO term response to cold were significantly overrepresented in all three genotypes only among genes that were upregulated, but not in genes that were downregulated after one hour at 4 °C. Comparison of these cold-responsive genes that were upregulated revealed some differences between the genotypes (**Figure 7D**), while only a few genes responded only in a single genotype, whereas most of the genes responded similarly in the three lines. Compared to the differences in overall upregulated genes

irrespective of their functions (**Figure 7A**) which showed a lot more variation between the lines, the cold-responsive genes appeared comparably uniform.

Table 1: Top five GO terms of differentially expressed genes in Col-0, *rap2.4c* and *rap2.4d* after one hour relative to unstressed plants of the same genotype.
Genes that were differentially regulated after one hour at 4 °C relative to unstressed plants were analysed for overrepresented GO terms via the GO term finder from the Princeton University (Boyle *et al*., 2004) in each of the three genotypes.

	Differentially regulated genes after one hour at 4 °C	
	GO term	p-value
Col-0	resp. to oxygen-containing compound	1.31^{-29}
	response to chemical	1.18^{-27}
	response to organic substance	1.73^{-27}
	response to stimulus	1.18^{-26}
	response to chitin	1.65^{-26}
rap2.4c	response to stimulus	6.16^{-40}
	response to stress	1.99^{-36}
	resp. to oxygen-containing compound	3.66^{-32}
	response to organic substance	1.96^{-27}
	response to chemical	2.88^{-27}
rap2.4d	response to stress	9.14^{-44}
	response to stimulus	7.75^{-43}
	defence response	5.49^{-42}
	resp. to oxygen-containing compound	1.44^{-36}
	response to external biotic stimulus	1.35^{-32}

Table 2: Top five GO terms of upregulated genes in *rap2.4c* and *rap2.4d* relative to Col-0 after one hour at 4 °C.
Genes that were differentially regulated in *rap2.4c* and *rap2.4d* relative to Col-0 plants after one hour in the cold were analysed for overrepresented GO terms via the GO term finder from the Princeton University (Boyle *et al*., 2004). No significant GO terms were found for genes that were downregulated in either of the two KO lines.

	Upregulated genes after one hour at 4 °C	
	GO term	p-value
rap2.4c	response to stress	2.16^{-10}
	response to acid chemical	1.37^{-09}
	multi-organism process	1.45^{-08}
	defence response	2.31^{-08}
	response to water deprivation	3.43^{-08}
rap2.4d	defence response	6.11^{-13}
	response to external biotic stimulus	1.09^{-07}
	response to other organism	1.09^{-07}
	response to biotic stimulus	1.11^{-07}
	defence response to other organism	1.01^{-06}

3.1.3.3 Transcriptome data of plants after three hours at 4 °C

After three hours in the cold, all genotypes showed more upregulation of genes than downregulation relative to unstressed plants of the same genotype (**Figure 7A**). Deregulation was more pronounced after three hours than after one hour. The overlap of genes that were up- or downregulated after one and three hours was approximately 50 % of the genes that were already differentially regulated after one hour in the cold in all genotypes. This indicates that the other 50 % were back to control levels after three hours, suggesting only transient differential expression of these genes. Furthermore, the majority of genes that were differentially regulated after three hours had not yet responded after one hour at 4 °C and are specific to a slightly slower cold response.

Both up- and downregulated genes showed differences between the genotypes, while the largest share of genes responded in the same way in all three lines. The similarities were supported by a GO term analysis among the genes that were differentially regulated in the single lines relative to unstressed plants of the same genotype (**Table 3**).

Table 3: Top five GO terms of differentially expressed genes in Col-0, *rap2.4c* and *rap2.4d* after three hours relative to unstressed plants of the same genotype.
Genes that were differentially regulated after three hours at 4 °C relative to unstressed plants were analysed for overrepresented GO terms via the GO term finder from the Princeton University (Boyle *et al.*, 2004) in each of the three genotypes.

	Differentially regulated genes after 3 hours at 4 °C	
	GO	p-value
Col-0	response to stimulus	2.36^{-50}
	response to chemical	1.03^{-43}
	resp. to oxygen-containing compound	3.11^{-40}
	response to abiotic stimulus	1.74^{-39}
	response to organic substance	1.20^{-36}
rap2.4c	response to stimulus	3.14^{-54}
	response to abiotic stimulus	9.63^{-44}
	resp. to oxygen-containing compound	2.85^{-42}
	response to stress	3.20^{-40}
	response to chemical	1.25^{-38}
rap2.4d	response to stimulus	1.55^{-47}
	response to abiotic stimulus	1.53^{-37}
	response to chemical	4.34^{-36}
	resp. to oxygen-containing compound	5.29^{-36}
	response to organic substance	2.49^{-34}

The top five GO terms were similar in the three genotypes. When genes, that were up- and downregulated in *rap2.4c* and *rap2.4d* relative to Col-0 after three hours at 4 °C were analysed separately, more distinct GO terms were found. Genes, that were upregulated in *rap2.4c* did not result in any significant GO terms, while those, that were upregulated in *rap2.4d* contained genes that were overrepresented in the three GO terms hormone-mediated signalling pathway (p-value $1.08x10^{-3}$), cellular response to hormone stimulus (p-value $4.7x10^{-3}$) and cellular response to endogenous stimulus (p-value $5.63x10^{-3}$). Genes, that were downregulated in the KO lines after three hours at 4 °C relative to Col-0 belonged to similar general stress-related GO terms with only one difference between the two KO lines (**Table 4**).

Table 4: Top five GO terms of downregulated genes in *rap2.4c* and *rap2.4d* relative to Col-0 after three hours at 4 °C.
Genes that were differentially regulated in *rap2.4c* and *rap2.4d* relative to Col-0 plants after three hours in the cold were analysed for overrepresented GO terms via the GO term finder from the Princeton University (Boyle *et al*., 2004). No significant GO terms were found for genes that were upregulated in either of the two KO lines.

	Downregulated genes after three hours at 4 °C	
	GO	p-value
rap2.4c	response to stimulus	9.45^{-18}
	response to stress	8.97^{-16}
	resp. to oxygen-containing compound	9.20^{-14}
	response to drug	7.99^{-13}
	defense response	1.27^{-12}
rap2.4d	response to stimulus	1.20^{-05}
	response to stress	6.99^{-05}
	response to drug	7.99^{-05}
	defense response	0.00026
	toxin metabolic process	0.00029

Again, genes that were differentially regulated within the three genotypes after three hours in the cold relative to unstressed plants were specifically analysed for genes belonging to the GO term "response to cold". As it was observed for the genes that were differentially regulated after one hour, cold-responsive genes were not significantly overrepresented in the group of genes that were downregulated after three hours in the cold. However, genes that were upregulated after three hours at 4 °C, were significantly overrepresenting the GO term "response to cold" (**Figure 7D**), displaying a similar pattern as after one hour in the cold with even more overlap. All three genotypes had a few differentially regulated genes that were unique to them, while the majority of genes was similarly regulated. Genes that are associated with the induction of cold acclimation were significantly overrepresented after three hours, but not after one hour at 4 °C in all genotypes. Only a single gene

(*ATRZ-1A*, AT3G26420), which encodes an RNA-binding protein that enhances freezing tolerance was unique to Col-0, while all other genes were common to the three lines (**Figure 7E**), indicating a very similar early cold acclimation response. Thus, it is likely, that the enhanced cold acclimation capacity of *rap2.4c* that was observed in the electrolyte leakage experiment (**Figure 2**), either depended on signalling that took place at a later time during cold acclimation or that it depended on a pathway that was not assigned to the GO term cold acclimation.

The groups of genes that were differentially regulated in *rap2.4c* and *rap2.4d* compared to Col-0 differed strongly between the different durations of the cold stress (**Figure 7C**), which was also reflected in the GO terms, that were found among the differentially regulated genes of both KO lines. The dynamics highlighted a putative function of RAP2.4d in cold-induced biotic defence responses and in hormone-related signalling pathways. Defence-related GO terms were downregulated before the stress but upregulated after one hour at 4 °C. Although the GO terms were widely identical, the overlap between the single defence-related genes that were downregulated before and upregulated one hour after the transfer to 4 °C was only 15 %, this pattern reveals that widely different sets of defence-related genes were differentially regulated in in unstressed and cold-stressed *rap2.4d* plants. Hormone-related GO terms were upregulated after three hours in *rap2.4d*. *rap2.4c* showed more general stress-related GO terms, that were less homogenous than those, that were found in *rap2.4d*. However, GO terms that are related to biotic defence responses were also found in cold-stressed *rap2.4c* plants.

Comparison of the gene sets that were up- or downregulated in either of the two KO lines relative to the wild type at the different sampling times revealed that there was hardly any overlap in deregulated genes between the sampling times within either of the KO lines (**Figure 7C**). Depending on the duration of the cold exposure, both transcription factors seem to (directly or indirectly) regulate entirely different sets of genes with only little overlap with the other cold stress durations. Considering the fast up- and downregulation of both genes upon transfer to 4 °C (**Figure 6**) and the time-specific GO terms that were overrepresented in *rap2.4d*, depending on the duration of the cold stress exposure, it can be assumed, that both transcription factors contribute to different responses in a highly context and timing-dependent manner.

3.1.3.4 Analysis of the robustness of the transcriptome data

For three well characterised cold-responsive genes, *LEA7*, *CBF1* and *ZAT10*, the robustness of the RNAseq data and especially the differences between the genotypes, were analysed by RT-qPCR in cDNA that was synthesized from the same RNA material that was used for the RNAseq experiment

(**Figure 8**). Overall, the RT-qPCR results strongly resembled the RNAseq data, thereby confirming the technical robustness of the RNAseq results.

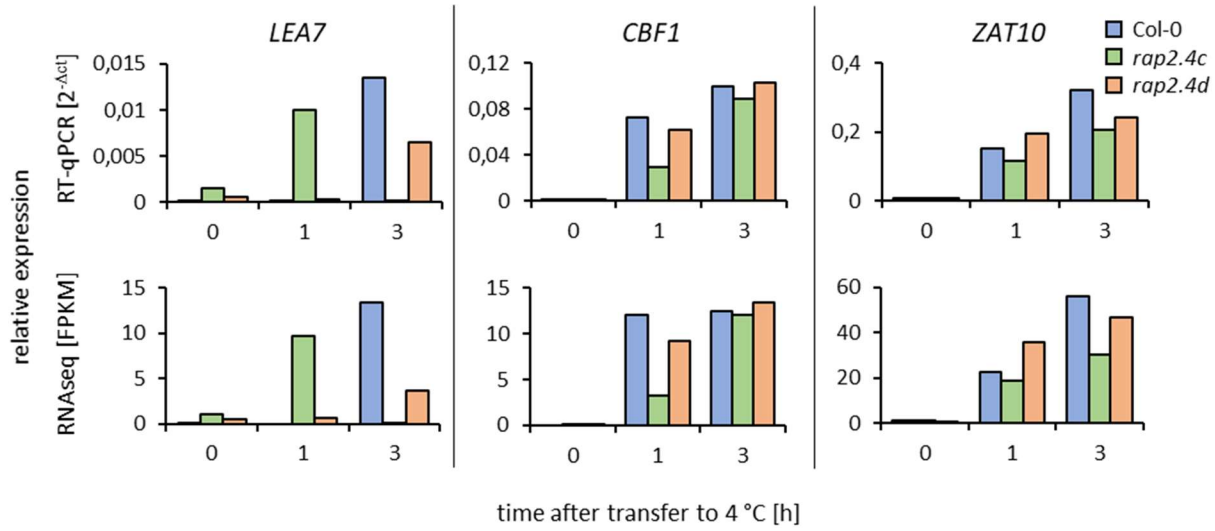


Figure 8: Comparison of RNAseq transcript data with RT-qPCR transcript data.
Transcript data of three representative genes obtained from the same RNA samples by RNAseq and RT-qPCR to confirm technical robustness of the RNAseq data. The experimental procedure was the same as described in the legend of **Figure 7**.

3.1.3.5 Comparison of RAP2.4d-interacting promoters with the RNAseq data

The RNAseq data were analysed in comparison to the results from the Yeast-One-Hybrid assay in which promoters were identified that interacted with RAP2.4d and that contained the identified putative DNA-binding motifs (**Figure 4B** and **Figure 5B**). Only a single one of these genes showed differential gene expression in the *rap2.4d* KO line relative to Col-0. The hypothetical gene AT1G32460 (**Figure 5B**) with no reported functions failed cold-induced upregulation in *rap2.4d* as it was observed in Col-0 after one hour at 4 °C.

In a next step, genes that were similarly deregulated in *rap2.4c* and *rap2.4d* (overlaps in **Figure 7B**) were analysed with the MEME tool for common DNA motifs within 1000 bp upstream of their transcription start. No motifs were found with an E-value of 0.05 or smaller.

Only one out of the 23 genes whose promoters contain either of the two or both putative DNA binding motifs (**Figure 4** and **Figure 5**) showed differential expression in *rap2.4d* compared with Col-0. As these target sequences were identified in a yeast system, *in planta* binding of RAP2.4d to these motifs is not guaranteed. Either the residual 22 promoters are no natural targets of RAP2.4d in Arabidopsis or it is not involved in their regulation during cold stress signalling. Expression of *RAP2.4d* is induced upon various abiotic stress events (Kilian *et al.*, 2007). The genes that lie downstream of the identified

interacting promoters (**Figure 4B** and **Figure 5B**) may be regulated by RAP2.4d as a response to other stressful stimuli, while only the hypothetical gene AT1G32460 is a target of RAP2.4d in the cold.

3.1.3.6 Analysis of expected transcriptional changes in the RNAseq data in *rap2.4c* and *rap2.4d*

sAPX and *tAPX* levels have been reported to be reduced in the *rap2.4c* and *rap2.4d* KO lines under standard conditions and cold induction of *COR47* and *COR15a* to be inhibited in *RAP2.4c* and *RAP2.4d* overexpressor lines after 24 hours at 4 °C (Bulcha, 2013; Rudnik *et al*., 2017). In the present comparison of *rap2.4c*, *rap2.4d* and Col-0, *sAPX, tAPX, COR47 and COR15a* expression levels revealed less differences between Col-0 and the KO lines (**Figure 9**). *tAPX* and *sAPX* transcript levels were similar in the two KO lines and Col-0 prior to the cold. *tAPX* levels were transiently reduced in both KO lines after one hour in the cold and *sAPX* levels were slightly reduced in both KO lines after three hours. *COR47* and *COR15a* displayed slightly reduced transcript levels in *rap2.4c* relative to Col-0 after one hour at 4 °C.

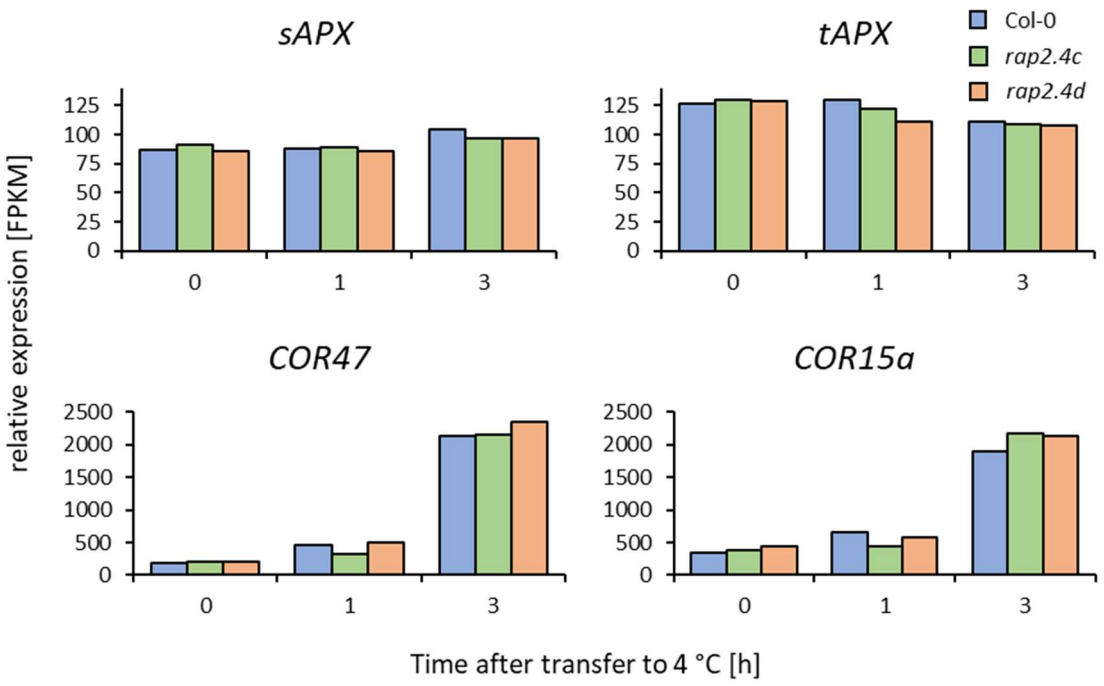


Figure 9: RNAseq data of two plastid *APX* genes and two *COR* genes at 4 °C.
The experimental procedure was the same as described in the legend of **Figure 7**.

To analyse the expression levels of *COR* genes and their upstream regulatory *CBF* genes, as well as plastid *APX* genes, the experiment performed for the RNAseq analysis was independently repeated three more times with additional sampling after 24 hours at 4 °C, but this time transcripts were analysed via RT-qPCR. The data obtained in this experiment confirmed the RNAseq results (**Figure 10**).

sAPX, *tAPX*, *COR47* and *COR15a* showed no significant differences between Col-0 and the KO lines at any time. Only the cold master regulator genes, *CBF1* and *CBF3*, that mediate *COR* gene expression displayed small and significant differences after 24 hours in the cold in one or both KO lines relative to Col-0. Expression of both genes was reported to peak after three hours in the cold and then to greatly decline over time (Fowler *et al*., 2005). *CBF1* was additionally elevated in *rap2.4d* after one and three hours at 4 °C, but this was not significant. None of these differences in *CBF1* or *CBF3* expression were reflected in elevated expression of the two analysed target *COR* genes, but a negative effect of RAP2.4c and RAP2.4d on the cold response was evident as the electrolyte leakage was decreased in *rap2.4c* and *rap2.4d*, respectively (**Figure 2**).

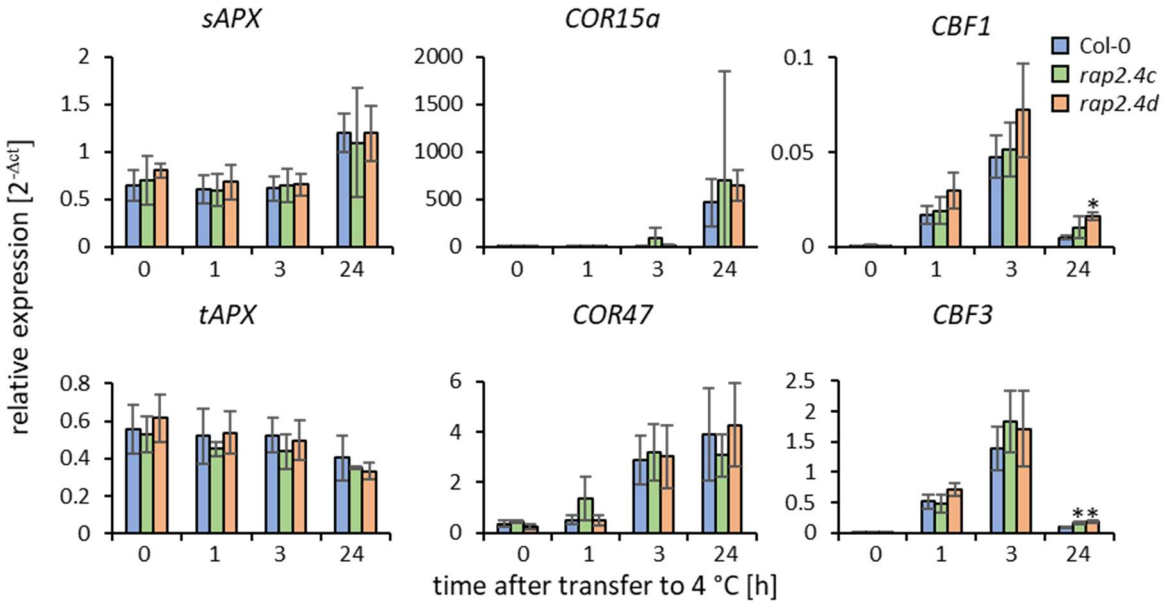


Figure 10: Relative expression of cold related genes determined by RT-qPCR.
Four-week-old Col-0, *rap2.4c* and *rap2.4d* KO plants were transferred to 4 °C and harvested before and one, three and 24 hours after the transfer. n=3±SD, student's t-test KO line vs. Col-0 at the same time, $p<0.05$.

