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Durian G, **Rahikainen M**, Alegre S, Brosché M, Kangasjärvi S. (2016) Protein Phosphatase 2A in the Regulatory Network Underlying Biotic Stress Resistance in Plants. *Frontiers in Plant Science*, 7, 812.

Rahikainen M, Pascual J, Alegre S, Durian G, Kangasjärvi S (2016) PP2A phosphatase as a regulator of ROS signalling in plants. *Antioxidants,* 2, 5.

Trotta A, Wrzaczek M, Scharte J, Tikkanen M, Konert G, **Rahikainen M**, Holmström M, Hiltunen HM, Rips S, Sipari N, Mulo P, Weis E, von Schaewen A, Aro EM, Kangasjärvi S (2011) Regulatory subunit B'γ of protein phosphatase 2A prevents unnecessary defense reactions under low light in Arabidopsis. *Plant Physiology,* 156, 1464-1480

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ABREVIATIONS

1MO-I3M GSL	1-methoxy-indol-3-yl-methyl glucosinolate
4MO-I3M GSL	4-methoxy-indol-3-yl-methyl glucosinolate
ABA	abscisic acid
ACO1/2/3	ACONITASE 1/2/3
ACS2/6	ACC SYNTHASE 2/6
ACT2	ACTIN 2
ADK	ADENOSINE KINASE
AMC	activated methyl cycle
AOX	alternative oxidase
ATP	adenosine triphosphate
BAK1	BRI1-ASSOCIATED RECEPTOR KINASE 1
BiFC	Bimolecular fluorescence complementation
BR	brassinosteroid
BZR1	BRASSINAZOLE-RESISTANT 1
CAT2	CATALASE 2
CBB cycle	Calvin-Benson-Basham cycle
CIMS	cobalamin-independent methionine synthase
CPK	calcium-dependent protein kinase
CSD2	COPPER/ZINC SUPEROXIDE DISMUTASE 2
DAB	3,3'-diaminobenzidine tetrahydrochloride
DDA-MS	data-dependent acquisition mass spectrometry
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ERF6	ETHYLENE RESPONSE FACTOR 6
F_m	maximal chlorophyll fluorescence in dark adapted leaf
FNR	ferredoxin $NADP^+$ oxidoreductase

F_v	variable fluorescence
G3P	gyseraldehyde-3-phophate
GSL	glucosinolate
HPLC	high performance liquid chromatography
HR	hypersensitive response
I3M GSL	indole-3-methyl glucosinolate
IGMT	indole glucosinolate methyltransferase
ICDH	isocitrate dehydrogenase
JA	jasmonic acid
KCN	potassium cyanide
mETC	mitochondrial electron transport chain
MKK9	MAP KINASE-KINASE 9
MPK	mitogen-activated protein kinase
MS	mass spectrometry
MT	methyltransferase
MTHFR	methyl tetrahydrofolate reductase
MYB	MYB-domain protein
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
OST1	OPEN STOMATA 1
PAMP	pathogen-associated molecular pattern
PEN2/3	PENETRATION 2/3
PP2A	protein phosphatase 2A
PPKL	protein phosphatases with Kelch-like repeat domains
PPM	Mg^{2+} or Mn^{2+} dependent protein phosphatase
PPP	phosphoprotein phosphatase
PR1/5	PHATOGENESIS-RELATED GENE 1/5
PSII/I	photosystem II/I
PTM	post-translational modification

PTP	phospho-tyrosine phosphatase
PVDF	polyvinylidene difluoride
RH	relative humidity
RK	receptor kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
RuBisCO	ribulose 1,5-bisphosphate carboxylase/oxygenase
SA	salicylic acid
SAH	S-adenosyl homocysteine
SAHH	S-adenosyl homocysteine hydrolase
SAM	S-adenosyl-L-methionine
SAMS/MAT	S-adenosyl-L-methionine synthase
SDS/CN-PAGE	sodium dodecyl sulphate/clear native-polyacrylamide gel electrophoresis
SHAM	salicylhydroxamic acid
SIMR	stress-induced morphogenic response
SLP-phosphatase	*Shewanella*-like protein phosphatase
SRM-MS	Selected reaction monitoring mass spectrometry
TCA cycle	tricarboxylic acid cycle
TF	transcription factor
TGG1	THIOGLUCOSIDE GLUCOHYDROLASE 1
YFP	yellow fluorescent protein

ABSTRACT

Environmental alterations challenge the plant growth and reproduction in nature and in crop fields. Plants harbour acclimatory and defensive mechanisms that become activated under unfavourable conditions to ensure the plant survival. Recognition of stress factors triggers signalling cascades in plant cell that activate changes in gene expression, induce hormonal signals and modulate plant metabolism via post-translational modification of enzymes. Reversible protein phosphorylation, carried out by counteracting pairs of protein kinases and phosphatases, presents a major mechanism in signal transduction as well as in the control of metabolic enzymes in plant defence and stress acclimation.

Protein phosphatase 2A (PP2A) is a trimeric phosphatase composed of a scaffold subunit A, catalytic subunit C and regulatory subunit B, all of which are encoded by multiple genes in the model plant *Arabidopsis thaliana*. PP2A regulatory subunit B'γ (PP2A-B'γ) has been previously identified as a negative regulator of salicylic acid associated defence and cell death in Arabidopsis leaves. In this PhD thesis, PP2A-B'γ target proteins and its role in regulation of plant acclimation and metabolism were further studied.

PP2A-B'γ and its closest homologue PP2A-B'ζ were found to modulate the plant growth and stress acclimation under normal growth conditions and under severe abiotic stress. Moreover, PP2A-B'γ was shown to regulate enzymes in plant primary and secondary metabolism. PP2A-B'γ interacted with ACONITASE 3 (ACO3) and controlled its phosphorylation. ACO3 phosphorylation was further connected to accumulation of ACO3 protein. Both PP2A-B'γ and ACO3 abundance were discovered to affect the accumulation of mitochondrial alternative oxidase at post-translational level thus contributing to the control of cell redox balance. In addition to modulation of primary metabolism, PP2A-B'γ was found to influence the formation of 4-methoxy-indole-3-yl-methly glucosinolate (4MO-I3M GSL), a defence compound with antimicrobial activities. Moreover, PP2A-B'γ interacted with activated methyl cycle (AMC) enzymes linked to production of 4MO-I3M GSL. PP2A-B'γ regulated the protein complex formation of AMC enzymes exerting its control over the cell methylation capacity.

These findings provide new information of plant acclimation under abiotic stress and regulation of stress associated adjustments in plant metabolism. Detailed knowledge of plant acclimatory and defensive mechanisms and stress induced adjustments in plant metabolism is valuable in the development of more tolerant and nutritious crops.

TIIVISTELMÄ

Vaihtelevat ympäristöolot vaikuttavat kasvien kasvuun ja lisääntymiseen sekä luonnossa että viljelmillä. Kasveilla onkin puolustus- ja sopeutumismekanismeja, jotka käynnistyvät epäsuotuisissa oloissa ja auttavat kasvia selviytymään. Stressitekijöiden havaitseminen aktivoi kasvisolussa viestejä, jotka aikaansaavat muutoksia geenien ilmenemisessä, käynnistävät hormonaalisia signaaleja ja säätelevät kasvin aineenvaihduntaa translaation jälkeisen säätelyn avulla. Vastavaikuttavien kinaasi-fosfataasiparien katalysoima palautuva proteiinifosforylaatio on tärkeä sekä viestinvälitystä ajava että aineenvaihdunnan entsyymejä ohjaava mekanismi kasvin puolustuksessa ja sopeutumisessa ympäristönmuutoksiin.

Proteiinifosfataasi 2A (PP2A) on trimeerinen fosfataasi, joka koostuu rakennealayksiköstä A, katalyyttisestä alayksiköstä C ja säätelyalayksiköstä B. Jokaista alayksikköä koodaa usea geeni mallikasvi lituruohon (*Arabidopsis thaliana*) genomissa. PP2A:n säätelyalayksikön B'γ (PP2A-B'γ) on aiemmin havaittu estävän salisyylihaposta riippuvaisia puolustusvasteita ja solukuolemaa lituruoholla. Tässä väitöskirjassa on tutkittu tarkemmin PP2A-B'γ:n kohdeproteiineja ja merkitystä kasvin stressisopeutumisessa ja aineenvaihdunnan säätelyssä.

PP2A-B'γ:n ja tämän homologin PP2A-B'ζ:n osoitettiin ohjaavan kasvin kasvua ja stressisopeutumista normaaleissa kasvuolosuhteissa ja vakavan abioottisen ympäristöstressin aikana. Lisäksi PP2A-B'γ:n havaittiin säätelevän entsyymejä sekä kasvin perusaineenvaihdunnassa että puolustusaineenvaihdunnassa. PP2A-B'γ:n osoitettiin vuorovaikuttavan akonitaasin ACO3 kanssa ja säätelevän tämän fosforylaatiota. Lisäksi ACO3:n fosforylaation havaittiin olevan kytköksissä ACO3 proteiinin kertymiseen. Sekä PP2A-B'γ:n että ACO3:n todettiin vaikuttivat vaihtoehtoisen oksidaasin määrään translaation jälkeisellä tasolla, ja sitä kautta solun hapetus-pelkistystasapainoon. Perusaineenvaihdunnan ohella PP2A-B'γ:n havaittiin säätelevän taudinaiheuttajille haitallisen 4-metoksi-indoli-3-yyli-metyyliglukosinolaattin (4MO-I3M glukosinolaatti) muodostusta. Lisäksi PP2A-B'γ vuorovaikutti 4MO-I3M glukosinolaatin muodostusta edesauttavan aktiivisen metyylikierron entsyymien kanssa. PP2A-B'γ:n osoitettiin säätelevän aktiivisen metyylikierron entsyymien muodostamia proteiinikomplekseja ja solun metylaatiokykyä.

Tämä tutkimus lisää tietämystämme kasvien sopeutumisesta abioottiseen stressiin sekä ympäristöstressin aikaansaamien aineenvaihdunnan muutosten säätelystä. Yksityiskohtainen tieto kasvin sopeutumis- ja puolustusmekanismeista sekä ympäristöstressin aikaansaamista muutoksista kasvin aineenvaihdunnassa on tärkeää puolustuskykyisempien ja ravinteikkaampien viljelykasvien jalostuksessa.

1. INTRODUCTION

1.1 Plants in a changing environment

The majority of plants have evolved in environments where the physical, chemical and biological attributes of their surroundings undergo constant changes. Thus, plants have evolved mechanisms that monitor their growth environment and adjust their physiology to best adapt to the prevailing conditions, hence ensuring their growth and reproduction. The 10 000-year history of plant breeding has altered the genetic basis of environmental acclimation and stress responses in crop plants, but even the intensely bred crops activate acclimatory measures under environmental changes (Asano et al. 2011, Mickelbart et al. 2015). Currently, climate change alters the growth environment of both wild and domestic plants and poses new challenges for the crop production. Changes in precipitation rates exposes plants to more frequent and severe drought on certain areas, while the altered distribution of rainfall and increasing temperatures may facilitate the spreading of pests and pathogenic microbes to new land areas. Detailed knowledge of plant stress signalling pathways and responses is the key to understanding how plants endure the environmental fluctuations and helps in the modern precision breeding of more tolerant crop plants (Carmody et al. 2016).

Plant stress research has taken advantage of the model plant *Arabidopsis thaliana* (thale cress or mouse-ear cress, hereafter Arabidopsis). Arabidopsis has dominated as a model plant of dicotyledons in basic plant research since its genomic sequence became available for researchers in 2000 (Kaul et al. 2000). Moreover, the feasibility of genetic transformation and availability of T-DNA mutant collections has enabled the forward and reverse genetic approaches in characterizing the role of individual genes and proteins in plant defence and stress tolerance. At the same time, development of omics methodologies has made it possible to gain holistic understanding of plant physiology under stress. Currently, the introduction of CrispR/Cas methodology greatly facilitates the translation of the knowledge to crop plants (Arora and Narula 2017, Yin et al. 2017).

