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PP2A: AT THE CROSSROADS BETWEEN GROWTH AND DEFENCE IN PLANTS

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following articles, which are referred in the text by their corresponding Roman numerals.

- I 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 Physiol* 156: 1464–80.
- II Rasool B, Karpinska B, Konert G, Durian G, Denessiouk K, Kangasjärvi S, Foyer CH (2014). Effects of light and the regulatory B-subunit composition of protein phosphatase 2A on the susceptibility of *Arabidopsis thaliana* to aphid (*Myzus persicae*) infestation. *Front Plant Sci* 5: 405.
- III Konert G, Rahikainen M, Trotta A, Durian G, Salojärvi J, Wrzaczek M, Kangasjärvi J, Aro E-M, Göhre V, Glawischnig E, Denessiouk K, Kangasjärvi S. Structure-function analysis of *Arabidopsis* PP2A phosphatase/CPK1 kinase interactions in plant immunity. *Manuscript*.
- IV Konert G, Trotta A, Kouvunen P, Rahikainen M, Durian G, Blokhina O, Fagerstedt K, Muth D, Corthals GL, Kangasjärvi S (2014). PP2A regulatory subunit B^γ interacts with cytoplasmic ACONITASE 3 and modulates the abundance of AOX1A and AOX1D in *Arabidopsis thaliana*. *New Phytol* doi: 10.1111/nph.13097.
- V Konert G, Rahikainen M, Trotta A, Salojärvi J, Durian G, Kobishev S, Tyystjärvi E, Kangasjärvi S (2014). Protein Phosphatase 2A subunits B^γ and B^ζ regulate photo-oxidative stress responses and growth in *Arabidopsis thaliana*. *Submitted*.

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ABBREVIATIONS

ACO	aconitase
AOX	alternative oxidase
ATP	adenosine triphosphate
ATPase	ATP synthase
BAK1	BRI1-ASSOCIATED RECEPTOR KINASE
BES1	BRI1-EMS-SUPPRESSOR 1
BIFC	bimolecular fluorescence complementation
BR	brassinosteroid
BZR1	BRASSINAZOLE-RESISTANT 1
cDNA	complementary DNA
CFU	colony forming units
CN	clear native
COX	cytochrome c oxidase
CPK	calcium dependant protein kinase
cRNA	complementary RNA
Da	Dalton; unified atomic mass unit
DAB	3,3'-diaminobenzidine
DNA	deoxyribonucleic acid
DSP	dual-specificity phosphatase
EMS	ethyl methanesulfonate
ETI	effector-triggered immunity
ETS	effector triggered susceptibility

F_0	minimal fluorescence
FDR	false discovery rate
F_m	fluorescence minimum
F_v	variable fluorescence ($F_m - F_0$)
GL	growth light
GUS	β -glucuronidase
HL	high light
KCN	potassium cyanide
LCMT	leucine carboxyl methyltransferase
MAMP	microbial associated molecular pattern
mitETC	mitochondrial electron transport chain
mnSOD	mitochondrial superoxide dismutase
MS/MS	tandem mass spectrometry
NAD(P)H	nicotinicamide adenine dinucleotide (phosphate)
O_2^-	superoxide
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular pattern
PCR	polymerase chain reaction
PDFV	polyvinylidene fluoride
PEA	plant efficiency analyser
PIN	pin-formed proteins; auxin transporters
PME	PP2A-specific methyl esterase
PP2A	protein phosphatase 2A

8 | ABBREVIATIONS

PPKL	protein phosphatase with Kelch-like repeat domains
PPM	metallo-dependent protein phosphatase
PPP	serine/threonine protein phosphatase
PRR	pattern recognition receptor
PTI	PAMP-triggered immunity
PTP	phosphotyrosine protein phosphatase
RH	relative humidity
RNA	ribonucleic acid
ROS	reactive oxygen species
SA	salicylic acid
SDS	sodium dodecyl sulfate
SHAM	salicylhydroxamic acid
SLP	<i>Shewanella</i> -like protein phosphatase
SPYCE	split YFP C-terminal fragment expression
SPYNE	split YFP N-terminal fragment expression
SRM	selected reaction monitoring
TAIR	The Arabidopsis Information Resource
TCA	tricarboxylic acid cycle (Krebs cycle, citric acid cycle)
YFP	yellow fluorescent protein

ABSTRACT

Living organisms manage their resources in well evolutionary-preserved manner to grow and reproduce. Plants are no exceptions, beginning from their seed stage they have to perceive environmental conditions to avoid germination at wrong time or rough soil. Under favourable conditions, plants invest photosynthetic end products in cell and organ growth to provide best possible conditions for generation of offspring. Under natural conditions, however, plants are exposed to a multitude of environmental stress factors, including high light and insufficient light, drought and flooding, various bacteria and viruses, herbivores, and other plants that compete for nutrients and light. To survive under environmental challenges, plants have evolved signalling mechanisms that recognise environmental changes and perform fine-tuned actions that maintain cellular homeostasis. Controlled phosphorylation and dephosphorylation of proteins plays an important role in maintaining balanced flow of information within cells.

In this study, I examined the role of protein phosphatase 2A (PP2A) on plant growth and acclimation under optimal and stressful conditions. To this aim, I studied gene expression profiles, proteomes and protein interactions, and their impacts on plant health and survival, taking advantage of the model plant *Arabidopsis thaliana* and the mutant approach. Special emphasis was made on two highly similar PP2A-B regulatory subunits, B'γ and B'ζ.

Promoters of B'γ and B'ζ were found to be similarly active in the developing tissues of the plant. In mature leaves, however, the promoter of B'γ was active in patches in leaf periphery, while the activity of B'ζ promoter was evident in leaf edges. The partially overlapping expression patterns, together with computational models of B'γ and B'ζ within trimeric PP2A holoenzymes suggested that B'γ and B'ζ may competitively bind into similar PP2A trimers and thus influence each other's actions. *Arabidopsis thaliana pp2a-b'γ* and *pp2a-b'γζ* double mutants showed dwarfish phenotypes, indicating that B'γ and B'ζ are needed for appropriate growth regulation under favorable conditions. However, while *pp2a-b'γ* displayed constitutive immune responses and appearance of premature yellowings on leaves, the *pp2a-b'γζ* double mutant suppressed these yellowings. More detailed analysis of defense responses revealed that B'γ and B'ζ mediate counteracting effects on salicylic acid dependent defense signalling. Associated with this, B'γ and B'ζ were both found to interact *in vivo* with CALCIUM DEPENDENT PROTEIN KINASE 1 (CPK1), a crucial element

of salicylic acid signalling pathway against pathogens in plants. In addition, B γ was shown to modulate cellular reactive oxygen species (ROS) metabolism by controlling the abundance of ALTERNATIVE OXIDASE 1A and 1D in mitochondria.

PP2A B γ and B ζ subunits turned out to play crucial roles in the optimisation of plant choices during their development. Taken together, PP2A allows fluent responses to environmental changes, maintenance of plant homeostasis, and grant survivability with minimised cost of redirection of resources from growth to defence.