Thus, the freezing tolerance effects (**Figure 2**) did not correlate with the tested *COR* genes as observed in *rap2.4d* after short term cold exposure (**Figure 9**) or with those in *rap2.4c* after two or three days of cold acclimation (**Figure 3**). In cold acclimation and in the regulation of freezing tolerance, the ICE1-CBF-COR pathway is by far the most studied pathway. Only 12 % of cold-responsive genes were found to be clearly regulated by the CBFs (Fowler and Thomashow, 2002), showing that the majority of cold-responsive genes is regulated by CBF-independent pathways. One such alternative pathway involves the WUS class transcription factor HOS9 (Zhu *et al*., 2004). *hos9* mutants exhibited increased upregulation of CBF-independent cold-responsive genes upon cold-treatments. Among these genes, there were *ERF* genes (*ERF5*, *ERF6* and *ERF11*), *WRKY* transcription factor genes (*WRKY33*, *WRKY40*

and *WRKY46*) and the plastid O_2^--responsive gene *BAP1*. *RAP2.4c* and *RAP2.4d* belong to the ERF-Ib subfamily of the ERF transcription factors that are also cold-responsive (Nakano *et al*., 2006; Kilian *et al*., 2007), but they are not differentially regulated in *hos9* (Zhu *et al*., 2004).

RAP2.4 genes strongly respond to a variety of environmental stimuli (Kilian *et al*., 2007; Rudnik *et al*., 2017). Furthermore, members of the *RAP2.4* gene family act highly redundantly in many respects (Iwase *et al*., 2011; Rae *et al*., 2011; Rudnik *et al*., 2017). The knock-out of any of the single members led to the altered expression of several other members (Rudnik *et al*., 2017) likely leading to compensatory effects of redundantly acting proteins. In the RNAseq analysis performed in the present study, the expression profiles of most *RAP2.4* genes (**Table 5**) were consistent with the previous study (Rudnik *et al*., 2017). Only *RAP2.4f* and *RAP2.4g* were upregulated in unstressed KO plants in the present study, while they were downregulated in the same KO lines previously, and *RAP2.4a* showed an inverse pattern in *rap2.4c*. Minor changes in the growth conditions, such as altered light quality or watering habits, that may have differed between the two studies, and the interplay within the partly redundant *RAP2.4* gene family could explain the differences in gene expression that were observed.

Table 5: Expression ratios of members of the *RAP2.4* gene family in *rap2.4c* and *rap2.4d* relative to Col-0 at 4 °C.
Expression levels [FPKM] of *RAP2.4* genes in *rap2.4c* and *rap2.4d* KO plants were divided by FPKM values of Col-0 at the same time before and one and three hours after the transfer to 4 °C. Transcripts of *RAP2.4e* and *RAP2.4h* were not detected in any of the samples, so they are not displayed in this table. Blue background indicates upregulation in the KO line relative to Col-0, red indicates downregulation. The experimental procedure was the same as described in the legend of **Figure 7**.

	0 hours at 4 °C		1 hour at 4 °C		3 hours at 4 °C	
Transcript	*rap2.4c* KO	*rap2.4d* KO	*rap2.4c* KO	*rap2.4d* KO	*rap2.4c* KO	*rap2.4d* KO
RAP2.4a	0.36	1.29	1.17	2.78	3.00	2.11
RAP2.4b	0.92	0.92	0.99	1.41	0.98	1.43
RAP2.4c		0.77		2.92		1.81
RAP2.4d	1.13		0.69		1.01	
RAP2.4f	1.41	1.26	0.85	1.05	0.78	0.73
RAP2.4g	1.27	0.60	1.05	1.05	0.78	0.80

3.1.3.7 Analysis of hormone related gene expression

The analysis of differentially regulated genes in *rap2.4c* and *rap2.4d* repeatedly yielded GO terms that revolved around defence responses against biotic stimuli (chapter 3.1.3.1, **Table 2** and chapter 3.1.3.3). For this reason, single genes that play a role in these responses were analysed in more detail. First, an overrepresentation of genes that are involved in the jasmonic acid (JA) pathway were found to be

upregulated in *rap2.4d* one hour after the transfer to 4 °C. This included the JA master regulator gene *MYC2* and various of its target genes, such as *VSP1*, *JAV1*, *JAZ1*, *JAZ8* and *CHIT*. Such a pattern was also found in *rap2.4c* with fewer genes following this pattern. A similar pattern was found in genes that are involved in the combined JA and ethylene (ET) pathway, including the master regulator genes *ORA59* and *ERF1* and their target genes *PDF1.2a*, *PDF1.2c*, *ERS2* and *DYL1*. Furthermore, genes involved in the salicylic acid (SA) pathway were affected. These genes included *PR1*, *PR2*, *WRKY38* and *WRKY18*, but not their regulators *NPR1* and *ICS1*. Examples of such upregulated hormone-related genes are shown in **Figure 11**. This figure also demonstrates that upregulation of gene expression after one hour at 4 °C is seen in only a few of these genes in the *rap2.4c* line and always to a smaller extent compared with *rap2.4d*. Upregulation of the SA downstream genes *PR1*, *PR2* and *WRKY38* was particularly strong.

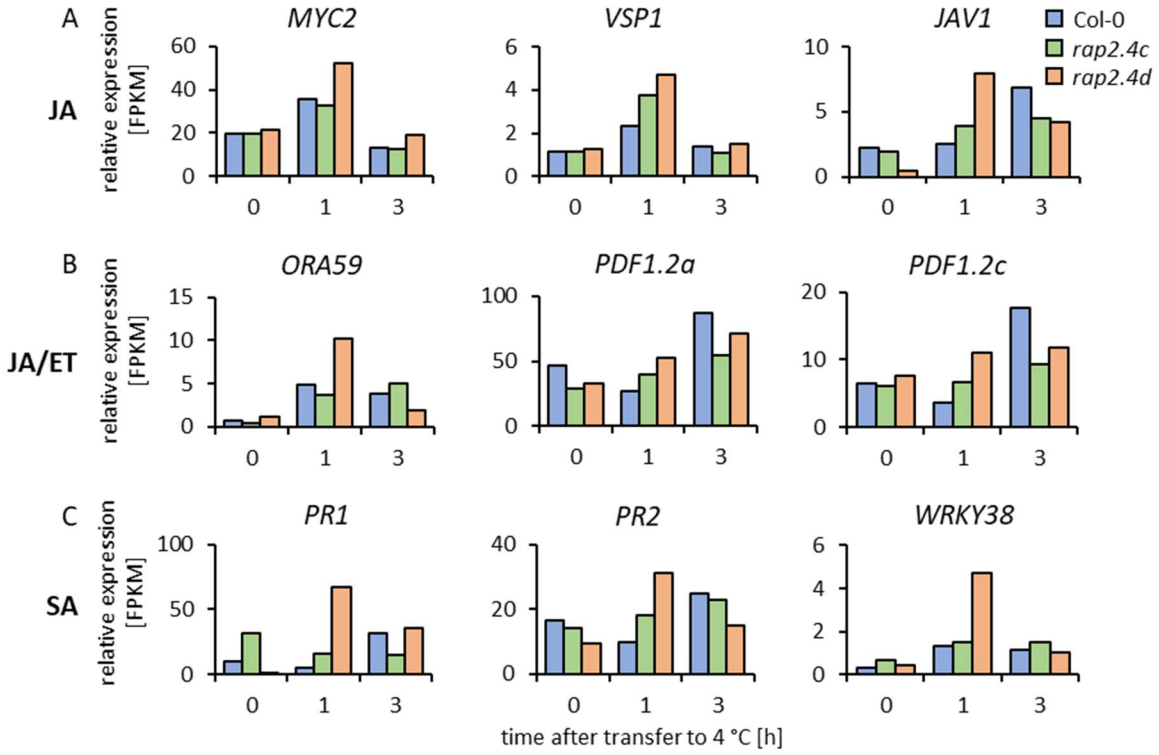


Figure 11: RNAseq data of representative hormone related genes at 4 °C.
A: JA-related genes. **B:** JA/ET-related genes. **C:** SA-related genes. The experimental procedure was the same as described in the legend of **Figure 7**.

The comparison between *rap2.4c* and *rap2.4d* regarding affected hormone-related genes (**Figure 12**) reports genes that were upregulated less than two-fold that was chosen as the standard cut-off in this study, to emphasise the pattern. The majority of upregulated genes belongs to the JA pathway. JA/ET genes show the mildest differential expression and none of them is upregulated more than five-fold, as opposed to JA and SA genes.

The RT-qPCR confirmed the trend towards upregulated expression of hormone-related genes in *rap2.4d* after one hour at 4 °C, although the differences were not significant for all genes (**Figure 13**).

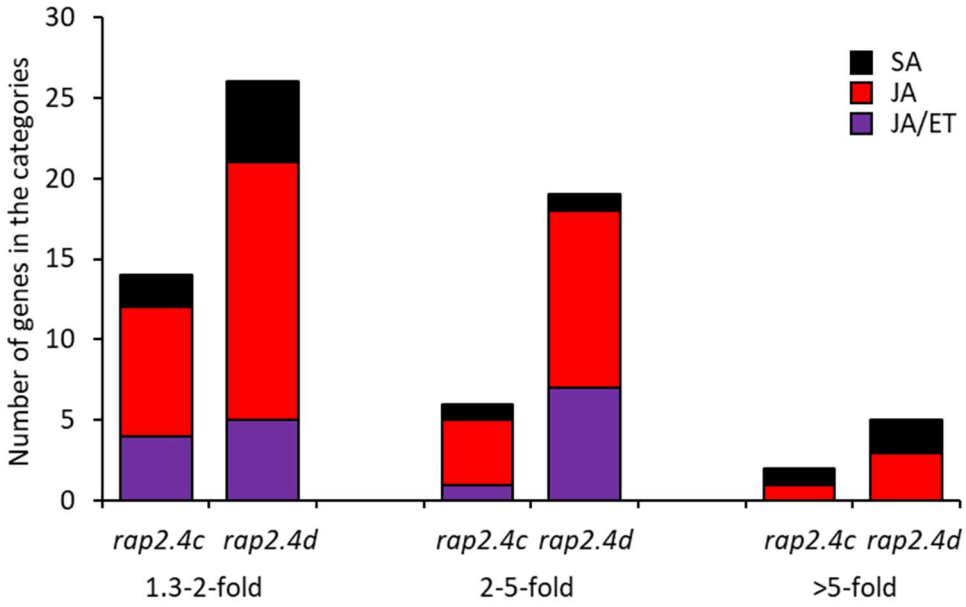


Figure 12: Relative RNAseq data of hormone related genes after one hour at 4 °C in the KO lines.
Hormone-related genes that were upregulated in *rap2.4c* and *rap2.4d* one hour after the transfer to 4 °C are grouped depending on their fold change in expression relative to Col-0 at the same time and depending on the hormone pathways that they belong to. Data are shown as (FPKM *rap2.4*)/(FPKM Col-0) ratios. The experimental procedure was the same as described in the legend of **Figure 7**.

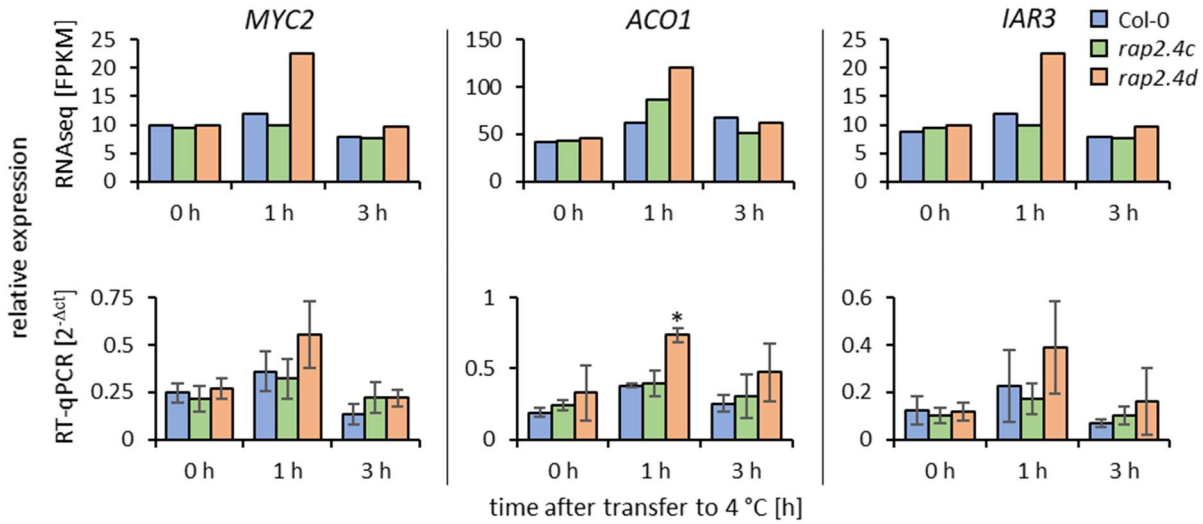


Figure 13: Comparison of RNAseq transcript data with RT-qPCR transcript data.
Transcript data of representative JA-related genes obtained by RNAseq (n=1, the experimental procedure was the same as described in the legend of **Figure 7**) and by RT-qPCR run in three independent biological replicates to confirm biological robustness of the RNAseq data. n=3±SD, student's t-test KO line vs. Col-0 at the same time, $p<0.05$.

The JA/ET, the JA and the SA signalling pathways all mediate defence responses against different types of attackers. The JA/ET pathway mainly confers resistance against necrotrophic pathogens, JA signalling predominantly induces defence mechanisms upon herbivory and SA strongly mediates resistance against biotrophic pathogens (Spoel *et al*., 2003; Pieterse *et al*., 2012), while these pathways control each other by feedback inhibition (Koornneef and Pieterse, 2008; Song *et al*., 2014; Caarls *et al*., 2015). A simplified model showing the crosstalk between these three hormone signalling pathways is shown in **Figure 14**. The feedback control prevents the simultaneous activation of more than one of these pathways under most conditions. JA-response regulation is largely controlled by the transcriptional master regulator MYC2 that leads to the expression of its target genes enhancing resistance against herbivores (Howe and Schilmiller, 2002). At the same time, MYC2-dependent signalling inhibits expression of genes that belong to the JA/ET and the SA pathways (Kazan and Manners, 2013). The other way around, pathogen-induced signalling leads to the activation of the SA response master regulator NPR1. Expression of NPR1 downstream genes enhances pathogen resistance and induces systemic acquired resistance, while JA signalling is inhibited, rendering plants more vulnerable to herbivore attacks (Cao *et al*., 1994; Spoel *et al*., 2003).

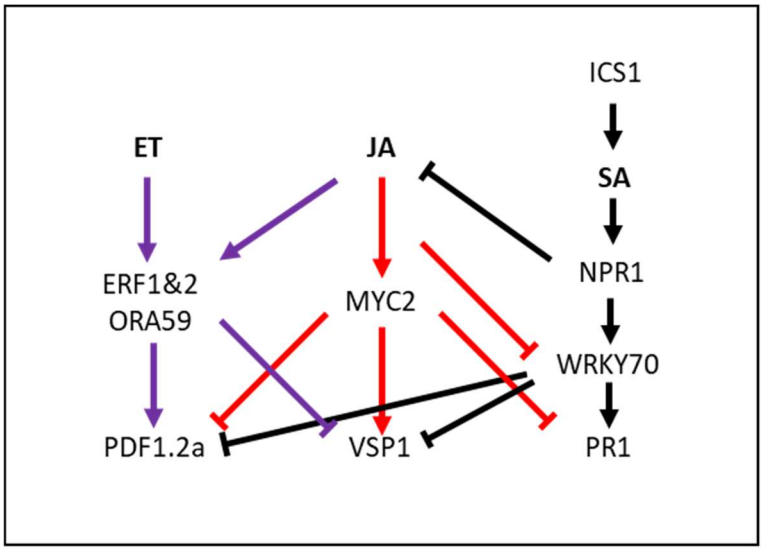


Figure 14: Simplified crosstalk scheme of the JA/ET, JA and SA hormone pathways.
Purple lines indicate effects of the JA/ET pathway, red lines represent the JA pathway and effects of the SA pathway are shown in black. Modified after Walley at al. (2008).

Figure 15 shows RNAseq data of key genes that are shown in the crosstalk scheme in **Figure 14** to exemplarily demonstrate the crosstalk pattern in the *rap2.4d* KO line relative to the wild type. One hour after Col-0 plants were transferred to 4 °C, they showed a classical MYC2 dominated expression

pattern. *MYC2* transcript levels increased, which led to the elevated expression of its target gene *VSP1* and to the inhibition of *PDF1.2a* and *PR1* gene expression. The three regulatory genes *ORA59*, *MYC2* and *NPR1* were all moderately induced after one hour. These three genes showed a very similar expression pattern in Col-0 and *rap2.4c*. In *rap2.4d* however, *MYC2* was induced to a higher level resulting in strongly enhanced *VSP1* induction. While this should have led to an enhanced inhibition of *PDF1.2a* and *PR1*, both genes were induced instead, indicating simultaneous activation of all three hormone pathways. *PR1* was even induced 58-fold, while it was downregulated two-fold in the wild type. One of its upstream regulators, *WRKY70,* was upregulated as well (data not shown), but only to a moderate extent, which failed to inhibit both *PDF1.2a* and *VSP1*. These data indicate impairments in the JA/ET-JA-SA crosstalk in the *rap2.4d* line.

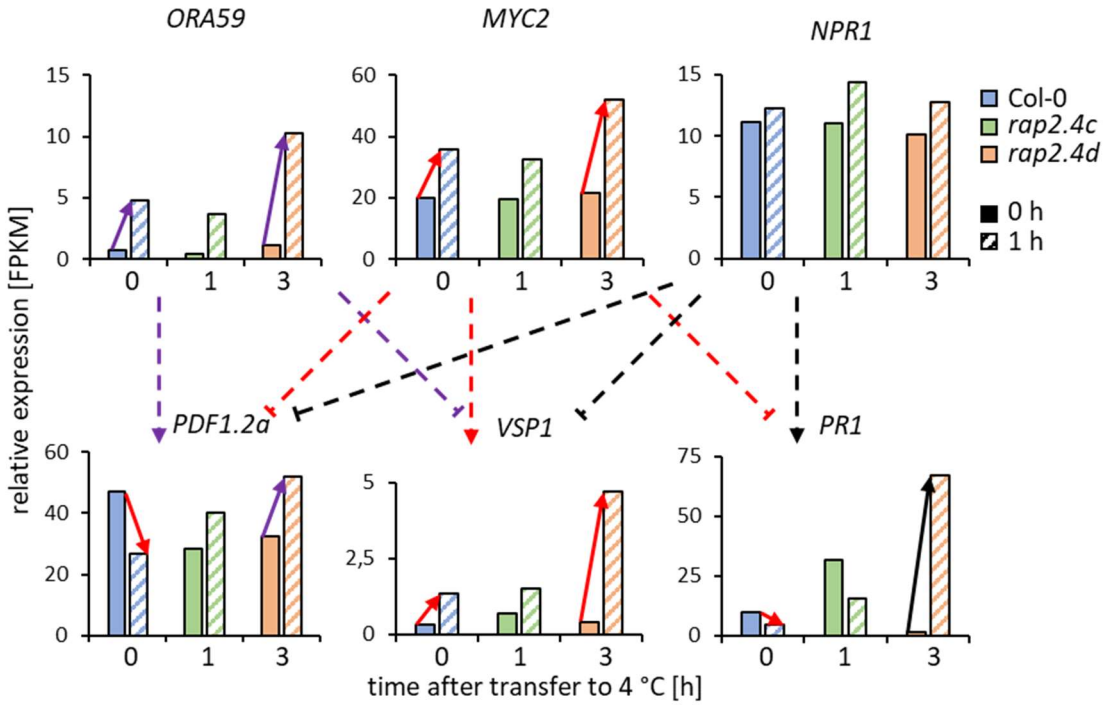


Figure 15: RNAseq data of hormone master regulator genes and their target genes.
RNAseq data of master regulator genes of the JA/ET (*ORA59*), JA (*MYC2*) and SA (*NPR1*) pathways and of representative target genes. Only samples before (solid bars) and one hour after the transfer to 4 °C (striped bars) are shown. Purple lines indicate effects of the JA/ET pathway, red lines represent the JA pathway and effects of the SA pathway are shown in black. Striped lines between the graphs represent interactions of the genes as they can be seen in **Figure 14**. Solid arrows within the individual graphs illustrate the upstream hormone that would induce the reaction in Col-0. The experimental procedure was the same as described in the legend of **Figure 7**.