1.2 Overview of plant defence signalling

Stress can be defined as any deviation of the plant growth conditions that has a negative influence on plant metabolism, growth, development and reproduction (Lichtenthaler 1996). Stressors are often divided to abiotic factors, such as availability of water, excess light, heat or cold, and to biotic stressors like pathogenic microbes and herbivorous insects. Stress is dose-dependent and thus the duration of stress as well as the severity of stress play a role in plant acclimation. Low and short-term stress may be overcome

by physiological compensations and repair mechanisms, while severe or chronic stress leads to considerable damage, cell death or even death of the whole plant. Stress and strain are routine in plant life and the limit between moderate environmental changes overcome by physiological adjustments and more severe stress remains vague (Lichtenthaler 1996).

The readiness for activation of stress responses is crucial for plant survival. A plant cell senses its environment by receptor molecules situated at multiple cellular compartments. Plant pathogens are sensed by plasma membrane receptor kinases (RK), which bind the epitopes of conserved pathogen associated molecular patterns (PAMP) like flagellin and chitin that are present in microbes. Upon epitope recognition, RKs form oligomeric complexes and the message is relayed to the cytoplasm by phosphorylation of the receptor's intracellular kinase domain. Activated RK sends a signal to the plasma membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, which catalyse a rapid apoplastic burst of reactive oxygen species (ROS), a characteristic hallmark in plant defence (Dubiella et al. 2013, Sierla et al. 2013). In the cytoplasm, the message is relayed as a phosphorylation cascade by mitogen activated protein kinases (MPKs) and calcium dependent protein kinases (CPKs) (Asai et al. 2002, Boudsocq et al. 2010). At the end of the signalling network lay the transcription factors (TF) controlling the nuclear gene expression (Figure 1). The activation of the TFs leads to transcriptional changes and triggers necessary defensive measures. The initial and transient onset of defence gene expression is followed by more persisting changes driven by hormonal signalling. Salicylic acid (SA), jasmonic acid (JA), and ethylene production activates new signals leading to further transcriptional reprogramming. Plant defence is further manipulated by the effector molecules secreted by microbial and viral pathogens to interfere and inhibit plant defensive measures (Zabala et al. 2015, Ivanov et al. 2016).

Plant defence responses are adjusted by the prevailing light conditions and light plays a crucial role in the activation of defence associated programmed cell death called the hypersensitive response (HR) (Trotta et al. 2014, Zabala et al. 2015). PAMP-triggered defence signals as well as secreted pathogen effectors cause disturbances in the photosynthetic electron transport chain and trigger ROS production in photosystem II (PSII) and photosystem I (PSI) (Göhre et al. 2012, Stael et al. 2015). Through so-far poorly understood mechanisms of retrograde signalling, chloroplast ROS production initiates signals that further modulate the gene expression in the nucleus (Sierla et al. 2013, Gollan et al. 2015). Furthermore, chloroplast redox state is tightly connected to mitochondria and peroxisomes via co-operational metabolic circuits. Cytoplasmic regulatory networks are crucial in combining the messages from different cellular compartments and in determining the final defence responses. In addition to triggering changes in gene expression, the cytoplasmic regulatory network can directly regulate

metabolic enzymes by post-translational modifications. Thus, the cell can quickly fine-tune its metabolism to adapt to the prevailing environmental changes.

The activation of defensive measures is costly and greatly inhibits the plant growth. This is exemplified by many Arabidopsis mutant lines demonstrating constitutive activation of pathogenesis responses accompanied with stunted growth phenotype (van Wersch et al. 2016). The outcome of defence measures depends on the balance between activating signals and the counteractive negative regulators inactivating these signals (Durian et al. 2016). Negative defence regulators are crucial in preventing unnecessary defence activation and restoring the physiological balance when the stress recedes. This negative regulation is attributed to protein phosphatases, which inactivate and attenuate the phosphorylation cascades driven by protein kinases.

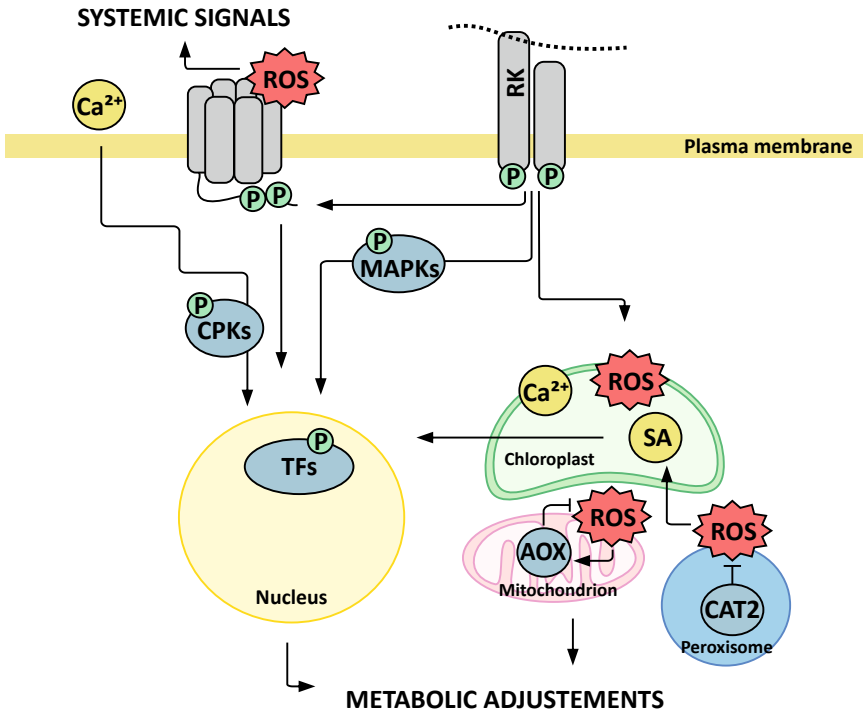

Figure 1. Schematic overview of plant defence signalling. PAMPs are recognised by the plasma membrane receptor kinases that initiate further signals to organelles and nucleus. The activation of plasma membrane NADPH oxidases catalyses a ROS burst that spreads to adjacent cells and triggers systemic signals. In the cytoplasm, the message is relayed by phosphorylation cascades employing MPKs and CPKs. Moreover, organellar ROS signals and defence hormones contribute to the activation of defensive measures. Downstream of the defence signals, plant metabolism is adjusted by transcriptional changes and post- translational regulation of the metabolic enzymes. Modified from Durian et al. (2016).

1.3 Plant defence metabolism

1.3.1 Modulation of plant primary metabolism in stress exposed tissues

Exposure to environmental stress triggers adjustments in plant primary metabolism to maintain the cell homeostasis under prevailing stress and to redirect metabolic fluxes towards production of protective and defensive metabolites. Chloroplasts and mitochondria harbour crucial primary metabolic circuits that are the basis of cell energy metabolism. In chloroplasts, light energy is harvested and captured into chemical energy as adenosine triphosphate (ATP) and reducing equivalents as NADPH by ATP synthase and ferredoxin $NADP^+$ oxidoreductase (FNR), respectively. ATP and NADPH are further utilized in carbon assimilation in Calvin-Benson-Basham (CBB) cycle yielding gyseraldehyde-3-phosphate (G3P), which is further metabolised to glucose and other organic compounds. In plants, glycolysis converting glucose to pyruvate takes place both in the cytoplasm and in plastids. In mitochondria, the stored energy enters tricarboxylic acid (TCA) cycle in the form of pyruvate, malate or oxaloacetate and nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide ($FADH_2$) and ATP are produced by catabolism of the carbon backbones. NADH and $FADH_2$ are oxidised in the mitochondrial electron transport chain (mETC) with a concurrent pumping of protons to the mitochondrial matrix by complex I, III and IV. The proton gradient is utilized by the ATP-synthase to produce ATP. The energy balance and redox state of mitochondria and chloroplast are tightly linked to primary metabolism in cytoplasm and peroxisomes and the metabolic intermediates are actively transported between the different cellular compartments (Igamberdiev and Eprintsev 2016). Reducing power is transported between organelles as organic acids, especially malate. In the so called malate valve, malate dehydrogenases catalyse the conversion of oxaloacetate to malate using NAD(P)H. Malate is transported between organelles and enters the TCA cycle and produces NADH for mETC. Under high light, the excess reducing power is quenched in mitochondria by the non-phosphorylating mETC bypasses like alternative oxidase (AOX). Moreover, coordinated actions and metabolite transport between chloroplast, peroxisomes and mitochondria are needed in photorespiration where 2-phosphoglycolate, produced by the oxygenase activity of RuBisCO in the chloroplast, is sequentially metabolized to glycolate, glyoxylate, glycine, serine, pyruvate and glycerate and re-enters the CBB-cycle as 3-phosphoglycerate.

In addition to their role as major metabolic hubs, chloroplasts, mitochondria and peroxisomes also comprise major sites of ROS production and are therefore crucial in defence and stress signalling. Moreover, they form connections between ROS signalling and primary metabolism. Stress induced changes in metabolism take place before transcriptional changes in the expression of genes encoding metabolic enzymes can be

detected (Baxter et al. 2007). It has been suggested that the metabolic changes are caused by direct oxidative regulation of specific enzymes. In mitochondria, the TCA cycle enzymes aconitase, pyruvate dehydrogenase and 2-oxogutarate dehydrogenases are sensitive to oxidation by ROS and their oxidative inactivation can diminish the biosynthesis of amino acids that require TCA cycle intermediates as precursors (Verniquet et al. 1991, Sweetlove et al. 2002, Baxter et al. 2007). In addition to redox regulation, enzymes are controlled by other post-translational modifications. The abundance of multiple enzymes that operate in cell energy metabolism or primary metabolism has been shown to be modulated at early time points in response to stress, suggesting that they are under direct post-translational regulation (Alqurashi et al. 2017). Hence, the homeostasis of primary metabolism is controlled by the quick post-translational regulation and the later transcriptional control. However, the knowledge of the regulatory networks controlling the metabolic adjustments has remained poorly understood.

1.3.2 Plant secondary defence metabolism

Upon pathogen infection or insect infestation, primary metabolism is adjusted to provide carbon backbones for the secondary defence metabolism. Plants produce a vast variety of secondary defence metabolites, which vary between taxonomic groups. Moreover, defence metabolites have been widely utilized as pharmaceuticals and chemicals. Currently, synthetic biology approaches are being utilized to harness these metabolic pathways for production of high value compounds (Facchini et al. 2012). In Arabidopsis, phytoalexin camalexin and glucosinolates (GSLs) have documented antimicrobial effects and have been used as reporter molecules for activation of chemical defence (Bednarek 2012). In addition to camalexin and GSL biosynthesis, recognition of pathogens has been shown to trigger the transcription of genes needed for biosynthesis of anthocyanins, lignins and flavonols (Truman et al. 2007). Moreover, activation of MPK3 and 6 alone was demonstrated to induce biosynthesis of camalexin and GSLs highlighting the role of phosphorylation cascades in the regulation of defensive secondary metabolism (Lassowskat et al. 2014).

1.3.3 Glucosinolates and the associated transmethylation reactions

GSLs form a large group of defence compounds with more than 120 different characterized structures (Halkier and Gershenzon 2006). They are typical for order Brassicales and have gained recent research interest as degradation products of specific glucosinolate species 4-methylsulfinylbutyl glucosinolate (4MSB GSL) and indole-3-ylmethyl glucosinolate (I3M GSL) have been shown to possess anticancerogenic activities (McGrath and Spigelman 2008). GSLs are divided to two major structural groups: aliphatic and indole GSLs, which are synthetized from methionine and

tryptophan, respectively. In the biosynthesis of aliphatic GSLs, methionine undergoes side chain elongation, core GSL synthesis steps and side chain modifications reactions to produce a diversity of chemical structures. In the biosynthesis of indole GSLs, tryptophan is metabolised to I3M GSL, which is further modified by methylation reactions to two major indole GSLs, 1-methoxy-indol-3-yl-methyl glucosinolate (1MO-I3M GSL) and 4-methoxy-indol-3-yl-methyl glucosinolate (4MO-I3M GSL) as well as to other indole GSL species. The specific activities of different GSLs are provided by the different functional group substitutions in their core structure at the final stage of the biosynthetic pathway (Figure 2) (Agerbirk et al. 2009, Sønderby et al. 2010).