TIIVISTELMÄ

Elolliset organismit hyödyntävät käytössään olevia resursseja kasvaakseen ja tuottaakseen jälkeläisiä. Tämä piirre on evolutiivisesti vanha ja pätee myös kasveihin. Siemenvaiheesta lähtien kasvien on aistittava ympäristönsä muun muassa välttääkseen itämisen epäsuotuisassa paikassa. Suotuisissa olosuhteissa kasvit suuntaavat fotosynteesin lopputuotteita kasvuun ja kehitykseen lisääntymismenestyksen takaamiseksi. Luonnollisessa kaivuympäristössään kasvit ovat kuitenkin alttiina myös monille ympäristön stressitekijöille, kuten riittämättömälle tai liian kirkkaalle valolle, kuivuudelle, liialliselle veden saannille, erilaisille bakteereille ja viruksille, kasvinsyöjille sekä muille, samoista resursseista kilpaileville kasveille. Selvitäkseen näistä ympäristön asettamista haasteista kasveille on evoluution kuluessa kehittynyt viestimekanismeja, jotka tunnistavat ympäristön stressitekijöitä ja sopeuttavat solujen toimintaa vallitsevien olosuhteiden mukaisesti. Proteiinien fosforylaatioon perustuva soluviestintä on tärkeä solujen toimintaa säätelevä tekijä. Tutkimukseni selvitti proteiinifosfataasi 2A:n (PP2A) toimintaa kasvien kasvun ja stressivasteiden säätelyssä. Tutkimuksessani selvitin geenitoiminnan muutoksia sekä proteiinien välisiä vuorovaikutuksia käyttäen hyväksi mallikasvina tunnettua lituruohoa (*Arabidopsis thaliana*). Tutkimukseni kohdistui erityisesti kahden PP2A:n säätelyalaysikön, B'γ ja B'ζ, toimintaan. B'γ ja B'ζ geenien promootterit olivat samalla tavalla aktiivisia kehittyvissä kasvinosissa. Täysikasvuisissa lituruohon lehdissä sen sijaan havaitsin, että B'γ-geenin promootteri oli aktiivinen laikkumaisissa alueissa kun taas B'ζ-promootterin aktiivisuus oli voimakasta lehden reunamilla. Osittain päällekkäisten B'γ ja B'ζ geenien ilmentymisalueiden sekä proteiinirakennemallituksen perusteella päätelin, että B'γ ja B'ζ saattavat vaikuttaa toistensa toimintaan kilpailemalla trimeeristen PP2A-holoentsyymien muodostuksesta. Lituruohon *pp2a-b'γ* ja *pp2a-b'γζ* kaksoismutanttien ilmiasu oli pienempikasvuinen kuin villityypin kasveilla, mikä viittasi siihen, että B'γ ja B'ζ edistävät kasvua suotuisissa olosuhteissa. Toisaalta *pp2a-b'γ* kellastui ennenaikaisesti, kun taas *pp2a-b'γζ* kaksoismutantti säilyi villityypin kasvien tapaan vihreänä. Yksityiskohtaisempi tarkastelu osoitti, että B'γ ja B'ζ aikaansaavat vastakkaisia vaikutuksia salisylihappovälitteisessä soluviestinnässä. Tähän liittyen B'γ- ja B'ζ-alaysiköiden havaittiin säätelevän kalsiumriippuvaisen proteiinkininaasi 1:n (CALCIUM DEPENDENT PROTEIN KINASE 1; CPK1) toimintaa. Tämä oli huomattava havainto, koska CPK1 on salisylihappoon perustuvan soluviestinnän keskeinen osatekijä. Osoitin myös, että B'γ säätelee solujen reaktiivisiin happilajeihin perustuvaa viestintää

vaikuttamalla mitokondrioiden vaihtoehtoiisiin elektroninsiirtomekanismeihin, erityisesti vaihtoehtoiisiin oksidaaseihin (ALTERNATIVE OXIDASE) 1A ja 1D. Proteiinifosfataasi siis mahdollistaa kasvien tehokkaan sopeutumisen erilaisiin ympäristön olosuhteisiin ja varmistaa tehokkaan kasvun ja lisääntymisen ympäristön stressitekijöistä huolimatta.

1. INTRODUCTION

1.1. Homeostasis through plant lifetime

Keeping internal processes in balance allows organisms to maintain conditions in which life sustainable chemical reactions can occur. This homeostasis lies in the very beginning of life. Control mechanisms act on many levels, including whole organism, cells and organelles.

Despite their rigid appearance and stagnant growth habitat, plants adapt remarkably well to their environment. Growth plasticity is achieved in part by perception of the quality and quantity as well as direction of solar radiation. Highly important issue are losses in crop yield due to herbivores and pathogens. Proper recognition and transduction of environmental signals to the response mechanisms allow plants to adapt and survive.

1.2. The function of signalling in plants

1.2.1. Growth and reproduction

Modification of plant growth and development is an evolutionary adaptation to adjust to environmental changes. As sessile organisms, they cannot avoid stresses by changing their growth habitat. Major processes that plants have to maintain are photosynthesis, respiration, and transpiration. It is important to note that plants can sacrifice cells, tissues, and organs in order to enable new beginning through the generation and propagation of seeds. Young tissues and, most importantly, reproductive structures are the sites of the strongest induction of defence responses upon pathogen or herbivore attack (Stamp 2003). Importantly, given tissue might be threatened and protected as it is important during one developmental moment while upon growth its importance diminishes. In the end, what matters to the plant is the tight balance between how much can be sacrificed before death, in order to move its genes forward.

1.2.2. Light signalling and acclimation

Plants use light to grow and maintain their life functions. However, light is not only nectar dripping from the skies, it also causes lots of damage. Damage comes with too high or too low irradiation or, as often happens in nature, with its fluctuations or general spectra (Konert et al. 2013). Perceiving the light condition and transducing the signal between organelles to induce proper

response, allowing the plant to adjust, change processes inside the cell, inside the tissue and even in the shape of the plant itself to avoid, cope or sometimes gather more resources.

Specific light signals are perceived and transduced into developmental modifications (Genoud et al. 2002; Barnes et al. 1995).

Utilisation of solar radiation in the plant photosynthesis reactions, perceived also as a signal to adjust developmental feedback to the environment changes is the finest example of perfect signalling capacity and the well-oiled plant cellular mechanism. Chloroplasts are commonly considered as a system to harvest light energy and convert it into carbohydrates. The photosynthetic machinery of chloroplasts can also perceive changes in the light conditions and launch self-regulatory signals that may lead to structural and functional rearrangements of protein complexes by alterations in the expression of nuclear genes (Allen et al. 1995; Larkin 2014).

Plants may utilise a variety of strategies to acquire the most of the sun, and to cope and survive in the end. Correctly perceived light intensity and quality causes morphological changes in plant organs to either twist away or bend towards the light (Goyal et al. 2013).

Under high light stress (HL), commonly occurring in nature in combination with increased temperature and limited water supply, chloroplasts become a primary site of reactive oxygen species (ROS) production (Hideg et al. 1998, Karpinski et al. 2012). Imbalance between incoming energy and the capacity of its consumption may lead to photoinhibition and create oxidative damage to the photosystems (Suorsa et al. 2012, Tyystjärvi 2013). Other roles of ROS have been underlined in numerous studies, highlighting important relationships between its place of origin, and of its signalling impact on nuclear gene expression and for acquiring stress resistance in photosynthetic organisms (Karpinski et al. 1999, 2003, Pogson 2008, Karpinski et al. 2010, Kangasjärvi et al. 2013).

On the cellular and tissue levels, high light exposed plants produce molecules that may control rising levels of ROS. The observable colour changes in plants long exposed to high light is due to the accumulation of antioxidant pigments such as anthocyanins (Archetti et al. 2009). Plants are armed with systems that

prevent cellular damage caused by increasing light irradiation. These systems include ascorbate, glutathione and catalase antioxidant systems, all of which include many intermediate steps in the removal of potentially aggressive molecules (Mhamdi et al. 2010, Foyer and Noctor 2011). Cells increase the levels of ROS scavenging molecules to help prevent internal oxidation damage to DNA, proteins and lipids, and disturbing inner metabolic pathways (Roldán-Arjona and Ariza 2009).

1.2.3. Defence reactions

Plant resistance to infections and the way in which plants can cope with them is an important area in environmental physiology. Plants are prone to diseases caused by fungi, viruses and bacteria, as well as mechanical damage caused by insects and roundworms.