Whereas a similar, but milder phenotype was observed in the *rap2.4c* line as well, clearly fewer genes were affected in this line, compared to *rap2.4d*. In *rap2.4c*, the expression patterns of *ORA59*, *MYC2*, *NPR1* and *VSP1* resembled the wild type, while *PDF1.2*a resembled the pattern in *rap2.4d*, only slightly

weaker. Additionally, the extent of upregulation of such hormone-related genes in *rap2.4c* was low compared to *rap2.*4d (**Figure 11** and **Figure 12**). For this reason, this phenotype will be mainly addressed in *rap2.4d*. *rap2.4c* will be analysed in comparison, as it displayed an intermediate phenotype. Increased activation of these three pathways, especially of the JA and the SA pathways at the same time, is an intriguing new phenotype of the *rap2.4d* (and to a minor extent in the *rap2.4c*) KO lines.

3.1.4 Hormone content analysis

Various hormone-related genes were upregulated in *rap2.4d* (and in *rap2.4c*) relative to the wild type after one hour at 4 °C. This may be due to alterations in phytohormone contents. To address this hypothesis, an experiment identical to the RNAseq was conducted with additional 24-hour samples, followed by quantification of the putative main regulators JA and SA (**Figure 16**). In addition to the already mentioned hormones, abscisic acid (ABA) levels were quantified, because the JA master regulator gene *MYC2* and its downstream targets that were upregulated in *rap2.4d* are also responsive to ABA (Kazan and Manners, 2013). The analysis of hormone contents revealed no significant differences at any time between the wild type and *rap2.4c* or *rap2.4d* for any of the analysed hormones. Apart from an increase of ABA after 24 hours in all genotypes, which is a well-known response to cold stress (Daie and Campbell, 1981; Chen and Gusta, 1983), there were no clear changes in hormone contents over time. Very minute differences include a slightly increased upregulation of JA in *rap2.4d* after three hours and a minor upregulation of SA after three hours in all three genotypes. But none of this was significant. Also, the minute increase in JA levels in *rap2.4d* rather appears to be a consequence of the observed transient gene regulation instead of its cause, as induced gene expression of the MYC2 pathway (one hour) which leads to the biosynthesis of JA (Chini *et al*., 2009), preceded JA induction (three hours). This is also in line with the finding, that genes that were upregulated in *rap2.4d* after three hours at 4 °C were assigned to GO terms that revolved around responses to hormones (chapter 3.1.3.3). The early samples before the cold treatment and after one hour at 4 °C were of particular interest, as they might have contributed to the failed crosstalk in the hormone pathways, but no differences were observed in these data.

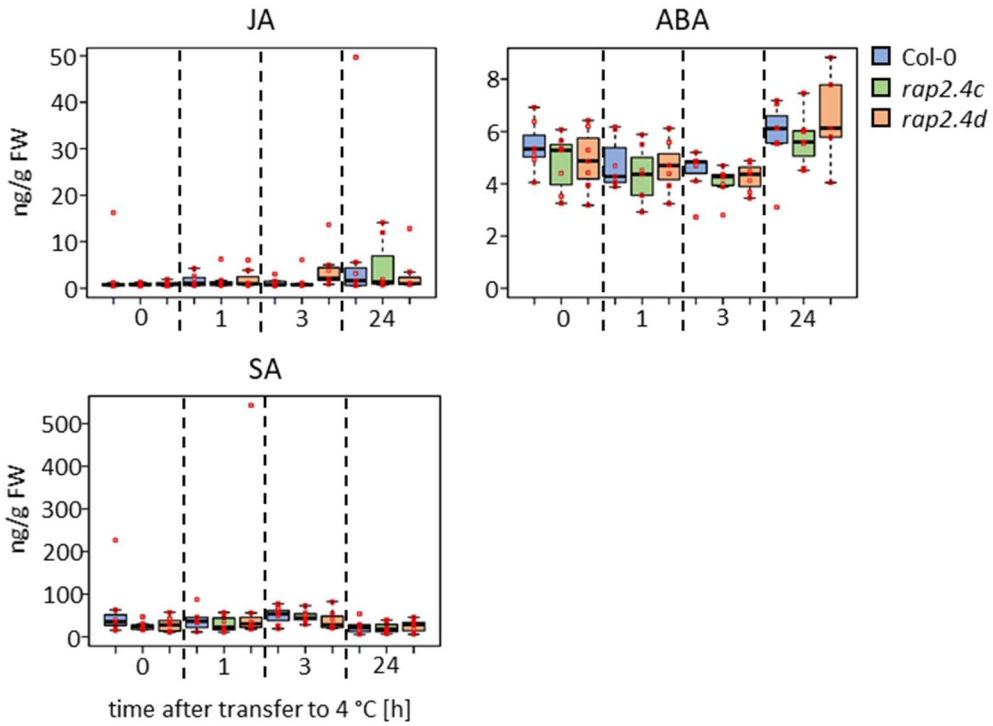


Figure 16: Hormone contents of SA, ABA and JA in Col-0, *rap2.4c* and *rap2.4d* at 4 °C.
Four-week-old plants were transferred to 4 °C and samples were taken before the transfer and after one, three and 24 hours. Hormones were extracted from plant material and contents were quantified by UPLC-MS/MS and normalized by internal SA, ABA and JA standards. Red dots represent individual samples, n=7.

To determine ET-effects, the RNAseq data of the characterised ET-inducible genes *ACONITASE 2* (*ACO2)*, *ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN* (*EBP*) and *ETHYLENE RESPONSE SENSOR 1* (*ERS1*) (Gomez-Lim *et al*., 1993; Büttner and Singh, 1997; Nemhauser *et al*., 2006) were analysed for ET effects. Only minor variation in expression between the KO lines and the wild type was observed, that did not indicate ET signalling (**Figure 17**). Various genes of the JA/ET pathway were upregulated in *rap2.4d* along with an array of JA responsive genes (**Figure 11**), while genes specifically inducible by ET did not show this response, indicating that elements acting in the JA pathway were the driving force behind the upregulation of JA/ET genes. Genes from the JA/ET pathway, that are usually inhibited by JA alone (**Figure 14**), can also be induced by JA, while not at the same intensity as by JA/ET signalling (Leon-Reyes *et al*., 2009; Zander *et al*., 2010). This may be particularly true in the disturbed crosstalk background that was observed in *rap2.4d* (**Figure 15**). The TGACG SEQUENCE-SPECIFIC BINDING PROTEIN (TGA) transcription factors TGA2, TGA5 and TGA6 interact with the SA regulatory protein NPR1 in an SA-independent manner to induce downstream gene expression (Fan and Dong, 2002). Zander *et al*. (2010) reported that *PDF1.2* expression was abolished in *tga2/5/6* triple mutants. Apparently, both JA and SA signalling have a direct influence on JA/ET gene expression in the absence of JA/ET signalling that may contribute to the upregulation of JA/ET downstream genes in *rap2.4d*.

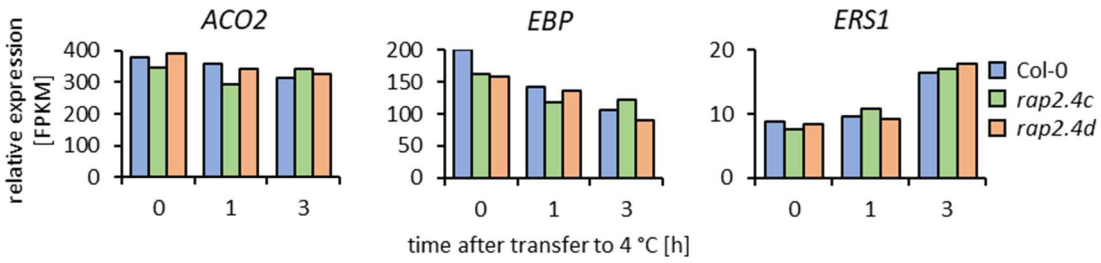


Figure 17: RNAseq data of ET-responsive genes at 4 °C.
The experimental procedure was the same as described in the legend of **Figure 7**.

3.1.5 Hormone treatments

Hormone level quantification (**Figure 16**) gave no evidence that the increased expression of hormone related genes in *rap2.4d* was not caused by alterations in hormone contents. An alternative explanation might be a change in the sensitivity or in the responsiveness to the hormones. Firstly, *RAP2.4c* and *RAP2.4d* gene expression could be responsive to hormone treatments and secondly, the response of hormone-related genes could be altered in the KO lines of both transcription factors. To test these hypotheses, the three genotypes were sprayed with JA, ABA or SA. A set of plants that was grown in parallel was sprayed with a mock solution as a control (**Figure 18**). The data revealed that in Col-0 plants both *RAP2.4c* and, to a smaller extent, *RAP2.4d* were inducible by ABA within one hour after the treatment (**Figure18A**). Transcript levels of both genes were back to control levels after three hours. Additionally, *RAP2.4d* was slightly induced by JA treatments after one hour and inhibited by SA. Because the JA-responsive genes showed the strongest effect in the RNAseq data, the JA master regulator gene *MYC2* and its direct target gene *JAZ1* were chosen as readout genes. Neither of them showed significant differences between Col-0 and the KO lines, except for a minimal, but significant increase of *JAZ1* after three hours in *rap2.4c* (**Figure 18B**). Thus, the data did not indicate a function of the two *RAP2.4* genes in hormone signal transduction explaining the increased gene expression detected in the RNAseq one hour after the transfer to 4 °C.

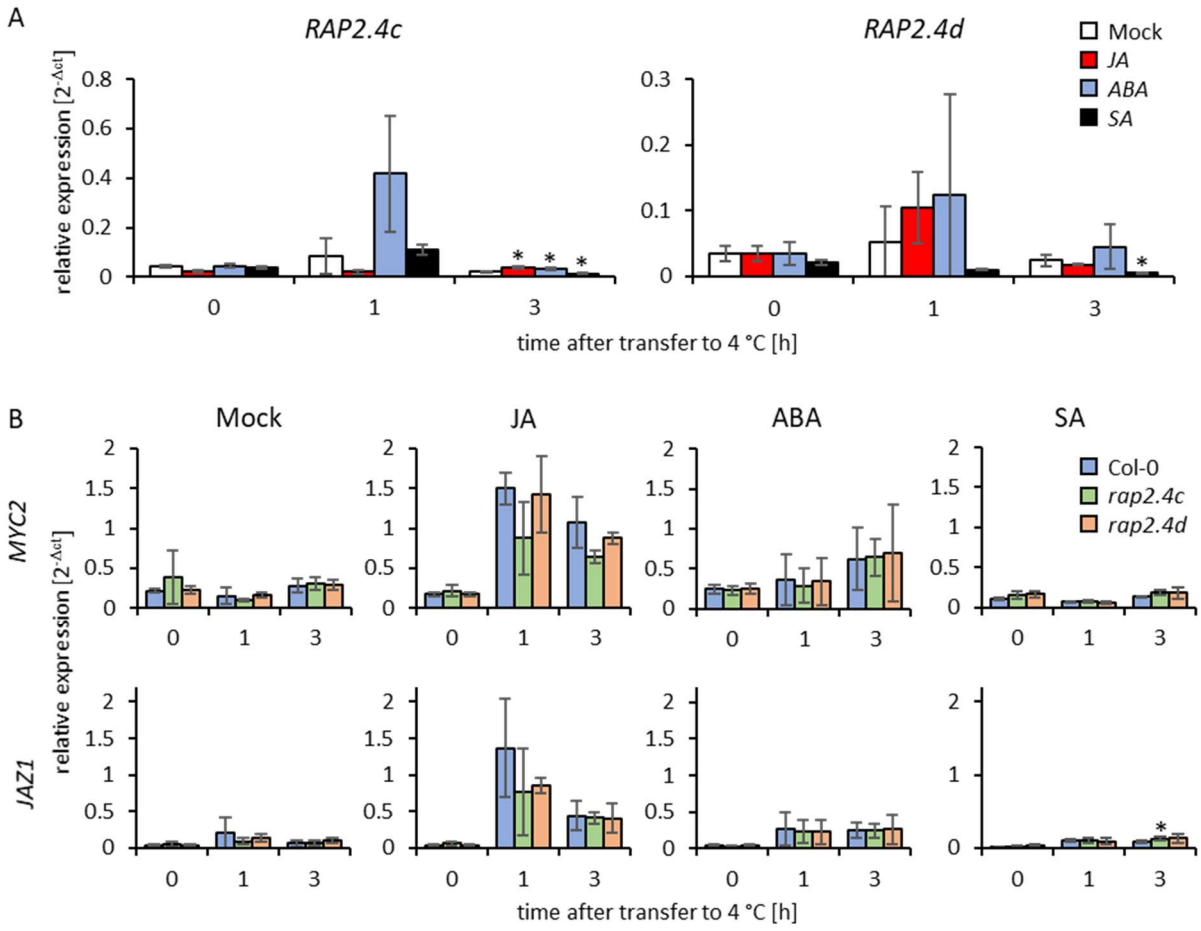


Figure 18: Relative transcript levels of *RAP2.4c*, *RAP2.4d*, *MYC2* and *JAZ1* in hormone treated plants.
Four-week-old Col-0, *rap2.4c* and *rap2.4d* plants were sprayed with a mock solution, 50 µM JA, 100 µM ABA or 1 mM SA. Samples were taken before and one and three hours after the treatments and transcript levels were analysed via RT-qPCR. **A:** Comparison of the relative expression of *RAP2.4c* and *RAP2.4d* after hormone treatments in Col-0 plants. n=3-4±SD, student's t-test relative to mock treated samples at the same time, $p<0.05$. **B:** Relative expression of the JA-responsive genes *MYC2* (top) and *JAZ1* (bottom) after hormone treatments in Col-0, *rap2.4c* and *rap2.4d*. n=3-4±SD, student's t-test KO line vs. Col-0 at the same time, $p<0.05$.

3.1.6 Transient overexpression of *RAP2.4c* and *RAP2.4d*

Since the analysis showed that modulation of hormone contents and of downstream signalling were not likely responsible for the alterations in hormone related gene expression patterns in *rap2.4d*, the data pointed towards an explanation more specifically linked to the expression of *RAP2.4d*. This was tested by transiently inducing *RAP2.4c* and *RAP2.4d* overexpression using estradiol-inducible transgenic lines.

For the generation of these inducible overexpressor lines, both full length transcript sequences were amplified via PCR from genomic DNA using gene specific primers (*RAP2.4c* forward: AGAGCTTCAGAACAAGCAAC; reverse: AAAAAGAGAGAACTAAAAGAAGA; *RAP2.4d* forward: CCACGCCTCCTATATAAACA; reverse: GTTCTTTTGTGCCAATTTAACT). The inserts were ligated into the pCR8/GW/TOPO vector and subsequently transformed into *E. coli* Top10 cells. After propagation, the

inserts of these plasmids were sequenced and plasmids with correct sequences were used to transfer their inserts into the estrogen-inducible expression vector pMDC7 via LR-reaction. Plasmids were transformed into *Agrobacterium tumefaciens* GV3101 (pMP90). These cells were used to integrate the T-DNA into the genome of *Arabidopsis thaliana* Col-0 plants using the floral dip technique (Bernhardt *et al*., 2012). Seedlings (T1) of transformed plants were selected on MS agar plates containing 15 µg/ml Hygromycin B (Harrison *et al*., 2006) and subsequently tested by PCR using a forward primer binding to the vector directly upstream of the insert (GGACACGCTGAAGCTAGT) and gene specific reverse primers that were used before.

Seeds (T2) of these plants were again selected on Hygromycin B. Surviving seedlings were allowed to produce seeds (T3). T3 seeds were plated following the same selection protocol and their survival rates were observed. 100 % survival indicated homozygous T2 plants. T3 seeds of homozygous T2 plants were considered homozygous and were used in the following steps.

Strong overexpression of inserted genes requires strong expression of the constitutively expressed chimeric XVE transcription factor encoded on the transformed pMDC7 plasmid. To identify plants with strong *XVE* expression, RNA was extracted, and reverse transcribed into cDNA. A PCR with *XVE* specific primers (forward: AGATCACAGACACTTTGATCCACC, reverse: GAGAGGATGAGGAGGAGCTGG) with 25 cycles allowed for quantitative comparison of *XVE* transcript levels. Plants with the strongest expression were chosen for experiments (*RAP2.4c* OE: line 8, *RAP2.4d* OE: line 10) (**Figure 19**).

First, induction intensities of *RAP2.4* transcripts were tested after estradiol treatment over a course of five days. Both genes were strongly induced upon estradiol treatments (**Figure 20**). *RAP2.4c* transcript levels gradually increased and reached a maximum of 80-fold upregulation after five days, while *RAP2.4d* peaked after three days at eight-fold induction and then slightly decreased to seven-fold after five days.

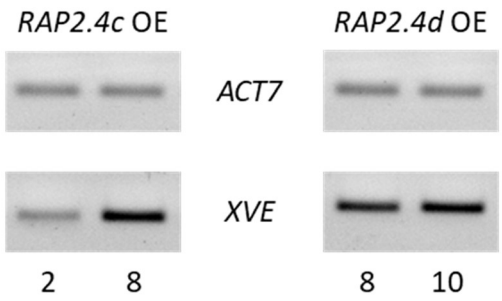


Figure 19: Semi-quantitative PCR for the determination of *XVE* expression.
RNA was extracted from homozygous four-week-old transgenic plants and transcribed into cDNA. PCRs were performed using *XVE* and *ACT7* specific primers.

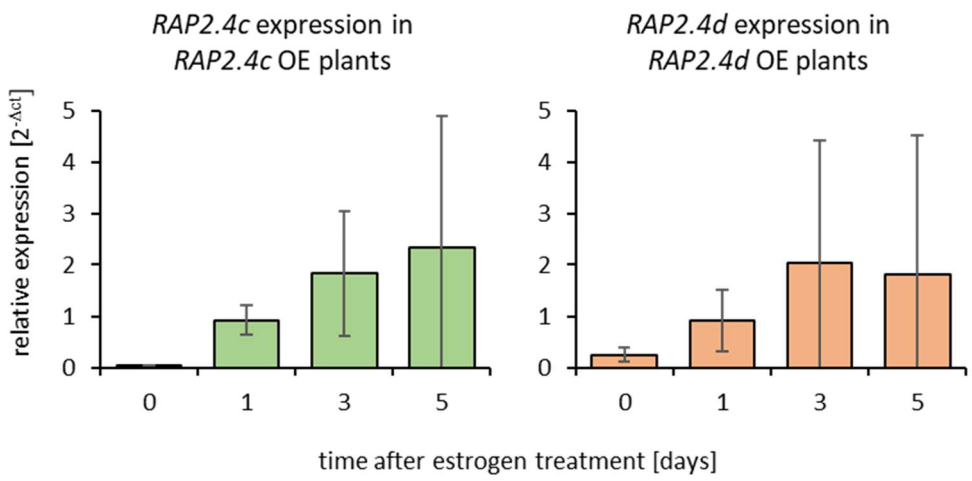


Figure 20: Analysis of inducible overexpression of *RAP2.4c* and *RAP2.4d* determined by RT-qPCR.
Four-week-old inducible *RAP2.4c* and *RAP2.4d* inducible overexpressor plants were sprayed with 100 µM 17β-estradiol for induction of overexpression. RNA was extracted and reverse transcribed into cDNA. *RAP2.4* expression was monitored via RT-qPCR over five days. n=3±SD.

After successful confirmation of both overexpression systems, Western blots were conducted to test overexpression on the protein levels. Sera extracted from rabbits that were injected with HIS-tagged *RAP2.4c* or *RAP2.4d* overexpressed in *E. coli* and purified via the tags were used as specific primary antibodies. Various attempts did not yield any signals on the blots. Even highly enriched nuclear fractions resulted in membranes with no signals. This may be explained by weak antibodies that generated weak signals even in *E. coli* overexpression samples in combination with low protein levels that may be insufficient for immunological detection.

Similar inducible overexpression lines were generated for *sAPX* and *tAPX* (see chapter 3.2.2). These genes exhibit stronger expression levels with well detectable protein levels. In these systems, successful overexpression could be confirmed on both the transcript and the protein levels to similar extents. These results made it reasonable to assume that induction of *RAP2.4c* and *RAP2.4d* at least transiently lead to increased protein abundances. Thus, both lines were used for experiments.

To investigate the influence of transient *RAP2.4c* and *RAP2.4d* overexpression on the cold independent expression of *MYC2* (JA-pathway) and *ORA59* (JA/ET-pathway) and their downstream genes *VSP2* and *PDF1.2a*, Col-0 and both inducible overexpression lines were grown for four weeks under standard conditions and then sprayed with estradiol to induce overexpression. Samples were taken before the treatment and after one, three and six hours. This short time frame was chosen over the five days span used to test overexpression, because the cold effect on hormone related genes in *rap2.4d* was observed after one hour at 4 °C when *RAP2.4d* would have been at its peak of expression. The data

revealed that overexpression of both genes started quickly after treatments and increased over time and that *RAP2.4d* expression was elevated already before the induction (**Figure 21**).