GSLs form a functional defence system with myrosinase enzymes. In intact tissues, glucosinolates are separated from myrosinases by compartmentalization inside a cell or to separate tissue structures (Koroleva et al. 2000, Husebye et al. 2002, Frerigmann et al. 2012). Upon tissue damage by herbivore eating or pathogen infection, GSLs come into contact with myrosinases and are hydrolysed to various reaction products including epithionitriles, nitriles, isothiocyanates and thiocyanates. These products are typical for the different GSL species and harbour specific antimicrobial and insect deterring activities (Agerbirk et al. 2009, Clay et al. 2009, Pfalz et al. 2009, Sun et al. 2009). However, the mechanistic background behind the specific effects of the different GSL degradation products remains elusive. In recent years, the enzymes catalysing the biosynthesis of different GSLs and their transcriptional regulation has been studied in more detail, but the post-translational regulation of the enzymes has gained less attention (Frerigmann et al. 2015, Sønderby et al. 2010, Xu et al. 2016).

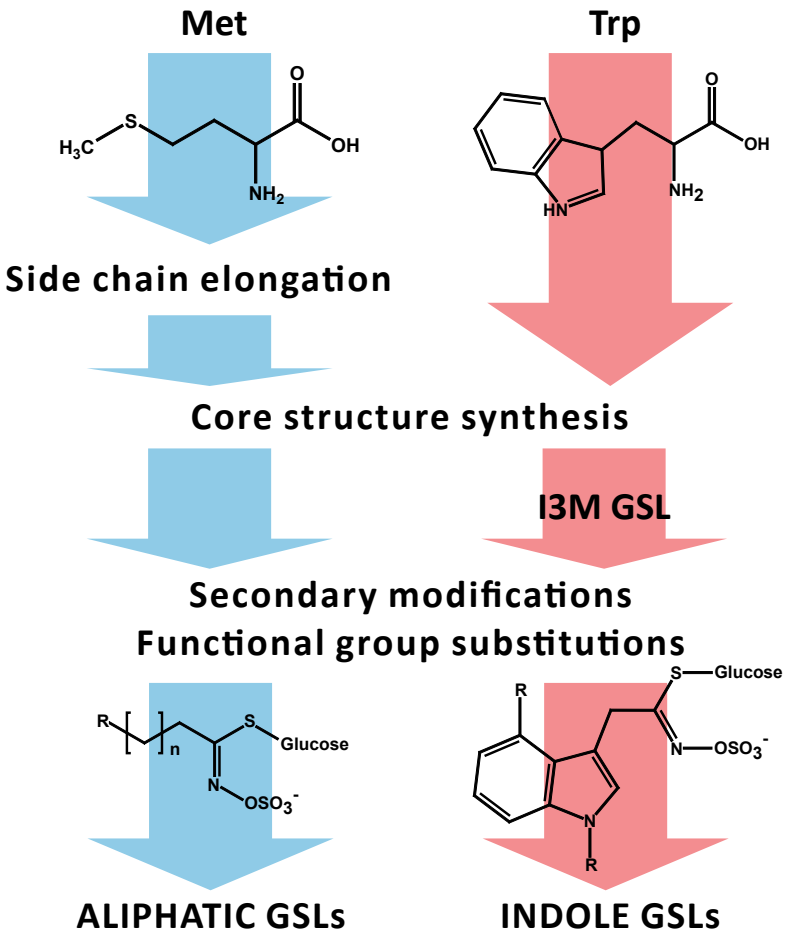

Figure 2. Overview of aliphatic and indole GSL biosynthesis pathways. In the aliphatic GSL synthesis, methionine undergoes chain elongation steps, core structure synthesis and various secondary modifications to produce a variety of chemical structures. In indole GSL synthesis tryptophan is metabolised to I3M GSL which undergoes functional group substitutions to provide structures with specific biological activities.

Methylation reactions of indole GSLs grant their specific biological activities (Agerbirk et al. 2009). GSL methylation, like nearly all transmethylation reactions in the cell, is dependent on the so-called activated methyl cycle (AMC). Methyltransferases (MTs) use a universal substrate S-adenosyl-L-methionine (SAM) as methyl group donor. The reaction yields an end product S-adenosyl homocysteine (SAH), which is highly toxic to the cell because it inhibits MT activity by binding and occupying their active site. Thus, SAH is actively hydrolysed by S-adenosine homocysteine hydrolase (SAHH). In the

reaction, SAHH produces homocysteine, which is further metabolized to methionine by cobalamin-independent methionine synthase (CIMS) and recycled to SAM via SAM-synthase (SAMS/MAT). Moreover, AMC reactions are further interconnected to other metabolic pathways like folate metabolism and de-novo methionine synthesis. In addition to its role as a methyl group donor, SAM is a precursor for ethylene synthesis. As external factors greatly affect the different sinks of SAM, AMC must be tightly coordinated under stress to optimize its function and maintain the cell transmethylation capacity.

1.4 Post-translational regulation of proteins

Post-translational regulation of metabolic enzymes offers a quick and versatile way to respond to changing environmental conditions and to help maintain cell homeostasis. Post-translational modifications (PTMs) change the activity, localization, turnover and complex formation of enzymes offering an important layer of regulation on top of the transcriptional and post-transcriptional regulation. PTMs include various different types of modifications like phosphorylation, acetylation, methylation, nitration, S-nitrosylation, glucosylation and sumoylation. Of these, phosphorylation is one of the most abundant PTM with almost 5000 identified phosphoproteins and close to 15 000 phosphorylation sites found in Arabidopsis proteome (Vlastaridis et al. 2017).

Reversible protein phosphorylation is governed by counteracting protein kinases and phosphatases. The Arabidopsis genome encodes around 1050 catalytic protein kinases and more than 150 catalytic protein phosphatases identified by their sequence homology, highlighting the importance of protein phosphorylation (Uhrig et al. 2013). Historically, protein kinases have gained more research attention than protein phosphatases. Moreover, the specificity of protein kinases has facilitated their characterisation. Catalytic protein phosphatases show wider range of target proteins and acquire regulatory proteins to specify their targets further complicating their research (Brautigan 2013).

1.4.1 Plant protein phosphatases

Protein phosphates are divided to four groups: phosphoprotein phosphatases (PPP), Mg^{2+} or Mn^{2+} dependent protein phosphatases (PPM) also called protein phosphatase 2Cs (PP2C), phospho-tyrosine phosphatases (PTP), and aspartate-dependent phosphatases. PPPs and PP2Cs dephosphorylate serine and threonine residues and the PPPs are further grouped to protein phosphatase 1 (PP1), PP2A, PP4, PP5, PP6, PP7, *Shewanella*-like protein phosphatase (SLP phosphatase), and protein phosphatases with Kelch-like repeat domains (PPKL) (Uhrig et al. 2013) Moreover, PPPs may form holoenzyme complexes and recruit regulatory proteins to direct their target specificity.

Individual phosphatases from all these groups have characterised functions in cellular processes like defence, growth and development (Rayapureddi et al. 2005, Feng et al. 2010, Uhrig et al. 2013, Shankar et al. 2015). Protein phosphatases have been considered as the counteractors of protein kinases and have a crucial role in attenuating and preventing the activation of programmed cellular processes like stress responses and developmental changes. Regulation of defence responses requires a fine balance of the networks controlling the activation and deactivation of the defence responses. However, protein phosphatases can also function as positive regulators and activate signals by dephosphorylation (Tang et al. 2011). Importantly, protein kinases and phosphatases form counteracting regulatory pairs that modulate plant metabolism via post-translational regulation of metabolic enzymes. However, few such kinase-phosphatase pairs have been characterised in plants (Lee et al. 2009, Samol et al. 2012).

1.4.2 Role of protein phosphatase 2A in plant defence

PP2A is a trimeric protein phosphatase consisting of a scaffold subunit A, which binds the catalytic subunit C and regulatory subunit B (Figure 3A). In Arabidopsis genome, these subunits are encoded by three, five and 17 different genes, respectively. The B subunits are further divided to subgroups B, B' and B" based on their sequence characteristics. The B-subunits determine the target specificity of the trimeric holoenzyme. Their subcellular localization, tissue specific expression and binding to different A and C subunits enables the multiplicity of the PP2A function. In addition to the A, B and C subunits, PP2A can recruit other regulatory proteins. In Arabidopsis, TAP46 protein has been shown to bind the PP2A catalytic subunit and control PP2A function in regulation of autophagy and cell death but the mode of regulation remains elusive (Ahn et al. 2011). Similar regulatory interactions have been characterized also in yeast and mammals indicating that some of the regulatory interactions are widely conserved (Chen et al. 1998, Jiang and Broach 1999).

In mammals, PP2A is an important regulator of oncogenic signalling cascades and its role as a tumour suppressor is under active research (Sontag et al. 1993, Suganuma et al. 1988). In plants, PP2A has been shown to regulate various developmental processes and stress responses (Ahn et al. 2011, Durian et al. 2016, Michniewicz et al. 2007, Tang et al. 2011, Waadt et al. 2015). Moreover, distinct PP2A subunits have been associated to plant defence signalling. B'η and B'ζ were shown to restrict the activation of BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), a co-receptor needed for the defence activation by multiple plasma membrane RKs (Segonzac et al. 2014). Moreover, peroxisome localized B'θ has been associated with resistance to bacterial pathogen *Pseudomonas syringae pv. tomato* (Kataya et al. 2015). In addition, reduced PP2A activity has been connected to increased expression of defence marker genes PATHOGENESIS-RELATED GENE 1 (PR1) and PR5 and cell death in tobacco plants (He et al. 2004),

whereas a pharmacological approach using PP2A inhibitor cantharidin suggested that PP2A is also involved in the control of the apoplastic ROS burst via regulation of NADPH oxidases (Segonzac et al. 2014). To strengthen the picture placing PP2A as a multifaceted regulator of plant defence, PP2A has been recognized as a possible target of AvrE bacterial effectors (Degrave et al. 2015, Jin et al. 2016).

PP2A subunits have also been shown to control various hormonal signalling pathways in plants. In ethylene biosynthesis, ACC SYNTHASE 2 and 6 (ACS2, ACS6) are under negative regulation by PP2A (Skottke et al. 2011). In contrast, PP2A with specific B' regulatory subunits directly controls the brassinosteroid (BR) activated gene expression by dephosphorylating the transcription factor BRASSINAZOLE-RESISTANT 1 (BZR1) and thus enabling its translocation from cytoplasm to nucleus (Tang et al. 2011). More recently, PP2A was shown to interact with the Sucrose Non-fermenting 1-Related Protein Kinase OPEN STOMATA 1 (OST1) in abscisic acid (ABA) signalling and to mediate various ABA dependent processes, such as germination and stomatal closure in Arabidopsis (Waadt et al. 2015). Altogether, PP2A controls a variety of defence and stress related processes in plants. However, the specific trimeric holoenzyme compositions targeting the specific phosphoproteins remain mostly unknown.