The ability of pathogens to cause disease in plants is not a widely observed rule and rather an exception. The defence mechanisms of plants are composed of multiple, complex layers that set barriers to diverse pathogens. Both physical and chemical barriers block the entrance and spreading of an infection. Upon pathogen recognition, plants are capable of inducing a wide variety of defence mechanisms comprised of diversity of morphological changes, biochemical alterations including oxidative bursts, induction of defence-related genes and rise of antimicrobial compounds, and cell death (van Loon et al. 2009).

PAMP triggered immunity (PTI) is a result of perception of microbial- or pathogen associated molecular patterns (MAMPs or PAMPs) by plant pattern recognition receptors (PRRs). Effector triggered susceptibility (ETS) occurs when pathogens secrete effectors that suppress PTI. Recognition of the pathogen effectors (either directly or indirectly) activate in plants effector-triggered immunity as a counter defence leading to disease resistance (Chisholm et al. 2006, Jones and Dangl 2006).

PTI and/or ETI enhance plant disease resistance and limit the growth of pathogens. Thus, recognition of an invading microorganism at the right time, followed by an effective signal transduction to induce proper defence responses is crucial for plant survival.

It should be noted, that plants have a limited pool of resources from which they need to allocate some to produce defensive metabolites as constant production

of them in all plant tissues is costly and some plant parts seems to be more valuable in given developmental stage, and need to be better protected.

Tissue value and the probability of attack are the factors that determine the investments in defensive metabolites. Therefore, any defensive allocation pattern has to be evaluated in the context of the plant's developmental status. Besides toxic defence-active secondary metabolites, primary metabolism and particularly the content of amino acids is an important emerging field in plant defence. Aconitase, for example, as an important branching point enzyme in the citric acid cycle, modulates oxidative stress and cell death in higher plants (Moeder et al. 2007). The mechanisms driving primary metabolism response, however, are not well understood. Plants have also evolved mechanisms where amino acids mediate protective effects against invading pathogens or herbivores (Zeier 2013). The mechanism of action might include misincorporation into proteins, interference with metabolism or, in case of insects, interfering with neurological processes (Huang et al. 2011).

1.2.4. Crosstalk between light and immunity signalling

Plant metabolic pathways are susceptible to variations in environment, and any caused imbalances might generate an oxidative stress in cells by inducing the generation of ROS, increasing oxidation of proteins, lipids, nucleic acids, and others, inhibiting metabolic pathways and disrupting integrity of the organelles (thus unbalancing homeostasis). However, it is now increasingly recognised that ROS also mediates important signalling functions in plant cells (Laloi et al. 2004). The redox state of organelles like mitochondria and the chloroplasts requires a delicate balance between production and consumption of the energy. Redox harmony is important for the need of avoiding imbalanced production of ROS. Extreme environmental conditions are the cases in which maintaining balance is especially critical. HL intensity, heat, drought or a combination of these are a good example of such circumstances.

The multitude of redox and ROS signals require a fine-tuned regulation and equilibrium between developmental metabolic reactions and cellular signalling. All plant processes, including growth and defence, require investment of energy. This explains why unstressed plants invest in growth, while stress-exposed plants may halt growth and invest in protective mechanisms until environmental conditions improve.

1.3. Protein phosphorylation

1.3.1. Protein phosphorylation in plants

Reversible protein phosphorylation is one of the key posttranscriptional mechanisms by which all living organisms cope with changes. Organisms adjust by transducing, modulating and reacting to the stimuli coming from the environment. Phosphoproteins are present in all plant compartments such as chloroplasts (Reiland et al. 2009), mitochondria (Ito et al. 2009), nuclei and cytosol (Olsen et al. 2006), as well as extracellular (Tagliabracci et al. 2012). Protein kinase-phosphatase pairs are important in maintaining homeostasis. A number of protein kinases, such as the plasma membrane receptor kinases and mitogen activated protein kinases have been discovered and functionally characterised in the plant kingdom (Oh et al. 2014, Bojar et al. 2014). Plant calcium dependent protein kinases (CPKs) possess a calmoduline-like calcium binding domain and a serine/threonine protein kinase domain. CPKs are involved in diverse cellular processes, including hormonal growth regulation, perception of abiotic stresses and pathogen defence (Asano et al. 2012, Ludwig et al. 2004, Coca and San Segundo 2010, Schulz et al. 2013). CPK1 has been shown to be a major component of SA signalling and plays a role in resistance to different types of plant pathogens (Coca and San Segundo 2010).

Recent research and discoveries on protein phosphatases have provided novel insights into the cellular importance and the incredible variability of both the enzymes themselves and the regulatory process in which they are involved. The delay of research interest in protein phosphatases, compared to the wealth of studies on protein kinases might partially stem from the fact that protein kinases tend to show substrate specificity based on the amino acid sequence of the target protein, while catalytic subunits (domains) of protein phosphatases do not show such clear discrimination. Interestingly, it is now clear that protein dephosphorylation is a tightly controlled regulatory action, which may activate or inactivate the target phosphoprotein (BZR1; Cohen 1992, 2000, He et al. 2002, Uhrig et al. 2013).

Due to active recent discoveries, classification of protein phosphatases in plants has evolved during last decade. Protein phosphatases are found by similarities in their active sites sequences (Kerk et al. 2008) and are mainly divided according to their target specificity and their requirements for divalent cations (Figure 1; Moorhead et al. 2009, Uhrig et al. 2013):

- Serine/threonine protein phosphatases (PPPs): PP1, PP2A, PP2B (not in plants), PP4, PP5, PP6, PP7, SLPs (*Shewanella*-like protein phosphatases), PPKLs (protein phosphatases with Kelch-like repeat domains)
- Metallo-dependent protein phosphatases (PPMs): PP2C clusters
- Aspartate-bases protein phosphatases, controlling the serine/threonine phosphorylation status of RNA polymerase II
- Dual specificity phosphatases (DSPs)
- Phosphotyrosine protein phosphatases (PTPs)

The variety of different protein phosphatases in plants reflects the importance of the signalling processes they are involved in, and that tight control by low abundance protein phosphatases may (and does) influence processes in the whole plant physiology.

serine / threonine	PPP	PP1
		PP2A
		PP4
		PP5
		PP6
		PP7
		SLP
		PPKL
	PPM	PP2C
	Asp-based	FCP-like
HAD family		
EYA		
DSP	DSPs	
PTP	nonreceptor	
	DPSs	
	LWPTP	
		tyrosine

Figure 1. Classification of plant protein phosphatases by substrate specificity. PPP, phosphoprotein phosphatase; PPM, metallo-dependent protein phosphatase; DSP, dual specificity phosphatase; PTP, protein tyrosine phosphatase; Asp-based, aspartate-based phosphatases.

1.3.2. Protein phosphatase 2A: structure, activation and assembly

PP2A is a highly conserved family of serine/threonine phosphatases. It is trimeric with scaffold A, catalytic C and highly variable regulatory B subunits encoded by many genes which leads to many possible combinations of trimers. Arabidopsis 17 regulatory B subunits divide into B, B' and B'' subunit families (Shi et al. 2009). The particular combinations of the trimers are still largely unknown and, despite the many interesting endeavours to reveal the role and importance of particular subunits in the composition and localisation of the holoenzyme, it remains a complete mystery (Gentry, 2002, Slupe 2011, Wei et al. 2014).