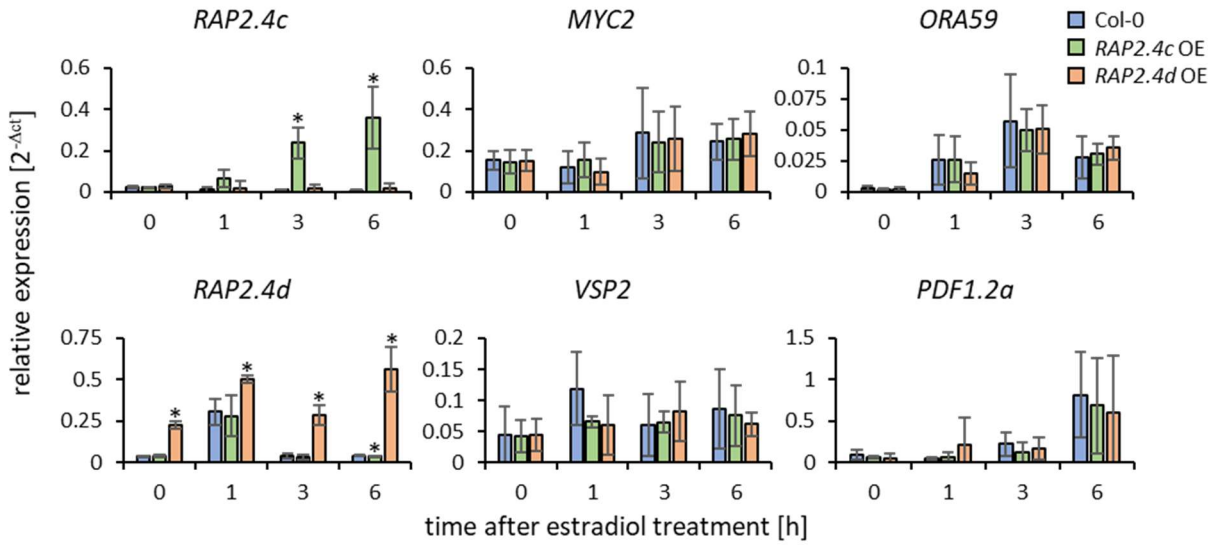


Figure 21: Relative expression of *RAP2.4c* and *RAP2.4d* and four readout genes determined by RT-qPCR. Four-week-old Col-0, *RAP2.4c* and *RAP2.4d* inducible overexpression plants were sprayed with 100 µM 17β-estradiol for induction of overexpression. Samples were harvested before the treatment and after one, three and six hours. RNA was extracted and reverse-transcribed into cDNA. Gene expression was monitored via RT-qPCR. n=3±SD, student's t-test relative to zero hours of the same genotype $p<0.05$.

RAP2.4d expression showed a short decline in transcript abundance after three hours before increasing again after six hours. This peak after one hour and the decline after three hours is a common expression pattern for *RAP2.4d* for a variety of abiotic stresses (Kilian *et al.*, 2007). Apparently, this negative regulation of *RAP2.4d* transcript stability is also active after estradiol treatment, while the artificial induction of gene expression was strong enough to overcome this transcript degrading destabilizing quickly. While overexpression of both genes was successful on the transcript level, none of the analysed readout genes showed any significant differences between the overexpression lines and the wild type. This could have several reasons. As previous studies have found, members of the RAP2.4 family may act as negative regulators of gene expression (Lin *et al.*, 2008; Bulcha, 2013). Also in the present study, particularly RAP2.4d seemed to have a negative influence on the expression of several hormone-related genes, as the lack of this gene led to the elevated expression of such genes (**Figure 12**). This negative regulation might be achieved by the RAP2.4d-dependent inhibition of a positive regulator that facilitates the expression of the hormone-related genes, which would fail in *rap2.4d* leading to the induction of target genes. In such a case, overexpression of *RAP2.4* genes might not further increase inhibition of gene expression of a gene that is already effectively inhibited. Another

reason might be a to date unknown mechanism that prevents RAP2.4c and RAP2.4d protein accumulation despite successful accumulation of their transcripts. However, a potential mechanism that was hypothesized in such a context involves the BTB⁄POZ-MATH (BPM) proteins that assemble with CUL3a and CUL3b and are hypothesized to act as adapter proteins to cullin-based E3-ligases. Members of this family have been demonstrated to bind RAP2.4b and RAP2.4d with RAP2.4b being quickly degraded in a 26S proteasome-dependent manner (RAP2.4d was not tested for this degradation) (Weber and Hellmann, 2009).

3.1.7 Assessment of JA dependent phenotypes

Neither hormone contents, nor direct responsiveness to hormone treatments, nor transient overexpression of the transcription factors revealed any differences between Col-0 and the KO/overexpressor lines, indicating a more subtle or context specific function. Thus, as JA related genes showed the most pronounced alterations, various JA dependent traits were analysed, comparing the KO lines with the wild type.

One such phenotype that is influenced by JA, is the regulation of senescence. While JA application induces early senescence (He *et al*., 2002), the precise mechanism is not clear and may depend on the modulation of ET signalling (Kim *et al*., 2015a). The influence of RAP2.4c and RAP2.4d on the senescence phenotype was tested by allowing Col-0, *rap2.4c* and *rap2.4d* plants to grow until they developed plenty of siliques and by visually observing differences between the genotypes. No visible differences could be determined in the onset or progress of leaf browning. But both T-DNA lines appeared to have more siliques and taller shoots (**Figure 22 A**). Both traits were quantified and analysed statistically (**Figure 22B**). The data revealed that *rap2.4d* plants had significantly more siliques than Col-0 and their shoots were taller, while this difference was not significant. *rap2.4c* plants once again, displayed an intermediate phenotype between Col-0 and *rap2.4d* plants with an intermediate number of siliques and *rap2.4d*-like shoot lengths, while none of both traits revealed a significant difference to Col-0.

Another plant trait that is modulated by JA is the root length. Elevated JA levels lead to reduced root growth (Barrera-Ortiz *et al*., 2018). To analyse whether RAP2.4c and RAP2.4d play a role in context of root length regulation, seeds of Col-0, *rap2.4c* and *rap2.4d* KO lines were sterilized and planted individually onto MS agar plates in a line. These plates contained either 1 µM JA or no JA as a control and were incubated in the light in an upright position for eleven days. Root length was determined for both groups (**Figure 22C**). Overall, the addition of JA to the growth medium significantly reduced root growth in all genotypes relative to the control samples. On those plates with no JA supplement, no

differences in root lengths could be observed between the genotypes. Presence of 1 µM JA on the other hand led to significantly increased root growth inhibition in *rap2.4d* seedlings compared with Col-0, leading to shorter roots, while *rap2.4c* seedlings displayed an intermediate phenotype that was not significant.

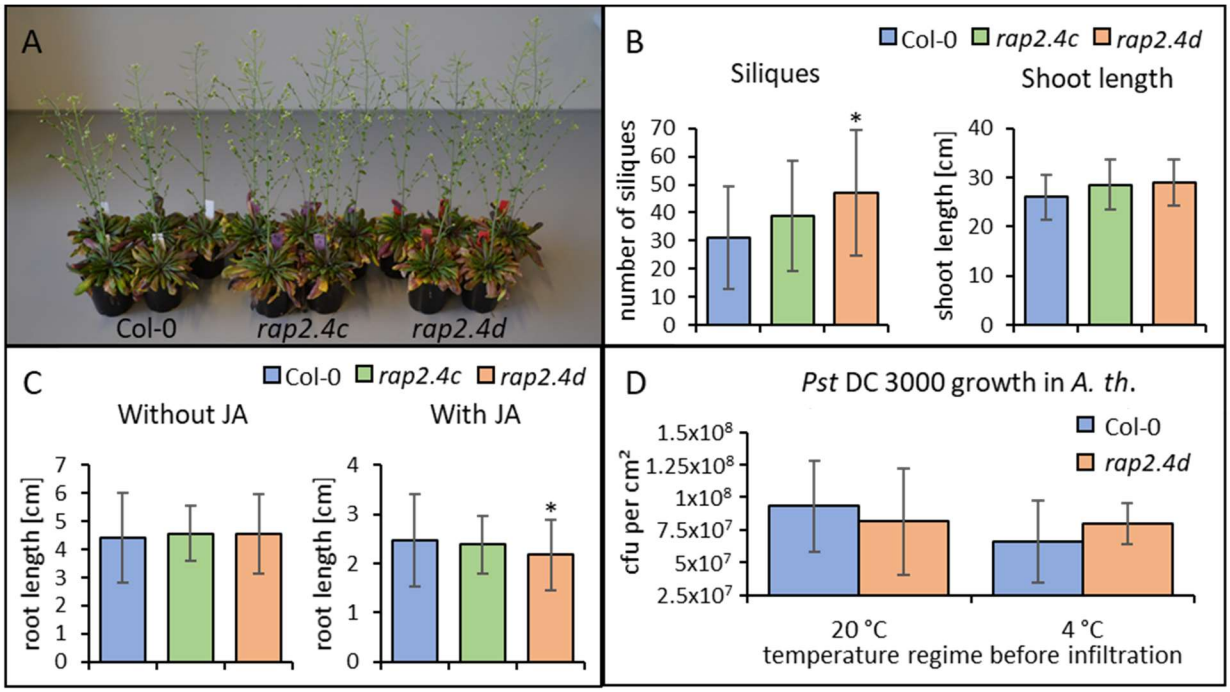


Figure 22: Evaluation of JA dependent phenotypes in Col-0, *rap2.4c* and *rap2.4d*.
A: Representative senescent plants that were used to quantify siliques and determine shoot lengths. **B:** Sterilised seeds of Col-0, *rap2.4c* and *rap2.4d* were planted on MS agar plates containing no or 1 µM JA and grown in an upright position. Root growth was determined after eleven days of growth. n≥46 individuals in three biological replicates ±SD, student's t-test KO lines vs. Col-0, $p<0.05$. **C:** Number of siliques and shoot length of senescing plants were determined. n≥15 individuals in three biological replicates ±SD, student's t-test KO lines vs. Col-0, $p<0.05$. **D:** Single leaves of four-week-old Col-0 and *rap2.4d* plants were infected with *Pst* DC3000 and normally grown for three days. Bacteria were isolated and grown on plates for three days and colony forming units were determined. n=5±SD, student's t-test *rap2.4d* vs. Col-0, $p<0.05$.

Regulation of both root length (Staswick *et al*., 1992) and senescence (He *et al*., 2002) are developmental processes in which JA more and more emerges to play a role. But the classical and probably best characterised function is the regulation of defence responses (reviewed by Howe and Jander (2008)). Activation of the JA pathway leads to enhanced resistance to herbivores, while increases in SA levels induce resistance towards biotrophic pathogens. Induction of either of the pathways inhibits the other pathway (reviewed in Pieterse *et al*. (2012)). Therefore, increased resistance against herbivores (JA pathway) comes at the cost of reduced resistance to pathogens (SA pathway). This way, JA has an indirect but strong influence on pathogen resistance. *Pseudomonas syringae* (*Pst*) DC3000 is a bacterial pathogen that injects the phytotoxin coronatine, a JA analogue,

into the host cell, leading to the activation of the JA pathway and repressing the SA pathway, thus repressing host resistance (Nomura *et al*., 2005). This way malfunctions in the JA signalling pathway in the *rap2.4d* line may influence pathogen resistance against *Pst* DC3000.

For this experiment, single leaves of Col-0 and *rap2.4d* KO plants were infiltrated with *Pst* DC3000 cells. Half of the plants had been pre-exposed to 4 °C for one hour to induce the induction of JA and SA genes that had been observed to be upregulated in *rap2.4d* in the RNAseq data. Infected plants were subsequently grown for three days, bacteria were isolated from leaf discs of uniform diameters and colony forming units (cfu) were determined (**Figure 22D**). Independent of pre-infiltration temperature treatments of the plants, no differences in bacterial growth could be observed. As the RNAseq data had already indicated, this cold-induced transcriptional re-programming in *rap2.4d* was very transient, as most of the upregulated genes were back to wild type levels after three hours of cold stress (**Figure 11**). As *rap2.4d* pathogen resistance was not affected by cold pre-treatment, the induction of JA and SA genes had no long-term effect on pathogen resistance over a course of three days.

Apparently, the lack of RAP2.4d fostered the JA induced root growth inhibition and led to an increased production of siliques during senescence, while it had little to no influence on shoot length and resistance to *Pst* DC3000. These data strengthen the notion that RAP2.4d has a very context dependent function in hormone signalling. These hormone-related phenotypes were mainly observed in plants that lacked *RAP2.4d*, while *rap2.4c* plants showed such phenotypes in a comparably mild form (**Figure 22**). This observation is consistent with the RNAseq data and with data obtained in other experiments: For example, only *RAP2.4d*, but not *RAP2.4c* was inducible by JA treatments (**Figure 18**). A similar observation was published by Iwase *et al*. (2011), where *RAP2.4d* (*WOUND INDUCED DEDIFFERENTIATION 2, WIND2*), but not *RAP2.4c* was induced by wounding. Wounding is an inevitable consequence of insect feeding and JA levels increase quickly as a response to induce defence reactions (Creelman *et al*., 1992; Bell *et al*., 1995). Consequently, induction of *RAP2.4d* upon wounding is most likely due to an increase in JA levels, while this hypothesis was not addressed in their study or in several follow-up studies. In accordance with this, Iwase *et al*. (2011) demonstrated that constitutive overexpression of *RAP2.4d* but not of *RAP2.4c* induced massive cell de-differentiation and formation of callus tissue in healthy seedlings. Callus formation is usually regulated by the phytohormones auxin and cytokinin (Skoog and Miller, 1957), but the RNAseq data did not show any differences in genes that are responsive to these hormones (data not shown). These consistent differences between RAP2.4c and RAP2.4d suggest a function of RAP2.4d in hormone crosstalk that is not fulfilled by RAP2.4c to the same extent.

3.1.8 NPR1 translocation into the nucleus may be regulated by RAP2.4d

None of the experiments investigating the role of RAP2.4c and RAP2.4d in the hormone crosstalk yielded any clues on how the disturbed crosstalk may be explained in the KO lines. Most of the experiments had focused on *MYC2* because of its central position in hormone crosstalk, that explained both the *PDF1.2a* and the *PR1* expression patterns (**Figure 15**). Shifting the focus on NPR1, which has a similar potential to explain the failed crosstalk, resulted in another hypothesis.

NPR1 is a well characterised master regulator gene in the SA-dependent regulation of plant basal and systemic acquired resistance against pathogens (Cao *et al*., 1994; Delaney *et al*., 1995; Fu and Dong, 2013). Under standard conditions, it is predominantly located in the cytosol forming a homo-oligomer that is stabilized via intermolecular disulfide bonds (Mou *et al*., 2003). Pathogen attack leads to an increase in SA levels (Malamy *et al*., 1990). This in turn results in a biphasic change in cellular reduction potential that leads to the dissociation of the NPR1 oligomer and to the translocation of the monomers to the nucleus (Mou *et al*., 2003). As a transcriptional co-activator which does not interact with DNA on its own, nuclear NPR1 binds transcription factors such as members of the TGA (Zhang *et al*., 1999; Despres *et al*., 2000; Fan and Dong, 2002) and WRKY families (Saleh *et al*., 2015). These complexes then regulate gene expression. Two of the most prominent NPR1/TGA target genes that are induced upon pathogen attack are the *PATHOGENESIS-RELATED* (*PR*) genes 1 and 2 (Zhang *et al*., 2003; Kesarwani *et al*., 2007). These two genes were strongly induced in the *rap2.4d* KO line after one hour at 4 °C in the present RNAseq data set (**Figure 11**), indicating nuclear localisation of NPR1 in this sample. In the wild type sample that was treated equally, both genes were downregulated instead, supporting cytosolic localisation of NPR1 and active inhibition of *PR* gene expression via the JA response (Walley *et al*., 2008).

In addition to explaining the strongly induced *PR* gene expression in *rap2.4d*, the hypothetical nuclear localisation of NPR1 may at the same time be an explanation for the increased activation of *MYC2* and its downstream genes, among them *VSP1* (**Figure 15**). JA signalling is actively inhibited by cytosolic NPR1, while nuclear NPR1 exhibits no such function (Spoel *et al*., 2003). In *rap2.4d*, cold-induced translocation of NPR1 to the nucleus may lead to a depletion of cytosolic NPR1, as NPR1 protein levels are very low under standard conditions. A lack of cytosolic NPR1 may abolish its inhibitory function on the JA-pathway leading to the increased expression of *MYC2* and its downstream genes that was observed in *rap2.4d* in the cold. This way, translocation of NPR1 to the nucleus may explain increased expression of both SA genes (*PR1* and *PR2*) and JA genes (*MYC2* and *VSP1*).

Olate *et al*. (2018) have identified 71 cold-inducible genes that were downregulated in *npr1* mutants relative to Col-0 after 24 hours at 4 °C, identifying NPR1 as a co-activator of their expression in the cold. Comparison of these 71 genes with genes that were upregulated in *rap2.4c* and *rap2.4d* in the

cold resulted in 27 and 43 matches, respectively (Appendix) (**Figure 23A**). 21 out of the 71 genes were found to be upregulated in both KO lines, showing quite some similarities between *rap2.4c* and *rap2.4d*. Whereas only six genes were exclusively upregulated in *rap2.4c* but not in *rap2.4d*, 22 genes were exclusive to *rap2.4d*. This supports the notion, that *rap2.4d* displays the broader effect on the hormone crosstalk with *rap2.4c* being affected to a smaller extent (**Figure 11** and **Figure 12**).

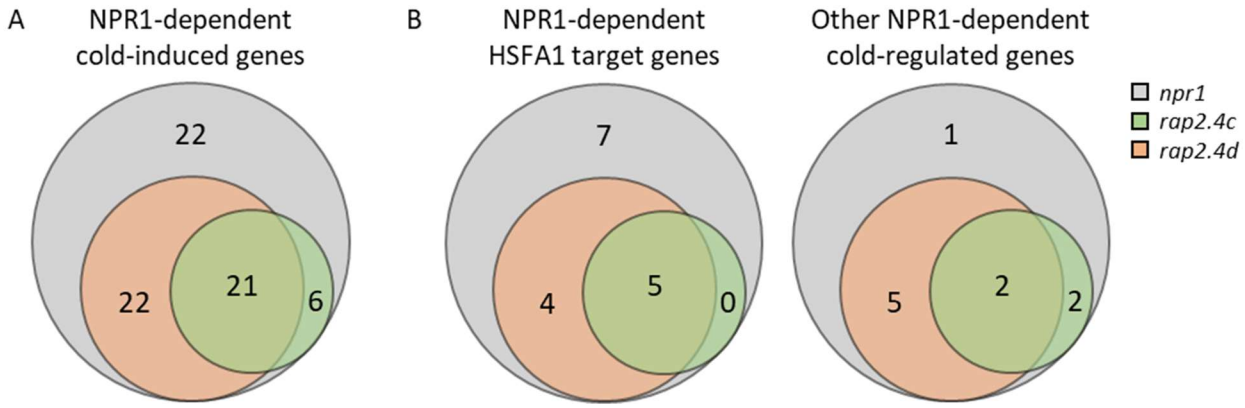


Figure 23: Comparison of cold-responsive NPR1-dependent genes with genes that were upregulated in *rap2.4c* and *rap2.4d* in the cold.
A: The Venn diagram shows the overlap of genes that were upregulated in *rap2.4c* and in *rap2.4d* after one or three hours at 4 °C relative to Col-0 (RNAseq data, experimental procedure in **figure 6**) within the 71 cold-inducible NPR1-dependent genes (Olate *et al*., 2018). **B:** Sub-groups out of the 71 NPR1-dependent genes are shown comparing the same three genotypes.

In the same study, Olate *et al*. (2018) have analysed two groups of NPR1 target genes in more detail. Firstly, the target genes of the HEAT SHOCK TRANSCRIPTION FACTOR 1 (HSFA1) and secondly, a group of other heterogenous cold-responsive genes that were downregulated in *npr1* in the cold. As shown in their study, nuclear NPR1 interacts with HSFA1 which induces expression of its downstream genes. A comparison of genes from both groups with the genes that were upregulated in the RNAseq data in both KO lines, revealed a familiar pattern (**Figure 23B**): Out of the 16 analysed HSFA1 targets, five and nine genes were upregulated in *rap2.4c* and *rap2.4d* in the cold, respectively. The list of the heterogenous group of cold-responsive NPR1-dependent genes (ten genes) contained four and seven such genes, respectively, revealing a higher correlation of NPR1 target genes with genes that were upregulated in *rap2.4d* than in *rap2.4c*. Also, genes from both lists were among the upregulated genes in both KO lines, indicating that the lack of *RAP2.4d* (and *RAP2.4c*) did not lead to the activation of a specific NPR1 downstream response, but to a more general activation of NPR1 responses.

The 16 HSFA1 target genes encode heat-shock proteins that are primarily associated with heat stress, but also have functions in other abiotic stresses. Heat shock proteins are mostly molecular chaperones

that assist in the correct folding of proteins during their synthesis or that help in maintaining native structures under certain conditions, such as dehydration-related stresses (water deficiency, osmotic, cold) (Nishizawa *et al*., 2006). With this in mind, it is noteworthy, that *npr1* mutants only exhibited reduced cold tolerance but were indistinguishable in their responses to drought and salt (Olate *et al*., 2018), indicating a specific NPR1 function in the cold response.