A specific PP2A regulatory subunit B'γ functions as a negative regulator of defence and cell death (Trotta et al. 2011). It localizes to the cytoplasm, where it controls the SA dependent defence responses and organellar ROS signals (Li et al. 2014, Trotta et al. 2011). Moreover, a study utilizing a *cat2 pp2a-b'γ* mutant, which has reduced levels of PP2A-B'γ and triggers organellar ROS signals due to deficiency of the peroxisomal CATALASE 2 (CAT2), revealed that PP2A-B'γ governs the ROS induced metabolic changes in Arabidopsis leaves (Li et al. 2014). Arabidopsis knock-down *pp2a-b'γ* exhibits premature senescence and cell death lesions after four weeks of age when grown in 8-hour light period under 130 μmol photons $m^{-2}s^{-1}$ and 50 % relative humidity (RH) (Figure 3B). The phenotype is accompanied by constitutive expression of SA dependent defence related genes and enhanced resistance to the fungal pathogen *Botrytis cinerea* and green peach aphid *Myzus persicae* (Trotta et al. 2011, Rasool et al. 2014). Interestingly, the *pp2a-b'γ* phenotype is highly dependent on the plant growth conditions. The lesion formation is restricted if plants are grown in higher light intensity or higher humidity, while the cell death phenotype becomes stabilized by the ROS accumulation in *cat2 pp2a-b'γ* double mutant (Trotta et al. 2011, Li et al. 2014, Rasool et at. 2014). These results suggest that PP2A-B'γ works in the signalling network integrating various environmental signals and controlling plant growth and defence.

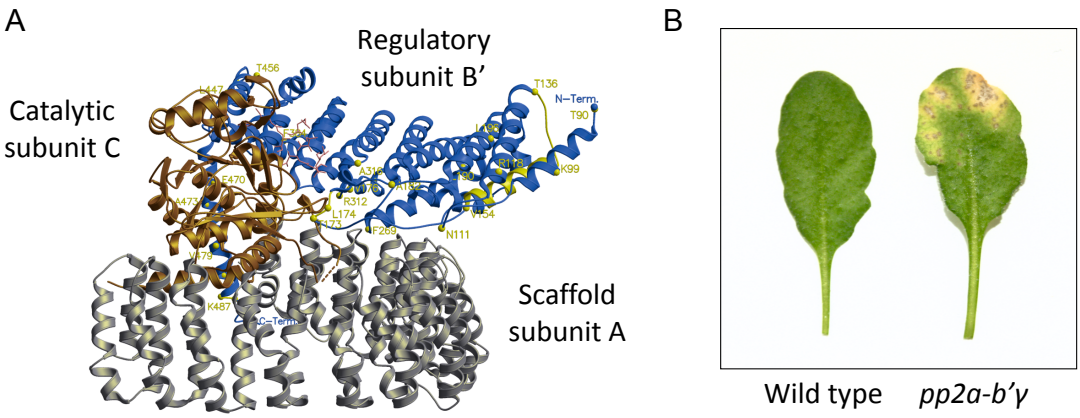

Figure 3. PP2A regulatory subunit B'γ. (A) Computational model of the trimeric PP2A holoenzyme with regulatory subunits B'γ or B'ζ. Model modified from Rasool et al. (2014). (B) Cell death phenotype of Arabidopsis *pp2a-b'γ* knock-down mutant.

2. AIMS OF THE STUDY

Plant growth and survival under unfavourable environmental conditions is controlled by intracellular regulatory networks. Positive defence promoting and the counteracting defence attenuating factors determine the final outcome of the stress responses. Protein phosphatases have been recognised as negative regulators of stress signalling preventing the unnecessary activation or exaggeration of defence, but have gained less research attention than the defence activating kinases. PP2A regulatory subunit B'γ has been associated with plant growth and defence, but its target proteins have remained elusive. The main aim of my PhD thesis was to characterise the PP2A-B'γ protein interactors and target processes in plant defence in the model plant Arabidopsis. Moreover, I investigated the defence related adjustments in plant primary and secondary metabolism. More specifically, the aims of my thesis were:

1. To resolve how PP2A-B'γ and its closest homologue PP2A-B'ζ control plant stress resistance and growth under normal growth conditions and under abiotic stress.

2. To reveal how PP2A-B'γ regulates ACONITASE 3 (ACO3) and if ACO3 plays a role in plant tolerance to stress.

3. To elucidate how PP2A-B'γ modulates the plant GSL content and what are the associated regulatory interactions.

3. METHODOLOGY

3.1 Plant growth conditions and treatments

Model plant *Arabidopsis thaliana* wild type (Col-0) and homozygote mutant lines *pp2a-b'γ* (At4g15415, SALK_039172, Trotta et al. 2011), *pp2a-b'γ 35S::PP2A-B'γ* complementation line (Trotta et al. 2011), *pp2a-b'ζ1-1* and *pp2a-b'ζ1-2* (At3g21650, SALK_107844C and SALK_150586 respectively, Rasool et al. 2014), *pp2a-b'γζ* double mutant (SALK_039172/SALK_107844C, Rasool et al. 2014) and *aco3* (At2g05710, SALK_013368, (Arnaud et al. 2007) were used in the study.

Plants were grown in 8-hour light period under 130 μmol photons $m^{-2}s^{-1}$, 50% RH and 22°C if not otherwise mentioned in the publications. For abiotic stress treatments, plants were grown for two weeks under normal growth conditions and then transferred to 800 μmol photons $m^{-2}s^{-1}$ and 28°C temperature for two weeks (Paper I). For drought treatment, plants were first watered normally for two weeks after which the watering was limited for two weeks (Paper I). High light and fluctuating light were utilized to assess plant tolerance to light stress. In high light, plants were grown under 600 μmol phot m^{-2} s^{-1} and in fluctuating light under 50/500 μmol phot m^{-2} s^{-1} with 5/1-minute fluctuation (Paper III, Tikkanen et al. 2010).

To induce oxidative stress, plants were sprayed with 100 μM methylviologen and kept under normal growth conditions for four hours or germinated and grown on ½ MS supplemented with 1,5 μM or 3,0 μM methyl viologen for 7 d in normal growth conditions (Paper III). 5 mM potassium cyanide (KCN) and 10 or 12 mM salicylhydroxamic acid (SHAM) were used to inhibit the cytochrome and AOX pathways of mitochondrial electron transport respectively (Paper II). Pathogen infections were carried out using a necrotrophic fungus *B. cinerea.* Plants were sprayed with 1 x 10^6 spores suspension and kept in 100% RH to facilitate the fungal infection. Rosettes were harvested 24 h post-infection (Paper IV).

3.2 Histochemical staining of H_2O_2

Localized H_2O_2 production in plants was studies utilizing 3,3'-diaminobenzidine tetrahydrochloride (DAB), which forms a visual brown precipitate upon oxidation by H_2O_2. Plants were incubated in 0,1% DAB overnight and transferred to light for 2 or 3 h as indicated in papers II and III.

3.3 Measurement of PSII activity

Maximal chlorophyll fluorescence (F_m) and variable chlorophyll fluorescence (F_v), indicating the difference between maximal fluorescence F_m and the initial fluorescence F_0, were used to assess the PSII activity. F_v/F_m ratio was measured after 30 min dark adaptation from detached Arabidopsis leaves with Hansatech PEA fluorometer.

3.4 Analysis of protein interactions

Yeast two-hybrid screen was used to identify putative protein interactors for PP2A-B'γ. The screen was conducted with HybridHunter (Invitrogen) kit and yeast train L40. PP2A-B'γ fused to LexA DNA binding domain in pHybLex plasmid was used as a bait against cDNA library enriched in stress induced transcripts (Jaspers et al. 2009) in pYESTrp2 plasmid. Tryptophan auxotrophic yeast were plated on -His selection media supplemented with 10 mM 3-aminotriazole to prevent autoactivation. Colonies were grown for 4 d in 28 °C and tested for β-galactosidase activity. Putative interactors were identified by sequencing.

BiFC was utilized for further analysis of putative protein interactions. Protein coding sequences of PP2A-B'γ, PP2A-B'ϰ, AOX1A, ACO3, INDOLE GLUCOSINOLATE METHYLTRANSFERASE 1-4 (IGMT1-4), SAHH1, CIMS1 and THIOGLUCOSIDE GLUCOHYDROLASE 1 (TGG1), were cloned to pSPYNE/SPYCE expression vectors (Walter et al. 2004, Waadt and Kudla 2008) creating C-terminal fusions with N- and C-terminal fragments of the yellow fluorescent protein (YFP). Vectors were verified by sequencing and transformed to *Agrobacterium tumefaciens* strain GV3101 PMP90.

For analysis of protein interactions, the studied proteins were heterologously expressed in *Nicotiana bethamiana* leaves. To facilitate the heterologous expression of the studied proteins, a tombusvirus RNA interference suppressor p19, encoded by *A. tumefaciens* strain pGV2260 pBin61-p19, was co-expressed with the studied Arabidopsis proteins. *A. tumefaciens* carrying the selected gene constructs were suspended to activation buffer containing 150 μM acetosyringone and infiltrated to *N. benthamiana* leaves for transient protein expression. Protein interactions were studied 2-4 days after infiltration with confocal microscope.

3.5 Generation of transgenic Arabidopsis lines

The role of serine phosphorylation in Arabidopsis ACO3 was studied by mutating the phosphorylated serine residue to either alanine or aspartate depleting the phosphorylation site or mimicking the constitutive phosphorylation of the protein

(Figure 4). Transgenic Arabidopsis phosphomutant lines were generated by cloning the ACO3 protein coding sequence together with 2439 nucleotide ACO3 promoter area to pGREENII0029 vector and pGREENII0029 vector with YFP. ACO3 serine 91 (Ser91) was mutated to aspartate or alanine using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies).

Figure 4. Schematic presentations of the ACO3 gene constructs. Mitochondrial targeting sequence is separated with dashed line and Ser91 is marked with solid line. Figure from paper III.

PP2A-B'γ and *PP2A-B'ζ* promoter activity was studied with GUS reporter system. *proPP2A-B'γ:uidA* has been previously characterized in Trotta et al. (2011). For *proPP2A-B'ζ:uidA* 2811 bp region upstream of the start codon was cloned in pGREENII0029 vector (Rozhon et al. 2010) with *uidA* gene encoding β-glucuronidase.

Plasmid sequences were verified by sequencing and transferred to Arabidopsis wild type (*proPP2A-B'ζ:uidA*) or *aco3* mutant background (ACO3 constructs) by *A. tumefaciens* mediated transfection using floral dipping. Kanamycin selection was used to select the plants carrying the transgene. GUS staining of *proPP2A-B'γ:uidA* and *proPP2A-B'ζ:uidA* was performed according to Weigel & Glazebrook (2006).

3.6 Confocal microscopy

Phosphomutated ACO3-YFP fusion proteins were imaged in Arabidopsis protoplasts prepared with tape-Arabidopsis sandwich method as described in Wu et al. (2009). Bimolecular fluorescence complementation (BiFC) protein interactions were imaged in *N. benthamiana* leaf discs. BiFC protein interactions and YFP fusion proteins were imaged with confocal microscopes Zeiss LSM510 and LSM780. C-Apochromat 40x/1.20 W Korr M27 objective was used to monitor Arabidopsis samples and Plan-

Apochromat 20x/0.8 objective was used to study *N. bentamiana* leaf discs. YFP was excited with 514 nm laser and detected between 518-621 nm, 535-590 nm or 530-600 nm as indicated in publications. Chlorophyll A was excited with 633 nm light and the detected at 647-721 nm. Confocal images were created with Zeiss Zen 2012 software.

3.7 Targeted analysis of plant metabolites

Arabidopsis GSLs were analysed with high performance liquid chromatography (HPLC) followed by mass spectrometry (MS) as previously described in Mikkelsen and Halkier (2003). For analysis of amino acids and AMC intermediates, rosettes were ground in liquid nitrogen and metabolites were extracted in 50% acetonitrile, 25 mM HCl and centrifuged for 15 min with 12 000 g in 4°C. The supernatant was collected and SAM and SAH were analysed as described in Castor et al. (2002) and Loiseau et al. (2007) and amino acids were analysed with HPLC as described in Kreft et al. (2003).