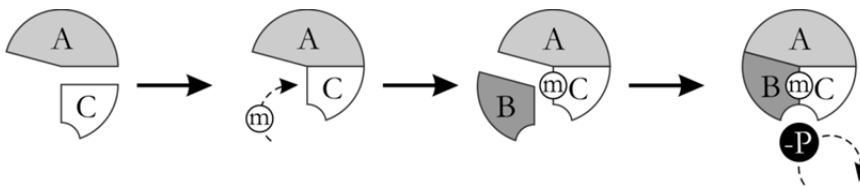


Figure 2. PP2A trimer assembly. Scaffold subunit A binds catalytic subunit C. The formed core enzyme is methylated on Leu³⁰⁹ of subunit C. This allows a conformational change that leads to a high affinity towards regulatory B subunits. A-B-C PP2A holoenzyme is fully activated and ready to dephosphorylate target proteins.

The PP2A core enzyme (A and C subunits bound together) is not just a transitional state towards formation of a PP2A holoenzyme but rather a relevant cellular structure (Kremmer et al. 1997). As it was shown in mammals, to form the PP2A holoenzyme, the core enzyme requires methylation on the Leu³⁰⁹ in the conserved TPDYFL³⁰⁷ motif of the C subunit. This methylation step increases affinity towards regulatory B, B' or B'' subunit (Ikehara et al. 2007, Ogris et al. 1997, Tolstykh et al. 2000, Wei et al. 2001, Xing et al. 2006, Xu et al. 2006, 2008) to bind, forming active PP2A holoenzyme (Figure 2).

The methylation step is fully reversible through the action of a PP2A-specific methyl esterase (PME1), which prevents the formation of active PP2A and thus regulates its actions (Lee et al. 1996). Inhibition of PP2A takes place also by phosphorylation of Tyr²⁰⁴ and Tyr³⁰⁷ of the C subunit (Janssens and Goris 2001). *In vitro* studies of Gentry et al. (2005) and Longin et al. (2007) suggested that methylation is not always required for PP2A holoenzyme assembly. However, the clear (and undisputed) result of binding experiments showed that

methyated PP2A core enzymes exhibit higher affinity towards B subunits than unmethyated PP2A (Xu et al. 2008). B, B' and B'' subunits are present in various plant tissues at different levels (Janssens and Goris 2001, Lechward et al. 2001). Taking this into account, the B subunits not only determine overall substrate specificity but also spatial and temporal functions of PP2A. The function of the B subunit determines the regulation of the whole PP2A holoenzyme localisation within tissue and cell, and modulates its action, with different B subunit combinations leading to the possible modulations of many signalling pathways (Kamibayashi et al. 1994; Yoo et al. 2007; Latorre et al. 1997; Jonassen et al. 2011).

1.3.3. PP2A interactions and functions

PP2A is involved in a number of signalling processes in plants. It is considered a key component in the regulation of signal transduction in defence responses (He et al. 2004, Segonzac et al. 2014), hormonal signalling (He et al. 2002, Kwak et al. 2002), abiotic stress responses to water deficit (Xu et al. 2007), cold (Monroy et al. 1998) as well as mechanical wounding (Rojo et al. 1998). PP2A has also been shown to directly interact with a plethora of cellular components and modulate their actions. These include for example, the auxin efflux carrier PIN (Michniewicz et al. 2007) and the brassinosteroid (BR) responsive transcription factors BZR1 and BES1 (Tang et al. 2011, DiRubbo et al. 2011). Recently, PP2A was also shown to control the activation of plasma membrane pattern recognition receptor complexes by interacting with the co-receptor and positive regulator BAK1 (Segonzac et al. 2014). These findings exemplify the distinct roles that PP2A trimers play in plant cells to keep the flow of signals in proper equilibrium for maintaining homeostasis.

2. AIMS OF THE STUDY

The aim of this study was to uncover the physiological role of Protein Phosphatase 2A in light acclimation and defence signalling in plants. To achieve this aim, I studied gene expression profiles, proteomes and protein interactions in *Arabidopsis thaliana* and its mutants and evaluated impacts on plant health and survival.

3. METHODOLOGY

3.1. Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia wild type and mutants were grown under growth light ($130 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) and high light ($800 \mu\text{mol photons m}^{-2}\text{s}^{-1}$). Homozygote *pp2a-b'* γ (SALK_039172 for AT4G15415), *pp2a-b'* ζ 1-1 and *pp2a-b'* ζ 1-2 (SALK_107944C and SALK_150586 for AT3G21650, respectively) mutant lines were identified from the SALK collection by PCR analysis (Alonso et al. 2003). A *pp2a-b'* $\gamma\zeta$ double mutant was obtained by crossing the mutant lines SALK_039172 and SALK_107944C. Experiments were conducted with four week old plants.

3.2. Construction of transgenic plants

PP2A-B' γ gene (S69317; Yamada et al. 2003) was cloned into the pGREENII0029 vector under the control of 35S cauliflower mosaic virus promoter to construct genetic complementation line for *pp2a-b'* γ mutation. For construction of GUS lines, wild type *Arabidopsis thaliana* plants were transformed with a promoter:uidA fusion construct containing a 2800 or 2811 bp region of genomic DNA upstream of the translational start codon of the *PP2A-B'* γ or *PP2A-B'* ζ coding sequences, respectively, in pGREENII002 (Rozhon et al. 2010).

3.3. Microscopy

YFP fluorescence was imaged with a confocal laser scanning microscope (Zeiss LSM510 META; <http://www.zeiss.com>) with excitation at 514 nm and detection at 535-590 nm. Chlorophyll fluorescence was excited at 543 nm and detected with a 650-nm passing emission filter. Images were created with Zeiss Zen 2012 software Version 8.0.0.273 (<http://www.zeiss.com>).

3.4. Protein analysis

3.4.1. Isolation of leaf extracts

Total leaf extracts isolation was performed in the presence of protease (Complete-Mini; Roche) and phosphatase inhibitors (PhosSTOP; Roche) using the methods described by Kangasjärvi et al. (2008).

3.4.2. Isolation of mitochondria

Arabidopsis thaliana mitochondria were isolated according to Sweetlove et al. (2007) with modifications of plant material whereby soil grown, 4 weeks old plants were kept in darkness for 24h prior to isolation. Material was fractionated using 60-35-20% Percoll-sucrose gradient. The mitochondrial band was collected from the top of 60% fraction.

3.4.3. Clear native/SDS-PAGE and protein blotting

Clear native (CN)-PAGE followed by SDS-PAGE in the second dimension was performed according to Peltier et al. (2006) with modifications for buffers as described in Rokka et al. (2005), except that only 0.05% deoxycholate was used as a detergent on CN gels.

Proteins in gels were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore), blocked with 5% (w/v) specific fat-free bovine serum albumin (Sigma), and probed with polyclonal antibodies diluted in 5% (w/v) fat-free bovine serum albumin, Tris-buffered saline, and 0.1% Tween 20. Immunoblotting with protein-specific antibodies was performed as described in Kangasjärvi et al. (2008).

3.4.4. Mass spectrometry

After imaging the gels with SYPRO Ruby (Invitrogen), mass spectrometry was performed as described by Peltier et al. (2006) using a liquid chromatography-electrospray ionization-MS/MS system (QTOF Elite; AB Sciex). MS/MS spectra were analysed with an inhouse installation of Mascot (www.matrixscience.com), with searches restricted to the Swissprot Arabidopsis database, allowing Met oxidation, Cys carboamidomethylation, and Ser/Thr phosphorylation as possible modifications.

Mitochondrial samples were separated on SDS-PAGE and used in in-gel digestion to obtain peptides. SRM peak groups were manually selected to match *dotp* and retention time values from SRM runs. ACO3 unique peptides were chosen according to Taylor et al. (2014). Using the in gel digested samples lowered the amount of co-eluting peptides in each injection. Phosphorylated and non-phosphorylated ACO3 peptides were detected within same injection and the relative abundance was calculated.