The HSFA1 target genes and the heterogenous group of genes from the *npr1* study contained genes that were not differentially regulated in *rap2.4d* (**Figure 23B**). Probably, the differences in cold exposure durations may contribute to explain this. Olate *et al*. (2018) analysed differentially expressed genes after 24 hours at 4 °C in an *npr1* mutant, while in the present study the upregulation of hormone-related genes was observed after one hour in the cold in *rap2.4d*. Accordingly, the transcriptomic and proteomic states in both systems were vastly different. The populations of transcriptional regulators in the nucleus differed strongly. In a transcriptomic study, Lee *et al*. (2005) demonstrated that in the early cold response after three hours at 4 °C, 128 and four genes were up- and downregulated, respectively. Later, after 24 hours in the cold, 581 and 265 genes were up- and downregulated, respectively. Including another six-hour sample, a total of 655 genes were upregulated within the first 24 hours in the cold. Out of these, 113 (17.3 %) were annotated to have a function in transcription. Furthermore, transcription factors were mainly upregulated in the early cold response, probably modulating the later response. Consequently, the potential for interactions between NPR1 and its interaction partners differs strongly between the early and the later cold response. Against this background, the correlation of differentially regulated genes in *rap2.4d* and *npr1* in the cold is not perfect but strikingly high to assume a common mechanism behind both phenomena.

The strong overlap in gene expression of NPR1 target genes in the cold with genes that were upregulated in the cold in *rap2.4d* (**Figure 23**), suggests that cold-induced translocation of NPR1 to the nucleus might be actively inhibited in the wild type by RAP2.4d. A lack of RAP2.4d in the KO line would then lead to the accumulation of NPR1 in the nucleus and to a depletion of cytosolic NPR1. This in turn would activate both transcriptional SA responses and, due to a lack of MYC2 inhibition, JA responses. This notion is supported by the strong induction of two of the most prominent NPR1 target genes, *PR1* and *PR2* in the *rap2.4d* KO line after one hour at 4 °C (**Figure 11C**).

In order to narrow down options in this putative signalling pathway, several pilot experiments were designed. Firstly, the hypothetical translocation of NPR1 was analysed via Western blots. Col-0 and *rap2.4d* plants were grown as for the RNAseq experiment and samples were harvested following the same sampling scheme. Due to very low NPR1 protein levels and thereout resulting expectable detection difficulties, several wild type plants were sprayed with 1 mM SA to induce *NPR1* expression as a positive control and were harvested 24 hours after the treatment which was shown to

substantially increase NPR1 protein levels in the nucleus (Mou *et al*., 2003). Nuclei were extracted from all samples and identical amounts of proteins were analysed via Western blots. Detection of histone-H3 proteins served as loading controls and showed strong bands that were absent in nuclei depleted samples, indicating successful extraction of the nuclei. However, NPR1 detection failed in all samples, even in the SA-treated positive controls, leaving the question of increased nuclear NPR1 accumulation open. To better address the hypothesis of increased cold-induced nuclear translocation of NPR1 in *rap2.4d*, a future experiment should be conducted with a tagged version of NPR1 that facilitates detection in a *rap2.4d* KO background.

3.1.9 PKS5, a common upstream regulator of RAP2.4d and NPR1

The PROTEIN KINASE SOS2-LIKE 5 (PKS5, also called SnRK3.22) has previously been shown to phosphorylate NPR1 and to be essential for pathogen-induced expression of *PR1, WRKY38* and *WRKY62* which requires nuclear translocation of NPR1 (Xie *et al*., 2010). This kinase additionally phosphorylates RAP2.4d (Lumba *et al*., 2014) establishing a direct connection between RAP2.4d and NPR1. RAP2.4d can additionally be phosphorylated by the closely related PKS24 (also called SnRK3.15) (Lumba *et al*., 2014). Posttranslational modifications of NPR1, such as phosphorylation, ubiquitination and sumoylation are essential for the regulation of NPR1 activity and localisation (Mou *et al*., 2003; Spoel *et al*., 2009; Xie *et al*., 2010; Lee *et al*., 2015; Olate *et al*., 2018). This includes the regulation of the oligomer/monomer equilibrium, the transport of the monomers to the nucleus and their stability.

PKS5 appears to phosphorylate NPR1 to allow for its translocation to the nucleus (Xie *et al*., 2010). As the putatively NPR1-dependent induction of hormone-related genes in the cold was observed only in *rap2.4d* and not in Col-0, it is possible that this reaction may only take place in the *rap2.4d* KO line, not in the wild type under the conditions that were tested in the RNAseq experiment. Cold-induced upregulation of *RAP2.4d* in Col-0 may potentially prevent cold-induced PKS5-dependent phosphorylation of NPR1 that would otherwise lead to the translocation of NPR1 into the nucleus.

Proteins from the calcium binding CALCINEURIN B-LIKE PROTEIN (CBL) family are, in addition to RAP2.4d and NPR1, further targets of various PKS kinases (Du *et al*., 2011). The interaction of various PKS and CBL proteins exhibits an intriguing feature. For example, phosphorylation of CBL1 by PKS5 stabilizes the interaction of both proteins neutralizing the inhibitory effect of PKS5 on a membrane bound ATPase, as the kinase is competitively bound and not available for further enzyme reactions (Du *et al*., 2011). In a similar way, phosphorylation of cold induced RAP2.4d may stabilize the PKS5/RAP2.4d interaction scavenging away free PKS5 leaving NPR1 unmodified in the cytosol. Du *et al*. (2011) also investigated the phosphorylation site in the CBL proteins, while it is unknown in RAP2.4d and NPR1.

The highly conserved PFPF domain, which is exclusive to this family, is located at the very C-terminal end and harbours an FPSF sequence of amino acids. The serine (CBL1: S201) was reported to be the phosphorylation site and the conserved flanking sequence may represent the recognition site for PKS kinases. A highly similar sequence, YPSY, can be found in the RAP2.4d protein, as well at the very C-terminus as in the CBL proteins. Phenylalanine (F) and tyrosine (Y) are identical in their molecular structures, except for a single additional hydroxyl group in tyrosine. In terms of steric properties, both recognition sites may be recognised by PKS5 with similar affinity, with the hydroxyl group modulating the intensity of this interaction. The PS core of this site is conserved in the entire RAP2.4 protein family and is located at the very C-terminus, as in the CBL family, with a clear overrepresentation of phenylalanine and tyrosine directly flanking this core (**Figure 24**). Valine (V) and methionine (M) are represented more than once as well. These directly flanking amino acids may potentially regulate kinase binding affinities.

Figure 24: Alignment of the C-terminal amino acids of all Arabidopsis RAP2.4 proteins.
The alignment was created with the Clustal Omega tool. Conserved identical amino acids are shown on a black background. Phenylalanine and tyrosine amino acids flanking the PS core are shown on a grey background. Numbers on the right indicate the position of the last shown amino acid.

NPR1 has only a single PS core (S414). This PS core is embedded in a PPSF sequence which shares the downstream phenylalanine but replaces the upstream phenylalanine that is found in CBL1 by another proline. This amino acid exchange may result in a weaker affinity of PKS5 to NPR1 than to RAP2.4d, enabling RAP2.4d to bind free PKS5 protein leaving NPR1 unmodified.

To investigate whether RAP2.4c or RAP2.4d can stably interact with PSK5 and PKS24, a Yeast-Two-Hybrid assay was conducted with modified versions of both RAPs. The S residues (RAP2.4c: S247, RAP2.4d: S245), which are the putative targets for phosphorylation, were substituted by alanine (A) which cannot be phosphorylated and by aspartic acid (D) or glutamic acid (E) which are the two phosphomimic amino acids, simulating a permanent phosphorylation, due to their steric properties and their negative charge. To achieve this, the codons encoding the specific S residues of the *RAP2.4c* or *RAP2.4d* genes were replaced by codons encoding A, D or E using site directed mutagenesis PCRs

(Montemartini *et al*., 1999). EcoRI and PstI overhangs were included in the PCR products and used to clone the inserts into the EcoRI and PstI sites of the pGBKT7 vector. PKS5, PKS24, CBL1 and NPR1 full length coding sequences were amplified from Arabidopsis cDNA samples using primers including 5' BamHI or BglII sites and 3' SacI or SalI sites. Hydrolysis with BamHI or BglII on the one hand and SacI or SalI on the other hand, leads to identical overhangs, allowing for ligation of all inserts into the BglII and SacI sites of the pGADT7 vector. Yeast cells were co-transformed with all 45 possible combinations of pGBKT7 (containing *RAP2.4c, RAP2.4c S247A, RAP2.4c S247D, RAP2.4c S247E, RAP2.4d, RAP2.4d S245A, RAP2.4d S245D, RAP2.4d S245E* and no insert) and pGADT7 (*PKS5, PKS24, NPR1, CBL1* and no insert). In addition to the analysis of differences of binding intensities, direct binding of all RAP2.4c and RAP2.4d variants with NPR1 and CBL1 were tested in this assay (**Figure 25**).

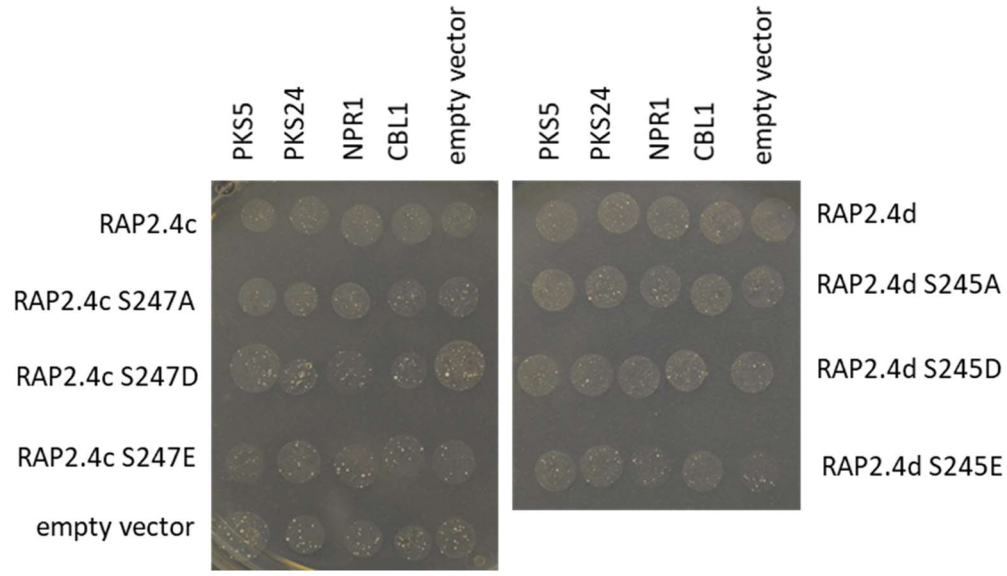


Figure 25: Yeast-Two-Hybrid experiment investigating RAP2.4 protein-protein interactions.
Yeast cultures expressing RAP2.4c or RAP2.4d in their native forms or with substituted serine residues (RAP2.4c: S247, RAP2.4d: S245, encoded on pGBKT7 plasmids) and either of the proteins PKS5, PKS24, NPR1 or CBL1 (encoded on pGADT7 plasmids) were dropped on SD-LTH agar plates containing 10 mM 3-AT. Serine residues were substituted by alanine (A), aspartic acid (D) or glutamic acid (E). Representative results of one out of three replicates are shown.

Cell growth was strongly inhibited on medium containing 10 mM 3-AT. At this density of colonies, comparison of cell growth could be conducted. But on all plates, cell growth was very similar for all yeast strains. Particularly, the strain carrying both empty plasmids showed similar growth to all other strains, indicating all growth to be due to leaky HIS3 expression exclusively, instead of successful protein-protein binding. Because no strain yielded better growth than this negative control, none of the tested protein pairs exhibited strong interactions. Even the pair that has a published interaction,

RAP2.4d and PKS5, showed no enhanced binding, indicating this phosphorylation might be a very short-lived interaction that only happens for the brief moment of the reaction that is insufficient to induce *HIS3* expression in this system. Also, the phosphomimic variants of both RAP2.4 proteins did not show enhanced binding, so there did not seem to be a stabilized interaction upon phosphorylation of the RAPs by the PKS kinases as it was found for the PKS5/CBL1 interaction. These results indicate that the FPSF motif in CBL1 may exhibit stronger binding affinity for PKS5 than the FPSV and YPSY versions in RAP2.4c and RAP2.4d.

Such yeast-based experiments represent a good model for protein-protein interactions that take place in plant cells, as both are eukaryotic organisms. But some interactions which may exist in plants *in vivo* may not give positive results in yeast-based assays due to wrong folding of the proteins or lacking further interaction partners or posttranslational modifications that are required for successful interaction (Deeds *et al*., 2006). Thus, the PKS/RAP2.4d interaction could not be confirmed in this study, but it can't be ruled out yet, either.

3.1.10 Analysis of the PKS5 phosphorylation site in RAP2.4c and RAP2.4d

As mentioned in chapter 3.1.9, RAP2.4d and RAP2.4c share a similar amino acid sequence with the published PKS phosphorylation site in the CBL family (Du *et al*., 2011). To analyse whether this YPSY (FPSV) sequence is the site of phosphorylation by PKS5 and PKS24, a kinase assay was conducted. In this assay, reactions of PKS5 and PKS24 with RAP2.4c and RAP2.4d and their S247A/S245A variants lacking the serine in question were conducted. In addition, CBL1 was used as a positive control for the reaction.

Coding sequences of all mentioned genes were amplified via PCR with primers that added a KpnI site at the 5' end and an MscI site at the 3' end. PCR products were cloned into the pOPINF vector, transformed into Top10 *E. coli* cells and analysed by sequencing for their correct sequence. Plasmids from clones with correct sequences were transformed into BL21 (DE3) pLysS *E. coli* cells for overexpression. Proteins expressed from the pOPINF vector carried an N-terminal 6x HIS tag for purification and detection.

Overexpression was tested via Western blot (data not shown, but expression can be seen in **Figure 26** with the very proteins samples that were used for a kinase assay). For the kinase assay, all steps, starting with the protein overexpression and ending with the electrophoretic separation of the proteins after the kinase reactions, were carried out in one day to ensure native and fresh proteins and prevent protein denaturation, particularly of the kinase proteins.

All proteins that were used for the kinase assay were freshly overexpressed in BL21 (DE3) pLysS cells. Previous experiments have shown that all these proteins tended to form inclusion bodies during the overexpression. For this reason, after overexpression, proteins were recovers from the pellets of lysed *E. coli* cells instead of the supernatants and a specialized protein extraction protocol was applied that efficiently recovered proteins from inclusion bodies. Compared with other protocols (Dürauer *et al*., 2009), this modified protocol avoided chemicals that denature proteins in order to obtain native solubilized proteins. These proteins from solubilised inclusion bodies were subsequently HIS-tag purified. The imidazole from the elution buffer poses a problem for cation-dependent enzyme reactions, such as the conducted kinase assay. Purified protein solutions were desalted and finally used for the kinase assay. Additionally, proteins were detected via Western blot to confirm successful expression of the proteins and their recovery throughout the purification procedures (**Figure 26**).

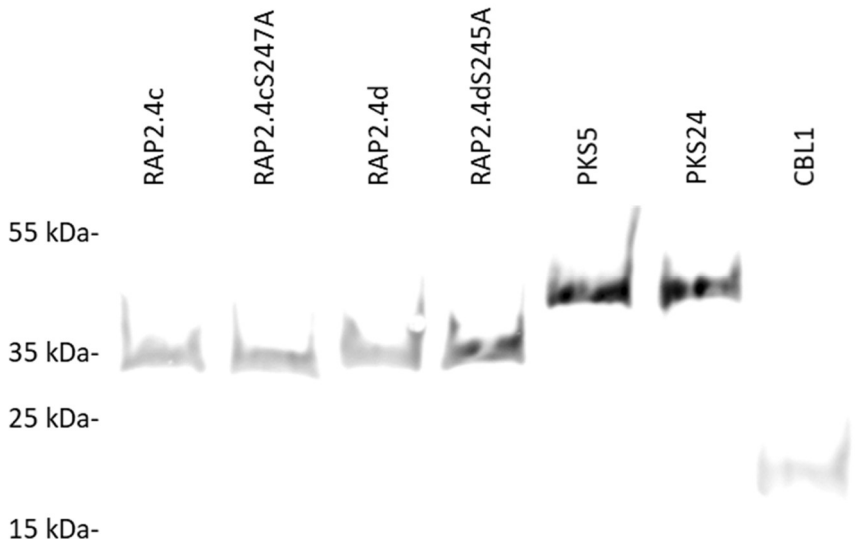


Figure 26: Immunological detection of proteins after overexpression in E. coli and HIS-tag purification.
Proteins were freshly overexpressed in BL21 (DE3) pLysS cells, extracted from the cells, HIS-tag purified and desalted. After electrophoretic separation, proteins were transferred to a membrane and immunologically detected with the α-HIS-tag antibody.

For the kinase assay, PKS5 or PKS24 proteins were mixed with RAP2.4c, RAP2.4c S247A, RAP2.4d, RAP2.4d S245A or CBL1. In a separate reaction, CBL1 was incubated alone as a negative control. All reactions were stopped with SDS running buffer and electrophoretically separated. The gels were exposed to phosphoimaging films for two to five days and detected. No signal could be detected in three independent experiments. Even the native RAP2.4d and CBL1 proteins that had been reported to be PKS5 targets did not reveal any phosphorylation signals. The Western blot clearly confirmed sufficient protein overexpression, but the kinase assay did not give evaluable results.

Kinase assays require native kinase enzymes that are still able to bind their target proteins and carry out their enzymatic reaction. Both kinase enzymes and the analysed target proteins formed inclusion bodies during their overexpression in *E. coli* cells. Purification of proteins from inclusion bodies, subsequent HIS-tag purification and de-salting of the samples required lengthy procedures which may have taken too long to sustain native enzymes throughout the experiment. Western Blot analysis confirmed the presence of proteins of the correct in all samples (**Figure 26**) but confirming the native status of the proteins was not possible.

3.1.11 RAP2.4d may inhibit cold-induced expression of *TRXH5* and *SnRK2.8*

Interaction of RAP2.4d with PKS5 could not be confirmed as the mechanism regulating the accumulation of NPR1 in the nucleus in *rap2.4d* at 4 °C (chapters 3.1.9 and 3.1.10). However, a very recent paper led to a new hypothesis that may explain nuclear accumulation of NPR1. Olate *et al*. (2018) have shown that the cytosolic THIOREDOXINs (TRXH) 3 and 5 and the SNF1-RELATED PROTEIN KINASE (SnRK) 2.8 are necessary for cold-induced translocation of NPR1 to the nucleus as the *snrk2.8-1* and the *trxh3trxh5* double KO lines almost completely failed to import NPR1. Among the eight members of the *TRXH* gene family, *TRXH3* is the one with the strongest constitutive expression (Reichheld *et al*., 2002), while *TRXH5* is the only one that is strongly induced upon oxidative stress and infection with *Pst* DC3000 (Laloi *et al*., 2004). A similar expression pattern was observed in the present RNAseq data in Col-0 (**Figure 27**): *TRXH3* exhibited an expression level that was substantially higher than that of *TRXH5*, whereas *TRXH5* expression slowly increased over time in Col-0. In *rap2.4d* however, *TRXH5* transcript levels strongly peaked after one hour. *SnRK2.8* transcript levels decrease after the transfer of Col-0 to 4 °C. In *rap2.4d*, this downregulation was impaired during the first hour at 4 °C but was visible after three hours in a weaker form compared with Col-0. Elevated transcript levels of both *TRXH5* and *SnRK2.8* in *rap2.4d* indicate nuclear localisation of NPR1 after one hour at 4 °C.