3.8 Proteomic analysis

3.8.1 Sample extraction

Aconitase and AOX protein abundance and phosphorylation were studied in Arabidopsis mitochondria (Paper II) and total leaf extract (Paper III). For isolation of mitochondria, four-week-old Arabidopsis were kept in darkness for 24 h and the mitochondria were isolated on 60-35-20% Percol-sucrose gradient as described in Sweetlove et al. (2007). Arapidopsis total leaf extracts were isolated with 20 mM Tris, pH 7,8 and 2% sodium dodecyl sulphate (SDS) in the presence of protease and phosphatase inhibitors and shaken in 37°C for 20 min. Other proteomic studies were carried out using Arabidopsis total soluble leaf extract isolated in the presence of protease (Complete-Mini, Roche) and phosphatase (PhosSTOP, Roche) inhibitors as descried in Kangasjärvi et al. (2008). Protein concentrations in extracts were determined using BioRad Protein Assay Kit.

3.8.2 Polyacrylamide gel electrophoresis

Various gel electrophoresis methods were used to study Arabidopsis proteins and protein complexes including denaturing SDS-PAGE, non-denaturing clear native (CN)-PAGE and CN-PAGE followed by SDS-PAGE in second dimension as described in papers I-IV. Prior to MS analysis, proteins were detected by staining the gel with Coomassie Brilliant Blue or Sybro RUBY (Invitrogen) or by partially blotting the proteins on membrane followed by immunodetection of the studied protein. Western blotting was used to investigate the abundance and complex formation of specific

proteins. Proteins were transferred on polyvinylidene difluoride (PVDF) membrane (Immobilion-P, Millipore) and detected by protein specific antibodies. Protein bands were visualized by horse radish peroxidase (HRP) conjugate and enhanced chemiluminescence (ECL) substrate and exposed on X-ray films.

3.8.3 Mass spectrometry

MS was utilized for identification of proteins and phosphorylation sites. For data-dependent acquisition (DDA) MS analysis, protein spots and bands were excised from the gels and subjected to in-gel trypsin digestion as described in Li et al. (2014). Peptides were analysed either with QTOF Elite (AB ScieX) or Q-Exactive (Thermo Fisher Scientific) mass spectrometers as previously described in Li et al. (2014). When appropriate, an inclusion list of *in silico*- digested tryptic peptides was used to increase the probability of identification of the proteins of interest. The obtained spectra were searched against Arabidopsis database (TAIR 10) using Mascot search engine as described in Li et al. (2014) (QTOF Elite data), or against Arabidopsis Uniprot database using Proteome discoverer v1.4 (Thermo Fisher Scientific) as an interface software (Q-Exactive data).

Selected reaction monitoring (SRM) MS was used to quantify the phosphorylated and non-phosphorylated forms of the ACO3 peptide in paper II. The transitions monitored in SRM were selected using Skyline software (MacLean et al. 2010) and quantified using TSQ Vantage MS (Thermo Fisher Scientific).

3.9 Transcriptomic analysis

Transcript abundance of selected genes was studied using reverse transcriptase-polymerase chain reaction (RT-PCR). Arabidopsis total RNA was isolated with innuPREP Plant RNA Kit (Analytik Jena AG) or Agilent Plant RNA Isolation Mini Kit (Agilent) followed by DNAse treatment with Ambion Turbo DNA-free Kit (Thermo Fisher Scientific). cDNA was synthetized using Invitrogen SuperScript III First-Strand Synthesis SuperMix (Thermo Fisher Scientific). Specific sequences were amplified with gene specific primers described in publications using Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific). ACTIN 2 (ACT2) was used as control gene. Amplified PCR products were separated on 1% agarose gel, stained with Midori Green Advanced DNA stain (NIPPON Genetics) and detected with Geliance 1000 Imaging System (PerkinElmer).

Global transcriptomic changes were studied with Agilent Arabidopsis (V4) Gene Expression Microarrays, 4x44K. RNA was isolated using Agilent Plant RNA Isolation Mini Kit (Agilent). RNA was Cy-3 labelled and processed with Agilent One-Color Low

Input Quick Amp Labeling Kit (Agilent) and RNA Spike-In Kit (Qiagen) respectively. Samples were hybridized to Agilent Arabidopsis (V4) Gene Expression Microarrays, 4x44K and scanned with Agilent Technologies Scanner G2565CA with AgilentHD_GX_1Color profile. Numeric gene expression data were produced with Agilent Feature Extraction program, version 10.7.3 and analysed with R statistical software version 3.1.1.

4. OVERVIEW OF THE RESULTS

4.1 PP2A-B'γ controls plant growth and acclimation

Plants sense the quality and severity of stress and take coordinated actions to acclimate or activate cell death signals. Previous studies have shown that PP2A-B'γ controls the activation of cell death but its functions depend on the prevailing growth conditions (Trotta et al. 2011, Li et al. 2014). The effects of PP2A-B'γ and its closest homologue PP2A-B'ζ on growth and cell death were further studied in paper I. Plants were grown in normal growth conditions under 50% RH for two weeks and then acclimated to higher 85% RH for one week. Transfer back to 50% RH triggered bleaching and cell death at the periphery of leaves in *pp2a-b'γ* but not in *pp2a-b'ζ* or *pp2a-b'γζ* double mutants, suggesting that PP2A-B'ζ is needed for initiation of cell death in *pp2a-b'γ*.

The roles of PP2A-B'γ and PP2A-B'ζ in plant stress acclimation were further investigated under high light and elevated temperature. Tissue specific promoter activity of PP2A-B'γ and PP2A-B'ζ was examined using GUS-reporter lines *proPP2A-B'γ:uidA* and *proPP2A-B'ζ*. Promoter activities were detected at overlapping areas in the fast growing tissues of the rosette and the expression pattern did not differ between normal growth conditions and high light. In normal growth conditions *pp2a-b'γζ* double mutant showed smaller rosette size and leaf area compared to *pp2a-b'γ* and *pp2a-b'ζ* single mutants or wild type plants. However, when plants were first grown under normal growth conditions for two weeks and then grown two weeks under high light of 800 μmol phot m^{-2} s^{-1}, elevated temperature of 28°C and drought, in order to cause more severe abiotic stress, the growth differences levelled off. These results suggested that PP2A-B'γ and PP2A-B'ζ are needed to optimize plant growth under normal growth conditions but their role is diminished under severe stress. However, *pp2a-b'γζ* demonstrated enhanced tolerance to combined high light and drought and showed higher F_v/F_m values, reflecting the functional status of PSII, than the single mutants or wild type. Such enhanced stress tolerance was supported by alterations in gene expression, as microarray analysis revealed elevated abundance of transcripts related to photoprotective mechanisms in high-light-acclimated *pp2a-b'γζ*. While PP2A-B'γ had been previously characterized as a negative regulator of pathogen defence, these results proposed that PP2A subunit composition affects the decision between cell death and acclimation in plants exposed to abiotic stress.

4.2 PP2A-B′γ regulates ACONITASE 3 and the expression of alternative oxidase at post-translational level

A yeast two-hybrid screen utilizing a cDNA library enriched in stress induced transcripts (Jaspers et al. 2009) was used to investigate the direct target proteins of PP2A-B'γ (Paper II). This lead to identification of aconitase isoform ACO3 as a putative interactor of PP2A-B'γ. Arabidopsis genome encodes three aconitase isoforms that catalyse the reversible conversion of citrate to isocitrate via the intermediate reaction product cis-aconitate. The catalytic centre of the protein possesses an $[4Fe{-}4S]^{2+}$ cluster rendering aconitases sensitive to oxidative inactivation by ROS (Verniquet et al. 1991). Arabidopsis ACO1 is localized to cytoplasm whereas ACO2 and ACO3 carry a mitochondrial targeting peptide. Interestingly, ACO3 is dually localized to cytoplasm and mitochondria (Bernard et al. 2009, Hooks et al. 2014). In mitochondria, aconitase forms a part of the TCA cycle, whereas in cytoplasm it participates in other citrate associated metabolic pathways including nitrogen and fatty acid metabolism (Hooks et al. 2014). Interaction between PP2A-B'γ and ACO3 and regulation of ACO3 were further studied in papers II and III.

4.2.1 PP2A-B′γ interacts with ACONITASE 3 and regulates its level of phosphorylation

PP2A-B'γ-ACO3 interaction was confirmed using BiFC (Walter et al. 2004). N and C-terminal halves of yellow fluorescence protein (YFP) were cloned to the C-terminus of the ACO3 and PP2A-B'γ respectively. The fusion proteins were transiently co-expressed in *N. benthamiana* leaves. The fluorescence signal indicative of protein interactions between ACO3 and PP2A-B'γ localized to cell cytoplasm. No interaction was detected between PP2A-B'γ and AOX isoform 1A, another mitochondrial protein identified in the yeast-two hybrid screen for putative interactors of PP2A-B'γ (Paper II).

To investigate if ACO3 is post-translationally regulated by phosphorylation, the total soluble leaf extract of wild type and *pp2a-b'γ* were separated on SDS-PAGE, aconitase bands were excised from the gel and the proteins were analysed by MS. The analysis identified a unique phosphopeptide, which is present in ACO3 but not in the other Arabidopsis aconitase isoforms. ACO3 was found to be phosphorylated at Ser91. Moreover, SRM-MS was used to quantify the portion of the phosphorylated and non-phosphorylated peptide in wild type and *pp2a-b'γ* mutant. Phosphorylation of Ser91 was increased in *pp2a-b'γ* compared to wild type. 3% and 6% portions of phosphorylated ^{89}TFSSMASEHPFK^{100} peptide were detected in wild type and *pp2a-b'γ* respectively, suggesting that PP2A-B'γ regulates the ACO3 phosphorylation at Ser91 (Paper II).

4.2.2 Phosphorylation of serine 91 regulates ACONITASE 3 protein abundance

Transgenic Arabidopsis lines harbouring mutations either mimicking the constitutive phosphorylation or depleting the phosphorylation site Ser91 were used to study the role of phosphorylation in ACO3 (Paper III). Mutations were introduced to the ACO3 coding DNA sequence so that Ser91 was replaced by aspartate or alanine respectively. Endogenous promoter sequence of ACO3 was used to drive the expression of the transgenes. To investigate if the phosphorylation controls ACO3 localization, YFP was fused to the C-terminus of the ACO3 sequence. Moreover, the ACO3 phosphomutant constructs were transferred to *aco3* mutant background with *A. tumefaciens* mediated transfection.

Fluorescence microscopy of *pACO3::ACO3-YFP, pACO3::ACO3^{S91A}-YFP* and *pACO3::ACO3^{S91D}-YFP* demonstrated that in four-week-old plants ACO3 is localized to mitochondria independent of the phosphorylation of Ser91. However, *pACO3::ACO3^{S91D}-YFP* showed stronger fluorescence signal than *pACO3::ACO3-YFP* or *pACO3::ACO3^{S91A}-YFP* suggesting differences in ACO3 abundance between the transgenic lines. Western blot analysis of the transgenic ACO3 lines confirmed that phosphorylation of Ser91 regulates the accumulation of ACO3-YFP fusion proteins at post-translational level. ACO3^{S91D}-YFP mimicking the constitutive phosphorylation accumulated in greater amounts than ACO3-YFP and ACO3^{S91A}-YFP, the latter showing the lowest level of ACO3-YFP fusion protein. Corresponding differences were not observed in the transcriptional activity of the gene constructs analysed by RT-PCR indicating that the abundance of ACO3-YFP fusion proteins is regulated at post-translational level (Paper III).