3.4.5. In-gel kinase assay

Total soluble leaf extracts corresponding to 30 µg of protein were heated at 95°C for 5 minutes and thereafter fractionated in 10% SDS-polyacrylamide gels containing 0.25 mg/mL histone type III-S (Sigma). The CPK activity assay was then conducted according to Gao et al. (2013).

3.5. Analysis of protein interaction

3.5.1. Yeast two-hybrid

Yeast two-hybrid screening was conducted with HybridHunter (Invitrogen, <http://www.invitrogen.com>) using L40 yeast strain and LexA DBD fusion of PP2A-B'γ in pHybLex as bait against a cDNA library enriched for stress-related factors as described in Jaspers et al. (2009). About 25 CFU of tryptophan auxotrophic yeast was placed on –His selection supplemented with 10 mM 3-aminotriazole (3AT; Sigma-Aldrich, <http://www.sigmaaldrich.com>) to remove autoactivation. Picking of colonies took place 4 days after in growth at 28°C and tested for β-galactosidase activity according to the HybridHunter manual. Found interaction partners in the pYESTrp2 library plasmid were confirmed by sequencing.

3.5.2. Bimolecular fluorescence complementation

All constructs for BiFC analyses were introduced into pGPTVII backbone vectors and verified by sequencing (Walter et al., 2004; Waadt and Kudla, 2008). Complete protein coding regions were amplified by PCR and fused to C-terminal fragment (SPYCE) or N-terminal fragment (SPYNE) of YFP.

3.6. Microarray analysis

Rosettes of wild type, *pp2a-b'γ*, *pp2a-b'ζ1-1* and *pp2a-b'γζ* double mutant plants were collected four hours after the onset of light period. RNA from four biological replicates was isolated using Agilent Plant RNA isolation mini kit and 200 ng of total RNA was amplified and Cy-3 labelled using Agilent one-color Low Input Quick Amp Labelling kit (Product number 5190-2331) and processed with the RNA Spike in kit (Product number 5188-5282). RNA/cRNA quality control was performed using Agilent 2100 bioanalyzer RNA 6000 Nano kit (Product number 5067-1511). 1.65 µg Cy-3 labelled samples were hybridized to Agilent Arabidopsis (V4) Gene Expression Microarrays, 4x44K (Design ID 021169) according to the manufacturer's

instructions, and finally scanned with Agilent Technologies Scanner G2565CA with a profile AgilentHD_GX_1Color. Numeric data was produced with Agilent Feature Extraction program, version 10.7.3.

Gene expression data was analysed using scripts in the R software package. First, the average of processed signal of probes targeting each gene was computed. Then, a linear model with genotype, treatment and their interaction as fixed effects was estimated. The p-values from the linear model were then subjected to false discovery rate correction using qvalues package. Genes with absolute \log_2 fold change >1 and FDR corrected p-values <0.05 were considered significant. Model comparisons and their p-values were estimated using multcomp package in R (Storey, 2002; Hothorn, 2008). Gene ontology annotations were derived from TAIR (<http://www.arabidopsis.org>), and used as gene sets in Gene Set Analysis (Efron and Tibshirani, 2007), carried out in R using GSA package. Gene sets with FDR-corrected p-values <0.05 were considered significantly enriched.

3.7. Analysis of promoter activity

Glucuronidase (GUS) staining of plants expressing the *proPP2A-B'*:*uidA* and *proPP2A-B'*:*uidA* fusions was performed according to Weigel and Glazebrook (2002).

3.8. Pharmacological approaches

Accumulation of H_2O_2 in the leaves was detected by using DAB (3,3'-diaminobenzidine; Sigma-Aldrich) as a substrate (Thordal-Christensen et al. 1997) with modifications as described in Kangasjärvi et al. (2008). For the inhibition of COXII and AOX, 5 mM potassium cyanide (KCN) and 10 or 12 mM salicylhydroxamic acid (SHAM), respectively, were used in the 0.1% DAB solution. Plants were incubated o/n on DAB solutions in the presence or absence of SHAM, and thereafter either kept in darkness or subjected to three hours $130 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ treatment for induction of ROS production.

3.9. Measurement of chlorophyll fluorescence

Photosynthetic activity was measured under normal growth light ($130 \mu\text{mol photons m}^{-2}\text{s}^{-1}/22^\circ\text{C}$) with the CIRAS-1 combined infrared gas analysis system (PP Systems) equipped with an *Arabidopsis thaliana* pot chamber. PSII

photoinhibition in intact leaves was recorded as the ratio of variable to maximal fluorescence (F_v/F_m , where F_v is the difference between maximum fluorescence [F_m] and initial fluorescence [F_0]), measured with a Hansatech PEA fluorometer after a 30 min dark incubation.

4. RESULTS

4.1. PP2A-B'γ and PP2A-B'ζ are highly similar regulatory subunits and form similar trimers

The protein phosphatase 2A-B family consists of 17 distinct regulatory subunit members. In *Arabidopsis thaliana* PP2A-B'γ (AT4G15415) and PP2A-B'ζ (AT3G21650) share 80% amino acid sequence identity; in addition, they are the closest relatives within the B' subfamily.

The sequence identity is not equal among the entire length of the proteins, with variation between T136-V154 and S157-A175 in PP2A-B'γ and PP2A-B'ζ respectively (Paper II). PP2A-B'ζ also possesses an 18 amino acid insertion which is not present in PP2A-B'γ, and there are several single amino acid differences in the N- and C- termini. On the other hand, PP2A-B'γ and PP2A-B'ζ also show highly conserved areas, which do not contain any major changes in chemical properties and are predicted to interact with PP2A-A and PP2A-C (Paper II). These observations suggest that PP2A-B'γ and PP2A-B'ζ may form similar PP2A trimers (Paper II).

4.2. PP2A-B'γ and PP2A-B'ζ share partially overlapping tissue localisation

My results clearly show that *PP2A-B'γ* and *PP2A-B'ζ* are expressed in all *Arabidopsis* organs, including young, developing leaves, root system and partially overlapping in expanded leaves (Papers I and V). Seedlings showed intense promoter activities of *PP2A-B'γ* and *PP2A-B'ζ* in whole root system and cotyledons, with less activity in hypocotyls (Paper V). During growth, *PP2A-B'γ* and *PP2A-B'ζ* promoter activities were highlighted in emerging lateral roots as well as in the whole lengths of the main root. Young seedlings of transgenic promoter-GUS lines showed high GUS activities in every tissue observed (Paper V). During maturation, the expression of *PP2A-B'γ* and *PP2A-B'ζ* began to decrease, with differences first presented in the leaf blades. The main difference between p*PP2A-B'γ*::GUS and p*PP2A-B'ζ*::GUS lines was found in the older leaves where p*PP2A-B'γ* driven GUS activity was expressed in lateral patterns of premature yellowings in *pp2a-b'γ* mutant leaves (Paper I), while the *PP2A-B'ζ* promoter was mainly found to be active in thin edges of the leaf blade. Upon maturation, p*PP2A-B'γ*::GUS activity was barely detectable in fully expanded leaves (Paper V).

4.3. *pp2a-b'ζ* suppresses the yellowing phenotype of *pp2a-b'γ*

PP2A-B'γ and PP2A-B'ζ deficient mutants showed astonishingly diverse phenotype of mature, 4 week old rosettes. When grown in 130 μmol photons m⁻²s⁻¹ and 55% RH, the smaller and slower growing *pp2a-b'γ* developed premature yellowing in peripheral patches of the mature leaf blades, yet displayed late flowering time (Paper I). *pp2a-b'ζ* showed neither of these *pp2a-b'γ* phenotypes, but exhibited slightly enhanced growth and premature bolting as compared to the wild type (Papers IV and V). It is also worth noting that when grown under high light (800 μmol photons m⁻²s⁻¹ and 60% RH), the leaf size did not differ between the *pp2a-b'γ* and *pp2a-b'ζ* single mutant lines, while the *pp2a-b'γζ* double mutant showed significantly reduced growth (Papers II and V).