Upregulation of these two genes could explain the putative elevated nuclear translocation of NPR1. The *TRXH5* promoter contains a DRE-motif, that was published as the RAP2.4d binding motif (Rae *et al*., 2011), 220 bp upstream of the transcription start and a CCCGT sequence that is part of the second motif that was found in the yeast system (**Figure 5**) 538 bp upstream of the transcription start. The *SnRK2.8* promoter does not contain a precise copy of the DRE-motif within 1000 bp, but four copies that include single bp exchanges in the 3rd or 4th bp of the motif (CC**GA**C) that reduce the DNA binding capacity of RAP2.4d but are dispensable for DNA-binding (Rae *et al*., 2011). Among these motifs, a CCGTC sequence which is located 652 bp upstream of the transcription start additionally strongly resembles the CCCGT motif that is present in the TRXH5 promoter. Both sequences lie in the CCCGTC

sequence shown in **Figure 5A** and are only shifted by a single base pair. Also, *SnRK2.8* was less induced than *TRXH5* which would be in line with a reduced RAP2.4d DNA binding affinity due to slightly different binding affinities to the slightly varying promoter sequences. These findings suggest RAP2.4d to be a negative regulator of *TRXH5* and *SnRK2.8* expression. In the *rap2.4d* KO line, the lack of *RAP2.4d* induction in the early cold response putatively enabled the expression of *TRXH5* and *SnRK2.8* which both have complete or slightly modified DRE-motifs and a similar CCCGTC motif closely upstream of their transcription starts. Induction of these genes may then result in the monomerization of NPR1 (facilitated by TRXH5) and in the translocation of NPR1 into the nucleus (facilitated by SnRK2.8). This in turn may explain the failed crosstalk of the JA/ET-JA-SA pathways and the particularly strongly induced expression of SA downstream genes (**Figure 11**). As the study demonstrating the NPR1-dependent cold-response and the roles of *TRXH5* and *SnRK2.8* therein was published (Olate *et al*., 2018) during the finalisation of the present study, the hypothesis that RAP2.4d is a negative regulator of *TRXH5* and *SnRK2.8* expression which modify nuclear translocation of NPR1 is an intriguing project for further studies.

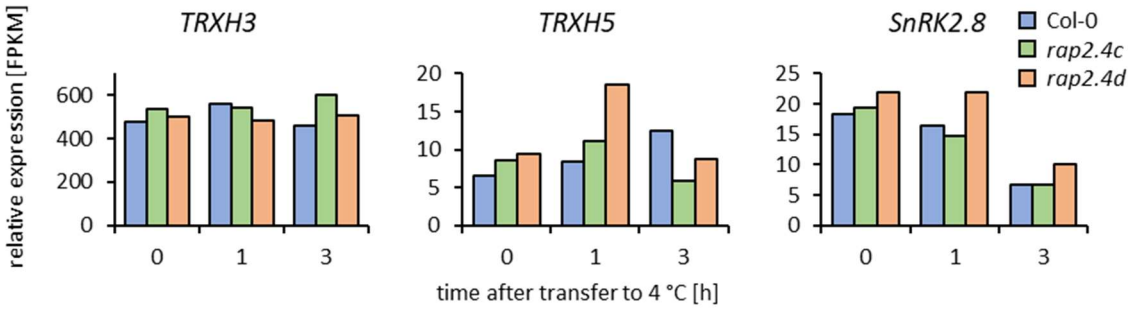


Figure 27: RNAseq data of *TRXH3*, *TRXH5* and *SnRK2.8* at 4 °C.
The experimental procedure was the same as described in the legend of **Figure 7**.

TRXH5 expression has been demonstrated to be enhanced by the transcription factor WRKY6 (Laloi *et al*., 2004). This study has shown that the last 1000 bp of the *TRXH5* promoter contain five copies of the W-box *cis*-element, which is a typical WRKY binding site that mediates expression during oxidative stress situations. Furthermore, *WRKY6* overexpressing plants were shown to exhibit strongly elevated *TRXH5* expression. Overexpression of *WRKY6* not only increased expression of *TRXH5*, but also of *PR1* (Laloi *et al*., 2004). The present RNAseq data indicate no differences in *WRKY6* transcript levels between the genotypes, while they double after one hour in the cold (data not shown). As *RAP2.4d* and *WRKY6* are both inducible within one hour at 4 °C and may inversely regulate *TRXH5* expression,

it is possible that they represent a mechanism for the finetuning of *TRXH5* expression in the early cold response and thus, for the regulation of NPR1 location and activity.

3.1.12 *rap2.4c* shows a weak *rap2.4d* phenotype

The analysis of various hormone-related phenotypes revealed a recurring pattern that was observed over and over: Significant differences in phenotypes and correlations were always observed in *rap2.4d* KO plants. *rap2.4c* plants usually showed a similar trend, but in a weaker, not significant form or showed less correlation with other phenomena. Relative to *rap2.4c*, *rap2.4d* plants exhibited more genes with a stronger upregulation of hormone-related genes in the RNAseq (**Figure 11** and **Figure 12**), a better correlation of these genes with the cold-induced NPR1-dependent genes (**Figure 23**) and stronger JA effects on root growth, shoot length and the number of siliques (**Figure 22**). *RAP2.4d*, but not *RAP2.4c*, was reported to have potentially JA-related functions in cell de-differentiation (Iwase *et al*., 2011) and expression of *RAP2.4d* was induced by JA treatment, while *RAP2.4c* was not (**Figure 18A**). The lack of *RAP2.4d* significantly improved freezing tolerance in naïve plants, whereas *rap2.4c* KO plants only showed a minor and not significant tendency towards improved tolerance and rather improving cold acclimation responses (**Figure 2**). Furthermore, *TRXH5,* which is hypothesized to be a direct target of RAP2.4d and whose induction may lead to the nuclear translocation of NPR1 is induced to an intermediate extent in *rap2.4c* (**Figure 27**). *RAP2.4d* expression quickly increased at 4 °C and then declined again (**Figure 6**). Such a cold-induced upregulation of *RAP2.4d* was also observed in the RNAseq data, but only in the wild type, not in *rap2.4c* (**Figure 28A**). *RAP2.4d* induction also failed in *rap2.4c* after JA treatments (**Figure 28B**). The data show that in *rap2.4c*, *RAP2.4d* transcripts were on a wild type level before the treatments, but their cold- or JA-triggered induction failed. Thus, stimulus-triggered induction of *RAP2.4d* seems to be RAP2.4c-dependent. Also, the timing of induction of *RAP2.4c* and *RAP2.4d* in the cold supports this notion, as induction of RAP2.4c precedes that of RAP2.4d in Col-0 (**Figure 6**). However, as inducible overexpression of *RAP2.4c* had no detectable effect on *RAP2.4d* expression (**Figure 21**), RAP2.4c would depend on specific conditions or interaction partners to induce RAP2.4d expression.

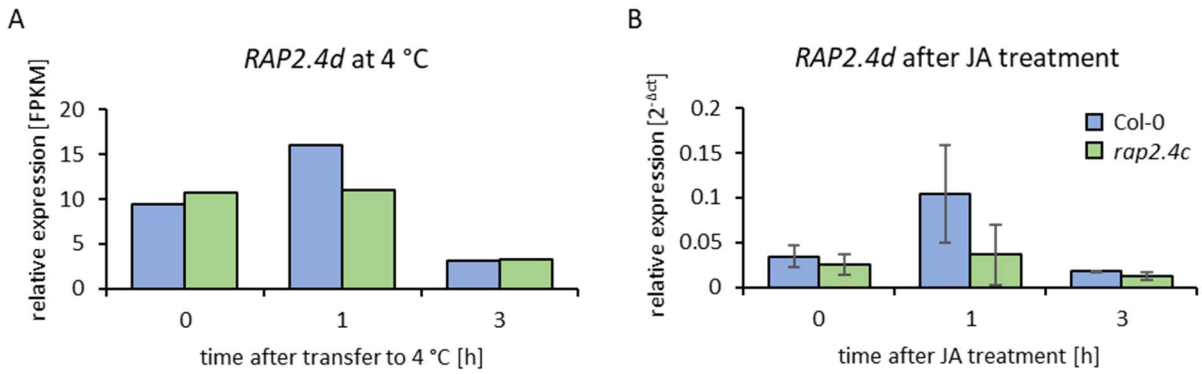


Figure 28: Transcript data of *RAP2.4d* after treatments that induce *RAP2.4d* expression.
A: RNAseq data of Col-0 and *rap2.4c* plants that were transferred to 4 °C (the experimental procedure was the same as described in the legend of **Figure 7**). **B:** RT-qPCR data of Col-0, *rap2.4c* and *rap2.4d* plants that were sprayed with 50 µM JA. Data were obtained in the experiment that is shown in **Figure 18**. n=3±SD, student's t-test relative to the wild type at the same time, $p<0.05$.

These data hint towards RAP2.4d as the central protein that causes the failed hormone crosstalk also in the *rap2.4c* KO line. As *rap2.4c* plants failed to induce *RAP2.4d* upon cold and JA treatments (**Figure 28**), but basal *RAP2.4d* levels were expressed in *rap2.4c*, the various intermediate phenotypes that were observed in *rap2.4c* relative to *rap2.4d* may be due to the intermediate levels of *RAP2.4d* in *rap2.4c*.

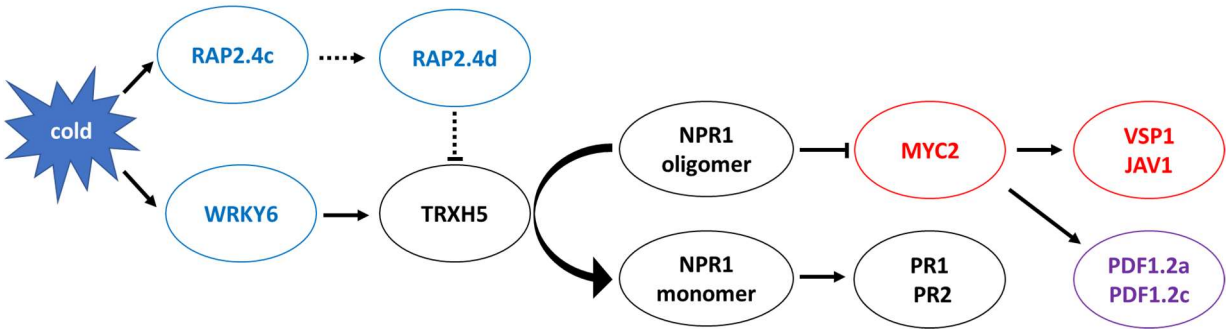


Figure 29: The proposed model for the function of RAP2.4c and RAP2.4d in the hormone crosstalk during the early cold response.
Cold exposure leads to the expression of *WRKY6*, *RAP2.4c* and *RAP2.4d*, while *RAP2.4d* expression may be dependent on RAP2.4c. Accumulation of RAP2.4d inhibits WRKY6-driven *TRXH5* expression (Laloi *et al*., 2004). TRXH5 catalyses the reduction of disulfide bonds in cytosolic NPR1 oligomers enabling their translocation into the nucleus (Olate *et al*., 2018). Nuclear NPR1 facilitates expression of its target genes *PR1* and *PR2 (Fan and Dong, 2002)*, while depletion of cytosolic NPR1 enables MYC2 downstream gene expression of JA-responsive (*VSP1* and *JAV1*) (Spoel *et al*., 2003) and JA/ET-responsive (*PDF1.2a* and *PDF1.2c*) genes (Leon-Reyes *et al*., 2009; Zander *et al*., 2010). Solid lines indicate published interactions and dotted lines indicate hypothesised interactions from the present study. Blue genes are cold-responsive, black genes are involved in SA, red genes in JA and purple genes in JA/ET signalling.

The data that were presented and discussed in the previous chapters lead to a regulatory scenario, in which cold-induced accumulation of RAP2.4d in Col-0 plants inhibits WRKY6-driven expression of *TRXH5*. This in turn would prevent TRXH5-dependent monomerization of NPR1 and consequently its translocation into the nucleus and the induction of SA and JA responses. In this model, the lack of *RAP2.4d* expression fails to inhibit *TRXH5* expression which results in the monomerization of NPR1 and in the induction of SA and JA responses (**Figure 29**).

3.2 RAP2.4c, RAP2.4d, sAPX and tAPX in cold priming

Plants can store information on a past stressful stimulus and modify their response to a future stress accordingly, a phenomenon which is called priming (Hilker *et al*., 2016). In the case of two 24-hour cold stimuli that were separated by five days under ambient temperatures, it was shown that plants that had experienced the first (priming) stimulus responded to the second (triggering) stimulus with a reduced induction of *ZAT10* gene expression than naïve plants (van Buer *et al*., 2016). Reduced expression of *ZAT10*, which is a marker gene for plastid H_2O_2, indicates H_2O_2 signalling to be involved in the regulation of priming. The same study investigated the role of two prominent plastid H_2O_2 detoxifying enzymes, sAPX and tAPX in cold priming. While *sapx* plants only slightly reduced their ability to inhibit triggering-induced *ZAT10* expression in primed plants, the lack of *tAPX* resulted in *ZAT10* expression that was significantly higher in primed plants than in naïve plants, suggesting the tAPX to be essential in the formation of the memory. *tAPX* transcript and protein levels started to increase, beginning at the end of the priming stimulus and were still elevated after five days of lag-phase compared to naïve plants. It was hypothesized, that elevated tAPX levels at the end of the lag-phase were responsible for improved ROS protection of the chloroplast leading to a reduced induction of *ZAT10* (van Buer *et al*., 2016).

3.2.1 Cold priming in *rap2.4c* and *rap2.4d*

Both RAP2.4c and RAP2.4d can bind the tAPX promoter in Yeast-One-Hybrid systems and modulate *sAPX* and *tAPX* expression *in planta* (Rudnik *et al*., 2017), suggesting that the two transcription factors RAP2.4c and RAP2.4d are candidates that may regulate tAPX-mediated cold priming of *ZAT10* expression. To analyse the influence of RAP2.4c and RAP2.4d on the expression of *sAPX*, *tAPX* and *ZAT10* at the end of the lag-phase and after cold triggering, standard priming experiments were carried out as previously performed by van Buer *et al*. (2016) (**Figure 30**). Four-week-old Col-0, *rap2.4c* and *rap2.4d* plants were either cold-primed for 24 hours or further grown at 20 °C. After a lag-phase of five days, naïve (C) and primed (P) plants were analysed for their gene expression patterns prior to

triggering. After 24 hours of cold exposure, transcript levels were analysed in triggered (T) and in primed and triggered (PT) plants.

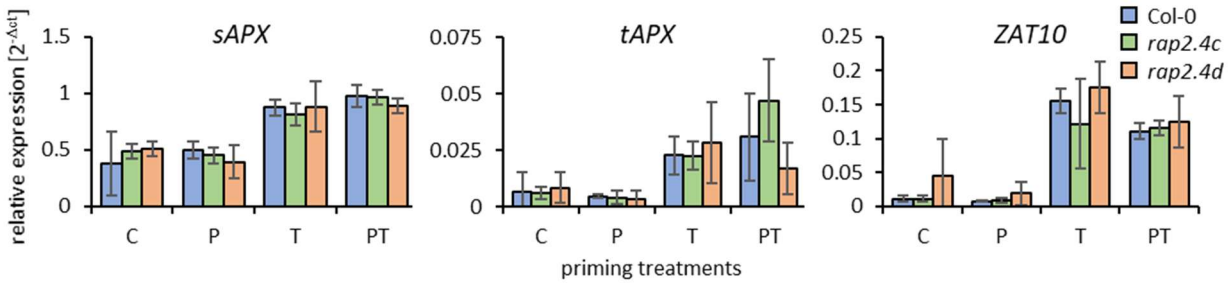


Figure 30: *sAPX*, *tAPX* and *ZAT10* transcript levels in cold-priming of Col-0, *rap2.4c* and *rap2.4d*.
Col-0, *rap2.4c* and *rap2.4d* were subjected to the standard priming experiment established by van Buer *et al*. (2016). C (naïve) and P (primed) plants after five days of lag-phase, T (only triggered) and PT (primed and triggered) plants at the end of the triggering stimulus. n=3±SD, student's t-test KO lines vs. Col-0 of the same treatment, p<0.05.

sAPX, *tAPX* and *ZAT10* transcript levels were induced by the cold treatment (T, PT) compared to plants that had grown under standard conditions for the last five days after priming (P) or constantly for 34 days (C). Compared to the study by van Buer *et al*. (2016), cold induction of *tAPX* was stronger and *sAPX* induction was weaker. In contrast to Rudnik *et al*. (2017), no significant differences of *tAPX* transcript levels were found between any of the KO lines relative to the wild type under control conditions.

sAPX, which was downregulated much stronger in *rap2.4d* than in *rap2.4c* in Rudnik *et al*. (2017), showed a similar slight upregulation in the present study, demonstrating that the effects are strongly conditional. After triggering, no differences in *sAPX* transcript levels were observable between the genotypes. Before the triggering stimulus, *tAPX* levels were similar in all lines. After triggering of *rap2.4d* plants, *tAPX* was slightly elevated in T plants and reduced in PT plants. In *rap2.4c* PT plants, *tAPX* levels were increased relative to Col-0.

ZAT10 levels were at least slightly increased in *rap2.4d* irrespective of the treatment. In *rap2.4c*, *ZAT10* was reduced only in T plants and was otherwise similar to Col-0. As *ZAT10* levels were similar in all three lines in PT plants, RAP2.4c and RAP2.4d appeared to have no influence on cold priming in Arabidopsis. Only cold-induced *ZAT10* expression in previously naïve plants (T) was impaired in *rap2.4c*, which is independent of priming. As *ZAT10* priming was not affected in *rap2.4c* and *rap2.4d* KO plants, they were not further analysed in the context of priming.

In contrast to the study by van Buer *et al*. (2016), *tAPX* transcript levels were not elevated in primed plants relative to naïve ones five days after priming and the *ZAT10* priming effect that leads to a reduced *ZAT10* induction in PT compared with T plants was weaker compared with the original finding (van Buer *et al*., 2016). An explanation for the difference could be that *tAPX* transcripts had already declined back to control levels after five days, while protein levels were still elevated to enable a mild *ZAT10* priming effect. To follow-up the observation by van Buer *et al*. (2016) that *ZAT10* priming is regulated by transient APX regulation, the roles of sAPX and tAPX in cold priming that were previously analysed in T-DNA KO lines, were investigated in more detail.

3.2.2 Generation of transgenic lines that transiently overexpress plastidic *APX*

T-DNA insertions leading to the knock-out of genes belonging to multi gene families often lead to compensatory effects by other family members during development, as it was shown for *sAPX* and *tAPX* (Kangasjärvi *et al*., 2008). To avoid such effects and to further clarify the roles of the sAPX and tAPX, 17β-estradiol inducible overexpression lines were generated.

These lines were made and tested for strong *XVE* expression following the same protocol that was used to generate and test the *RAP2.4c* and *RAP2.4d* lines in chapter 3.1.6, but with specific primers for *sAPX* and *tAPX* (*sAPX* forward: GTTGATCAACAATTAAACACAAAAAC; reverse: ACAAAACCAAGGGTGTGTAGTTATA; and *tAPX* forward: TCAGCTGATAGAAATCATTATCCA; reverse: AAGAAACTCACACTAATCTCAAAATTCT). Plants with strongest *XVE* expressions were chosen for further characterisation and experiments (*sAPX* OE: 3, *tAPX* OE: 16) (**Figure 31**). Inducible tAPX silencing plants were obtained from Shigeru Shigeoka (Maruta *et al*., 2012).

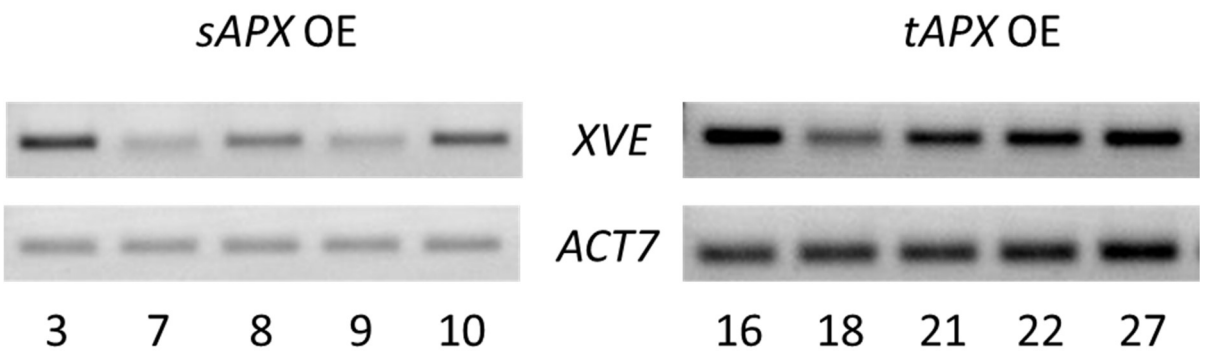


Figure 31: Semi-quantitative PCR for the determination of *XVE* expression.
RNA was extracted from four-week-old homozygous transgenic plants and reverse transcribed into cDNA. PCRs with only 25 cycles were performed using *XVE* and *ACT7* specific primers.