4.2.3 PP2A-B′γ and ACONITASE 3 control the expression of alternative oxidase

Previous studies have shown that *pp2a-b'γ* accumulates H_2O_2 in rosette leaves (Trotta et al. 2011). Further investigation of the origin of the ROS using inhibitors blocking the mitochondrial electron transfer pathways revealed that the oxidative stress in *pp2a-b'γ* is alleviated by modulation of the mitochondrial AOX (Paper II). A pharmacological approach utilizing AOX inhibitor SHAM demonstrated that the inhibition of AOX further increased the ROS accumulation in *pp2a-b'γ*. Moreover, western blot analysis of isolated mitochondria showed that *pp2a-b'γ* accumulates specific AOX isoforms AOX1A and AOX1D, detected by α-AOX antibody and further identified by MS analysis. Moreover, similar accumulation of AOX1A mRNA was not detected in RT-PCR analysis, which demonstrated slightly reduced levels of AOX1A mRNA and slightly higher levels of AOX1D mRNA in *pp2a-b'γ* compared to wild type. However,

these differences in AOX1A and AOX1D gene expression between *pp2a-b'γ* and wild type were not statistically significant. These results suggest that PP2A-B'γ negatively regulates the abundance of specific AOX isoform AOX1A and AOX1D in Arabidopsis leaves (Paper II).

In subsequent studies I found that also ACO3 affects the abundance of AOX (Paper III). Transgenic *pACO3::ACO3-YFP, pACO3::ACO3^{S91A}-YFP* and *pACO3::ACO3^{S91D}-YFP* lines showed higher levels of AOX than wild type, *aco3* or *pACO3::ACO3, pACO3::ACO3^{S91A}* and *pACO3::ACO3^{S91D}*. AOX abundance followed the accumulation of ACO3 in the YFP-fusion lines so that *pACO3::ACO3^{S91D}-YFP* exhibited also the highest levels of AOX, suggesting that ACO3 abundance is dynamically linked to AOX expression. Moreover, differences in AOX accumulation between *pACO3::ACO3-YFP, pACO3::ACO3^{S91A}-YFP* and *pACO3::ACO3^{S91D}-YFP* lines were not detected at the transcriptional level, indicating that AOX abundance was regulated at post-translational level. The high ACO3 and AOX levels in *pACO3::ACO3^{S91D}-YFP* plants were in line with the enhanced tolerance to methyl viologen induced oxidative stress in germinating seedlings (Paper III). These results suggest that ACO3 phosphorylation is connected to plant tolerance to oxidative stress possibly via modulation of AOX abundance in mitochondria.

4.3 Methylation of indole glucosinolates and the cell transmethylation capacity are under post-translational regulation by PP2A-B'γ

During pathogen infection, production of defence metabolites creates a new sink for SAM, the methyl group donor in the transmethylation reactions (Ranocha et al. 2001, Bekaert et al. 2012). Specific methylation reactions of indole GSLs determine their biological activity in plant-microbe and plant-insect interactions (Agerbirk et al. 2009). Moreover, the AMC responsible for the regeneration of SAM must be adjusted to support the production of the defence compounds upon pathogen infection of insect infestation (reviewed in Rahikainen et al. 2018). The role of PP2A-B'γ in the post-translational control of the AMC enzymes and production of 4MO-I3M GSL was investigated in paper IV.

4.3.1 PP2A-B'γ controls the abundance of 4MO-I3M GSL in Arabidopsis

The yeast two-hybrid screen for putative PP2A-B'γ target proteins identified an INDOLE GLUCOSINOLATE METHYLTRANSFERASE 4 (IGMT4) as a candidate interactor. IGMT1-4 catalyse the methylation reaction of I3M GSL to 4MO-I3M GSL, while IGMT5 is needed for synthesis of 1MO-I3M GSL (Pfalz et al. 2011, Pfalz et al. 2016). A BiFC study confirmed that PP2A-B'γ is able to interact with IGMT4 as well

as with IGMT1. Interestingly, MS analysis demonstrated that only IGMT4 isoform could be detected in the unstressed Arabidopsis leaves, while no unique peptides were identified for IGMT1-3. To investigate if the PP2A-B'γ-IGMT interaction contributes to the GSL biosynthesis, the main aliphatic and indole GSLs were analysed in Arabidopsis wild type, *pp2a-b'γ* and *pp2a-b'γ 35S::PP2A-B'γ* complementation line. Since 4MO-I3M GSL synthesis is induced upon defence activation, the GSL profiles were studied in vegetative state in 19-day-old plants, in 33-day-old plants when *pp2a-b'γ* undergoes defence associated cell death, and after infection by *B. cinerea* fungus. *pp2a-b'γ* demonstrated increased amounts of 4MO-I3M GSL compared to wild type and the complementation line. Moreover, infection by *B. cinerea* induced 4MO-I3M GSL biosynthesis in all studied plant lines compared to the mock treated plants while *pp2a-b'γ* still demonstrated the highest levels of 4MO-I3M GSL. Comparison of the portion of 4MO-I3M GSL to all measured indole GSLs clearly demonstrated that *pp2a-b'γ* specifically accumulated 4MO-I3M GSL in all tested growth stages and conditions. These results suggest that PP2A-B'γ controls the formation of 4MO-I3M GSL possibly via direct interaction with IGMTs.

4.3.2 PP2A-B'γ and indole glucosinolate methyltransferases form interactions with the activated methyl cycle enzymes

Formation of 4MO-I3M GSL is dependent of the methyl groups provided by the AMC. PP2A-B'γ has been previously shown to regulate the abundance of SAHH1, the AMC enzyme detoxifying SAH (Trotta et al. 2011, Li et al. 2014). Moreover, proteomic analysis of *cat2 pp2a-b'γ* double mutant identified a SAHH1 phosphopeptide not detected in wild type plants (Li et al. 2014). Thus the connection between PP2A-B'γ and SAHH1 was further studied in paper IV. A BiFC assay between PP2A-B'γ and SAHH1 identified SAHH1 as a putative target of PP2A-B'γ. Moreover, since SAHH1 is needed to hydrolyse the inhibitory reaction product SAH produced by IGMTs, also the interactions between SAHH1 and the IGMTs were studied. SAHH1 was found to interact with IGMT1, 3 and 4. In addition, an interaction was detected between PP2A-B'γ and SAHH1 and CIMS1, the ACM enzyme catalysing the regeneration of methionine from Hcy. Altogether, the BiFC analysis revealed mutual interactions between the AMC enzymes IGMT, SAHH and CIMS and showed that PP2A-B'γ exerts its regulation to the ACM enzymes SAHH and CIMS. These findings suggest that AMC is tightly connected to methylation of indole GSLs and that these two processes are co-regulated.

4.3.3 Plant transmethylation capacity and complex formation of activated methyl cycle enzymes are regulated by PP2A-B'γ

Analysis of the AMC intermediates SAM and SAH in wild type, *pp2a-b'γ* and *pp2a-b'γ 35S::PP2A-B'γ* demonstrated that *pp2a-b'γ* shows increased SAM/SAH ratio indicative of increase in the cell transmethylation potential. To further investigate the regulation of ACM, total soluble proteins of wild type and *pp2a-b'γ* were separated on CN-PAGE and blotted with antibodies recognizing SAHH and CIMS. SAHH was detected in multiple separate bands. The low molecular weight bands likely represented the SAHH monomer and dimer whereas the higher molecular weight complexes indicated SAHH presence in different oligomeric protein complexes. Complex 4 of roughly 200 kDa was the most abundant one and showed further accumulation in *pp2a-b'γ* compared to wild type. Moreover, CIMS presented one higher molecular weight protein complex whose abundance was decreased in *pp2a-b'γ* compared to wild type. In addition, a MS analysis revealed serine phosphorylation in both SAHH and CIMS in *pp2a-b'γ* protein extract. These results suggest that PP2A-B'γ controls the complex formation of SAHH and CIMS via post-translational regulation.

5. DISCUSSION

Plant growth environments are characterized by continuous alteration of favourable and unfavourable conditions. Moreover, different stress factors are not only present one at the time but are often combined and plant stress signalling pathways therefore employ partially overlapping mechanisms. For example, compartmentalized production of ROS and activation of MPK3 and 6 have been shown to play key signalling roles in both biotic and abiotic stress responses (Asai et al. 2002, Beckers et al. 2009, Besteiro et al. 2011, Choudhury et al. 2017). Furthermore, stress responses are coordinated reactions to signals from multiple sources that try to optimize the plant survival or reproduction under unfavourable environmental conditions. Plants may launch acclimation processes to overcome the prevailing stress or activate localized cell death to eliminate the stress-exposed tissues. Moreover, response to stress depends on the prevailing physiological state of the plant. Despite the recent research advances, the signalling network that receives stress signals from various receptor molecules and cellular compartments and controls the decision on the ultimate physiological responses is still poorly understood.

The cytoplasmic PP2A regulatory subunit PP2A-B'γ was found to control multiple processes in plant stress acclimation and cell death. Under favourable growth conditions PP2A-B'γ prevents the unnecessary activation of defence measures. Moreover, PP2A-B'γ works in a regulatory network that controls organellar ROS signals and components of plant primary metabolism as well as the closely connected reactions in the secondary defence metabolism (Papers II, III and IV). Under stress PP2A-B'γ modulates the extent of the acclimatory and defensive reactions depending on the type of the stress (Papers I and IV). Upon *B. cinerea* infection, PP2A-B'γ limits the production of 4MO-I3M GSL (Paper IV), whereas under high light PP2A-B'γ together with PP2A-B'ζ restricts the activation of photoprotective mechanisms (Paper I). Thus, PP2A-B'γ affects the decision between growth and defence under varying environmental conditions.

5.1 PP2A as regulator of plant growth, stress and acclimation

Previous studies have demonstrated that PP2A-B'γ is a negative regulator of defence associated cell death in Arabidopsis (Trotta et al. 2011, Li et al, 2014, Rasool et al 2014). PP2A-B'γ controls the SA mediated cell death that becomes activated by organellar ROS signals (Trotta et al. 2011, Li et al. 2014). Moreover, the development of the spontaneous cell death in *pp2a-b'γ* is highly dependent on the plant growth conditions. The role of PP2A-B'γ and its closest homologue PP2A-B'ζ was further studied under abiotic stress in paper I. Growth experiments and physiological measurements

demonstrated that PP2A-B'γ and PP2A-B'ζ are required for optimal growth under favourable conditions. Smaller and compact rosette morphology under stress has been described as stress-induced morphogenic response (SIMR) and ROS signalling has been associated with this phenotype (Potters et al. 2007). However, under combined abiotic stress the deficiency of PP2A-B'γ and PP2A-B'ζ induced photo-protective mechanisms that enhanced stress tolerance, indicating that under severe stress PP2A-B'γ and PP2A-B'ζ negatively regulate the activation of acclimatory measures.

Stress induced cell death is under tight genetic control and not simply the consequence of detrimental oxidative damage of cell structures (Mullineaux and Baker 2010, Brosche et al. 2014). Decision of cell death is an interplay of promoting and opposing factors. PP2A-B'γ controls the ROS accumulation under normal growth conditions but does not have an impact on the major antioxidants ascorbate and glutathione in the cell (Trotta et al. 2011, Li et al. 2014). Interestingly, organellar ROS accumulation in *cat2 pp2a-b'γ* induced SA signalling whereas the deficiency of PP2A-B'ζ in the *pp2a-b'γζ* double mutant triggered enhanced photoprotective mechanisms under high light. Thus it seems that the PP2A subunit composition has a role in determining the plant response to various stress signals. PP2A-B'ζ shares 80% sequence identity with PP2A-B'γ and structural analysis suggests that they are able to interact with similar PP2A-A and C subunits (Rasool et al. 2014). Thus, it is intriguing that they demonstrate distinct roles in plant stress signalling. The functional interaction between PP2A-B'γ and PP2A-B'ζ may be further complicated by possible competition of the PP2A A and C subunits between PP2A-B'γ and PP2A-B'ζ. Moreover, PP2A-B'γ and PP2A-B'ζ are likely to interact with multiple target proteins in the cell, some of which are unique while others may be shared by these two regulatory subunits. Further research of PP2A subunit composition and targets will elucidate the interconnections between the different PP2A regulatory subunits.