The phenotypic characteristics of *pp2a-b'γ* were partially suppressed in a *pp2a-b'γζ* double mutant grown in GL conditions, suggesting that the B'γ and B'ζ subunits of PP2A are functionally interconnected with opposing effects on the performance of Arabidopsis plants. Even though the *pp2a-b'γζ* double mutant still exhibited a dwarfed phenotype, it did not display symptoms of cell death (Paper III). What is more remarkable, is that *pp2a-b'γ* showed no yellowings when grown in high light conditions (Papers I and V).

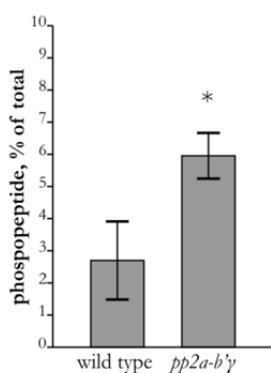
4.4. Combined drought and high light stress favour *pp2a-b'γ* and *pp2a-b'γζ* mutants compared to wild type

The light conditional phenotypes of the *pp2a* mutants suggest malfunctions in the regulation of photooxidative stress responses. To further assess the impact of PP2A on high light tolerance, wild type and the *pp2a* mutants were grown in different conditions and subjected to analysis of the level of photosystem II photoinhibition by measurement of chlorophyll fluorescence (Fv/Fm). In the GL and HL conditions there were no observable differences between the wild type and the *pp2a* mutant lines. However, when HL stress was combined with drought, statistically significantly higher Fv/Fm values were observed for *pp2a-b'γ* and for the *pp2a-b'γζ* double mutant, as compared to the wild type (Paper V).

4.5. PP2A-B'γ interacts with CPK1 and ACO3

Microarray analysis revealed that the levels of *CPK1* (*CALCIUM DEPENDENT PROTEIN KINASE 1*) mRNA were significantly elevated in both the *pp2a-b'γ* and *pp2a-b'γζ* double mutant plants, with no alterations in genes encoding other CPKs (Paper III). Yeast two-hybrid screening against a

stress induced library (Jaspers et al. 2009) suggested that CPK1 and ACO3 (ACONITASE 3) were possible interactors for PP2A-B γ (Paper III). This result was confirmed *in vivo* by bimolecular fluorescence complementation (BIFC), which showed clear interactions between PP2A-B γ and CPK1, and PP2A-B γ and ACO3 in Tobacco (*Nicotiana benthamiana*) leaves (Paper III). In-gel kinase assays confirmed increased CPK activities in PP2A-B γ deficient mutants. CPK bands with molecular weights of 68 and 60 kDa were constantly enhanced in *pp2a-b'\gamma* and *pp2a-b'\gamma\zeta* double mutant plants, with only minor activity detected in wild type and *pp2a-b'\zeta* plants (Paper III).



Finding ACO3 as a possible interactor of *pp2a-b'\gamma* *in vivo* prompted studies on the mechanistic influence of PP2A on the level of ACO3 phosphorylation. A combination of MS and SRM methods (Paper IV) revealed a lower ratio of phosphorylated to non-phosphorylated ACO3 unique peptides in *pp2a-b'\gamma* compared to the wild type (Paper IV; Figure 3). In the wild type, 3% of a unique ACO3 peptide was phosphorylated while in *pp2a-b'\gamma* this value doubled to 6%.

Figure 3. Percentage of $^{89}\text{TFSSMASEHPFK}^{100}$ peptide phosphorylation in wild type and *pp2a-b'\gamma*.

4.6. *pp2a-b'\gamma* and *pp2a-b'\gamma\zeta* accumulate H_2O_2

As shown in Paper I, *pp2a-b'\gamma* mutants demonstrated increased levels of H_2O_2 in patches, which closely resembled the localisation of the leaf yellowings. To begin understanding the role of mitETC in this phenomenon, I designed a pharmacological approach to test the involvement of mitochondria originated ROS in the total H_2O_2 levels observed in *pp2a-b'\gamma*. The *pp2a-b'\gamma* and *pp2a-b'\gamma\zeta* double mutants showed increased levels of H_2O_2 when plants were exposed to light in the presence of the AOX inhibitor salicylhydroxamic acid (SHAM). The *pp2a-b'\gamma* mutant already showed accumulation of H_2O_2 when grown in GL conditions, with only a fraction of this accumulation observed in the *pp2a-b'\gamma\zeta* double mutant (Paper IV). Both *pp2a-b'\gamma* and *pp2a-b'\gamma\zeta* double mutants suffered from high ROS production when mitochondrial alternative oxidase activity was compromised by the addition of the oxygen binding inhibitor SHAM. The

tissue localisation of H_2O_2 was not identical in the *pp2a-b'γ* and *pp2a-b'γζ* double mutants. The *pp2a-b'γ* plants showed patches similar to their premature yellowing and *PP2A-B'γ* promoter activity (Papers I and V), whereas *pp2a-b'γζ* double mutants patches were not so precisely localised (Paper IV).

4.7. PP2A-B'γ controls the abundance of alternative oxidases 1A and 1D in leaf mitochondria

Since *pp2a-b'γ* showed enhanced ROS staining in the presence of SHAM, an important question arose as to whether the levels of AOX proteins were altered in *pp2a-b'γ*. To answer this question, I used anti-AOX antibody on purified mitochondrial fractions from *pp2a* lines. Protein blotting showed increased levels of AOX1A protein of 34 kDa size in *pp2a-b'γ* and *pp2a-b'γζ* double mutants (Paper IV;

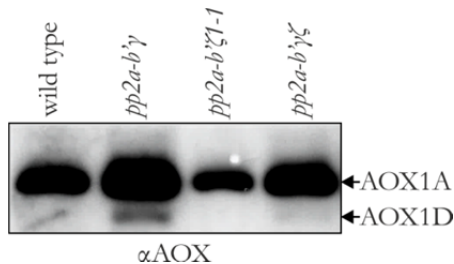


Figure 4. Overaccumulation of AOX1A in *pp2a-b'γ* and *pp2a-b'γζ*. Identified band of AOX1D highly increased in *pp2a-b'γ* and at lower levels in *pp2a-b'γζ*.

Figure 4). No consistent alteration in AOX1A abundance was observed in *pp2a-b'ζ*. Interestingly, in addition to increased levels of AOX1A in *pp2a-b'γ* and *pp2a-b'γζ* double mutants, I observed the appearance of an extra band sized of 29 kDa (Paper IV; Figure 4). To identify the AOX isoform, I separated the mitochondrial proteins by SDS-PAGE and partially blotted them to a PDFV membrane. The immunoblot with anti-AOX antibody was then overlapped with the remaining Coomassie-stained gel. This allowed localisation and cutting of both AOX1A and the 29 kDa AOX bands from the gel, and subsequent mass spectrometry analysis by ESI-hybrid quadrupole-orbitrap (Paper IV). In the three independent biological replicates analysed, the upper band was the AOX1A isoform as expected, while the 29 kDa band contained unique peptides for AOX1D. As I have shown in Paper IV, the transitions and corresponding retention times of the specific and common peptides for AOX1A and 1D were found by SRM analysis. Retention times of corresponding peptides were comparable with the shotgun results.