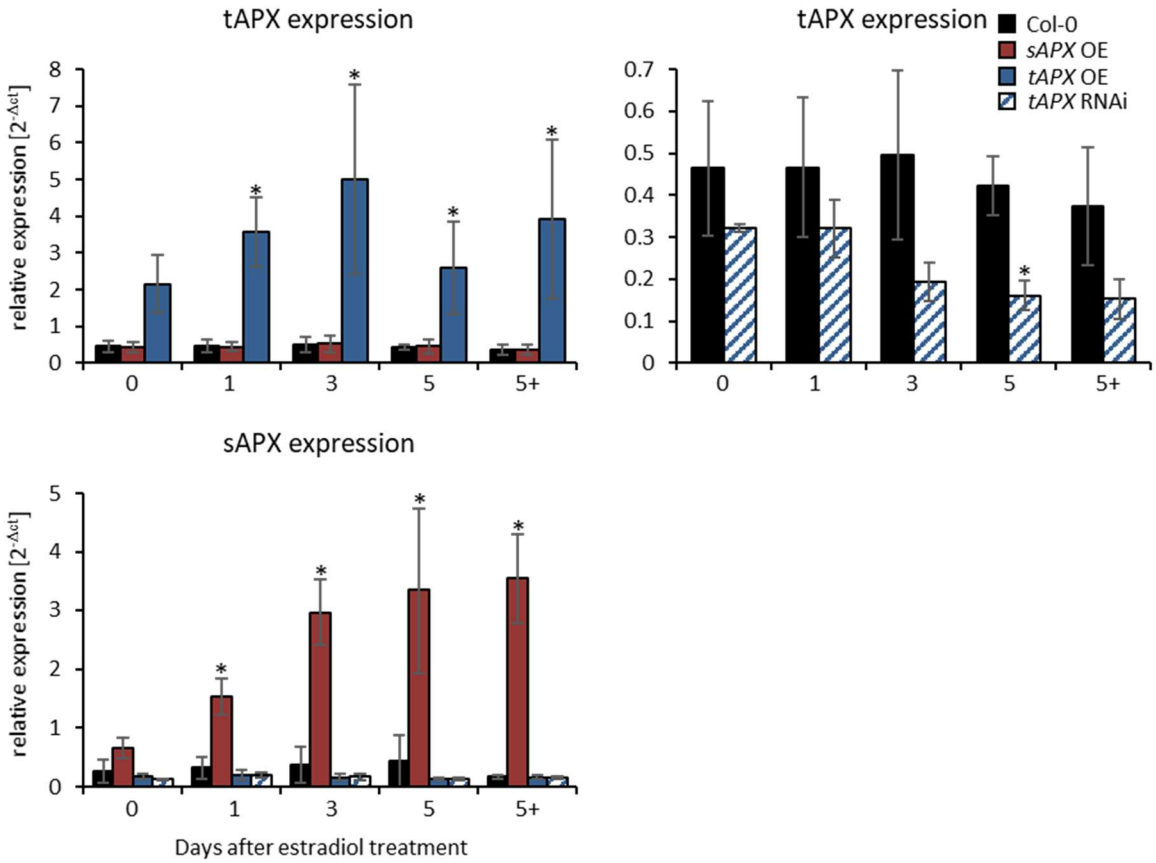


Figure 32: Relative expression of *sAPX* and *tAPX* determined by RT-qPCR.
Four-week-old plants were sprayed with 100 µM 17β-estradiol for induction of overexpression or silencing. Plants were either sprayed only once or a second time three days later (5+ samples). *APX* expression was monitored over five days after the treatments. The figures at the top show *tAPX* and the figure at the bottom shows sAPX expression. *tAPX* expression data are shown in two separate figures for better visibility of *tAPX* expression in the *tAPX* RNAi silencing line. n=3±SD, Student's t-test inducible lines vs. Col-0 at the same time, $p<0.05$.

To test *APX* overexpression, a 100 µM 17β-estradiol solution was sprayed on four-week-old plants to induce transcription of the inserts in the estrogen-inducible system. After three days, some of the plants were sprayed a second time to keep APX expression high for the entire lag-phase. *APX* expression was monitored over five days, as this would later be the length of the lag-phase in priming experiments. These plants were used for transcript analysis by RT-qPCR (**Figure 32**) and protein detection by Western blot (**Figure 33**). Transcript analysis confirmed successful overexpression of both transcripts and silencing in the RNAi line. Overexpression of *tAPX* and *sAPX* continually increased until day three or five, respectively. While the second estradiol treatment after three days did not influence *sAPX* overexpression, *tAPX* transcripts were clearly more abundant after five days if the plants were sprayed twice. *tAPX* silencing efficiency also increased over time, while the second treatment had no obvious effect. Because the second treatment had a positive effect on *tAPX* overexpression and no

negative effect on both other inducible systems, the second treatment was always conducted in priming experiments.

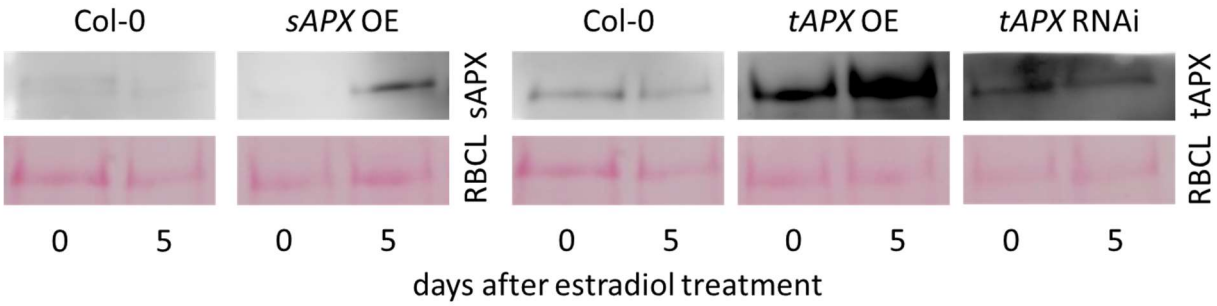


Figure 33: Immunological detection of sAPX and tAPX in non-induced and estradiol induced plants.
Four-week-old Col-0, estradiol-inducible *sAPX* and *tAPX* overexpression plants and estradiol-inducible *tAPX* silencing plants were sprayed with 100 µM 17β-estradiol for induction of overexpression or silencing (the treatment was repeated after three days). Samples were taken before (0) the treatment and after five days (5). Proteins were extracted from whole rosettes and analysed by Western blots.

3.2.3 Priming in *sAPX* and *tAPX* inducible lines

Priming experiments were conducted with Col-0, the inducible *sAPX* and *tAPX* overexpressors and the inducible *tAPX* RNAi line. As controls, all four lines were grown following the standard priming protocol consisting of four weeks of growth, 24 hours of priming at 4 °C, five days of lag-phase back under standard conditions and 24 hours of triggering at 4 °C (van Buer *et al*., 2016).

In a separate set of plants, instead of priming at 4 °C, Col-0 and the overexpressing lines were sprayed with estrogen at normal growth temperatures at the time the control plants were taken out of priming to mimic the priming event by inducing *APX* expression. *tAPX* silencing plants were cold primed and subsequently estrogen treated directly after the cold stress ended to suppress *tAPX* induction which starts as plants are transferred back to control conditions (van Buer *et al*., 2016). To ensure high levels of expression, the plants were sprayed again after three days. The trigger was uniformly applied as 24 hours at 4 °C.

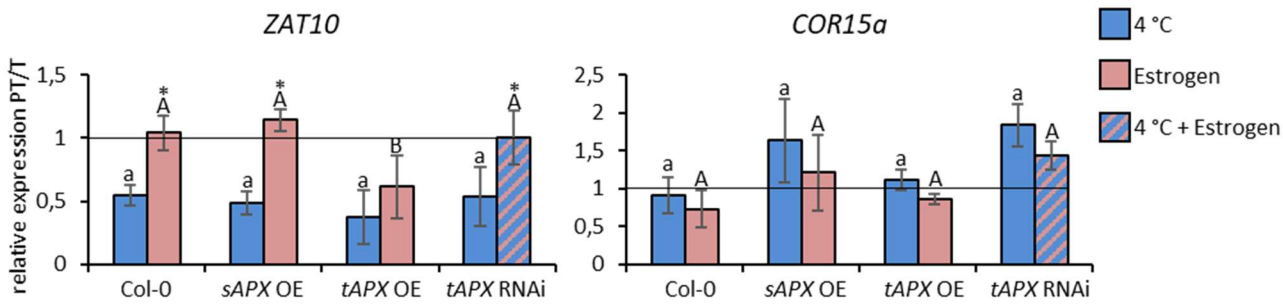


Figure 34: Relative gene expression after a priming experiment shown as PT/T ratios.
Col-0 and estradiol-inducible plants (*sAPX* OE, *tAPX* OE and *tAPX* RNAi) were grown for four weeks and subjected to a priming experiment. Col-0 and inducible APX overexpression (OE) plants were either not primed (only triggered, T), cold-primed or sprayed with 100 µM estradiol to induce *APX* overexpression to mimic priming (primed and triggered, PT). *tAPX* RNAi plants were either not primed, cold-primed or cold-primed and immediately sprayed to suppress *tAPX* expression during the lag-phase. Results are shown as the PT/T gene expression ratios of either 4 °C or estradiol primed plants. The PT/T ratios were calculated independently for each biological replicate first and their means ±SD are shown. Different small letters show significance of difference in cold primability, different capital letters show significance of difference in the cold response after estradiol spraying (ANOVA, Tukey tests, $p < 0.05$). The asterisks label significantly different results between cold- and estradiol-priming (student's t-test, $p < 0.05$).

Transcript levels of *ZAT10* and *COR15a* were determined at the end of the triggering stimulus (**Figure 34**). The cold marker gene *COR15a* that was shown to be not primable, again showed no significant priming effects in cold or estrogen primed plants, indicating no significant differences in the perception of cold stress in estrogen-treated plants (van Buer *et al*., 2016).

ZAT10 showed a reduction of transcript levels of approximately 50 % in all four lines when primed at 4 °C compared to naïve (only triggered) plants (ratio of primed and triggered plants (PT) and triggered only plants (T) ≈ 0.5), which indicates that all inducible lines were primable to a similar extent as the wild type. Estrogen treated wild type and *sAPX* OE plants had a PT/T ratio of ~1, indicating that the chemical treatment did not induce priming in Col-0 and that *sAPX* overexpression did not mimic a priming event. However, induction of *tAPX* overexpression at 20 °C five days before triggering resulted in a PT/T ratio that was similar to the ratio of plants that were primed at 4 °C. Thus, induction of the *tAPX* but not of the *sAPX* five days before a triggering cold stimulus successfully mimicked a cold priming stimulus in terms of *ZAT10* expression. Additionally, *tAPX* RNAi plants that had been cold primed and subsequently treated with estrogen to suppress cold-induced *tAPX* induction, did not show the cold-cold priming effect.

These data prove the hypothesis, that tAPX plays the predominant role in the formation of the cold memory (van Buer *et al*., 2016). This indicates that H_2O_2 scavenging directly at the site of its formation during photosynthesis is of key importance. During cold stress, H_2O_2 is generated at the photosystems in the thylakoid membrane of the chloroplasts (Genty and Harbinson, 1996). The tAPX is bound to the

thylakoid membrane and due to its large stromal domain, it is localised in the unstacked stretches that also harbour photosystem I (Miyake *et al*., 1993; Dekker and Boekema, 2005). This proximity of the H_2O_2 generating complexes (the photosystems) and the H_2O_2 scavenging enzymes (tAPX) seems to play a pivotal role in the cold priming process, as transient overexpression of the *sAPX* which has similar enzymatic properties in mature leaves (Kangasjärvi *et al*., 2008) but is localised in the stroma surrounding the photosystems was not able to mimic a priming event. Since both sAPX and tAPX scavenge plastidic H_2O_2 in neighbouring, yet different sub-compartmental locations and only tAPX modulates the *ZAT10* response in cold priming experiments, cold-induced H_2O_2 signalling differs depending on which of the APX isoforms is upregulated. *ZAT10* is encoded in the nucleus and is a marker gene for plastidic H_2O_2 as its expression is induced upon various stresses that elevate plastidic H_2O_2 levels (Sakamoto *et al*., 2000; Sakamoto *et al*., 2004; Davletova *et al*., 2005; van Buer *et al*., 2016). Thus, the signals of primed and unprimed plants as well as of *sAPX* or *tAPX* overexpressing plants differ in their H_2O_2-dependent signalling upon transfer to 4 °C. This in turn modulates gene expression of the nuclear gene *ZAT10*. The regulation of nuclear gene expression that is mediated by signals that originate from plastids or mitochondria a called retrograde signalling (Nott *et al*., 2006).

A possible mechanism for tAPX-dependent retrograde signalling involves the influence of H_2O_2 on the photosynthetic electron transport chain (PET). In the water-water cycle, as it was described by Asada (1999), electrons from the PET are transferred to oxygen, resulting in the formation of ROS. These ROS are detoxified by molecules, that were previously reduced by electrons from the PET as well. Thus, both the formation and detoxification of ROS drain electrons from the PET which reduces electron pressure during cold exposure. tAPX accumulation in primed plants enhances efficiency of this process, consuming ascorbate at elevated levels, which increases pools of its oxidised variants: Monodehydroascorbate (MDA) and dehydroascorbate (DHA). Both molecules are reduced to ascorbate at the expense of FER, NAD(P)H or glutathione (Miyake and Asada, 1994; Shimaoka *et al*., 2003; Sano *et al*., 2005), which are finally regenerated using electrons from NADPH, the final electron acceptor of the PET (Mhamdi *et al*., 2010). This way, tAPX accumulation leads to the elevated drainage of electrons from the PET that are used for the detoxification of ROS, reducing electron pressure and avoiding further ROS production.

tAPX/ascorbate-dependent enhanced drainage of PET electrons supports the redox state control of plastoquinone and particularly of FER and TRX downstream of PSI, as more oxidised electron acceptors are available in the form of MDA, DHA and oxidised glutathione. The redox state of the plastoquinone pool has been suggested to play a central role in retrograde signalling, as it carries information on the state of photosynthetic balance and the potential requirement for adjustments of nuclear gene expression (Karpinski *et al*., 1997; Lepetit *et al*., 2013). Also, the redox states of FER and TRX have been

reported to play a role in retrograde signalling, particularly in the early stress response (Piippo *et al*., 2006). The redox state of the former additionally represents the source of retrograde regulation of 2CPA expression (Baier *et al*., 2000; Baier *et al*., 2004).

The enhanced tAPX-dependent turnover of ascorbate may also play a role in retrograde signalling, as levels of reduced ascorbate may drop in the cold due to elevated production of H_2O_2 and low ascorbate levels have been demonstrated to influence retrograde signalling in an ABI4-dependent manner (Kerchev *et al*., 2011; Foyer *et al*., 2012; Kerchev *et al*., 2013).

Alternatively, H_2O_2 signalling may alter nuclear gene expression by the oxidation of plastidic proteins which alters their activity and leads to retrograde signalling. One example of a chloroplast located protein that directly or indirectly perceives various changes in the plastids is GENOMES UNCOUPLED 1 (GUN1) (Strand *et al*., 2003). GUN1-dependent signalling causes the nuclear located transcription factor ABA INSENSITIVE 4 (ABI4) to modify nuclear gene expression (Koussevitzky *et al*., 2007). As transient overexpression of *sAPX* did not mimic a priming effect, whereas *tAPX* overexpression did so, it is unlikely that H_2O_2 leaking from the chloroplasts and oxidation of stromal components play a role in cold-priming of *ZAT10* (**Figure 34**). Consequently, cold-induced H_2O_2 production at the photosystems that regulates cold priming would have to be perceived in close proximity. This includes sensor molecules that are bound to the thylakoid membrane and that may function in the photosynthetic electron transport chain (PET).

One such protein that is located at the thylakoid membrane is WHIRLY1 (WHY1) (Grabowski *et al*., 2008). This protein has been demonstrated to translocate from the chloroplasts into the nucleus where it induces expression of *PR* genes (Isemer *et al*., 2012). As WHY1 was proposed as a high light-sensitive protein that is localised in the thylakoid membrane and translocates into the nucleus where it induces gene expression, it was hypothesized as a chloroplast PET redox sensor involved in retrograde signalling (Foyer *et al*., 2014). In the chloroplasts, WHY1 forms oligomers that are likely to be stabilized by conserved cysteine residues that form disulfide bonds (Desveaux *et al*., 2002; Cappadocia *et al*., 2010; Cappadocia *et al*., 2012). As conditions such as high light and cold stress lead to both, a reduced plastoquinone pool and H_2O_2 production, the tAPX level-dependent efficiency of H_2O_2 scavenging modifies the ratio of both signals and may alter WHY1 monomeric/oligomeric states by modulating intramolecular disulfide bonds and thus, its signalling activity. The mechanism behind WHY1 transport to the nucleus remains elusive. But its oligomeric structure in the chloroplast and its potential monomerization upon redox signals suggests that redox signal-induced monomerization may facilitate WHY1 translocation in a similar way as it has been described for NPR1 (Mou *et al*., 2003).

An *sapx/tapx* double KO line has previously been shown to mediate retrograde signals after 30 minutes of exposure to high light, although their levels of H_2O_2 accumulation were only slightly higher than in the wild type, indicating that minute APX-dependent differences in plastid H_2O_2 levels are sufficient for the induction of retrograde signals (Kangasjärvi *et al*., 2008). The mechanism behind this phenomenon was not experimentally addressed in this study, but it was hypothesized that the altered phosphorylation state of the LIGHT HARVESTING COMPLEX II (LHCII) that was detected in their study may have resulted from the slightly elevated H_2O_2 levels in the *sapx/tapx* background. H_2O_2 has been shown to serve as an oxidant and to activate the LHCII kinase STATE TRANSITION 7 (STN7) (Martinsuo *et al*., 2003) that usually gets inactivated via reduction by TRX during high light stress, which is similar to cold stress in terms of electron pressure and TRX reduction levels (Huner *et al*., 1998; Rintamäki *et al*., 2000; Bellafiore *et al*., 2005; Rosso *et al*., 2006). As such, STN7 is regulated by the ratio of redox and H_2O_2 signals in a similar way as it was proposed above for WHY1.

Notably, activity of the LHCII complex and of STN7 have been linked with retrograde signalling processes (Bonardi *et al*., 2005; Kangasjärvi *et al*., 2008). *stn7* KO plants have been shown to fail long-term adjustment of nuclear encoded photosynthetic genes in different light regimes (Bonardi *et al*., 2005). In another study, *stn7* KO plants failed to adjust gene expression of approximately 800 nuclear encoded genes that were differentially regulated in Col-0 plants that were shifted between PSI and PSII stimulating light conditions (Bräutigam *et al*., 2009). Pesaresi *et al*. (2009) have demonstrated, that both short-term adaptations to changing light conditions via state transitions and long-term responses via regulation of nuclear gene expression depend on STN7, while only the former involves LHCII phosphorylation, suggesting a STN7 signalling function that is independent of LHCII phosphorylation. STN7 is bound to the thylakoid membrane (Bennett, 1979; Reiland *et al*., 2009) and is predominantly located in the unstacked areas, bound to the Cytochrome b_6f complex (Wunder *et al*., 2013; Shapiguzov *et al*., 2016; Kirchhoff *et al*., 2017), making it a prime candidate as a thylakoid membrane H_2O_2 sensor protein.

Cold-induced H_2O_2 may oxidise STN7 in naïve plants that do not accumulate tAPX to elevated levels. This putatively induces a retrograde signalling pathway that may or may not depend on phosphorylation of LHCII (Pesaresi *et al*., 2009). In plants that accumulate tAPX upon cold priming or chemical induction (van Buer *et al*. (2016) and **Figure 34**), cold-induced H_2O_2 that is produced at the photosystems is detoxified more efficiently. This protects STN7 from oxidation and retrograde signalling that leads to the induction of *ZAT10* may be suppressed.

Chi *et al*. (2013) hypothesized that plastid ROS and PET redox signals converge on the GUN1-ABI4 retrograde signalling pathway and that STN7 may contribute to both of these signals, as it is activated by H_2O_2 (Martinsuo *et al*., 2003) and inactivated by high reduction of TRX (Huner *et al*., 1998; Rintamäki

et al., 2000; Bellafiore *et al*., 2005). GUN1 and ABI4 are well-characterised proteins that take part in plastid retrograde signalling. Single KO lines of both genes were shown to delay and reduce high light-induced *ZAT10* and *ZAT12* induction (Koussevitzky *et al*., 2007), highlighting the importance of both proteins in plastid ROS/redox signal retrograde signalling. Plants lacking *ABI4* display a weaker *gun1* phenotype, supporting its GUN1 downstream function (Koussevitzky *et al*., 2007). However, as GUN1 has various regulatory functions in the chloroplast, several of these may influence ABI4 activity in the nucleus (reviewed in Colombo *et al*. (2016)). While the interaction of STN7 and GUN1 was only hypothesised and not experimentally tested, the discussed findings indicate the following retrograde signalling pathway: Priming-dependent tAPX levels regulate cold-induced H_2O_2 concentrations that leak from the photosystems and that modulate STN7 activity at the thylakoid membrane, which acts as an H_2O_2/redox sensor, transmitting its signals via the GUN1-ABI4 pathway resulting in the modulation of nuclear *ZAT10* expression (**Figure 35**).