5.2 ACONITASE 3 abundance is regulated at the post-translational level

Environmental fluctuations induce quick alterations in plant metabolism to maintain the cell homeostasis. ACO3 functions in basic energy metabolism in the TCA cycle and glyoxylate cycle as well as in the cytoplasmic citrate metabolism. TCA cycle enzymes malate dehydrogenase and isocitrate dehydrogenase (ICDH) have been shown to be regulated by phosphorylation, which inhibits their activity via binding to 14-3-3 proteins (Diaz et al. 2011). Moreover, especially aconitase was demonstrated to be sensitive to H_2O_2 and become inactivated by ROS signals (Verniquet et al. 1991).

Previously, Trotta et al. (2011) have shown that *pp2a-b'γ* accumulates aconitase in total soluble leaf extract, but the contribution of the three different isoforms to the increase could was not determined (Trotta et al. 2014). Moreover, in the non-symptomatic *pp2a-b'γ* plants the level of citrate was reduced, while increased oxidative stress in *cat2 pp2a-b'γ* resulted in an increase in citrate when compared to wild type plants (Li et al. 2014). A yeast-two hybrid screen together with BiFC analysis indicated that PP2A-B'γ physically interacts with ACO3 (Paper II). Moreover, a unique phosphopeptide was identified in ACO3 and this peptide is missing in the other two aconitase isoforms. Quantification of the phosphorylated and non-phosphorylated peptides in wild type and *pp2a-b'γ* demonstrated that PP2A-B'γ negatively affects the ACO3 phosphorylation in Ser91, making it a likely target for dephosphorylation by PP2A. In mammals, phosphorylation of mitochondrial aconitase in Ser690 adjacent to its active site increases the aconitase reverse activity converting isocitrate to citrate (Lin et al. 2009). Moreover, phosphorylation of Ser138 has been reported to destabilize the $[4Fe{-}4S]^{2+}$ cluster in the aconitase active site and contribute to the iron regulatory protein (IRP) functions found in mammalian aconitase (Deck et al. 2009). However, no role as an IRP could be demonstrated for plant aconitase (Arnaud et al. 2007). Moreover, Ser91 lies relatively far from the ACO3 active site and is thus unlikely to directly regulate the access of the substrate to the active centre (Paper III).

The role of ACO3 phosphorylation was further studied in paper III using transgenic phopshomutant lines. Interestingly, ACO3 harbours a mitochondrial target peptide but has been shown to be dually localized between mitochondria and cytoplasm (Bernard et al. 2009, Hooks et al. 2014). Inefficient translocation to mitochondria has been suggested to contribute to the cytoplasmic localization but reports indicating distinct differences in ACO3 localization in different developmental stages suggest that the subcellular distribution of ACO3 is under tight regulation (Carrari et al. 2003, Bernard et al. 2009, Hooks et al. 2014). ACO3 phosphorylation at Ser91 lies close to the predicted cleavage site of the target peptide and could thus potentially regulate the distribution of ACO3 between mitochondria and cytoplasm. However, in four-week-old plants ACO3-YFP was found to localize predominantly in mitochondria independent of the phosphorylation state of Ser91 (Paper III).

Fluorescence microscopy and proteomic approaches demonstrated that Ser91 phosphorylation in ACO3 affected the ACO3 protein abundance in the transgenic lines where phosphomutated ACO3 were fused to YFP. Phosphomimetic $ACO3^{S91D}$-YFP showed clear accumulation at protein level compared to ACO3-YFP and $ACO3^{S91A}$-YFP. Corresponding differences were not detected in the transcript amounts suggesting the phosphorylation might regulate the ACO3-YFP turnover. While no differences were found in the localization of ACO3-YFP fusion proteins between the phosphomutated lines, it cannot be ruled out that the phosphorylation of Ser91 controls

the ACO3 distribution via differential turnover in mitochondria and cytoplasm. Aconitase has been shown to accumulate in the total soluble leaf extract of *pp2a-b'γ* whereas no differences were found between wild type and *pp2a-b'γ* plants when isolated mitochondria were studied, suggesting that *pp2a-b'γ* controls the abundance of cytoplasmic aconitase (Trotta et al. 2011, Paper II). Moreover, PP2A-B'γ localizes to cytoplasm and the interaction with ACO3 was found in the cytoplasm of epidermal cells of *N. benthamiana*. However, the regulation of ACO3 abundance by phosphorylation of Ser91 does not explain the higher aconitase levels in the soluble protein fraction but not in isolated mitochondria between *pp2a-b'γ* and wild type. Moreover, mutations in Ser91 did not lead to evident changes in aconitase abundance in the transgenic lines lacking the YFP fusion protein. Furthermore, RT-PCR analysis suggested that the ACO3 transgenes without the YFP fusion failed to complement the ACO3 expression back to wild type levels (Paper III). Thus, further investigation of the transgenic ACO3 lines will be needed to solve the discrepancies found between the transgenic ACO3 and ACO3-YFP lines.

5.3 ACO3 is functionally connected to alternative oxidase and redox signalling

In addition to its role in the primary metabolism, aconitase contributes to regulation of cell redox balance. Arabidopsis ACO1 has been shown to bind the mRNA of the chloroplast COPPER/ZINC SUPEROXIDE DISMUTASE 2 (CSD2) in cytosol and regulate its stability (Moeder et al. 2007). Moreover, aconitase provides substrate for ICDH that catalyses the oxidative decarboxylation of isocitrate to α-ketoglutarate producing CO_2 and NADH. Thus, aconitase activity contributes to the availability of reducing equivalents in the cell. Furthermore, functions of ACO3 and the cytoplasmic ICDH have been linked to plant tolerance to oxidative stress and pathogen defence (Moeder et al. 2007, Mhamdi et al. 2010).

Interestingly, the protein abundance of AOX followed the ACO3 abundance and phosphorylation status in the phosphomutated ACO3-YFP lines, suggesting that the ACO3 abundance is linked to AOX expression (Paper III). *pACO3::ACO3^{S91D}-YFP* line showing the highest accumulation of ACO3 also showed the highest AOX abundance. Moreover, the high levels of ACO3 and AOX in *pACO3::ACO3^{S91D}-YFP* correlated with enhanced tolerance to oxidative stress in germinating seedlings. AOX functions as a safety valve of mETC and prevents the over-reduction and further ROS production in mETC by donating electrons to molecular oxygen forming water. TCA cycle is closely connected to AOX expression and activity (Vanlerberghe and McIntosh 1996, Gray et al. 2004, Selinski et al. 2018). Citrate, malate and 2-oxoglutarate have been shown to induce the AOX at the transcriptional level independently of mitochondrial ROS signals

whereas 2-oxoglutarate also regulates the activity of specific AOX isoforms AOX1A and AOX1D (Gray et al. 2004, Selinski et al. 2018). Interestingly, the phosphorylation status and the protein accumulation of ACO3-YFP in the transgenic lines were only linked to AOX accumulation at the protein level whereas similar changes were not detected at the transcriptional level (Paper III). Moreover, AOX abundance was affected in lines expressing ACO-YFP fusion proteins and not in the lines expressing ACO3 alone. If the YFP fusion has a role in the expression of the transgenes or AOX requires further research. As ACO1 has been previously shown to regulate the stability of CSD2 mRNA in cytosol (Moeder et al. 2007), it is possible that ACO3 would regulate the AOX expression through a similar mechanism. Moreover, no accumulation of H_2O_2 was detected in any of the transgenic ACO3 lines indicating that the increased AOX abundance is not due to elevated cellular ROS levels. Further metabolic analysis is needed to investigate if citrate of other TCA cycle intermediates play a role in the AOX expression in the phosphomutated ACO3-YFP lines.

Arabidopsis genome encodes five different AOX subunits called AXO1A-D and AOX2, of which the AOX1A is the predominant isoform (Clifton et al. 2006). The size of the AOX protein band accumulating in the ACO3 dependent manner supported the conclusion that AOX1A is likely to be the major isoform accumulating in the phosphomutated ACO3-YFP lines (Papers II and III). However, individual AOX isoforms have been reported to undergo specific transcriptional changes in response to various stress conditions (Clifton et al. 2005, Clifton et al. 2006). Interestingly, PP2A-B'γ was found to control the expression of specific AOX isoforms AOX1A and AOX1D at protein level, whereas similar accumulation was not detected in the AOX1A and AOX1D mRNAs (Paper II). Moreover, the AOX activity was connected to alleviation of the oxidative stress and ROS accumulation in *pp2a-b'γ* leaves. AOX activity contributes to the ROS signalling redox balance not only in mitochondria but also in other organelles via metabolic shuttles circulating the redox active metabolites between different cellular compartments. Altogether, these results suggest a role for PP2A-B'γ in the control of plant redox signalling and metabolism via post-translational regulation of ACO3 and AOX (Figure 5).

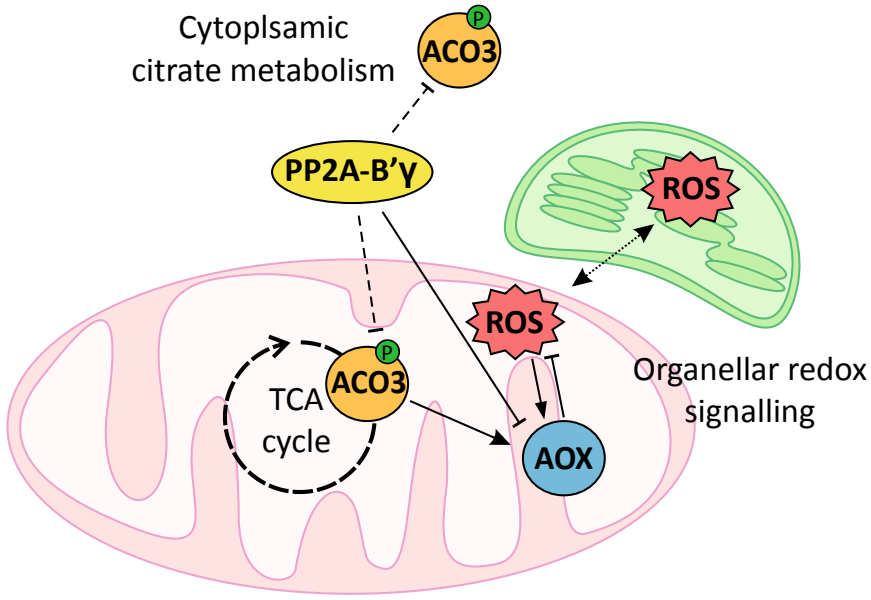

Figure 5. A simplified scheme depicting the interactions and functional connections between PP2A-B'γ, ACO3, AOX and organellar ROS signals. PP2A-B'γ regulates the phosphorylation of ACO3, which promotes its accumulation. ACO3 abundance and PP2A-B'γ further modulate the expression of AOX at the post-translational level.

5.4 Methylation of indole glucosinolates is connected to cell transmethylation capacity via interactions with activated methyl cycle

GSLs are structurally diverse defence compounds whose biosynthetic pathways have recently been studied in greater detail (Halkier and Gershenzon 2006, Pfalz et al. 2009, Bednarek et al. 2009, Sønderby et al. 2010, Pfalz et al. 2011, Bekaert et al. 2012). Different GSL structures are known to have specific defence and signalling roles although the mechanistic background is not yet known (Agerbirk et al. 2009). Recent reports have demonstrated that GSL biosynthesis is under transcriptional control. Genes encoding the GSL biosynthetic pathways are regulated by MYB34, MYB51 and MYB122 transcription factors (Frerigmann et al. 2015). MYB51 and MYB122 in turn are under the control of ETHYLENE RESPONSE FACTOR 6 (ERF6) that becomes activated by MPK3 and 6 upon infection by necrotrophic fungus *B. cinerea* (Xu et al. 2016). Moreover, CYP81F2 and IGMT1 and 2 catalysing the hydroxylation and subsequent methylation of I3M GSL to 4MO I3M GSL are controlled by ERF6 (Xu et al. 2016). Furthermore, *IGMT2* and *CYP81F2* become activated by chloroplastic H_2O_2 signals suggesting a role for organellar ROS signalling in 4MO I3M GSL production

(Sewelam et al. 2014). However, post-translational regulation of the GSL biosynthesis had not been reported until PP2A-B'γ was shown to influence the methylation of indole GSLs in paper IV.