5. DISCUSSION

5.1. PP2A is crucial for growth and development

Plants are not defenceless. In the process of evolution they have developed methods to repel and destroy their enemies, and they activate their own immune responses upon pathogen attack. The multiple routes to plant defence are reflected in the varying degrees of response that can be observed, not only between species, but also between individual specimens. Here, it is important to note that the price of constantly keeping up defence mechanisms is a large one to pay. Prolonged or even constant defence response leads to impaired growth, as all cell mechanisms are invested in keeping the plant alive. Investing in defence at the expense of biomass is worthwhile only when pathogens or abiotic stress conditions are present and are dangerous to the particular species. If the danger is sensed wrongly, either due to oversensitivity, or to the plant being incapable of perceiving the threat, then the plant will pay the highest possible price. Thus, correct signal perception and transduction is essential to the sessile plant, which cannot escape from threat.

As shown in papers I, III and V, PP2A is present in multiple plant tissues and cell compartments, including the roots and stem, leaves and flowers as well as seeds, and all in different developmental stages. The importance of PP2A in balancing growth has been evidenced in hormonal signalling with involvement of polar auxin transportation in *Arabidopsis* (Michniewicz et al. 2007). Proper redirection of auxin flux is crucial for plant growth upon light stimuli, allowing adjustment to the changing abiotic conditions. Furthermore, protein phosphatases support BR signal transduction inside cells by acting both on BRI1 turnover and by dephosphorylating BZR1, thus maintaining the correct growth (Di Rubbo et al. 2011). Moreover, as reported by Jonassen et al. (2011), *pp2a-b'aβ* mutants show a male fertility dependant phenotype associated with brassinosteroid perception in stalks.

As I have presented in Papers I and III, the promoter activities of *PP2A-B'γ* and *PP2A-B'ζ* are tightly correlated with the developmental stage of the tissue. In seedlings, as well as in young one to two week old plants, there is no observable difference between these two promoters in their expression patterns. Both promoters tend to be active in all tissues and organs, including young roots, cotyledons and small leaves. Upon maturation, in rosette leaves, the pattern of *PP2A-B'γ* and *PP2A-B'ζ* promoter activities mostly stays in

young, rapidly growing tissues. However, in older leaves the *PP2A-B'γ* promoter activity is observed in distal patches resembling the patches of premature yellowing observed in the *pp2a-b'γ* mutant (Paper I). The promoter of *PP2A-B'ζ*, on the other hand, is still active in the young tissues, as well as in lateral parts of the edges of old leaves.

The presence of both *PP2A-B'γ* and *PP2A-B'ζ* transcripts in young, developing tissues, together with the distinct phenotype of the *pp2a-b'γ* mutant, fit with the claim of PP2A being involved in proper growth regulation. The lack of *PP2A-B'γ* leads to malfunction of developmental processes, leading to a dwarfish phenotype and the premature yellowing of the older leaves of plants. This supports the theory that dephosphorylation of proteins by PP2A-B'γ is needed for maintaining proper development in plants; avoiding premature adulthood and most importantly, the premature onset of senescence.

5.2. PP2A is required for light acclimation and defence responses

In nature, high light stress rarely comes alone. Increased light exposure is often accompanied by heat and drought stress. Such conditions are extremely harsh to plants as they may cause photo-oxidative damage in light exposed chloroplasts, but also may limit the mechanisms by which the plant can defend itself. Formation of ROS in intra- and extracellular compartments initiates numerous stress responses (Bechtold et al. 2008, Sierla et al. 2013). From precise, localised responses to ROS molecules are created important evolutionary traits. The perception of ROS and corresponding signalling cascades of cell death or acclimation are determined through antagonistic actions of pro- and anti-cell death mechanisms (Mullineaux and Baker, 2010). Rigorous balance allows precise responses to be adjusted in response to numerous ROS mediated stimuli. Understanding the processes that branch plant reactions to cell death or acclimation may allow breeding of more stress tolerant cultivars. It is interesting to note that in GL *pp2a-b'γ* triggers cell death (Paper III), while the *pp2a-b'γζ* double mutant shows enhanced activation of photo-oxidative stress responses and acclimation to light stress (Paper V). This suggests that PP2A-B'γ and PP2A-B'ζ might be at the crossroads between growth and defence responses.

5.3. PP2A interacts with basic metabolism and signalling molecules

The importance of PP2A trimers in both growth and stress responses has been reported widely (Trotta et al. 2011, Michniewicz et al. 2007, Tang et al. 2011, Xu et al. 2007). The interaction of PP2A-B γ with ACO3 as reported here, is a new finding that shows the versatile nature of protein phosphatases (Paper IV). Through its enzymatic activity (isomerization of citrate to iso-citrate), aconitase plays an important role in achieving metabolite and redox balance under stress conditions (Moeder et al. 2007). Paper IV showed that PP2A-B γ is needed to dephosphorylate ACO3. Based on a unique phosphopeptide, the level of ACONITASE 3 phosphorylation between *pp2a-b γ* and the wild type was doubled (Paper IV). It is likely that this phosphorylation impacts the availability of active aconitase enzymes, especially when the spatial distribution of ACO3 is considered (Paper IV). It has also been shown that the inhibition of aconitase leads to induction of AOX (Gupta et al. 2012), hence forming a connection between the two metabolic enzymes.

5.4. PP2A is required for organellar crosstalk

Proper communication between cellular compartments is of basic importance in every living organism. Maintaining information exchange as to the current state of organelles provides information to the nucleus of the general status of the cell. Any disturbance of regular compartment metabolism must be precisely signalled to other parts of the cell to allow targeted reaction.

Any disturbance caused by an incidence of biotic or abiotic stress, alters the actions taking place inside the plant. Increased light intensity, for example, exerts its main impact through increased absorption of excitation energy in chloroplasts, sometimes increasing the capacity of the photosynthetic apparatus to fix carbon (Tyystjärvi 2013). This situation is highly likely to lead to the formation of ROS in chloroplasts, potentially leading to oxidative damage to thylakoid proteins (Aro et al. 1995, Hutin et al. 2003, Tikkanen et al. 2008). Organelles play different roles in cells but yet are tightly interconnected. Increased light intensities perceived by chloroplasts exposed to short-term high light stress result in a strong upregulation of mitochondrial AOXs (Vishwakarma et al. 2014, Gandin et al. 2012, Fu et al 2012, Yoshida et al. 2011, Zhang et al 2010). Mitochondrial reactions are power sources for all eukaryotes. The tricarboxylic acid cycle (TCA) uses photosynthesis delivered sugar power to create energy, by using pyruvate and creating CO₂ and NADH (Meléndez-

Hevia et al. 1996). The resultant reducing agent is consumed in the electron transport chain (mitETC), where electrons are moved between protein donors to acceptors, and ultimately to oxygen in creating water. The mitETC enzymes consist of seven core elements, NAD(P)H dehydrogenase (complex I), succinate dehydrogenase (complex II), ubiquinone, cytochrome bc1 (complex III), cytochrome c, cytochrome c oxidase (COX, complex IV) and ATPase. The electrons, passing from NADH to COX, are in the end used to reduce oxygen to water, although on their way, the energy being created is stored by pumping protons across the mitochondrial membrane using 3 enzymes: complex I, III and IV. That proton gradient is used by ATPase to create ATP by releasing ΔpH (Schertl and Braun 2013).

Upon increasing light irradiation, noticeable increases in reducing agents occur in chloroplasts. To prevent blocking of photosynthetic machinery this power has to be used, providing free NAD(P)⁺ for continuing photosynthesis. In conditions in which this will not be fulfilled, photosynthetic proteins that are blocked by electrons will not be capable of reducing NAD(P)⁺, thus creating the dangerous situation of increased ROS levels. The mitochondrial capacity of oxidising NADH has been known for a long time (Gardeström and Lernmark 1995, Taniguchi and Miyake 2012) and MitETC is considered to be a good valve to prevent the over reduction of the chloroplast stroma. Several mechanisms of shuttling reductants from the chloroplast to the stroma and mitochondria are known to date (Taniguchi and Miyake 2012, Linka and Weber 2004, Shimazaki et al 1989, Stocking and Larson 1969) but are not yet completely understood.