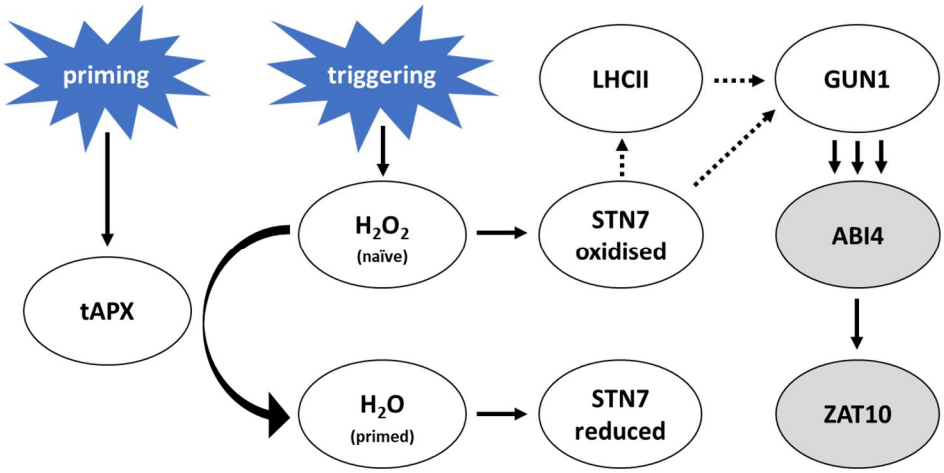


Figure 35: Proposed model for the tAPX-dependent regulation of cold priming of ZAT10 expression.
24 hours of cold priming lead to the induction of tAPX expression over the following five days (lag-phase) (van Buer *et al*., 2016). Elevated tAPX levels protect thylakoid membrane bound proteins from triggering-induced H_2O_2. In naïve plants, cold-induced H_2O_2 production leads to oxidation and activation of STN7 (Martinsuo *et al*., 2003) which triggers retrograde signalling, probably via a GUN1-ABI4 pathway (Chi *et al*., 2013) either directly or indirectly via phosphorylation of LHCII (Pesaresi *et al*., 2009). Nuclear ABI4 induces *ZAT10* expression in a GUN1-dependent manner (Koussevitzky *et al*., 2007). Chloroplasts of cold primed or tAPX induced plants are prepared to detoxify triggering-induced H_2O_2 and STN7 is protected from oxidation, omitting retrograde signalling and *ZAT10* expression. Solid lines, represent published interactions, dotted lines represent hypothesized interactions. Grey background indicates nuclear and white background indicates chloroplast localised interactions.

Irrespective of the mechanism that facilitates priming responses, priming is discussed to enable plants to respond in a more stress-specific way (Avramova, 2017). The author hypothesizes the response to the first stress to be a general “panic-like” response of the plant, that induces various genes that are not specific to the present stressful situation, but to a broader group of stressors. Such general

responses may involve any response that is induced by ROS signalling, which is activated upon various challenges. One such general response is the induction of *ZAT10* expression upon ROS signalling (Sakamoto *et al*., 2000; Sakamoto *et al*., 2004; Davletova *et al*., 2005; van Buer *et al*., 2016). Memory of the first stress would then enable plants to respond with a more specific and appropriate response to the second stress, avoiding costly induction of other non-specific responses. Following this hypothesis, reduced *ZAT10* expression in primed (or tAPX induced) and triggered plants may represent a mechanism that confers specificity to the plants' response to a second cold stimulus, focusing on the induction of more specific cold responses. The specific necessity of thylakoid bound APX in this response supports this notion, as it detoxifies its substrate directly at the site of its production during cold stress and thus confers specificity cold stress.

4 Conclusion

The two AP2/ERF-Ib transcription factors RAP2.4c and RAP2.4d have been reported to positively regulate the expression of the H_2O_2-detoxifying enzymes sAPX and tAPX under standard conditions (Rudnik *et al*., 2017) and to negatively regulate various *COR* genes in the cold (Bulcha, 2013). Both transcription factors are transiently inducible by various stresses, among them cold stress (Kilian *et al*., 2007). RAP2.4d and RAP2.4c both directly bind to the *tAPX* promoter (Rudnik *et al*., 2017) and they have been shown to bind the DRE-motif *in vitro* or a DRE-like motif *in vivo*, respectively (Rae *et al*., 2011; Bulcha, 2013).

This study demonstrates RAP2.4d to regulate the JA/ET, JA and SA crosstalk, as genes that are involved in these hormone pathways were upregulated in *rap2.4d* after one hour at 4 °C in a transcriptomic analysis, while these pathways inhibited each other in Col-0 (**Figure 11**, **Figure 12** and **Figure 15**). These genes strongly correlated with cold-inducible genes that require nuclear NPR1 for their expression (Olate *et al*., 2018), indicating RAP2.4d to function upstream of NPR1 in the regulation of cold-induced expression of hormone-related genes (**Figure 23**).

TRXH5, which is involved in quaternary structure regulation and, consequently, nuclear translocation of NPR1 (Olate *et al*., 2018), was strongly induced in *rap2.4d* compared with Col-0 after one hour at 4 °C, indicating RAP2.4d to be a negative regulator of *TRXH5* in the wild type (**Figure 27**). The *TRXH5* promoter contains a DRE-motif, which is a known RAP2.4d target (Rae *et al*., 2011) and another binding motif that was determined in the present study using a Yeast-One-Hybrid approach (**Figure 5**). In *rap2.4d*, cold-induced and TRXH5-dependent translocation of NPR1 into the nucleus enables expression of NPR1 target genes (Olate *et al*., 2018) and at the same time reduces its inhibitory effect on JA-signalling which requires cytosolic NPR1 (Spoel *et al*., 2003).

No differences in hormone contents and sensitivity to phytohormones could be found between *rap2.4c*, *rap2.4d* and Col-0 (**Figure 16** and **Figure 18**). Additionally, transient overexpression of *RAP2.4c* and *RAP2.4d* did not affect expression of hormone related marker genes (**Figure 21**). These results demonstrated that the failed hormone crosstalk in *rap2.4c* and *rap2.4d* was not caused by altered sensitivity to hormones or changes in their metabolism, but rather in RAP2.4d-dependent redox-signalling via the TRXH5-NPR1 pathway (**Figure 29**).

Furthermore, this study demonstrated that stress-induced expression of *RAP2.4d* is RAP2.4c-dependent, as *RAP2.4d* induction failed in *rap2.4c* in the cold and after JA-treatments (**Figure 28**). Failed *RAP2.4d* induction in *rap2.4c* can explain the weak phenotypes that were observed regarding the upregulation of hormone-related genes, shoot length, number of siliques, root growth (**Figure 22**),

freezing tolerance (**Figure 2**), *TRXH5* induction (**Figure 27**) and cell de-differentiation (Iwase *et al*., 2011).

Using knock-out plants, a previous study has found tAPX but not sAPX to be of central importance in the formation of a memory in cold-priming experiments (van Buer *et al*., 2016). Using estrogen-inducible transient *sAPX* and *tAPX* overexpressor and silencing (RNAi) lines, it was proven that the formation of the memory is located at the thylakoid membrane. Transient overexpression of *tAPX* at ambient temperatures five days before the cold stimulus resulted in *ZAT10* expression levels that were similar to those of plants that had been cold primed, while counteracting cold-induced *tAPX* accumulation in *tAPX* RNAi plants resulted in *ZAT10* expression similar to unprimed plants (**Figure 34**). *sAPX* overexpression did not mimic a cold-priming stimulus, demonstrating H_2O_2 detoxification specifically at the thylakoid membrane to be a key in the formation of the cold-memory. A putative signalling pathway in which tAPX-dependent detoxification of H_2O_2 specifically at the thylakoid membrane results in the modification of nuclear *ZAT10* expression via the STN7-GUN1-ABI4 pathway is shown in **Figure 29**.

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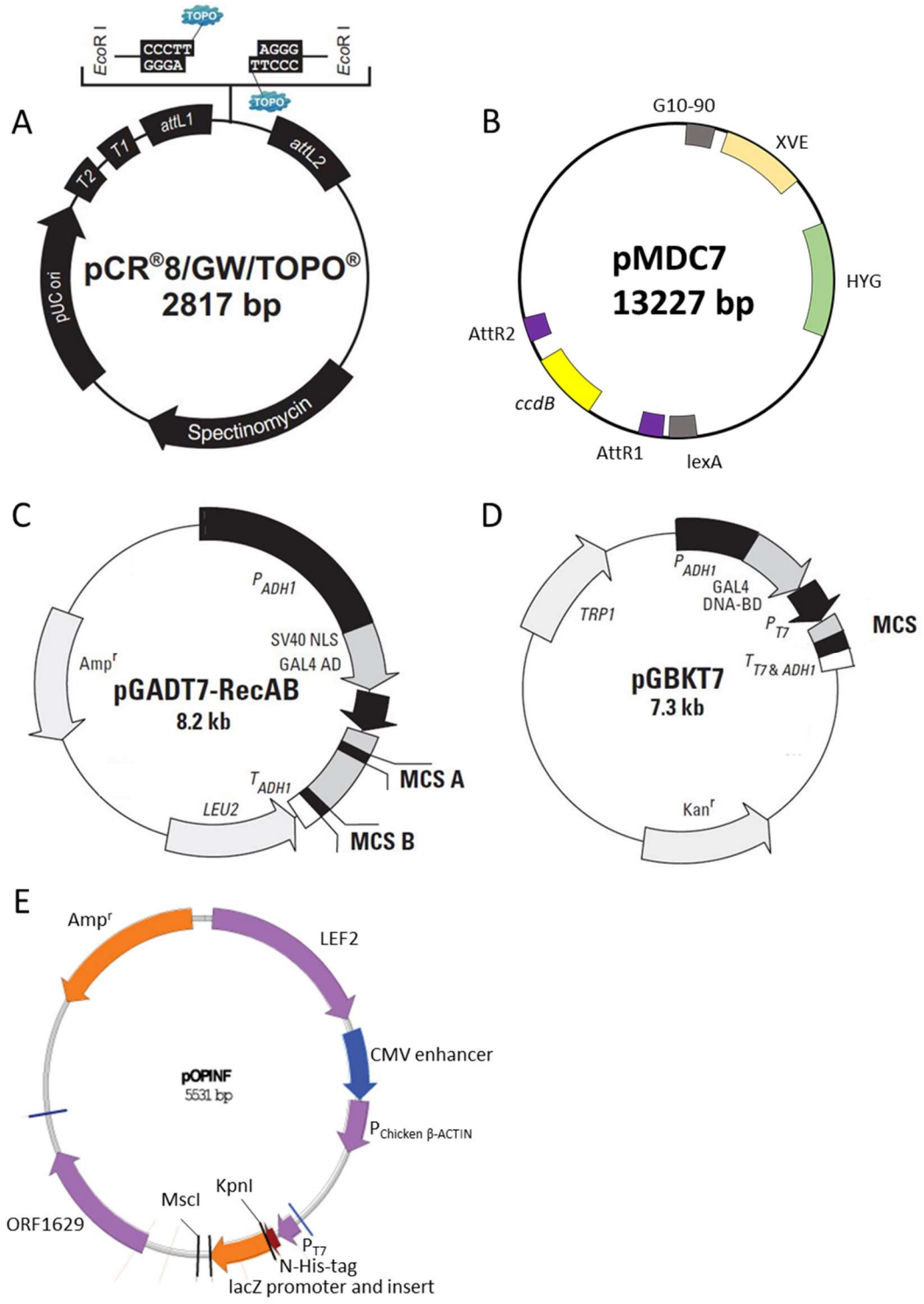
6 Appendix

Table S1: List of primers used in qRT-PCR experiments.

Gene	AGI code	forward primer	reverse primer
ACT7	At5g09810	GTTGCCATTCAGGCCGTTCTTTC	CAGAATCGAGCACAATACCGGTTG
AOC1	At3g25760	GGCTAATGATTTGCCACTGG	CAACTCCACTGGGCTTAAGC
CBF1	At4g25490	GCTCCGATTACGAGCCTCAAG	GACAACTCGTGGCCAACG
CBF3	At4g25480	CAACTTGCGCTAAGGACA	TCTCAAACATCGCCTCAT
COR15A	At2g42540	AACGAGGCCACAAAGAAAGC	CAGCTTCTTTACCCAATGTATCTGC
COR47	At1g20440	TCTTCGAGCGATGAAGAAGG	GTGTCCTGGGGACTTCTC
IAR3	At1g51760	CGCCGTACTTTGAAGTTAACG	ATCTTGTGGCCATGGAAGC
JAZ1	At1g19180	GAATGCTAACCACCCTCAAGC	TCAAAAAGCATGAAGATAGGAGC
LEA7	At1g52690	GAAGCACACTTTAGGGCTTCG	CCTCTGTGTCTCACGAGTAGTGG
MYC2	At1g32640	TGAAGATAATTGGTTGGGACG	CAACTCCAAATCCATCAACG
ORA59	At1g06160	ATCAGGCGGCTTTCGCTTTG	CTTCCGGAGAGATTCTTCAACGAC
PAL1	At2g37040	GCAGTGCTACCGAAAGAAGTGG	TGTTCGGGATAGCCGATGTTCC
PDF1.2a	At5g44420	TTTGCTTCCATCATCACCCTTA	GCGTCGAAAGCAGCAAAGA
PR1	At2g14610	GATGTGATCATGCATACACACG	ACATCCTGCATATGATGCTCC
PR4	At4g02520	GCGGCAAGTGTTTAAGGGTGAAG	CGTTGCTGCATTGGTCCACTATTC
RAP2.4c	At2g22200	TAGACGTAGCACAATCTCAAACC	CAGTGCCTTTGCCTTACG
RAP2.4d	At1g22190	CTCCAATTTCATACGGGTCG	GTTTAGCCGCGGAAGTACC
sAPX	At4g08390	AGAATGGGATTAGATGACAAGGAC	TCCTTCTTTCGTGTACTTCGT
tAPX	At1g77490	GCTAGTGCCACAGCAATAGAGGAG	TGATCAGCTGGTGAAGGAGGTC
VSP2	At5g24770	AGGACTTGCCCTAAAGAACGACAC	TCGGTCTTCTCTGTTCCGTATCC
XVE	non plant	AGATCACAGACACTTTGATCCACC	GAGAGGATGAGGAGGAGCTGG
YLS8	At5g08290	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT
ZAT10	At1g27730	TCACAAGGCAAGCCACCGTAAG	TTGTCGCCGACGAGGTTGAATG

Table S2: List of genes that were upregulated in rap2.4c and rap2.4d after one or three hours at 4 °C and that were also found to be upregulated in npr1 KO plants after 24 hours at 4 °C by Olate *et al*. (2018) (**Figure 22**).

rap2.4c		*rap2.4d*		
AT5G52640	AT3G55970	AT5G52640	AT3G57260	AT3G26210
AT3G28210	AT4G11890	AT3G12580	AT4G14365	AT3G55970
AT1G14870	AT4G18250	AT3G28210	AT1G24140	AT4G11890
AT1G57630	AT4G23140	AT5G12020	AT1G35230	AT4G18250
AT2G18690	AT4G23220	AT5G12030	AT1G47370	AT4G23140
AT1G52690	AT4G34770	AT1G14870	AT1G66090	AT4G23150
AT1G52890	AT5G10760	AT1G57630	AT1G74930	AT4G23220
AT3G57260	AT5G22380	AT4G12400	AT1G75050	AT4G34770
AT4G39670	AT5G24110	AT2G18690	AT2G17040	AT5G10760
AT1G75050	AT5G24530	AT1G02920	AT2G18660	AT5G24110
AT2G01170	AT5G27420	AT1G15010	AT2G20142	AT5G24530
AT2G18660	AT5G49120	AT5G25250	AT2G25735	AT5G27420
AT2G43570	AT5G66650	AT1G52690	AT2G34600	AT5G39670
AT3G17520		AT1G76650	AT2G43570	AT5G59220
			AT5G66650	



Supplemental Figure 1: Maps of vectors that were used in this study.
A: pCR8/GW/TOPO. T1 and T2: Transcription termination sequences; attL1 and attL2: Attachment sites 1 and 2 for LR-reactions; pUC ori: Origin of replication. **B:** pMDC7. G10-90: G10-90 promoter; *XVE*: chimeric estrogen-responsive transcription factor; *HYG*: Hygromycin B resistance gene; lexA: lexA -46 35S promoter; attR1 and attR2: Attachment sites 1 and 2 for LR-reactions; *ccdB*: Suicide gene. **C:** pGADT7. *Amp*^{r}: Ampicillin resistance gene; *LEU2*: Leucine synthesis gene; P_{ADH1}: constitutive ADH1 promoter; SV40 NLS: SV40 nuclear localisation signal; *GAL4 AD*: GAL4 activation domain; MSC A and MSC B: Multiple cloning sites A and B; T_{ADH1}: ADH1 transcription termination signal. **D**: pGBKT7. *TRP1*: Tryptophan synthesis gene; *GAL4 DNA-BD*: GAL4 DNA binding domain; P_{T7}: T7 promoter; T_{T7}: T7 transcription terminator; *Kan*^{r}: Kanamycin resistance gene. **E:** pOPINF. N-His-tag: N-terminal 6x histidine-tag. MscI/KpnI: Restriction recognition sites. $P_{Chicken\ \beta\text{-}ACTIN}$: Constitutive β-ACTIN promoter from chicken. *LEF2/ORF1629*: Baculoviral recombination sites.

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8 List of publications

Reviewed publications in scientific journals

Baier, M., Bittner, A., Prescher, A. and van Buer, J. (2018). Preparing plants for improved cold tolerance by priming. Plant, Cell & Environment **2018**, 1-19.

van Buer, J., Prescher, A. and Baier, M. (2019). Cold-priming of chloroplast ROS signalling is developmentally regulated and is locally controlled at the thylakoid membrane. Scientific Reports **9**, 3022.

Conference contributions

Talk

2018 10th Scandinavian Plant Physiology Society PhD student conference, Copenhagen, Denmark. *Two AP2/ERF-Ib transcription factors modulate the cold stress response*

Posters

2018 International conference on Arabidopsis Research (ICAR) 2018, Turku, Finland

Prescher, A., Lortzing, T., Steppuhn, A. and Baier, M. Two AP2/ERFI-b transcription factors modulate the cold stress response.

2018 International Symposium (CRC973): Bridging Ecology & Molecular Biology, Berlin, Germany

Prescher, A., Lortzing, T., Steppuhn, A. and Baier, M. Two AP2/ERFI-b transcription factors modulate the cold stress response.

2017 Botanikertagung, Kiel, Germany

Prescher, A., Bulcha, J., Bode, R., van Buer, J. and Baier, M. Two AP2/ERFI-b transcription factors modulate the cold stress response.

2015 Botanikertagung, Freising, Germany

Prescher, A., Bulcha, J., Bode, R., van Buer, J. and Baier, M. The Regulation of *sAPx* and *tAPx* and their Role in Cold Stress.

9 Curriculum vitae

Personal data

Name	Andreas Prescher
Dates of birth	31.01.1987 in Gräfelfing, Germany

Academic and occupational education

Since 01/2015	**Doctorate** at the Freie Universität Berlin, Germany in the Plant Physiology group headed by Prof. Dr. Margarete Baier
10/2012 – 12/2014	**Biology master studies** with emphasis on plant cell biology at the Christian-Albrechts-Universität Kiel, Germany • Masters thesis „Entwicklungsabhängige Veränderung im ptDNA-Gehalt und in der Menge und Verteilung des Nukleoidproteins WHIRLY1“ in the Biology of the Plant Cell group headed by Prof. Dr. Karin Krupinska (2014) • Internship in the ARC Centre of Excellence in the Plant Energy Biology group headed by Prof. Dr. Ian Small, University of Western Australia
10/2008 – 08/2012	**Biology bachelor studies** at the Christian-Albrechts-Universität Kiel, Germany • Bachelors thesis „Untersuchungen zur Expression und Aktivität der Acetohydroxysäuresynthase aus *Symphytum officinale*“ in the Biochemical Ecology and Molecular Evolution group headed by Prof. Dr. Dietrich Ober
11/2008 – 12/2014	**Activities as a student assistant** at the Christian-Albrechts-Universität Kiel, Germany • Supervision of students during lab courses (2012 – 2014) • Lab assistance in the Biology of the Plant Cell group headed by Prof. Dr. Karin Krupinska (2011 – 2013) • Lab assistance in the Developmental Physiology of the Plants group headed by Prof. Dr. Margret Sauter (2008 – 2009)

Occupational training and professional experience

01/2010 – 09/2010	Civil service at the Arbeiterwohlfahrt „Altes Volksbad“ Kiel, Germany
08/2006 – 07/2008	Occupational training as a Biological technical assistant Gewerbeschule 13 in Hamburg, Germany

School education

10/1997 – 06/2006	Secondary school Othmarschen, Hamburg, Germany
10/1994 – 06/1997	Primary school Wesperloh, Hamburg, Germany
10/1993 – 07/1994	Primary school Bookholzberg, Germany