4MO-I3M GSL has been shown to deter aphids and microbial pathogens and affect the oviposition preferences of crucifer-specialist lepidopterans (de Vos et al. 2007, Bednarek et al. 2009, Clay et al. 2009, Pfalz et al. 2009, Sun et al. 2009). Moreover, the enzymes catalysing the production and degradation of 4MO-I3M GSL are well conserved in different Brassicaceae species but the levels of 4MO-I3M GSL and its precursor I3M GSL upon pathogen infection or insect feeding differ greatly between species (Bednarek et al. 2011, Fahey et al. 2001). This highlights the role of 4MO-I3M GSL in determining the specific plant-insect and plant-microbe interactions (Bednarek et al. 2011). Moreover, 4MO-I3M GSL has demonstrated biological activity both as an intact compound and after hydrolysis by myrosinases (Bednarek et al. 2009, Clay et al. 2009). Interestingly, 4MO-I3M GSL degradation by myrosinase PENETRATION 2 and 3 (PEN2/3) induced intracellular defence signals that promote callose deposition, a defence measure against non-host fungal and bacterial pathogens (Clay et al. 2009). Moreover, GSLs have been associated also to plant resistance to abiotic stress. Under mild osmotic stress MKK9-MPK3/6 phosphorylation cascade activates the production of 4MO-I3 GSL (Zhao et al. 2017). The role of 4MO-I3M GSL in abiotic stress responses remains elusive but it seems plausible that it plays a role in plant stress signalling.

GSL analysis of wild type and *pp2a-b'γ* in two different developmental stages and under *B. cinerea* infection demonstrated that PP2A-B'γ negatively regulates the formation of 4MO-I3M GSL in Arabidopsis (Paper IV). PP2A-B'γ directly interacted with IGMT1 and 4 in BiCF assay suggesting that PP2A-B'γ regulates the last methylation step in 4MO-I3M GSL biosynthesis. *IGMT1-3* are transcriptionally induced upon green peach aphid infestation (De Vos and Jander 2009) whereas *IGMT1* and *IGMT2* become activated by *B. cinerea* infection (Xu et al. 2016). MS analysis in paper IV demonstrated that IGMT4 was constitutively present and detected in non-stressed Arabidopsis leaves whereas the unique peptides for the other IGMT isoforms could not be detected (paper IV). These results suggest that IGMT4 provides the basal activity for 4MO-I3M GSL methylation in unstressed plants whereas the other three isoforms are induced upon stress. The MS analysis did not detect phosphorylated peptides in IGMTs and thus their regulatory mechanism remains elusive. However, phospho-regulation has been previously demonstrated for O-methyltransferase catalysing the monolignol biosynthesis in poplar (Wang et al. 2015). Poplar 5-hydroxyconiferaldehyde O-methyltransferase 2 activity is switched off by phosphorylation whereas an opposite effect would be expected in regulation of IGMTs by PP2A-B'γ.

The methylation of 4MO-I3M GSL is tightly connected to the function of AMC, which hydrolyses the inhibitory reaction product SAH produced by the methyltransferases and regenerates the methyl group donor SAM (Figure 6). The function of AMC is crucial for the cell, since it supports the wide variety of cellular methylation reactions including DNA, RNA and histone methylation, protein methylation and biosynthesis of various metabolites (reviewed in Rahikainen et al. 2018). Upon stress, the biosynthesis of secondary defence compounds creates an increased methylation sink, while the DNA methylation has been reported to be reduced upon defence activation (Pavet et al. 2006, Trotta et al. 2011). Moreover, AMC enzymes have been reported to be upregulated at transcriptional and post-translational levels upon defence activation towards plant pathogens, including oomycetes, necrotrophic fungi and biotrophic bacteria (Elmore et al. 2012, Arasimowicz-Jelonek et al. 2013, Winterberg et al. 2014, Li et al. 2015,). In addition, SAHH and SAMS have been found to be targets for interference by viral effectors, further highlighting the role of AMC in plant immunity (Ivanov et al. 2016).

In addition to the interaction between PP2A-B'γ and IGMT1, a BICF assay revealed that PP2A-B'γ interacts with SAHH and CIMS of the AMC. Thus it seems that PP2A-B'γ exerts its regulation over the AMC. Interestingly, both SAHH and CIMS were found to form protein complexes, whose abundance was regulated by PP2A-B'γ. *pp2a-b'γ* showed accumulation of SAHH in a specific protein complex, whereas the abundance of the single detected CIMS complex was downregulated. Moreover, phosphopeptides were identified from SAHH1 and CIMS1. Hence, it seems possible that PP2A-B'γ regulates the phosphorylation of specific sites in these enzymes and thus controls their protein interactions (Figure 6). However, the physiological role of the identified complexes remains unclear.

Methyl tetrahydrofolate reductase (MTHFR) was found to associate with CIMS in the protein complex, which provides a link between AMC and folate metabolism. Moreover, mutual interactions were detected between SAHH1 and IGMT1, 3 and 4 and CIMS1 suggesting that the ACM may be organized to so-called metabolon formed by multiple enzymes catalysing closely connected metabolic reactions. This organization enhances the efficiency of the metabolic circuits by substrate channelling and provides control over metabolic fluxes. SAHH1 has been previously shown to interact with adenosine kinase (ADK) that phosphorylates adenine produced in the hydrolysis of SAH by SAHH (Lee et al. 2012). However, SAHH and CIMS did not associate in the same protein complex on a CN-PAGE, suggesting that their interactions are transient in nature.

SAHH1 has been previously shown to interact with mRNA cap MT required for efficient protein translation (Lee et al. 2012). Moreover, studies of allelic *sahh1* mutant lines harbouring specific point mutations suggest that certain amino acid residues in

SAHH are required for distinct transmethylation reactions in cell (Rocha et al. 2005, Mull et al. 2006). It seems possible that SAHH associates with different methyltransferases to ensure the efficient hydrolysis of SAH and thus maintains the methyltransferase activity. Specific interactions between SAHH and methyltransferases may be regulated via SAHH phosphorylation at distinct sites. Whether some of these sites are regulated by PP2A requires further investigation.

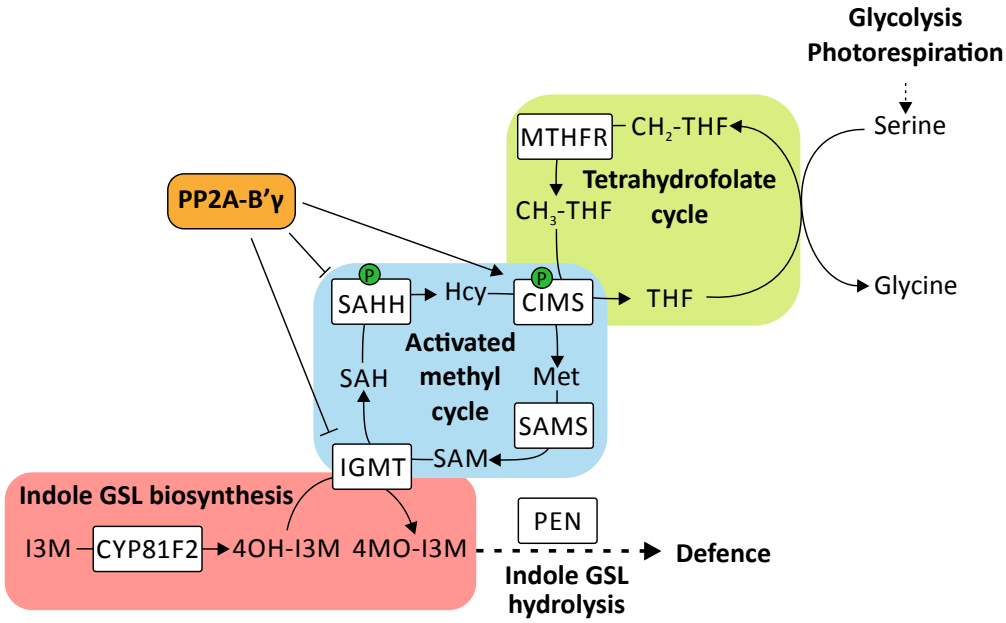

Figure 6. Overview of the regulation of 4MO-I3M GSL biosynthesis and AMC by PP2A-B'γ. The methylation of indole GSLs is closely connected to the AMC hydrolysing SAH and regenerating SAM. Methyl-groups for AMC are provided by tetrahydrofolate cycle. AMC enzymes SAHH and CIMS as well as IGMT catalysing the formation of 4MO-I3M GSL are regulated by PP2A-B'γ. Modified from Paper IV.

6. CONCLUDING REMARKS

The research presented in this PhD thesis contributes to our understanding of the regulation of plant stress and defence responses. PP2A-B'γ was shown to control plant acclimation under abiotic stress and regulate stress associated adjustments in plant metabolism (Figure 7). The following conclusions were made:

- PP2A-B'γ and PP2A-B'ζ influence the plant growth and stress acclimation under normal growth conditions and under severe abiotic stress.
- PP2A-B'γ modulates ACO3 phosphorylation and protein abundance, which is further connected to cell redox balance via post-translational regulation of AOX expression.
- PP2A-B'γ adjusts plant defence metabolism by controlling cellular transmethylation potential and production of 4MO-I3M GSL.

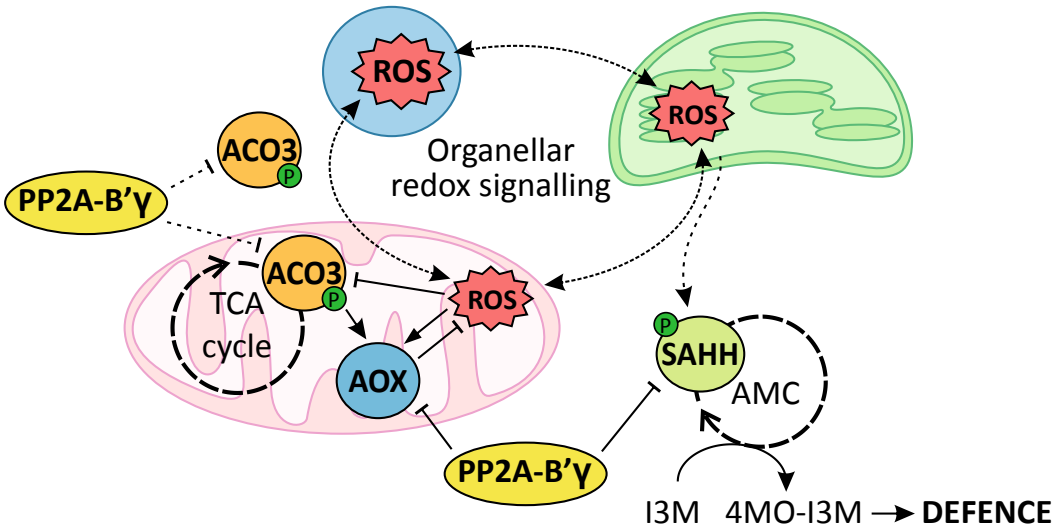

Figure 7. Regulation of plant defence metabolism by organellar ROS signals and PP2A-B'γ. Organellar ROS signals control enzymes in plant primary metabolism including ACO3. Moreover, ROS promote the activity and expression of AOX, whose abundance is further linked to ACO3 at the port-translational level. ACO3 phosphorylation and protein abundance as well as accumulation of specific AOX isoforms is controlled by PP2A-B'γ. Moreover, in secondary defence metabolism, PP2A-B'γ limits the synthesis of 4MO-I3M GSL by control over AMC and cell transmethylation capacity.

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Moona

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