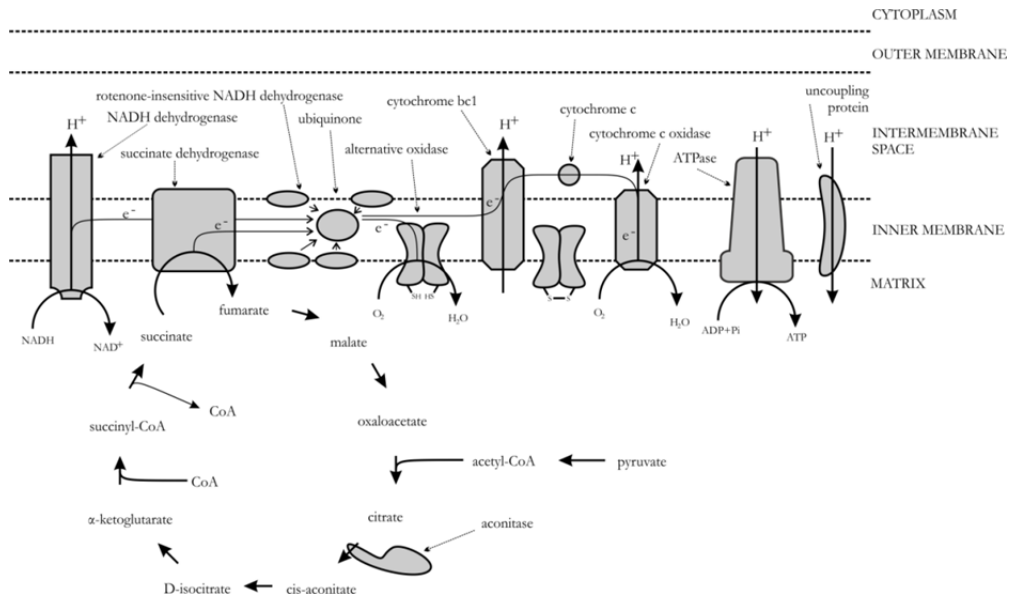


Figure 5. Mitochondrial electron transport chain with oxidative pathway.

Mitochondrial capacity to oxidise NADH is, as all processes in living organisms, limited. When mitochondrial enzymes, especially complex IV, are flooded with electrons from NADHs the risk of ROS creation increases heavily. The main ROS species created by complex IV is superoxide (O_2^-), which is almost instantly converted into H_2O_2 by the mitochondrial matrix *mnSOD*. Only the plant kingdom (with small exceptions in a few Protista) has evolved the specialised mitochondrial enzyme capable of accepting some electrons and helping mitETC to maintain proper work under a heavy shift. Cyanide-resistant oxidase (alternative oxidase, AOX) present in the inner mitochondrial membrane is capable of reducing oxygen to water without creating ΔpH . In this way, at least two steps of moving protons across the membrane are avoided (complex III and IV). While mitETC works at full capacity, ATPase is not able to dissipate all the energy from the proton gradient, leading to an increase of ΔpH and the blocking of mitETC enzymes. AOXes are not altered by ΔpH at all, thus being totally fully capable of accepting electrons from ubiquinol and allowing the swift oxidation of NADH. Increased AOXes activity diminishes the ROS production by intercepting electrons from complexes III and IV as AOXes are physically incapable of creating ROS (Shiba et al. 2013).

My results show that AOX protein levels are controlled by PP2A-B'γ (Paper IV). The *pp2a-b'γ* mutant contains increased levels of ROS arising from mitochondria. Employing a pharmacological approach allowed me to specifically block mitETC core enzyme COX (complex IV) by potassium cyanide (KCN). This reaction led to the misdirection of electron flow into AOX enzymes and as a result showed reduction of total ROS levels in every plant investigated, but mainly in *pp2a-b'γ*. While plants were exposed to the AOX inhibitor salicylhydroxamic acid (SHAM), it was clear that ROS levels were higher in the rosette leaves. The increase in H₂O₂ was observed not only in *pp2a-b'γ* but also in the *pp2a-b'γ ζ* double mutant. In addition, isolated mitochondrial fractions of PP2A mutant lines have shown that the *pp2a-b'γ* and *pp2a-b'γ ζ* double mutants had observable increases in levels of specific AOX1A and AOX1D isoforms (Paper IV). This is a remarkably new result, showing the tight regulation of AOX1D protein levels by the negative defence regulator PP2A-B'γ phosphatase.

6. CONCLUSIONS

Through the work undertaken in this thesis, I have shown that:

PP2A-B' γ and PP2A-B' ζ are highly similar proteins that co-regulate cellular processes and modulate each other's actions, possibly by competing for similar PP2A/C dimers. Promoter activities of *PP2A-B' γ* and *PP2A-B' ζ* show overlapping spatial and temporal tissue localisation through all growth stages, except in adult rosette leaves.

pp2a-b' γ and *pp2a-b' ζ* mutants present surprisingly contrasting phenotypes, *pp2a-b' γ* being small with premature senescence and *pp2a-b' ζ* with premature bolting. PP2A-B' γ interacts with AtCPK1, forming a direct link to salicylic acid signalling and defence (Coca and San Segundo 2010). In addition, PP2A-B' γ interacts with ACO3 and alters its phosphorylation status, with a potential impact on the stability and/or enzymatic activity of the enzyme. Inhibition of ACO3 activity also promotes up-regulation of *AOX1A* gene expression (Gupta et al. 2012). My study reveals that the mitochondrial AOX1A and, even more significantly, AOX1D protein levels are negatively controlled by PP2A-B' γ . By quenching excess reducing energy, AOX activity may essentially contribute to cellular ROS homeostasis and stress resistance in plants.

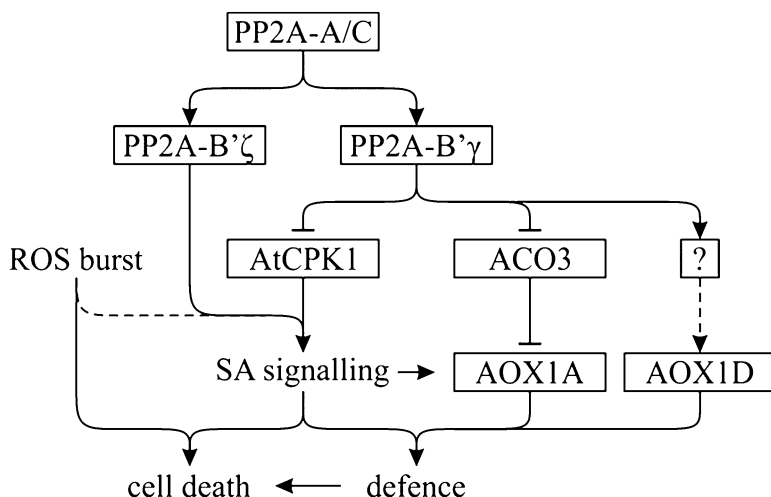


Figure 6. Schematic representation of PP2A-B' γ and PP2A-B' ζ signalling pathways. PP2A holoenzyme formation with PP2A-B' γ or PP2A-B' ζ influences its later substrate specificity and thus signalling processes.

Understanding the very mechanisms of plant defence response allows us to better apply our actions for plant well-being. Vital and resistant crops have been an aim of agriculture for centuries.

Unravelling the molecular puzzle behind plant perception of stress factors like drought, high light and pathogen attack will allow us to develop strategies to cope with increasing demands on the food production sector in future.

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